Turku sugar studies VII

Principal biochemical findings on whole saliva and plaque

KAUKO K. MÄKINEN & ARJE SCHEININ

Institute of Dentistry, University of Turku, Finland

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Plaque and whole saliva samples of the subjects of the Turku sugar studies were analyzed for several enzymes and biochemical compounds. Strict xylitol diet maintained throughout the study a 50 % lower quantity of plaque than the sucrose or fructose diets. Decreased plaque and whole saliva lactate concentration, diminished activity of salivary amylase, and reduced hydrolysis rate of sucrose in plaque and whole saliva were observed in relation to xylitol consumption. The xylitol diet also reduced the ratio of glucose to proteins in plaque. On the other hand, increased activity in plaque of α- and β-glycosidases (against *p*- and *o*-nitrophenyl derivatives), fucosidase and aspartate transaminase, as well as increased activity of proteinases and lactoperoxidase in saliva were found in connection with xylitol consumption. The fructose diet caused less clear differences when compared to sucrose, but the experiments indicated a selectivity of the effects of dietary carbohydrates on the biochemistry of whole saliva, plaque and salivary glands. The results contribute in explaining the cariostatic effects of xylitol and the lower cariogenicity of fructose when compared to sucrose.

Key-words: xylitol; fructose; sucrose; enzymes; dental caries

Kauko K. Mäkinen, Institute of Dentistry, University of Turku, SF-20520 Turku 52, Finland

The biochemical experiments of the two-year trial can be grouped into the following main categories:

I. *Determination on oral fluid (whole saliva), plaque and their composite fractions*

1. Proteins, total nitrogen, urea nitrogen, total sugars, sialic acid, glucose and fructose, and reducing sugars.
2. Nucleid acids (DNA and RNA).
3. Total keto acids, pyruvate and lactate; enzymes forming keto acids from L-djencolic acid.
4. Certain metals (Na, K, Mg and Ca), inorganic anions (F^-, I^-, and SCN^-), and inorganic phosphate.
5. Transaminases (aspartate aminotransferase and alanine aminotransferase) and aldolase.
6. Lactate, xylitol and alcohol dehydrogenases.
7. Collagenase, other proteinases and aminopeptidases.
8. Invertase-like enzymes, dextranase, and amylase.
9. Peroxidases and catalase.
10. pH, specific resistance, redox potential (E_h) and rH (hydrogen pressure above the solution).
11. Amino acid composition (and hydroxyproline separately).

12. Isoelectric focusing pattern of whole saliva proteins.
13. Immunoglobulins (A, G and M).

II. *Determinations on gingival crevice fluid*

1. Aminopeptidase activity (with special reference to aminopeptidase B), peroxidase, glycosidases, and proteins.
2. Evaluation of inflammatory reactions through microscopy of the hamster cheek pouch.

III. *Determinations on blood and serum*

1. Glucose, cholesterol, triglycerides, uric acid, pyruvate and lactate
2. Transaminases (as for saliva) and lactate dehydrogenase.
3. Hemoglobin, leucocytes and sedimentation rate of red blood cells.
4. Insulin (in the connection of a sugar tolerance test).
5. Bilirubin, ascorbic acid, Ca, Mg, Na, and K.
6. Alkaline and acid phosphatase, and amylase.
7. Proteins (isoelectric focusing and polyacrylamide electrophoresis) and immunoglobulings (IgA, IgG, and IgM).

IV. *Determinations on urine*

1. Uric acid.

Items listed below were subjected to a more detailed analysis:

1. *Aminopeptidase and proteinase activity of oral fluid*
   It has been earlier shown in this laboratory that in the presence of xylitol, and in the absence of fermentable sugars, the cells of *Streptococcus mutans* (Ingbritt) developed extracellular proteolytic and intracellular aminopeptidase-like enzymes (*Mäkinen*, 1972a; *Knuuttila & Mäkinen*, 1975a, b). These *in vitro* experiments have indicated that the polysaccharide-forming *Streptococcus mutans* could be converted into a »proteolytic organism». Consequently, a more detailed analysis on the proteinase activity of the saliva samples of the test persons was considered necessary. Furthermore, the involvement of aminopeptidase B in saliva was subjected to separate examination.
2. *Invertase-like enzymes of oral fluid.* These include enzymes cleaving sucrose, or producing reducing sugars from sucrose. Earlier papers have suggested that invertase-like enzymes may be induced by sucrose but not by xylitol diet (*Mäkinen & Scheinin*, 1971, 1972). Because the earlier methods did not differentiate between transglycosidases, true invertase and other enzymes, it was found necessary to perform a more detailed analysis about the nature of the enzymes involved.
3. *Oral peroxidases. Hoogendoorn* and his coworkers (*Hoogendoorn & Moorer*, 1973; *Koch, Edlund & Hoogendoorn*, 1973; *Hugoson et al.*, 1974) as well as *Morrison & Steele* (1968), have emphasized the possible importance of the salivary lactoperoxidase in the physiological defence mechanisms of the oral cavity. This item was thus given special consideration in the present study.
4. *The electrophoretic protein pattern of serum.* The fractionation pattern of salivary and serum proteins was determined using disc electrophoresis on polyacrylamide, and isoelectric focusing.
5. *Serum alkaline and acid phosphatase.* It has been claimed that rat serum

Table I. *List of chemical and other assays on plaque, saliva or their component fractions*), showing the point of performance in the course of the trial*

Assay	Point of performance (months) 0	3.5	7.5	13	16.5	20	24	Paper No.
Proteins (all individual supernatant fluids of whole saliva and all pooled samples)	×	×	×	×		×	×	VII
Total nitrogen (certain pooled samples)	×			×			×	VII
Total sugars (all pooled samples)	×	×	×	×		×	×	VII
Glucose (all pooled samples)	×	×	×	×		×	×	VII
Fructose (all pooled samples)	×	×	×	×		×	×	VII
Hydroxyproline (pooled supernatant fluids of whole saliva)				×	×		×	XIV,VII
RNA and DNA (all pooled samples)	×	×	×	×		×	×	VII
Total keto acids (all pooled samples)	×	×	×	×		×	×	VII
Pyruvate (all pooled samples)	×	×	×	×		×	×	VII
Lactate (all pooled samples)	×	×	×	×		×	×	VII
Na, K, Ca, Mg (individual supernatant fluids of whole saliva)				×			×	VII
Ionizable fluorine, iodine and thiocyanate (as for metals)							×	XII
Transaminases (all pooled samples)	×	×	×	×		×	×	VII
Lactate and xylitol dehydrogenases (all pooled samples)	×	×	×	×		×	×	VII
Alcohol dehydrogenase (all pooled samples)				×		×		VII
Collagenase (all pooled samples)	×	×	×	×		×	×	VII
Proteinase, hemoglobin as substrate (pooled supernatant fluids of whole saliva and 24-month individual saliva samples were tested)					×	×	×	VII
Aminopeptidase (all individual supernatant fluids of whole saliva), 3 different substrates (*N*-L-aminoacyl-2-naphthylamines)	×	×	×	×		×	×	VII
Enzymes forming keto acids from L-djencolic acid (pooled supernatant fluids of whole saliva)	×	×	×	×		×	×	VII
Invertase-like enzymes (all individual supernatant fluids of whole saliva and all pooled samples)	×	×	×	×	×	×	×	VII
Dextranase (as above)	×	×	×	×		×	×	VII
Aldolase (all pooled samples)	×	×	×	×		×	×	VII
Amylase (all individual supernatant fluids of whole saliva)							×	VII
Glycosidases (all pooled samples)							×	VII, XVIII
Peroxidase (all pooled samples)			×	×			×	XII
Catalase (pooled supernatant fluids of whole saliva)							×	XII
Amino acid composition (pooled supernatants of whole saliva)				×	×	×	×	XIV
Sialic acid (all pooled samples)				×		×	×	VII
pH (all uncentrifuged individual whole saliva samples)	×	×	×	×	×	×	×	VII
Eh and rH (as above)					×			XII
Specific resistance (pooled supernatant fluids of whole saliva)							×	VII
Enzymes resembling aminopeptidase B (all individual supernatant fluids of whole saliva)							×	VII
Phosphorus (all individual supernatant fluids of whole saliva)				×			×	VII
IgA, IgG and IgM (all individual supernatant fluids of whole saliva and serum)							×	VII

*)The biochemical analyses of gingival exudate are listed separately (*Paunio, Mäkinen & Scheinin*, 1975).

displays elevated alkaline phosphatase activity and bilirubin levels due to dietary xylitol (*Heraud*, 1973). Consequently, it was found necessary to elucidate this particular problem.

6. *Salivary amino acid composition.* The increased proteinase activity, observed *in vitro* with *Streptococcus mutans* as a result of a prolonged effect of xylitol, necessitated these experiments.
7. *Salivary glycosidase activity.* The proposed importance of glycosidases and particularly of the exo-type of β-*N*-acetyl-glucosaminidases in the metabolism of salivary glycoproteins and in the evolution of plaque (*Leach & Melville*, 1970; *Watanabe et al.*, 1974; *Mäkinen*, 1974), led to a separate study.

The present paper describes a major part of the biochemical results of the two-year trial. Additionally, the following separate papers of this series contain biochemical data:

— IX on the principal periodontal findings (*Paunio, Mäkinen & Scheinin*, 1975).
— XI on the principal blood and urine chemistry studies (*Huttunen, Mäkinen & Scheinin*, 1975).
— XII on oral peroxidases, ionizable iodine and fluorine, thiocyanate, and redox potential (*Mäkinen, Tenovuo & Scheinin*, 1975).
— XIII on certain clinico-chemical blood values, in addition to XI (*Mäkinen & Scheinin*, 1975b).
— XIV on the amino acid composition of oral fluid (*Mäkinen, Lönnberg & Scheinin*, 1975).
— XV on the inflammatory properties of gingival exudate (*Luostarinen et al.*, 1975).
— XVI on the disc electrophoresis of certain serum proteins (*Mäkinen, Mielityinen & Scheinin*, 1975).
— XVII on the fractionation and activity of oral glycosidases (*Mäkinen et al.*, 1975).

Table I provides a list and the timing of the chemical assays.

MATERIALS AND METHODS

1. *General methods*

General information on the materials and methods of the trial has also been given in previous papers (*Scheinin, Mäkinen & Ylitalo*, 1974, 1975; *Mäkinen & Scheinin*, 1975a).

In this study the following samples of oral origin were analyzed:

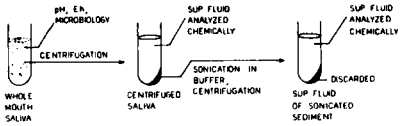
1. Aqueous extract of plaque collected *in situ.*
2. Supernatant fluid of sonicated plaque material.
   (The sonication was performed with the pellets after suspending them in a cold 0.9 % NaCl solution).
3. Supernatant fluid of whole mouth saliva (oral fluid).
4. Supernatant fluid of sonicated whole mouth saliva sediment.

In general, the saliva and plaque samples of the subjects were collected and analyzed for several biochemical properties six times including the base line determinations. One additional collection at the 16.5-month phase provided material for a more detailed study of the invertase-like activity. A number of chemical assays was carried out once or twice only. Fig. 4 (*Mäkinen & Scheinin*, 1975a) provides a general description, and Table I in the present paper shows in detail the timing of the chemical analyses carried out. The figures indicating the months in Table I stand for the start of the 2—4 week collection or analysis periods.

2. *Collection and treatment of saliva and plaque*

a. *General conditions.* The practice of the earlier short-term studies was followed in principle in the collection of the oral fluid and plaque samples (*Scheinin & Mäkinen*, 1971, 1972; *Mäkinen & Scheinin*, 1971, 1972). The procedure is described in detail below.

The subjects usually provided the saliva, plaque and other samples simultaneously with the clinical inspections. For practical reasons the samples were most often obtained between 8 a.m. and 3 p.m. For this reason it was not possible to arrange an identical timing of the individual sample collections at all analysis



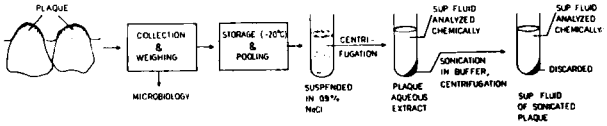


Fig. 1. A schematic presentation of the main preparative steps involved in the study of saliva and plaque

periods. Consequently, the time of collection of the samples may have influenced some of the results. The problem was given particular consideration in the determination of the redox potential of oral fluid (*Mäkinen, Tenovuo & Scheinin*, 1975). It was found, however, that during the course of the study the timing of the individual inspections and sample collections was evenly distributed, and in effect randomized among the three sugar groups.

The subjects were requested to refrain from all oral hygiene procedures for 12—24 hours before the sample collections. This period of neglected oral hygiene comprised the preceding night and the morning of the inspection day. Fig. 1 provides information about the preparation methods of plaque and whole saliva.

b. *Plaque.* The sampling was started with a 5 min plaque collection *in situ.* The whole saliva collection (10 ml, paraffin stimulation) was then carried out and the subjects were finally submitted for the clinical examination. Plaque samples were collected from all available surfaces as earlier described in detail (*Scheinin & Mäkinen*, 1971). Each plaque sample was immediately weighed with a Mettler balance (Type H15), and separate aliquots of approximately 1 mg were taken for microbiological studies.

The plaque samples were stored after weighing in cold (+4°C) and pooled at the end of each collection day (between 3 and 4 p.m.) to three pools and frozen at —20°C. In the course of the sampling period of approximately three weeks the final three complete plaque pools were gradually formed. The pools were 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 10 min at 23500 × *g* with a Sorvall RC-2B refrigerated centrifuge, unless otherwise mentioned. The three clear supernatant fluids were subjected to various analyses after each collection period. These preparations were kept at —20°C between experiments. The pellets remaining after the above centrifugation were stored at —20°C until they were thawed and treated with an ultrasonic disintegrator (described in paragraph *d*).

Consequently, the plaque material collected was divided into two parts, i.e. an aqueous extract and a water-insoluble (or sedimentable) phase, of which the latter was sonicated. All analyses carried out on these materials thus represented the plaque pools of the three sugar groups, and not the individual samples.

c. *Saliva.* The 10 ml oral fluid (whole saliva) samples were obtained using paraffin stimulation. The pH of the saliva samples was determined immediately at 25°C with glass and calomel electrodes. Simultaneously proper aliquots were removed from the oral fluid samples for microbiological studies. The samples were kept after this at +4°C, centrifuged within 2—3 hours after the collection and stored overnight at +4°C. The supernatant fluids were analyzed in the following morning for various compounds and enzymes before freezing the samples at —20°C for storage and further analyses.

The pellets obtained from the individual oral fluid samples at the end of each collection day were stored at —20°C for later pooling (after approximately 3 weeks). In this case no pooling solutions were used because the whole frozen pellets could easily be loosened from the centrifuge tubes with a glass rod. The centrifuge tubes were additionally washed with cold 0.154 M sodium chloride solution, used subsequently to suspend the three pellet pools. For material obtained from the sucrose and fructose groups 75 ml of the above cold NaCl solution was used. In the xylitol group the corresponding volume was 100 ml in order to compensate for the different size of the groups. The suspensions were treated with an ultrasonic disintegrator as described below.

d. *Sonication.* The ultrasonic treatments of the pooled salivary sediments were performed with a MSE Ultrasonic Disintegrator (Model 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 (tap water, + 10°C). The samples were treated for 10 min in 20 ml portions. The resulting mixtures were centrifuged for 15 min at 45000 × *g* (18000 rpm) and frozen at —20°C for later analyses. The 20 ml supernatant fluids in each sugar group were pooled before freezing. The pellets were discarded.

Table II. *List of chemical methods used in the present paper. For the methods used in the assay of other compounds or enzymes indicated in the Introduction section, see the references given*

Compound or enzyme to be assayed	Method, substrate and other remarks	References
Dextranase	In principle as for invertase, but replacing sucrose with a 5 % aqueous solution of dextran (Dextran T70, MW 70.000, light scattering; Pharmacia Fine Chemicals, Upsala, Sweden)	*Hostettler, Borel & Deuel* (1951); *Scheinin & Mäkinen* (1971)
Aldolase	The spectrophotometric assay of Warburg (with enzymatic auxiliary and indicator reactions). Fructose 1.6-diphosphate was used as substrate	p. 728*)
Lactate dehydrogenase (LDH)	The spectrophotometric assay using lactate and NADP	p. 736*)
Aspartate and alanine aminotransferase	The colorimetric method using 2.4-dinitrophenylhydrazine	p. 842*)
Xylitol dehydrogenase	The spectrophotometric method using xylulose as substrate (both enantiomorphic forms)	As sorbitol dehydrogenase and testing various coenzymes
Alcohol dehydrogenase	Spectrophotometrically using ethanol and NAD	In principle according to *Vallee & Hoch* (1955)
Collagenase-like activity	Colorimetrically using a synthetic collagenase substrate (4-phenylazobenzyloxycarbonyl-L-prolyl-L-leucyl-glycyl-L-prolyl-D-arginine)	*Wunsch & Heidrich* (1963)
Aminopeptidases	*N*-aminoacyl-2-naphtylamines of L-alanine, DL-arginine and L-proline	*cf. Mäkinen (1968)*
Proteinase	Denatured hemoglobin	In principle according to *Rich* (p. 80)*)
Amylase	Using starch as substrate (the 3.5-dinitrosalisylic acid method)	*Henry & Chiamori* (1960)
Catalase	A spectrophotometric method	*Lück* (1963)
Glycosidases	Using various *p*-nitrophenyl derivatives as substrate	In principle according to the method used by *Mäkinen & Knuuttila* (1971)
Invertase-like enzymes (β-fructofuranosidase)	The 3.5-dinitrosalisylic acid method	*Hostettler, Borel & Deuel* (1951); *Scheinin & Mäkinen* (1971)
Keto acid forming enzymes	A colorimetric assay using 2.4-dinitrophenylhydrazine	*Friedemann & Haugen* (1943); *Mäkinen* (1968)
Pyruvate	Spectrophotometrically with LDH and NADPH	p. 253*)
Total keto acids	A colorimetric assay using 2.4-dinitrophenylhydrazine	*Friedemann & Haugen* (1943); *Mäkinen* (1968)
Lactate	Two almost identical spectrophotometric methods using LDH and NADP	1) *Marbach & Weil* (1967); *Gloster & Harris* (1962); *Scholz et al.* (1959) 2) *Pfleiderer & Dose* (1955)**)
Proteins	Folin-Ciocalteau (bovine serum albumin as standard)	*Layne* (1963)

Total nitrogen and urea nitrogen	Micro-Kjeldahl (ammonium sulphate as standard)	Conventional methods
RNA	Orcinol method (D-ribose as standard)	*Bial* (1902); *Mejbaum* (1939)
DNA	Diphenylamine method (2-deoxy-D-ribose as standard	*Burton* (1956)
Total sugars	Anthrone method (glucose as standard)	*Dreywood* (1946); *Morris* (1948)
Glucose	With glucose oxidase	pp. 123—130*)
Fructose	Enzymatically	pp. 156—159*)
Na, K, Ca, Mg	Atomic absorption spectrophotometry	Perkin Elmer Manual (January 1964)***)
Ionizable fluorine and iodine	Electrometrically using ion specific electrodes	According to instructions provided by Beckman Co.
Thiocyanate	Colorimetrically	*Powell* (1945)
Sialic acid	The thiobarbiturate assay	*Warren, L.* (1959)****)
Phosphorus (inorganic)	Lowry-Lopez method	*Leloir & Cardini* (1972)
Amino acid analyses	With the Beckman Unichrom Amino Acid Analyzer using standard procedures given in the Manual	Beckman Manual
Disc electrophoresis of proteins	With the Canalco Modell 12 Electrophoresis Apparatus	*Ornstein* (1964)
Isoelectric focusing of proteins and enzymes	LKB Isoelectric focusing system as recommended by the manual of the supplier	LKB Produkter (Bromma, Sweden)
pH, Eh, and rH	Using standard procedures with glass and calomel, and calomel and platinum electrodes, respectively, according to the Radiometer Manuals	Radiometer, Copenhagen, Denmark
Hydroxyproline	Converting OH-proline to pyrrole which is determined with the *p*-dimethylbenzaldehyde method	*Stegemann* (1958), *Woessner* (1961)
IgA, IgG, IgM	Mancini technique	Orion Diagnostica, Orion, Helsinki, Finland

*) The page numbers refer to: *Bergmeyer, H.* (1963) Loc. cit.

**) See also: Chemical Methods of Medical Investigation, 10th Ed., E. Merck AG, Darmstadt, pp. 135—136.

***) The methods especially recommended for saliva were used except for Ca which was determined according to the general procedure (excluding TCA). It was found that in the present study both Ca-methods yielded equal results).

****) In most cases free sialic acid was assayed. In the determination of bound sialic acid a sulphuric acid hydrolysis method was used.

For pooled plaque material (paragraph *b*) the procedure was in principle the same, but a probe of 9 mm end diameter was used. The sedimentable and frozen plaque material of each sugar group was suspended into 10 ml of cold 0.154 M sodium chloride solution and the suspensions were treated with the disintegrator. The volume of the mixtures treated at a time was 5 ml. The supernatant fluids in each group were pooled after centrifugation. The samples were stored at —20°C. The pellets were discarded.

e. *Freezing and thawing of samples.* It is well known that freezing and thawing greatly affects the behaviour and structure of enzymes (cf. Federation Proceedings 24, Suppl. 15, S-55, 1968). All the present samples, stored at —20°C, were frozen and thawed in principle according to the »slow freeze» method (*Chilson, Costello & Kaplan*, 1968).

3. *Chemical assays*

Table II gives the methods used in the present biochemical studies excluding, however, the methodology in serum and urine analyses. The

gmmunoglobulin assays are described in parairaph 23. All chromatogrophy was descending.

4. *Number of subjects involved in separate experiments*

The exact number of the subjects or samples in a particular experiment varied slightly during the course of the trial. This was a consequence of the complex nature of the study, e.g. duration, number of subjects, occasional sicknesses, treatment with antibiotics, discontinuation, occasional loss of samples, etc. The sugar groups maintained, however, their size within a range of a few subjects.

5. *Expression of enzyme activities and concentrations*

Most enzyme activities in this paper are expressed as specific activities [as liberated μmoles of end product/(min × mg protein)]. This practice facilitated the comparison of the activities of the plaque and salivary preparations. In order to render this comparison possible, some of the values in the four-part figures shown later in the Results section, were all drawn in the same scale (e.g. Figs. 29 & 30). This resulted in curves passing close to the abscissa in the case of saliva supernatant fluid, which usually displayed lower concentrations and activities than the other preparations studied. If the salivary values were very low, the results obtained were indicated numerically in the figures instead of drawing curves which would not adequately fit to the scale involved. This was considered important in showing the fundamentally great differences of the specific activities and concentrations between the aqueous plaque extracts and the supernatant fluids of saliva (e.g. Fig. 43).

In one case (lactate dehydrogenase) the activity was also shown in such customary units (in U/ml) in which this enzyme is occasionally expressed in clinical chemistry. Because this does not, however, render a direct comparison between the different preparations possible, the activity of the above enzyme was also given as U/mg. Amylase activity was also given in U/l.

The usual expression of the concentration of certain chemical compounds (pyruvate, keto acids, etc.), i.e. to give them as μmoles per a unit of volume, does not allow a direct comparison between the different preparations. In such instances the effects of the sugar diets are readily comparable within solely one type of biological sample only (saliva, for example). The concentration of such compounds was, therefore, also expressed as mg proteins per μmoles, which is more illustrative for the present purposes. In a number of cases it was considered advantageous to give the ratio of protein to the compound involved (in mg × ml^{-1}/mg × ml^{-1}). This was performed for total sugars, RNA, and DNA, for example.

6. *Reagents and their sources*

As far as possible, all reagents were of analytical grade. The chemicals were mainly obtained from E. Merck AG (Darmstadt, Germany). A number of reagents was purchased from the following sources:

Nicotinamide nucleotides; C. F. Böhringer & Söhne GmbH (Mannheim, Germany).

Auxiliary enzymes used in a number of enzymatic determinations; Sigma Chemical Company (Los Angeles, Calif., U.S.A.).

N-L-Aminoacyl-2-naphthylamines, collagenase substrate and the corresponding chromophore; Mann Research Laboratories (New York, N.Y., U.S.A.).

The water used in the solutions was distilled and treated with an ion exchange resin. The specific resistance of the water was approximately 1 MΩ-cm.

7. *Comparative relation of the results during two years*

All chemical assays of the Turku sugar studies were performed, as far as possible, according to the following principles:

— The same technician or investigator performed a particular assay or analysis throughout the trial. Only a few unavoidable and insignificant deviations took place.

— The differences in the activities of auxiliary enzymes and properties of certain reagents (*p*-nitrophenyl phosphate, for example), representing separate lots, were taken into account.

8. *The number of experimental values in the graphic presentations*

In some figures of the Results section the number of experimental observations do not comprise all main six analysis phases. In a few cases values for the base line or for the last phases of the study are not shown, mainly because of occasional loss of the samples involved. For example, the bulk of the pooled sonicated plaque preparations in the fructose group of the 20 month phase was destroyed. Consequently, practically no values for this material are given. In this case the 13 and 24 month mean values were connected with a straight line.

RESULTS

1. *Salivary flow rate*

In the course of the trial it was found that the dietary regimen affected the concentration of several compounds and enzymes in whole saliva. The flow rate of saliva was thus examined, in order to evaluate the relationship between the chemical changes and the rate of secretion.

At the 16.5 month phase the subjects provided paraffin stimulated saliva samples, collected between 8 and 9 a.m. during a period of 10 min. All subjects were

studied within 10 days. Because the samples collected were also used for the determination of the salivary redox potential, the subjects were asked to refrain from all oral hygiene procedures, at least from the preceding evening until the collection. The saliva samples were obtained into graded centrifuge tubes, the volume recorded, the flow rate and the redox potential determined, and the samples centrifuged for the enzyme assays mentioned above.

The results of the flow rate determinations are shown in Fig. 2. The differences between the groups were not found significant, although the flow rate under paraffin stimulation was slighly lower in the F- and X-groups than in the S-group.

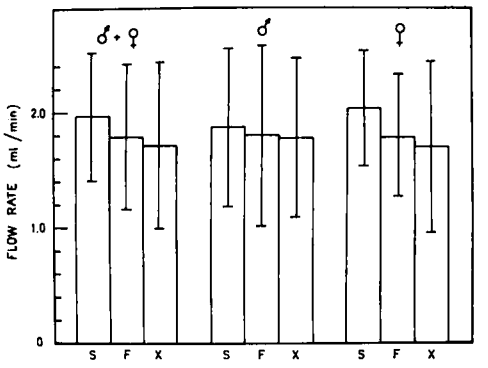


Fig. 2. The means and standard deviations of the paraffin-stimulated salivary flow rate in the three test groups determined approximately after 16.5 months' dietary regimen.

2. *Plaque wet weight*

Fig. 3 shows the development of the plaque wet weight. Xylitol consumption maintained the plaque wet weight at an approximately 50 % lower level when compared to the S-group. A detailed analysis showed that a high amount of plaque was found in a few individuals in the X-group, these subjects being habitual consumers of starch-containing products (potatoes, pastry, rice) in high quantities. The amount of plaque seemed in general to be characteristic to a subject irrespective of the sugar group.

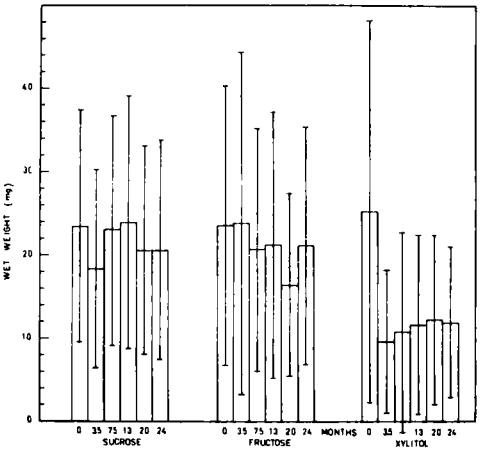


Fig. 3. The means and standard deviations of the development of plaque wet weight

The amount of plaque of the F-group was comparable to that determined in the S-group. The finding differs from the previous short-term studies; it is obvious that a prolonged F-consumption was not able to control plaque growth. A noticeable trait of the plaque determinations was the considerably high standard deviation of plaque wet weight.

The significance levels of differences between the sugar groups are given separately in Table XIII.

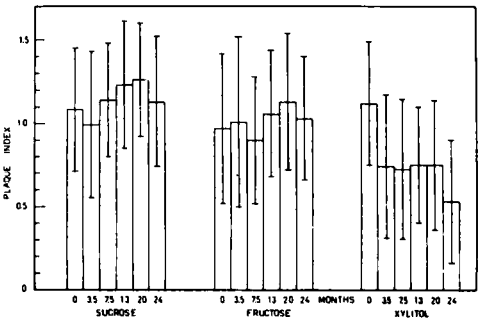


Fig. 4. The means and standard deviations of the development of the plaque index values.

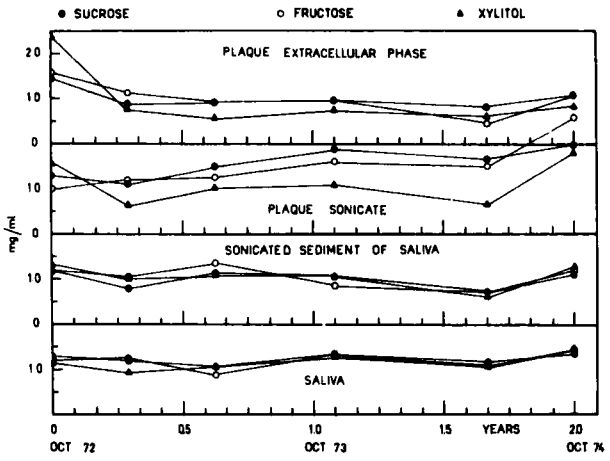


Fig. 5. Protein concentration of pooled samples.

3. *Plaque index*

Fig. 4 shows the means of the plaque index values of the three sugar groups. The indices remained approximately at the base line level throughout the study in the S- and F-groups. In the X-group lower values than in the base line examination were consistently recorded throughout the study. The significance levels of these differences appear in Table XIII.

4. *Proteins*

Most samples were studied for their protein content primarily for the calculation of the specific activities of salivary and plaque enzymes. However, the results can also be considered separately. Fig. 5 shows the protein content of the pooled samples. It appeared that the preparations yielded in most cases similar results in the three sugar groups throughout the study. The only exceptions were some values for the sonicated plaque and plaque extracellular phase, in which the X-group showed somewhat lower values than in the other groups. This might be due to the lower amount of plaque in the X-group. The three curves were practically identical for the salivary values, indicating that the low amount of plaque in the X-group did not affect the protein concentration of the supernatant fluid of saliva and the corresponding sediment (Fig. 5).

The values for the sonicated preparations remained practically unchanged in the course of the study. This can be considered to indicate a similarity between all sonications, resulting in an identical level maintained throughout the trial. The procedure, carried out at several months' intervals with a disintegrator which may not act in an similar way at all times, may easily result in preparations displaying noticeably varying protein concentrations. The values obtained for both types of sonicates entitles, however, to consider the expression of the specific enzyme activities in this and subsequent papers as reliable.

Fig. 6 shows the means of the protein concentrations in the individual supernatant fluids of whole saliva. There were no statistically significant differences between the groups. The identical stepwise appearance of the protein columns as a function of time in all test groups was obviously not a methodological trait. The concentration peaks occurred regularly

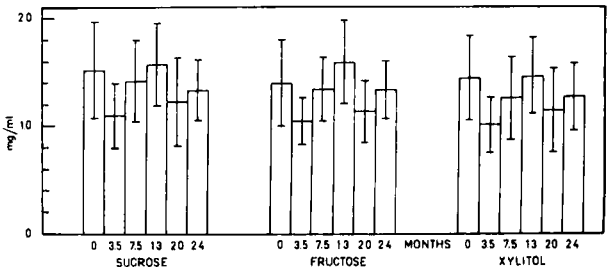


Fig. 6. The means and standard deviations of the protein concentrations of centrifuged whole mouth saliva.

in the autumn (0, 13 and 24 months), although the differences were not significant in all cases.

7. *Total nitrogen*

Total nitrogen was determined for pooled samples representing the three sugar groups. For practical reasons the samples reserved for the determination of total nitrogen were sealed in glass vials immediately after making the different pools at the end of each collection period. The samples were put in 200 × 8 mm glass tubes, usually used in the hydrolysis of protein samples. After sealing the tubes were stored at —20°C until the contents were analyzed within two days 4 months after the termination of the clinical trial. The micro Kjeldahl nitrogen determination may otherwise lead to erroneous results if the analyses would have been carried out at long time intervals. The results are shown in Fig. 7. The lowest total nitrogen values in plaque aqueous extract were obtained in the X-group, but the dietary regimens did not noticeably change these values. Additional experiments showed that urea nitrogen exhibited the same trend. It should be emphasized that although X-diet reduced the amount of plaque and plaque carbohydrates, it simultaneously seemed to increase the amino acid concentration of saliva (*Mäkinen, Lönnberg & Scheinin*, 1975). This phenomenon must be reflected in the plaque and saliva total nitrogen values as well. Therefore, no noticeable change was detected.

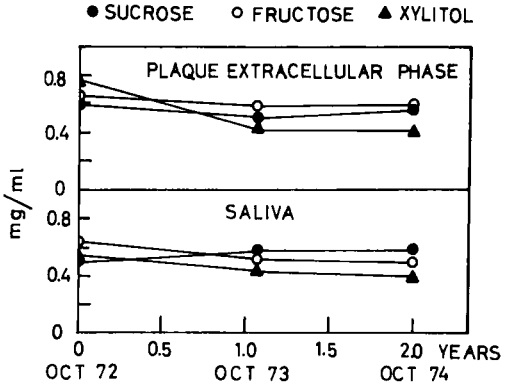


Fig. 7. Total nitrogen of pooled samples.

6. *Total carbohydrates and reducing sugars*

Total carbohydrates were determined with the anthrone method for pooled samples representing the three sugar groups. The results are given in Fig. 8 as mg per ml. This renders an examination of the behaviour of the sugar groups possible only for one type of sample at a time, because the four different preparations tested were handled in different ways. Furthermore, the four types of samples represent different biological features. There was an initial drop in the total sugar values in the plaque aqueous extract in all other groups except for the sucrose group in which the sugar values remained rather constant throughout the trial. The initial drop in the F- and X-groups may at least in part result from the change of the composition of the microbial flora of plaque. A similar observation was made for a number of other compounds in the aqueous plaque extract, as shown later. In the sonicated preparations the amount of total sugars seemed to remain rather constant during the study. There were no clear differences between the sugar groups in the sonicated salivary sediment. In the sonicated plaque material the X-group displayed the lowest values. In line with the protein concentration, the lower values partly resulted from decreased plaque wet weights in the X-group.

In centrifuged whole mouth saliva the total sugar values were rather low when compared to plaque aqueous extract, These values were rather similar in all sugar groups. The colour produced by centrifuged whole mouth saliva in the assay involving the anthrone method was to a certain extent disturbed by some salivary ingredients. However, the results were comparable between the three sugar groups and they were also in accordance with the previous studies carried out in this laboratory.

When the total sugar values were plotted as the ratio of proteins to sugars (both in mg/ml), the curves presented in Fig. 9 were obtained. The resulting curves provide a better concept than Fig. 8 about the relative amounts of sugars.

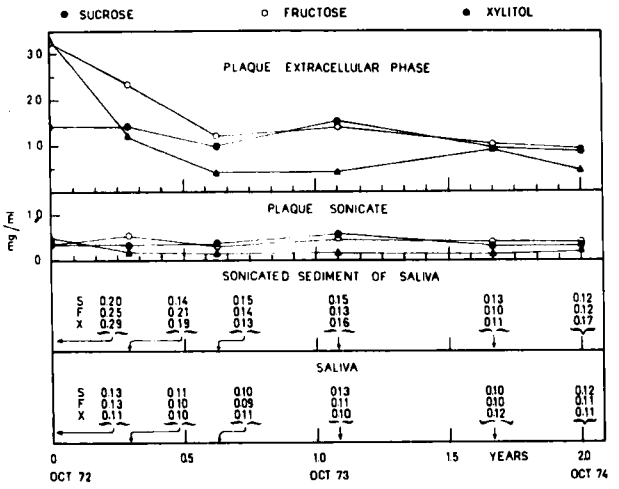


Fig. 8. Total carbohydrates of pooled samples (anthrone method) expressed as mg per ml.

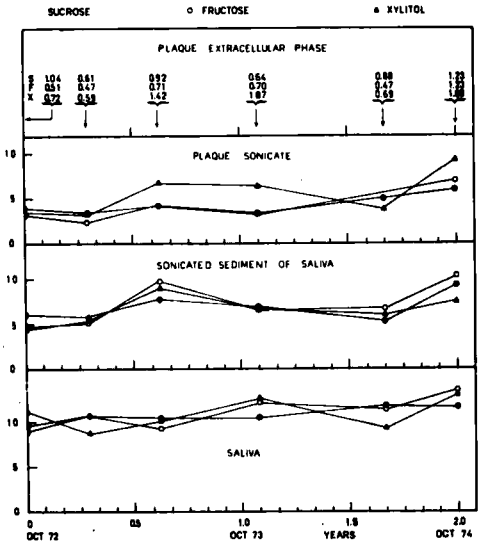


Fig. 9. Total carbohydrates of pooled samples (anthrone method) expressed as the ratio of proteins to sugars (both in mg per ml).

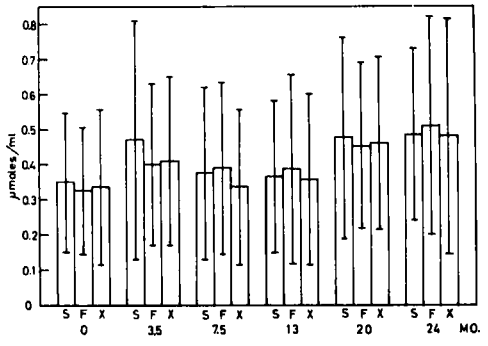


Fig. 10. The means and standard deviations of the concentration of reducing sugars of centrifuged whole mouth saliva.

With the exception of the two topmost figures, there were no noticeable differences between the groups. The ratio remained rather constant in saliva in all sugar groups throughout the study. The concentration of reducing sugars in the supernatant fluid of whole mouth saliva is shown in Fig. 10. There were no significant differences between the sugar groups.

7. *Glucose and fructose*

Figs. 11 and 12 give the results of the glucose and fructose determinations of the pooled samples. Glucose remained at a constant level in the aqueous extract of plaque of the S-group. The X-group yielded the lowest values at most analysis periods.

In the other samples the amount of glucose was considerably lower than in plaque aqueous extract. The enzymatic method involved works with rather low glucose concentrations, but the situation of Fig. 11 (A-D) was deliberately arranged to reveal the differences concerned. Least glucose was generally found in plaque obtained from the X-group.

The concentration of glucose in centrifuges saliva (D) corresponds to approximately 180 mg per liter which is close to the mean often reported for saliva. The lower part of Fig. 11 (E, F, G, H) gives the ratio of glucose to proteins (in μmoles per mg). The relative distribution revealed in the Fig. 11 (A-D) was not noticeably changed. The enzymatic glucose assay appeared to be somewhat sensitive; certain variations occurred (Fig. 11 (F-G).

The description of the fructose assays required the following considerations. The enzymatic method involved (Table II), comprises the determination of the extinctions of the reaction mixtures at several stages of the test. In this method the difference between E_3 and E_1 stands for the total concentration of fructose, glucose and the corresponding 6-phosphates. For the specific determination of fructose, several series of measurements were carried out (comprising

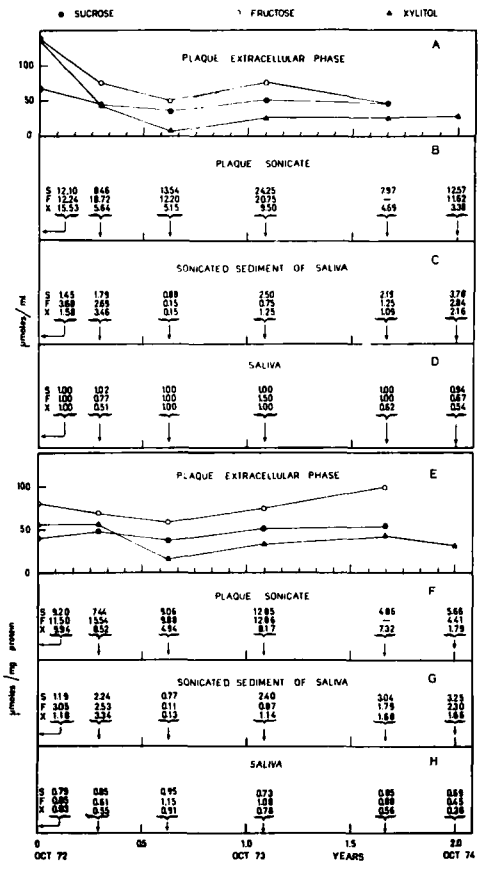


Fig. 11. D-Glucose concentration of pooled samples (A-D) and the ratio of glucose to proteins (E-F).

E_1, E_2, E_3, E'_1, E'_2 and E'_3). The determination of E_2 in the present study was performed at 5, 10 and 15 min.

The above assays were carried out at the first five main examination periods (thus excluding the 24 month period) with all pooled samples. It appeared that of the four types of samples only plaque extracellular phase (aqueous plaque extract) displayed sufficiently high and reliable extinctions, without concentration steps. Because such a measure would have affected the comparative relation between the four preparations (due to the small amount of plaque available, no concentration was possible), the presentation of the fructose assays will be here restricted to the total amounts of glucose, fructose and the 6-phosphates only. For practical reasons, this is shown in Fig. 12 as E_3-E_1. The values of E_2 (extrapolated to the time of the addition of glucose 6-phosphate dehydrogenase) were always very close to E_3. Fructose standards acted in the assays in a normal and expected way.

The results indicate that E_3-E_1 was very low in the preparations derived from saliva and

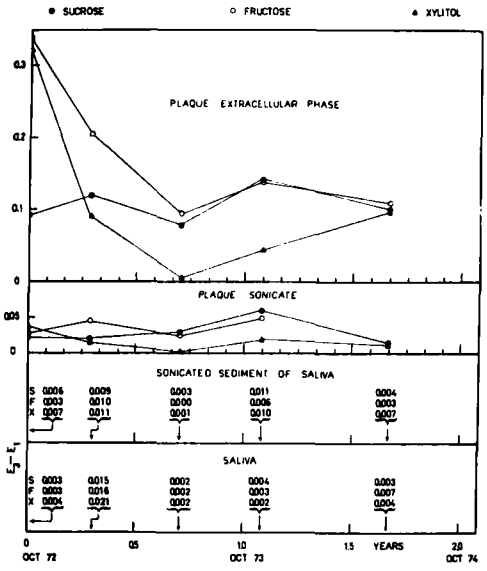


Fig. 12. Results of the fructose determinations (in E_3-E_1; see text). The values include fructose, glucose and the corresponding 6-phosphates.

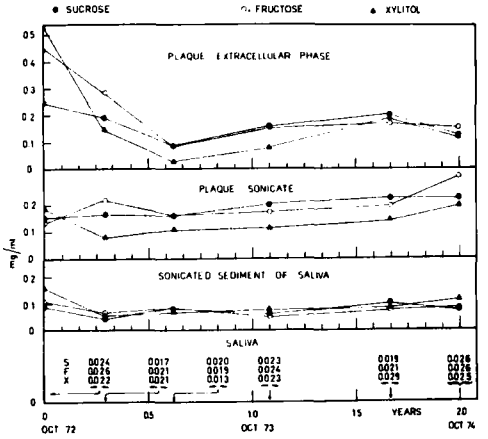


Fig. 13. The RNA content of the pooled samples expressed in mg per ml.

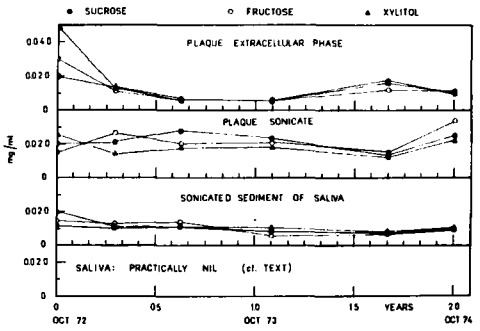


Fig. 14. The DNA content of the pooled samples expressed in mg per ml.

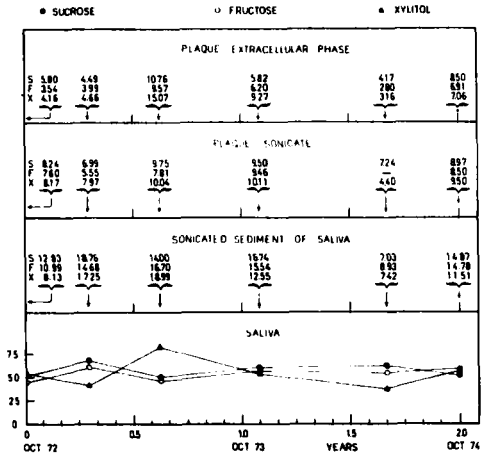


Fig. 15. The ratio of proteins to RNA in the pooled samples (both in mg per ml).

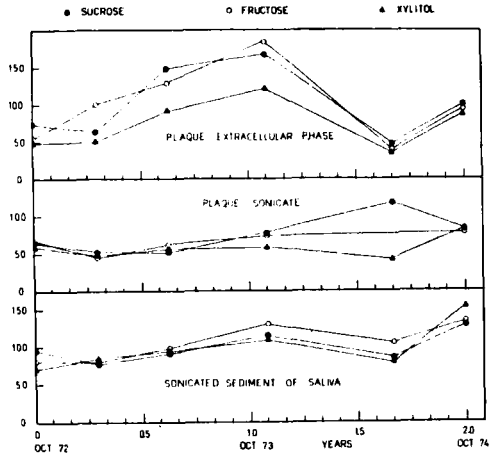


Fig. 16. The ratio of proteins to DNA in the pooled samples (both in mg per ml).

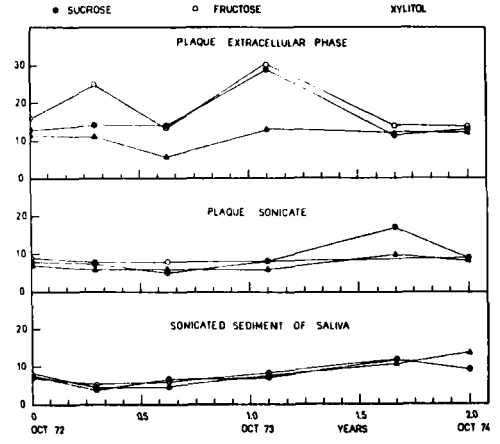


Fig. 17. The ratio of RNA to DNA in the pooled samples (both in mg per ml).

highest in the plaque extracellular phase, when compared on the basis of the volumes of the materials studied. The values of E_3-E_1 for the latter preparations were most often lowest in the X-group, except the base line examination and the 20-month phase.

8. *DNA and RNA*

The nucleic acids were assayed for pooled samples only. The results are shown in Figs. 13 & 14 in which the concentration of RNA and DNA is expressed in mg/ml. In line with the total sugar determinations, there was an initial drop in both nucleic acids in the plaque extracellular phase after the onset of the diet. The drop was more pronounced in the F- and X-groups than in the S-group. The DNA and RNA curves representing sonicated preparations run almost parallel to the abscissa, indicating basicly identical ultrasonic treatment throughout the study. There were no pronounced differences between the sugar groups, the values being lower in the X-group only in samples representing sonicated plaque. This partly resulted from the lower amount of plaque in this group. The concentration of DNA in the supernatant fluid of whole saliva was very low and difficult to determine on unconcentrated samples.

Figs. 15 & 16 show the ratio of proteins to RNA and DNA, respectively. There were the following three noticeable results:

1. In the plaque extracellular phase of the X-group the proteins to DNA ratios were lowest (Fig. 16).
2. There were changes as a function of time (Figs. 16, top, & 15, salivary sediment).
3. The proteins to RNA ratios were similar for all sugar groups in the supernatant fluid of whole saliva. For DNA no ratio to proteins is shown because the salivary DNA content was too low to permit reliable calculations. Fig. 17 shows the ratio of RNA to DNA. Except for the base line and 20- and 24-month determinations, the ratio in the plaque aqueous extract was clearly lowest in the X-group. The ratios were rather similar in the supernatant fluid of saliva in all groups throughout the study.

9. *Total keto acids, pyruvate and lactate*

These three groups of compounds were assayed for pooled samples representing the three sugar groups. The results are shown in Fig. 18 for total keto acids, in Fig. 19 for pyruvate, and in Fig. 20 for lactate. In all cases the concentrations were expressed in μmoles per ml. As regards the keto acids (both total and pyruvate) there was in most cases a trend of the X-group samples to show somewhat

lower values than the other two groups in the preparations derived from plaque (the two upper parts of Figs. 18 & 19.) The determination of the total keto acids yielded in one case (7.5 month phase, top part of Fig. 18) results which deviated considerably from the values of other assays. Fig. 18 indicates that there were also a few other sudden deviations or increasing and decreasing trends with regard to the keto acid concentrations. Careful examinations with known standards showed that the assay method acted adequately in all cases. It is possible that the deviation at the 7.5 month phase mentioned above may have resulted from certain differences in the storage and/or treatment of the samples concerned when compared to other phases of the study.

The ratio of the total keto acids to pyruvate is shown in Fig. 21. The ratio remained approximately between 1 and 2 throughout the study in all test groups, regardless of what material was studied (a few exceptions occurred). According to the present results, the bulk of free keto acids in the salivary and plaque samples was represented by pyruvate*).

Figs. 22—24 give the results when the concentrations were expressed with proteins. The picture already revealed by Figs. 18—20 was not changed noticeably. There were no clear differences between the sugar groups. Lactate was determined at two analysis periods (20 & 24 months) using an enzymatic method specifically used for blood (Table II) and at two analysis periods (13 & 20 months) using a similar method originally developed by *O. Warburg* (*Pfleiderer & Dose*, 1955).

*) The previous values of total keto acids and pyruvate of the supernatant fluid of whole saliva (*Mäkinen & Scheinin*, 1971, 1972) were too low as a result of lacking of exponents. The previous keto acid values have to be multiplied by 10^{-2} and those of pyruvate by 10^{-1}.

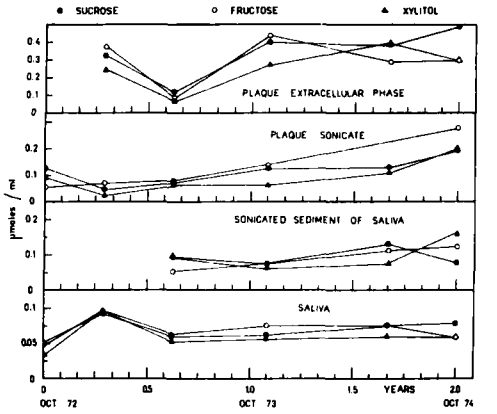


Fig. 18. Total keto acids of pooled samples.

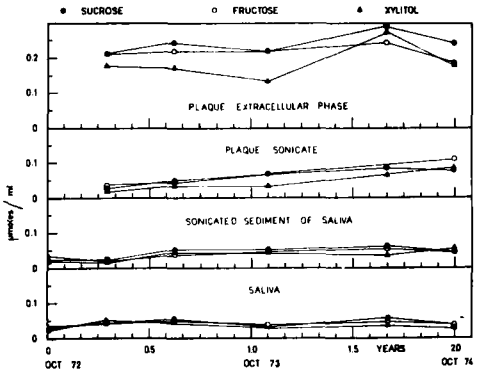


Fig. 19. The pyruvate concentration of pooled samples.

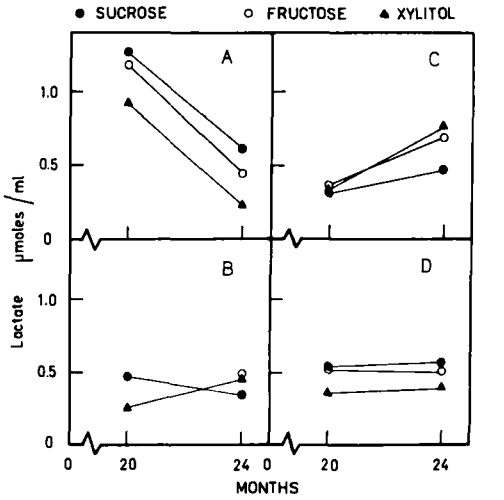


Fig. 20. The lactate concentration of pooled samples. A, plaque extracellular phase; B, plaque sonicate; C, sonicated sediment of saliva; D, saliva.

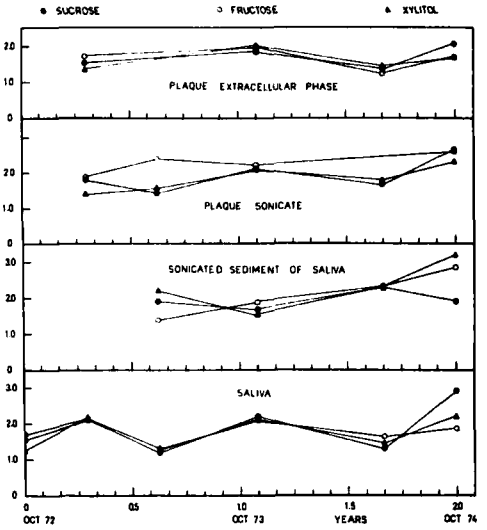


Fig. 21. The ratio of total keto acids to pyruvate (both in μmoles per ml) in the pooled samples.

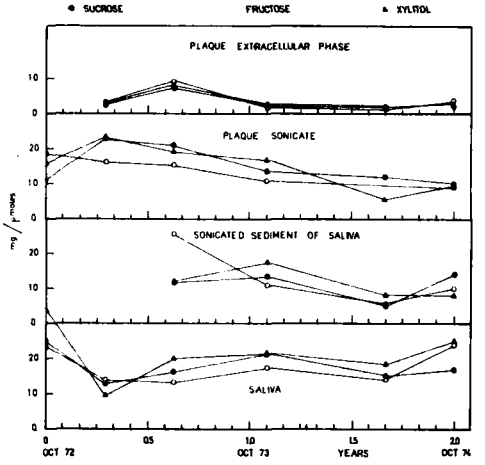


Fig. 22. The ratio of proteins to total keto acids (in mg per μmoles) in the pooled samples

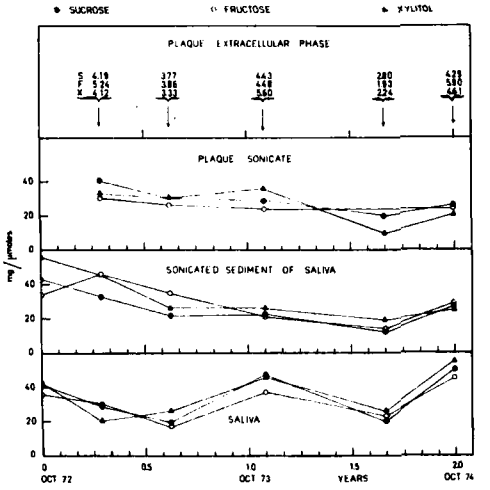


Fig. 23. The ratio of proteins to pyruvate (in mg per μmoles) in the pooled samples.

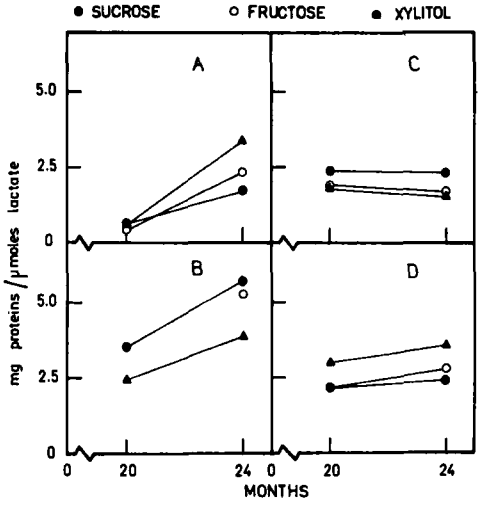


Fig. 24. The ratio of proteins to lactate (in mg per μmoles) in the pooled samples. A, B, C and D as for Fig. 20.

The differences between the two methods were in principle negligible and both yielded essentially the same results.

Figs. 20 & 24 show the results given by the former method. Both methods yielded similar values at the 20 month analysis period (which was the only phase of the trial, in which both procedures were used). The latter method gave the values shown in Table III.

The lactate values and proteins to lactate ratios deserve the following examination:

— The apparently abrupt increases (Fig. 24) and decreases (Fig. 20) in the figures should not solely be considered to indicate noticeable changes in the lactate concentration of the samples (particularly in plaque). The decrease in Fig. 20 A mostly resulted from

Table III. *The concentration of lactate in pooled samples at two phases of the dietary regimens*

	13 Months		20 Months	
Plaque aqueous extract				
	μmoles per ml	mg Proteins per μmoles	μmoles per ml	mg Proteins per μmoles
S	1.0	1.0	0.9	0.9
F	0.8	1.1	0.8	0.6
X	0.1	7.6	0.4	1.4
Sonicated plaque				
S	—	—*)	0.2	9.1
F	—	—	—	—
X	—	—	0.01	49.0
Sonicated salivary sediment				
S	0.2	4.7	0.07	10.9
F	0.2	4.3	0.14	4.8
X	0.1	9.3	0.04	16.7
Supernatant fluid of saliva				
S	0.2	6.5	0.36	3.3
F	0.2	6.3	0.31	3.7
X	0.1	11.4	0.14	7.8

*) — = Not analyzed.

differences in the length of the storage of the samples, and to a lower extent from actual concentration changes. The results between the sugar groups at each analysis period were, however, comparable. The longer the storage at —20°C, the lower the lactate content (24 month phase; Fig. 20). Preparations indicated in Figs. 20 A and C cannot be compared, because there were slight differences in the storage.

— In spite of the above variations, the X-group samples showed most frequently the lowest lactate values and the highest protein to lactate ratios. Of 15 separate assays (eight in Fig. 20 and seven in Table III) 13 yielded lower lactate values for the X-group than for the other sugar groups.

10. *Sodium, potassium, calcium, magnesium, and pH of oral fluid*

The atomic absorption spectrophotometric analyses for potassium, sodium, calcium, and magnesium were carried out on individual paraffin-stimulated samples of oral fluid of all subjects. These assays were performed after 13 months concerning K, Na, and Mg, and at the end of the study on all four elements. The results are shown in Fig. 25 and Table IV. There were no significant differences between the sugar groups as regards any of the elements studied.

The calcium concentration of the centrifuged oral fluid samples was somewhat lower than has been given in a number of other studies. It should be considered, however, that in the present study the centrifugation of saliva removed a substantial part of calcium, and also that many of the earlier values were determined titrimetrically. In the present study the standard method for calcium yielded the same results as obtained with the method recommended by the Perkin Elmer Manual for saliva (Table II). The present values are also in good accordance with later studies (cf. *Shannon, Suddick & Dowd*, 1974). Table V shows the pH values of paraffin-stimulated oral fluid samples obtained at the base line examination and at the subsequent collections during the study. There were no significant differences between the sugar groups.

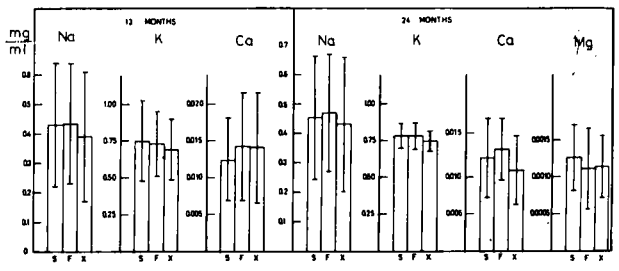


Fig. 25. The concentration of sodium, potassium, calcium and magnesium in the supernatant fluid of whole mouth saliva. For the concentration of calcium, see text and Table II.

Table IV. *The means and standard deviations for sodium, potassium, calcium, magnesium and inorganic phosphorus in the supernatant fluid of whole saliva (paraffin-stimulation)*

Element μg/ml	Sugar group	Months 13 $\bar{x}$	13 S.D.	24 $\bar{x}$	24 S.D.
Sodium	S	432	211	453	213
	F	434	205	468	202
	X	391	218	429	229
Potassium	S	750	271	783	186
	F	734	223	783	191
	X	690	207	745	173
Calcium	S	12.4	5.5	12.6	5.5
	F	14.2	7.3	13.9	4.3
	X	14.1	7.7	11.0	4.7
Magnesium	S			1.26	0.45
	F			1.12	0.56
	X			1.14	0.43
Inorganic phosphorus	S	125	29	117	27
	F	126	25	112	29
	X	120	26	109	25

Table V. *pH values of paraffin-stimulated oral fluid samples obtained during the course of the trial*

Sugar group	Months 0 $\bar{x}$	0 S.D.	3.5 $\bar{x}$	3.5 S.D.	7.5 $\bar{x}$	7.5 S.D.	13 $\bar{x}$	13 S.D.	20 $\bar{x}$	20 S.D.	24 $\bar{x}$	24 S.D.
Sucrose	7.37	0.19	7.32	0.20	7.37	0.23	7.33	0.21	7.27	0.22	7.33	0.23
Fructose	7.34	0.21	7.33	0.18	7.37	0.16	7.32	0.13	7.28	0.14	7.31	0.17
Xylitol	7.29	0.21	7.31	0.16	7.37	0.17	7.33	0.19	7.31	0.20	7.34	0.22

11. *Inorganic phosphorus*

Inorganic phosphorus of the individual supernatant fluids of whole mouth saliva was assayed with samples collected at two analysis periods (13 & 24 months). The results are shown in Fig. 26 and Table IV. There were no significant differences between the sugar groups.

12. *Sialic acid*

Free sialic acid (*N*-acetylneuraminic acid) was assayed with individual centrifuged oral fluid samples obtained after 24 months' diet. Fig. 27 shows that there were no significant differences between the sugar groups when the results were expressed in μmoles per ml, or as the ratio of mg proteins to μmoles of sialic acid (both in ml). Sialic acid was also assayed on certain pooled samples of the last (24-month) and certain earlier collection periods. The results of these experiments are shown in Table VI. The concentration of bound sialic acid was simultaneously determined. There were no significant differences between the sugar groups.

13. *Hydroxyproline*

The determination of salivary hydroxyproline was carried out on the 24 month samples. The results are shown in Table VII.

14. *Transaminases*

Both aspartate and alanine aminotransferase were assayed for pooled samples. The results are shown in Figs. 28 & 29. The rate of the aspartate aminotransferase-catalyzed reactions remained rather constant except for one value of the X-

Table VI. *The concentration of sialic acid in certain pooled samples (in μmoles per ml). The concentration of free and bound sialic acid is separately given*

Sugar group and sample	Free	Bound
7.5 Months		
Aqueous plaque extract		
S	0.09	0.08
F	0.08	0.05
X	0.06	0.07
13 Months		
Supernatant fluid of sonicated salivary sediment		
S	0.12	0.16
F	0.09	0.15
X	0.12	0.19
16.5 Months		
Supernatant fluid of sonicated salivary sediment		
S	0.12	0.12
F	0.12	0.13
X	0.11	0.09
20 Months		
Aqueous plaque extract		
S	0.11	—*)
F	0.09	—
X	0.09	—
Supernatant fluid of sonicated plaque		
S	0.03	0.04
F	—	—
X	0.03	0.03
Supernatant fluid of sonicated salivary sediment		
S	0.10	0.15
F	0.08	0.14
X	0.10	0.08
Serum		
S	0.02	2.7
X	0.02	2.7

*) — = Not analyzed.

Table VII. *Salivary hydroxyproline in relation to sugar group*

Sugar group	mg/ml	μmoles/ml
Sucrose	0.0019	0.0165
Fructose	0.0018	0.0156
Xylitol	0.0015	0.0130

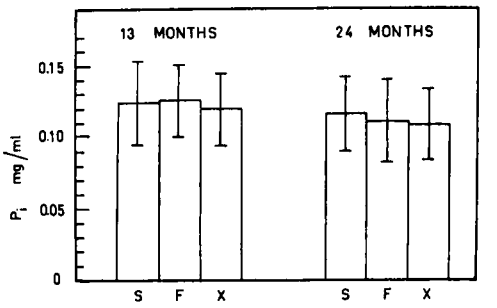


Fig. 26. The concentration of inorcanic phosphorus in the supernatant fluid of whole mouth saliva (*cf.* Table IV).

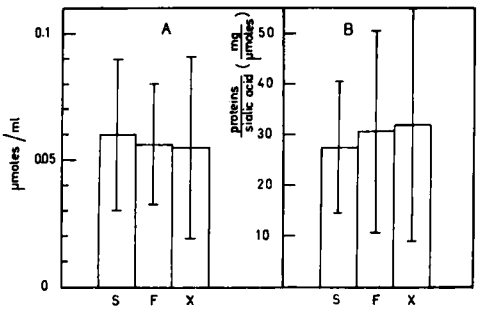


Fig. 27. The sialic acid content of the supernatant fluid of whole saliva (A) and the ratio of proteins to sialic acid (B). 24-month samples.

group at the 20 month phase (sonicated plaque). The values for the extracellular aqueous extract of plaque were somewhat higher in the X-group when compared to the two other test groups (Fig. 28). In the supernatant fluid of whole saliva the rates were low and the values therefore more subject to errors than in the other cases.

The activity of alanine aminotransferase (Fig. 29) was lower than that of aspartate aminotransferase. Only in the supernatant fluid of saliva the activities seemed to be more similar. Alanine aminotransferase displayed considerable changes in the plaque extracellular phase during the study in all sugar groups.

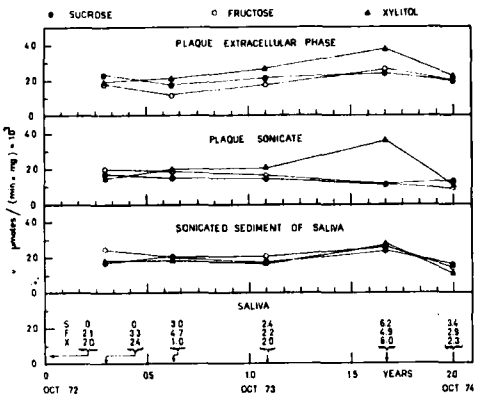


Fig. 28. The activity of aspartate aminotransferase in the pooled samples.

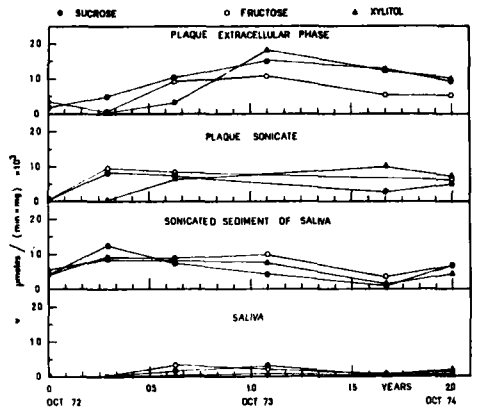


Fig. 29. The activity of alanine aminotransferase in the pooled samples.

Table VIII. *Activity of alcohol dehydrogenase of pooled preparations* [in Δ E/(min × mg) × 10^{-4})]

Sample	13 Months			20 Months		
	S	F	X	S	F	X
Plaque extracellular fluid	40.8	32.6	43.3	35.6	63.0	41.3
Sonicated plaque	—*)	—	—	15.2	—	27.3
Sonicated salivary sediment	5.0	3.9	5.9	8.1	6.5	10.9
Supernatant fluid of saliva	4.1	3.7	5.0	8.1	4.4	10.9

*) — =Not analyzed

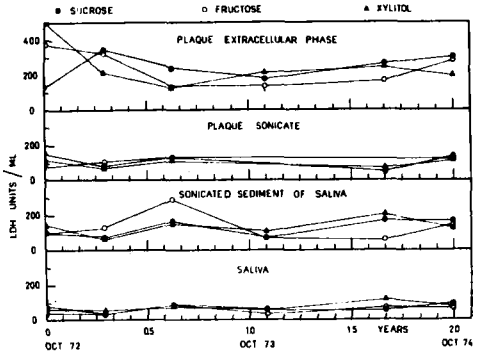


Fig. 30. The activity of lactate dehydrogenase in pooled samples (given in units per ml).

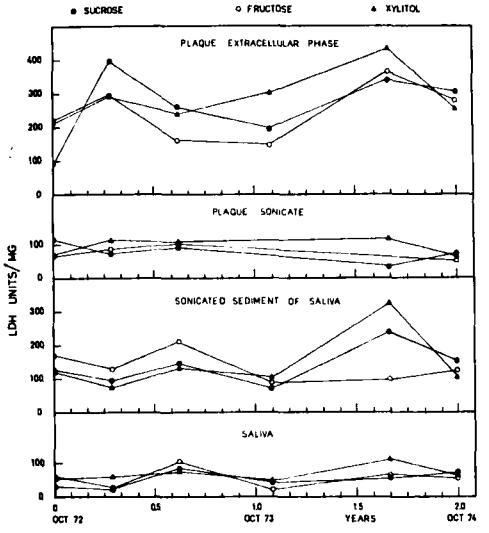


Fig. 31. The activity of lactate dehydrogenase in pooled samples (given in units per mg protein).

15. *Dehydrogenases*

Lactate, alcohol and xylitol dehydrogenase activity was analyzed with various pooled samples. The results are shown in Figs. 30—32 for lactate dehydrogenase and in Fig. 33 for xylitol dehydrogenase. Alcohol dehydrogenase determinations are shown in Table VIII.

The activity of lactate dehydrogenase, expressed in units per ml (Fig. 30), gives a comparable situation only in the case of the salivary supernatant fluid. All values were, however, similar in the sugar groups, except for the base line determinations relative to the plaque aqueous extract. The constant activity in the supernatant fluid of saliva is to be noticed.

When the activity was given in units per mg

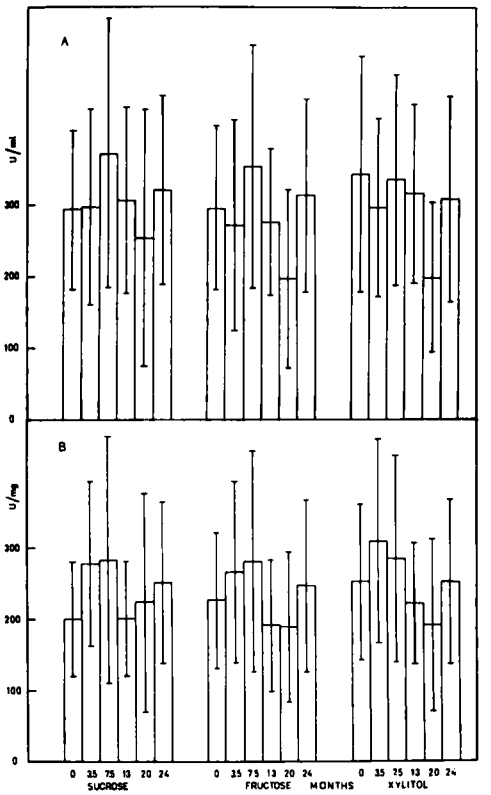


Fig. 32. The activity of lactate dehydrogenase of individual samples of the supernatant fluid of whole saliva given in units per ml (A) and units per mg (B).

protein (Fig. 31), a more realistic idea about the activity of lactate dehydrogenase in other samples was obtained. The highest activity was again found with the plaque aqueous extract. There was no repeatedly observable difference between the sugar groups. The variations in the protein concentrations led to variations in the ratios concerning particularly plaque extracellular phase. The ratios remained constant in the supernatant fluid of saliva.

Fig. 32 shows the results of the LDH assays on individual saliva samples. There was no significant difference between the sugar groups. The salivary LDH values of Fig. 31 were lower than those of Fig. 32. This resulted from the use of fresh samples in the assays of Fig. 32. The pools of Fig. 31 were analyzed after several weeks storage at —20°C.

Fig. 33 gives the results of the xylitol dehydrogenase assays. Except for the values of Fig. 33 D, there were no significant differences between the sugar groups. The activities were very low in all cases. The activity of alcohol dehydrogenase (Table VIII) was in most cases highest in the X-group.

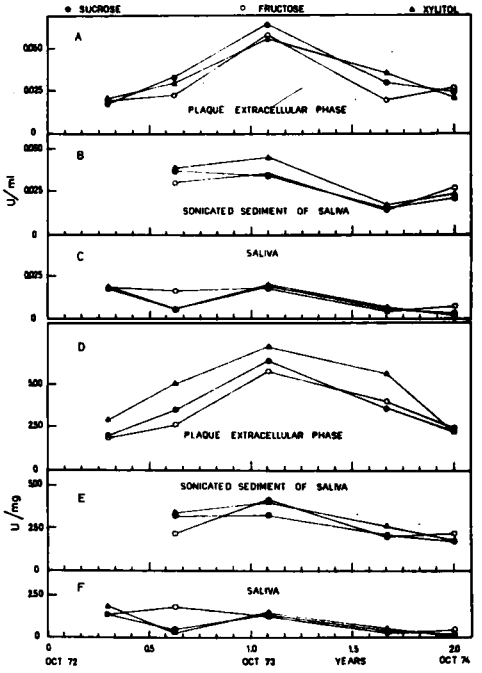


Fig. 33. The activity of xylitol dehydrogenase in pooled samples, given in units per ml (A, B, C) and in units per mg (D, E, F). Tested with xylitol and NAD.

16. *Aldolase*

Aldolase activity was assayed for pooled samples. The results are shown in Fig. 34. The activity varied considerably in the sonicated preparations. The activity was high in the aqueous plaque extract when compared to the supernatant fluid of saliva. There were no clear differences between the sugar groups.

17. *Amylase*

The supernatant fluids of all whole mouth saliva samples were determined for amylase activity at the end of the two-year test. For practical reasons the amylase assays were carried out approximately two months after the last sample collection. The centrifuged saliva samples were kept at —20°C until analyzed. The results are shown in Fig. 35. The activity was lowest in the X-group. The difference between the S- and X-groups and that between the F- and X-groups was statistically significant ($p < 0.05$ by Student's *t*-test).

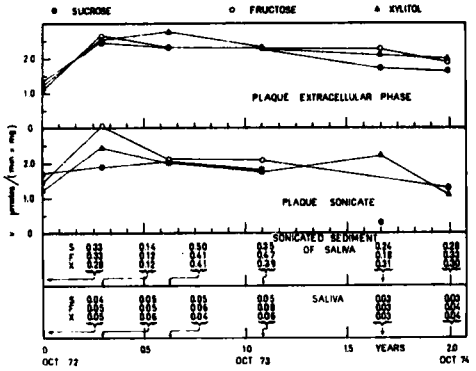


Fig. 34. The activity of aldolase in pooled samples.

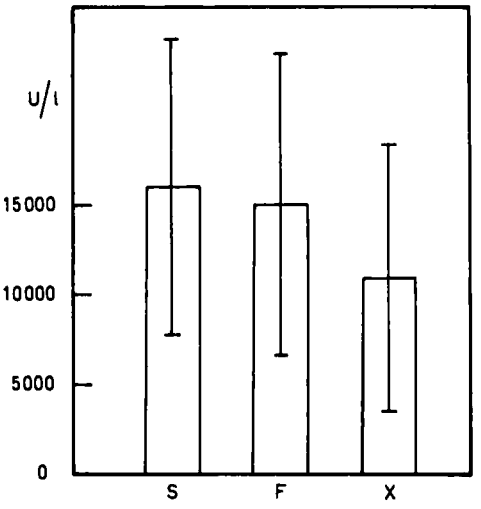


Fig. 35. The activity of amylase of the supernatant fluid of whole mouth saliva (in units per l). 24-month samples.

18. *Glycosidases*

The specific activity of α-D-glucosidase and β-D-galactosidase was determined using respective synthetic *p*-nitrophenyl derivatives as substrates. The results are shown in Figs. 36 and 37. The curves describing the observations regarding the two plaque preparations indicate that both activities toward the substrates were high in the X-group when compared to the S-group. For β-galactosidase the difference was particularly marked, and was also found between the F- and X-groups. No corresponding trait was encountered with whole saliva or its sonicated sediment. Thus an almost 100 % increase in the specific β-galactosidase activity in the plaque of the X-group during the first study year was considered one of the important enzymic changes revealed by the dietary regimens involved. The S-group exerted the lowest α-glucosidase activity at most analysis periods.

In centrifuged whole saliva the activity of both glycosidases was low and variable when compared to plaque or salivary sediment. The initial increase in the specific activities (Fig. 36) was due to the loss of activity of the base line samples at —20°C (prolonged storage). Due to the same reason no base line values for β-galactosidase are shown. The storage made certain assays on sonicated plaque unreliable (Fig. 37) and the results of these assays were not included. β-galactosidase activity was clearly sensitive to low temperature (—20°C) and/or to the freezing and thawing steps involved. The longer the storage at —20°C, the higher loss in enzyme activity. A two-month storage destroyed 75 % of the activity. In the cases which were excluded from Fig. 37, the X-group yielded at least two times higher values than the S- and F-groups.

Table IX shows the activity of α- and β-fucosidase. The activity of α-fucosidase was highest in the X-group. Except for a few cases this was also true for β-fucosidase, although the differences between the groups were not great. Both fucosidase activities were low.

19. *Peptidohydrolases*

The activity of certain peptidohydrolases of centrifuged oral fluid was studied with

Table IX. *The specific fucosidase activity of pooled samples (in μmoles/(min × mg) × 10^{-5})*

Sample	13 Months	20 Months		24 Months	
	α-Fucosidase	α-Fucosidase	β-Fucosidase	α-Fucosidase	β-Fucosidase
Plaque aqueous extract					
S	8.6	25.5	4.4	16.0	4.1
F	8.5	19.2	6.2	17.0	4.0
X	22.4	43.6	5.2	23.3	3.4
Sonicated plaque					
S	53.9	31.0	1.9	41.0	1.7
F	54.0	—*)	—	40.0	1.6
X	78.6	47.0	2.6	47.0	1.8
Sonicated salivary sediment					
S	31.6	28.8	4.7	19.3	2.1
F	36.1	16.6	3.1	21.6	2.6
X	50.7	36.1	4.9	21.2	2.6
Supernatant fluid of saliva					
S	1.5	4.4	1.9	1.5	0.5
F	1.5	4.4	2.0	1.5	0.5
X	1.8	4.5	2.3	1.5	0.5

*) — = Not analyzed.

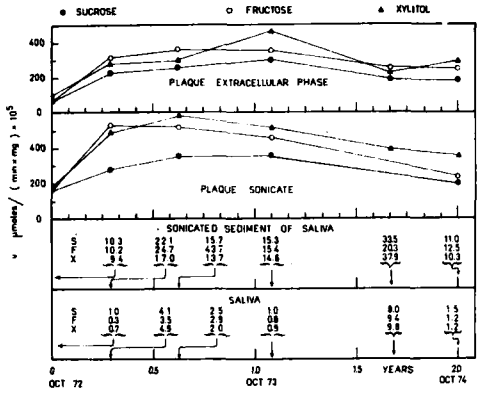


Fig. 36. The activity of α-glucosidase in pooled samples.

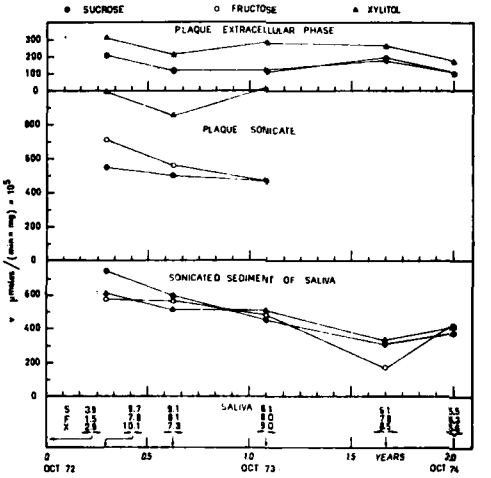


Fig. 37. The activity of β-galactosidase in pooled samples.

the following substrates: Three *N*-L-aminoacyl-2-naphthylamines (cf. Table II), a chromogenic collagenase substrate, and hemoglobin.

a. *Collagenase-like activity.* The synthetic collagenase substrate was not hydrolyzed at a measurable rate by any of the four types of enzyme preparations derived from plaque and saliva. This concerned all test groups and all analysis periods. However, a bacterial collagenase*) (*Cl. histolyticum*, Type I, Sigma

*) The commercial enzyme contained protease and peptidase activity.

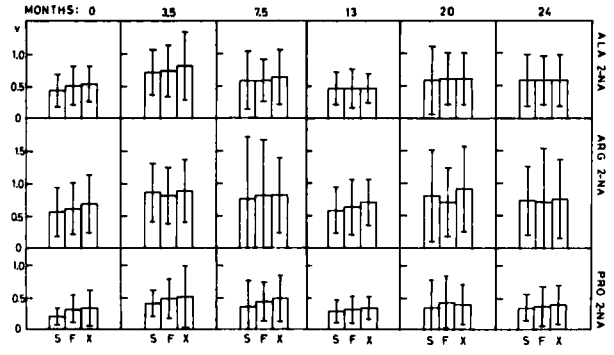


Fig. 38. Aminopeptidase activity, v [in μmol/(min × mg) × 10^{-3}], of the supernatant fluid of whole mouth saliva tested with the *N*-aminoacyl-2-naphthylamines of L-alanine, DL-arginine and L-proline

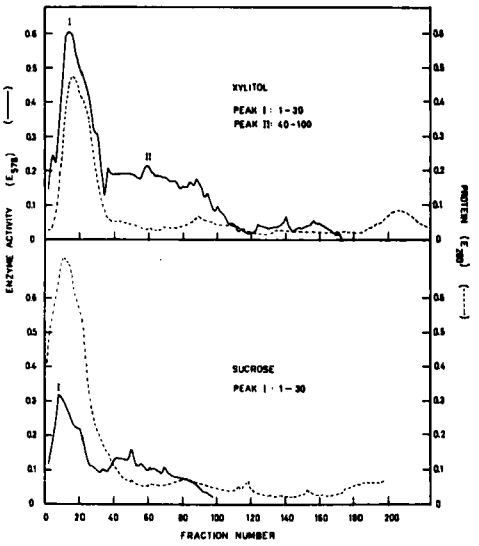


Fig. 39. Molecular permeation chromatography of salivary proteinases on Sephadex G-100 Superfine. Column: 1.5 × 138 cm; Elution buffer: 0.01 M $\beta\beta$-dimethylglutarate buffer, pH 7.2; Hydrostatic pressure: 15 cm; Temperature: +4°C; Sample: pooled samples of the supernatant fluid of whole mouth saliva of the sucrose and xylitol groups (representing mixed samples of 16.5- and 20-month phases). 200 ml of the combined supernatant fluids were concentrated in both test groups (Amicon ultrafiltration) to 50 ml and applied on the column. The enzyme activity was tested with denatured hemoglobin and it is given as extinctions formed in the tyrosine assay (Table II).

Chemical Company), tested in identical conditions rapidly hydrolyzed the cromogenic substrate. The reference enzyme was added to the assay mixture described by *Wunsch & Heidrich* (1963) in a concentration of 0.5 mg/ml. The results indicate that no active collagenase-like enzymes were involved in the test material (stored for 6 weeks at —20°C) of the sugar studies. Separate experiments with fresh saliva samples supported this idea.

b. *Aminopeptidase activity.* The results of the aminopeptidase studies (Fig. 38) showed, no significant differences between the effects of the three dietary regimens when comparing the whole sugar groups. However, when the quantities of the three sugars were taken into account, the highest specific activities were in many cases encountered in the X-group (paragraph 24, Table XI).

c. *Proteinase activity.* During the second half of the trial the combined pooled saliva samples taken at the 16.5-month phase and at the end of the study were analyzed and fractionated for protease activity using denatured hemoglobin as substrate. The results are shown in Fig. 39. In this experiment pooled supernatant fluid samples of the sucrose and xylitol groups were subsequently fractionated through one and the same Sephadex G-100 Superfine column. This single pair of experiments showed that the activity toward denatured hemoglobin was higher in the X-group when compared to S-group. The X-samples yielded two enzyme

peaks with higher activity than in the S-group, although equal volumes of saliva were studied. The F-group was not studied.

The enzyme pools resulting from the above molecular permeation chromatography were focused in a pH gradient from 3.5 to 10.0 (Fig. 40). The idea was to elucidate the contribution to the aminopeptidase pattern of enzymes being activated by 0.2 M NaCl. The X- and S-samples (Pool I) yielded essentially identical chromatograms (A and B) which were characterized by heterogeneity and a similar NaCl-effect. The X-peak II (Fig. 39) also exerted heterogeneity in focusing (Fig. 40 C). Fig. 40 indicates that there were no profound differences between the two sugar groups with regard to whole saliva aminopeptidases which were activated by Cl^- ions.

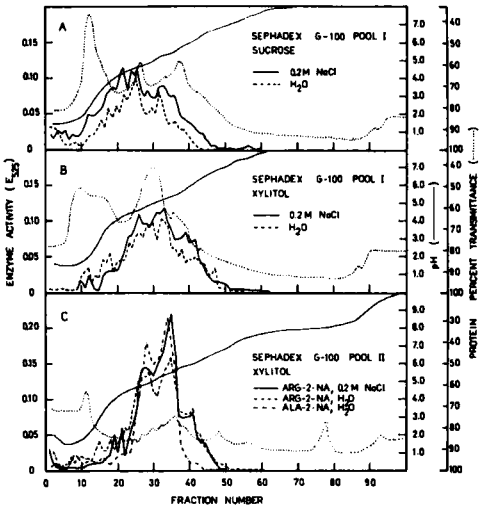


Fig. 40. Isoelectric focusing of the three enzyme preparations indicated in Fig. 39 (pH gradient: 3.5—10.0). Tested with *N*-L-arginyl-2-naphthylamine (A, B and C) and *N*-L-alanyl-2-naphthylamine (C), both in the presence of 0.2 M NaCl and without added salt.

d. *Chloride-activated arginine aminopeptidases.* A separate portion of the 24-month saliva samples was used to study the involvement of arginine aminopeptidases being activated by 0.2 M NaCl. As being discussed in more detail in the appropriate section, this particular analysis was considered necessary not only from the point of view of salivary enzyme spectrum, but also in view of explaining the origins of the corresponding enzyme activity in exudate (*Paunio, Mäkinen & Scheinin*, 1975). The results are shown in Fig. 41. The following observations were made:

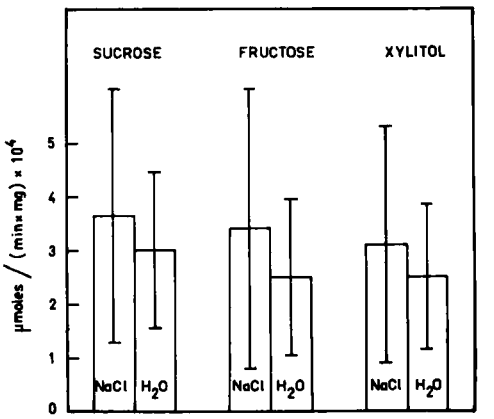


Fig. 41. The specific activity of arginine aminopeptidases of the supernatant fluid of whole mouth saliva determined at the 24-month phase. Tested in the presence of 0.2 M NaCl and without added salt. Substrate: *N*-L-arginyl-2-naphthylamine.

— The specific activities attained without added NaCl were almost comparable with those given in Fig. 38 for the same substrate (*N*-L-arginyl-2-naphthylamine).

— The presence of 0.2 M NaCl in the reaction mixtures led to a 25 % higher rate of hydrolysis when compared to conditions without added salt. This concerned all test groups.

— There was a slight (but not statistically significant) trend of the X-samples to display lower rates than the S-samples.

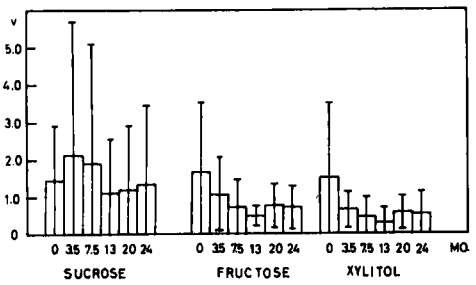


Fig. 42. The specific activity, v [in μmoles/(min × mg) × 10^3], of invertase-like enzymes of the supernatant fluid of whole mouth saliva.

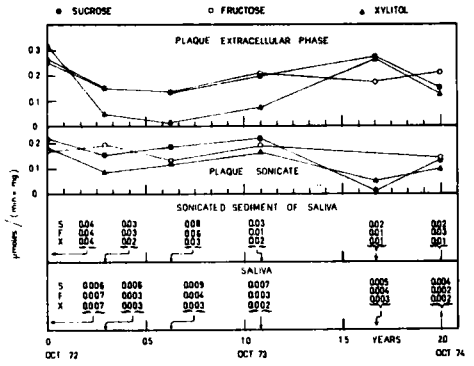


Fig. 43. The specific activity of invertase-like enzymes of pooled samples.

20. *Activity of enzymes yielding reducing sugars from sucrose and dextran (including invertase-like activity)*

a. *Activity in crude samples.* In earlier papers the term invertase or invertase-like enzymes was used to describe an enzyme activity toward sucrose, when the assay was based on the estimation of reducing sugars (*Mäkinen & Scheinin*, 1971, 1972). This method (Table II) has been used in the assay of yeast invertase. In the above papers it was also stated that this type of enzyme activity, determined on crude samples, most likely comprises transglycosidases and related enzymes as well. Therefore, the problem was investigated in the present study in a more detailed way.

Fig. 42 shows the ability of centrifuged oral fluid samples to yield reducing sugars from sucrose, which activity most likely includes also the potential invertases. The specific activity was lowest throughout the study in the X-group. As also earlier found with this enzyme activity, the standard deviations were high. The significance levels of the observed differences between the groups are given in Table XIII.

Fig. 43 shows the specific activities for the pooled samples. The base line values in the plaque extracellular phase were almost identical. During the subsequent 13 months the activity was lowest in the X-group. After this phase the activity difference was not observed. The other samples exerted more similar values throughout the study, except for one analysis period (20 months) with a prolonged storage of the samples at —20°C. It has to be emphasized that the activity in plaque preparations was much higher than in preparations derived from whole saliva.

b. *Chromatography.* Pooled samples of the supernatant fluid of whole saliva at the 16.5-month phase were investigated using gel permeation chromatography on Sephadex G-100 Superfine. The three pools were subsequently fractionated through one and the same column. The chromatographic tests were performed at approximately 10-day intervals. During this period the other samples were stored at —20°C. The first sample fractionated was the sucrose pool and this pool was also kept at —20°C for 60 days before fractionation. The F- and X-samples were fractionated next. Consequently, the two last mentioned samples were stored somewhat longer (more than 60 days) before chromatography, but the differences involved were found insignificant in view of the aim of the experiments. The results are shown in Fig. 44. The chromatograms indicated that all sugar groups yielded one sucrose-splitting enzyme peak (Peak I) in the void volume of the column. In the S-sample another peak (Peak II) appeared in fractions 65—120. In the F-sample the latter peak was considerably lower. In the X-samplet his particular enzyme peak was almost totally missing. Consequently, the X-diet removed from the salivary enzyme spectrum one enzyme (or group of closely related enzymes) capable of yielding reducing sugars

from sucrose. The F-diet only partly caused this phenomenon. The specific activities of the Sephadex peaks I and II (Fig. 44) were as shown below:

Sucrose I (fractions 8-35)	6.7×10^{-3} μmoles/(min × mg)
Sucrose II (fractions 65-120)	30.2×10^{-3} μmoles/(min × mg)
Fructose I (fractions 8-35)	2.5×10^{-3} μmoles/(min × mg)
Fructose II (fractions 65-110)	8.6×10^{-3} μmoles/(min × mg)
Xylitol I (fractions 10-30)	3.7×10^{-3} μmoles/(min × mg)

In the next step pools were thus formed from the two types of enzyme peaks shown in Fig. 44. The peaks designated as I were pooled from all three sugar groups to one single preparation because they were considered to represent practically identical enzymes. Because Peak II showed sufficiently high activity in the sucrose group only, this enzyme preparation was used to represent all peaks designated as II. The pools, their fractions given above, were dialyzed and concentrated in the Amicon Ultrafiltration system. The dialysis was carried out against a 10-fold volume of water and the concentration was performed to yield approximately a 10 ml final sample in each group and pool. The specific activities of the dialyzed and concentrated samples are shown below:

Pool I (Peaks I of S, F and X)	2.4×10^{-3} μmoles/(min × mg)
Pool II (Peak II of S)	10.7×10^{-3} μmoles/(min × mg)

The resulting samples were applied on a DEAE-cellulose column (Fig. 45). The chromatography showed that Pool II produced only one main enzyme peak with the assay method used (Fig. 45, top). This peak was designated as DEAE Peak IIIa. One main and two lower enzyme peaks (DEAE Peaks I, II and IIIb) were revealed with the Sephadex pool I (Fig. 45, bottom).

The resulting enzyme preparation (DEAE Peak IIIa and DEAE Peaks I, II and IIIb) were tested in a reaction mixture of the following composition: 4.0 ml of the enzyme solution, 1.0 ml of 0.01 M acetate buffer, pH 5.0, and 2.5 ml of a 0.3 M sucrose solution. After a 22 h reaction time the reducing sugars were determined with the 4.5-dinitrosalicylate method (Table II) using a 0.4 ml aliquot of the reaction mixtures. The specific activities of the four enzyme preparations are shown in Table X.

Simultaneously with the above »invertase assay» the remaining reaction mixtures were evaporated to dryness. 2.0 ml of water was added and the solid residue was dissolved by stirring with a glass rod. The resulting solutions were analyzed for glucose with the glucose oxidase method and for fructose with another enzymatic method (Table II). Similar reaction mixtures were also treated after the 22 h reaction time subsequently with 10 % TCA and ethanol, 1.5 times the volume of the sample, in order to analyze the products of the enzyme reactions by treating the alcoholic solution for 3 h at 100°C in 0.1 N or 2 N H_2SO_4. No precipitates were detected.

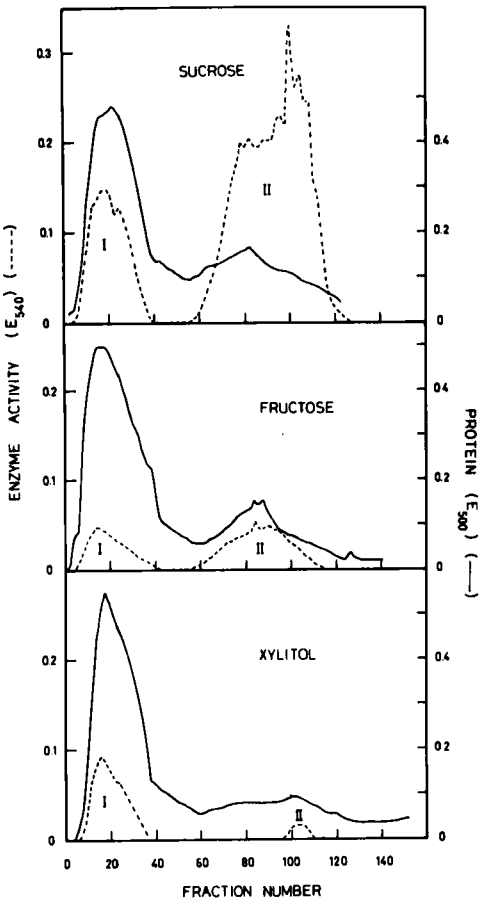


Fig. 44. Molecular permeation chromatography on Sephadex G-100 Superfine of invertase-like enzymes (determined as the liberation of reducing sugars from sucrose) of pooled supernatant fluids of whole mouth saliva. Column: 4.7 × 127 cm; Elution buffer: 0.01 M ββ-dimethylglutarate buffer, pH 7.2; Fraction volume: 6 ml; Hydrostatic pressure: 15—20 cm; Temperature: +4°C. Samples: 80 ml of concentrated pools representing the 16.5-month phase. The original volume of 500 ml was concentrated by an Amicon Ultrafiltration Apparatus. 5.0 ml of 0.1 % Blue Dextran was added. The activity is given as extinctions of the arrested reaction mixtures. Proteins are given as extinctions of the assay mixtures (Folin Ciocalteu reagent). Pooling: Peak I, 8—35 (sucrose and fructose, 10—30 (xylitol); Peak II, 65—120 (sucrose only).

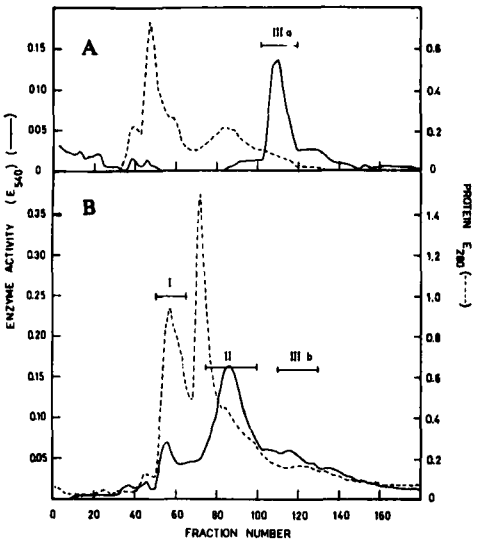


Fig. 45. Ion exchange chromatography of pooled enzyme peaks of Fig. 44 on DEAE-cellulose (Schleicher & Schüll; 230—270 mesh). Column: 1.7 × 23.5 cm; Elution buffer: 0.01 M ββ-dimethylglutarate buffer, pH 7.2; NaCl gradient: from 0 to 0.75 M (mixing volume 150 + 150 ml); Fraction volume: 1.6 ml; Hydrostatic pressure: 120 cm; Samples: Pooled active fractions of Fig. 44 (the pooling is indicated in the previous Fig.) The enzyme activity is expressed as extinctions of the assay mixtures after arresting the reaction. Proteins are given as extinctions of the assay mixtures. The DEAE peaks indicated (I, II, IIIa and IIIb) were investigated for their properties (see text). A, Peak II of Fig. 44; B, Peak I of Fig. 44.

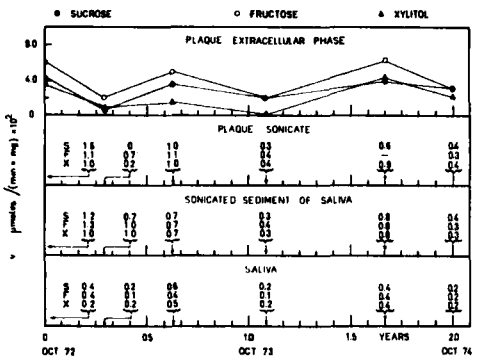


Fig. 46. The specific dextranase activity of pooled samples.

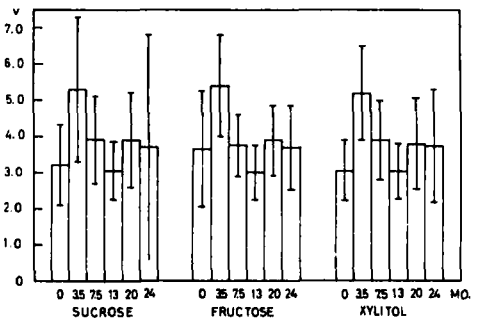


Fig. 47. The means and standard deviations of the specific dextranase activity, v (in μmoles/(min × mg) × 10^{-3}), of the supernatant fluid of whole saliva.

Table X. *The specific invertase-like activity [in μmoles/(min × mg) × 10^{-3}, assayed as reducing sugars in the reaction mixture] of enzyme preparations resulting from DEAE-cellulose chromatography**)*

DEAE Peak I	DEAE Peak II	DEAE Peak IIIa	DEAE Peak IIIb
1.0	4.6	61.6	6.7

*) Cf. Fig. 45.

In all cases using the four enzyme preparations the assays showed approximately 0.1—0.2 mM glucose concentration in the original reaction mixtures of 7.5 ml. This was found in spite of the differences in the specific activities shown in Table X. The amount of fructose was estimated to be roughly the same in all reaction mixtures, and equivalent to the concentration of glucose. As mentioned above, no polysaccharide precipitation was observed. Consequently, the enzyme preparations revealed in Fig. 44 seemed to be invertase-like.

Figs. 46 and 47 show the dextranase activity of the pooled and individual samples, respectively. The same differences between the sugar groups as encountered with the invertase-like enzymes were not observed. The specific activity was in some cases slightly lower in the aqueous extract of plaque of the xylitol group than in the other groups (Fig. 46). The enzyme activities in Fig. 46 were slightly lower

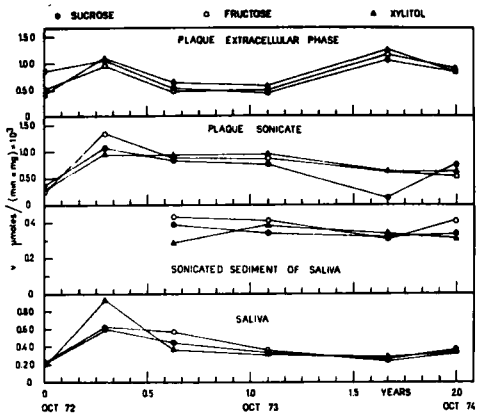


Fig. 48. The overall specific activity of enzymes in pooled samples capable of liberating keto acids from L-djencolic acid.

than in Fig. 47. This was due to prolonged storage of the pooled samples (Fig. 46) when compared to the individual ones which were analyzed almost immediately after collection.

21. *Enzymes forming keto acids from L-djencolic acid*

L-djencolic acid is a naturally occurring S-alkyl derivative of cysteine. Plaque and salivary enzymes attack it and readily transform it into keto acids and other products. This reaction proceeds also without added pyridoxal coenzymes. Fig. 48 shows the overall specific activity of enzymes yielding keto acids from L-djencolic acid. The highest specific activities were encountered in the plaque preparations. There were no significant and repeatedly observed differences between the sugar groups.

22. *Isoelectric focusing of salivary proteins*

Fig. 49 shows a series of chromatograms of isoelectric focusing of the supernatant fluid of whole saliva in two pH gradients. The pooled samples represented the 16.5-month phase. There were no significant differences between the sugar groups in the isoelectric focusing patterns of the salivary proteins (determined at 278 nm).

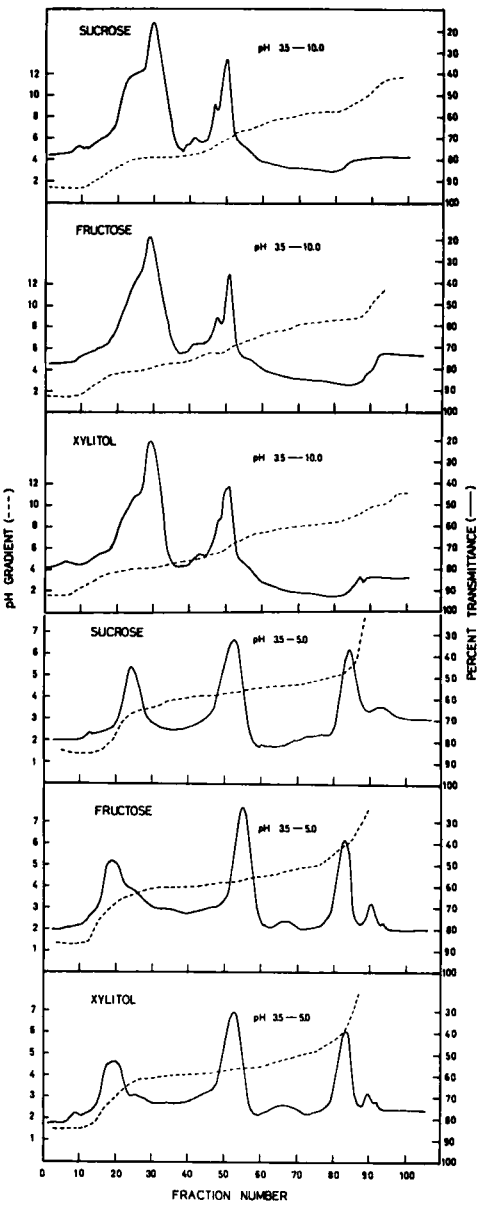


Fig. 49. Isoelectric focusing of pooled supernatant fluids of whole saliva sediment in two pH gradients (3.5—5 and 3.5—10.0).

23. *Assay of IgA, IgG,* and *IgM*

The determination of immunoglobulins A, G, and M in the supernatant fluid of whole mouth saliva was carried out only on the 24-month samples. The specimens were kept after the collection and centrifugation of saliva at —20°C for 2.5 months before the assays. For the determination of IgA and IgG no concentration step was necessary. For the assay of IgM 0.5 ml samples of the supernatant fluids were concentrated by freezedrying. The solid residue was dissolved in each case in 75 μl of water and the resulting mixtures were analyzed for IgM.

The determinations were based on the Mancini technique (Orion Diagnostica, Orion, Helsinki). The standards for the immunoglobulins were as follows: IgA, 1.0, 2.5, 5.0, and 10.0 mg per 100 ml; IgG, 1.0, 2.5, 5.0, and 9.0 mg per

Table XI. *The means and standard deviations of certain chemical values of all subjects calculated separately for the total sugar groups and subgroups ($n_S = 15$, $n_F = 16$, $n_X = 17$) of large scale consumers. The determinations were carried out with the supernatant fluid of paraffin-stimulated whole saliva at the end of the trial*

Determination			$\bar{x}$	S.D.
Ionized iodine M × 10^6	S	Tot	12.8	8.9
		Sub	13.8	8.6
	F	Tot	12.1	7.8
		Sub	13.4	9.0
	X	Tot	13.0	8.5
		Sub	10.6	6.4
Ionized fluorine ppm	S	Tot	0.151	0.101
		Sub	0.137	0.080
	F	Tot	0.149	0.072
		Sub	0.165	0.094
	X	Tot	0.128	0.048
		Sub	0.115	0.054
Thiocyanate μg/ml	S	Tot	89	51
		Sub	93	57
	F	Tot	105	75
		Sub	100	65
	X	Tot	83	54
		Sub	124	70
Aminopeptidase (Arg-2-NA with added NaCl, 0.2 mol/l) μmol/(min × mg) × 10^3	S	Tot	0.36	0.23
		Sub	0.39	0.28
	F	Tot	0.34	0.26
		Sub	0.27	0.13
	X	Tot	0.31	0.22
		Sub	0.35	0.29
IgA mg/100 ml	S	Tot	3.9	1.4
		Sub	4.1	1.0
	F	Tot	3.8	1.4
		Sub	3.6	1.3
	X	Tot	3.5	1.3
		Sub	3.9	1.6
IgG mg/100 ml	S	Tot	2.1	1.8
		Sub	2.4	2.5
	F	Tot	2.1	1.3
		Sub	2.3	1.0
	X	Tot	2.0	1.7
		Sub	1.8	1.6
IgM mg/100 ml	S	Tot	0.42	0.22
		Sub	0.35	0.16
	F	Tot	0.44	0.28
		Sub	0.47	0.27
	X	Tot	0.48	0.44
		Sub	0.44	0.42

Determination			$\bar{x}$	S.D.
Aminopeptidase (Arg-2-NA without added NaCl) μmol/(min × mg) × 10^3	S	Tot	0.30	0.15
		Sub	0.31	0.17
	F	Tot	0.25	0.15
		Sub	0.20	0.10
	X	Tot	0.25	0.14
		Sub	0.27	0.16
Amylase U/l	S	Tot	15945	8322
		Sub	14620	8339
	F	Tot	15169	8540
		Sub	13944	5904
	X	Tot	10899	7668
		Sub	8655	4791
Sialic acid μmol/ml	S	Tot	0.060	0.030
		Sub	0.069	0.034
	F	Tot	0.056	0.025
		Sub	0.053	0.025
	X	Tot	0.056	0.036
		Sub	0.056	0.028
Sialic acid mg proteins per μmol sialic acid	S	Tot	27.42	13.14
		Sub	25.27	13.47
	F	Tot	30.76	20.21
		Sub	35.51	25.08
	X	Tot	32.02	23.46
		Sub	31.98	26.88

100 ml; IgM, 2.5, 5.0, 7.5, and 10.0 mg per 100 ml. The standards, prepared by the supplier of the gels, were checked against the WHO standard. In the assay of IgA and IgG the samples were diluted with water in the ratio of 1 : 1. As mentioned above, in the assay of IgM 75 µl water was added onto the freeze-dried residues. After suspension the somewhat turbid samples were analyzed. After diffusion at 22°C the gels were treated for 30 min in a 0.9 per cent NaCl solution, stained for 10 min in 1.0 per cent Amido Black solution, and the diameters of the precipitate rings were determined. The diffusion times were: IgA and IgG, 50 h; IgM, 80 h.

It is natural that, as in the case with certain other determinations of the present study, the freezing and thawing step may have influenced the actual concentration of the salivary IgA, IgG and IgM. The results were, however, comparable between the three sugar groups. The results are shown in Fig. 50. There were no statistically significant differences between the sugar groups.

The three immunoglobulins were also assayed in serum. The standards varied from 57 to 288 mg per 100 ml. The samples were diluted in the case of IgA and IgM with water in the ratio of 1 : 1 (1 : 11 for IgG). The diffusion at 22°C was 50 h for IgA and IgM (50 h for IgG). Otherwise the method was as for the salivary immunoglobulins. The results are shown in Fig. 50. There were no statistically significant differences between the test groups. The immunoglobulin values were comparable to those given in the literature, except for IgM which yielded higher values in the present study than reported by *Brandtzaeg* (1972) and *Shillitoe* (1972). The IgM values given for serum are, however, normal for Finnish subjects.

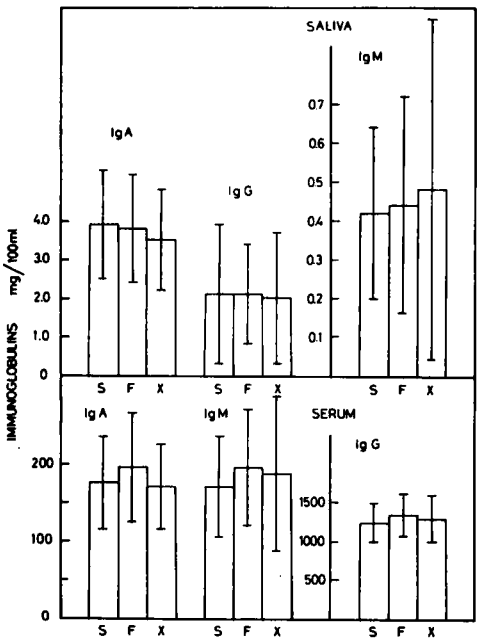


Fig. 50. The concentrations of IgA, IgG, and IgM in the supernatant fluid of whole saliva and serum. 24-month phase.

24. *The chemical values in relation to substantial consumption of sugars*

The contribution to the chemical results of subjects consuming the three sugars in substantial amount was separately elucidated. Tables XI and XII show the results for a number of variables which were determined on individual samples and subjects. The two means comprise the determinations of the samples of the subjects who consumed the highest amounts of sugars and those of the whole sugar groups. The following participants were included in the subgroups, the amount, in kg, of the sugars consumed during two years given in brackets; see also *Mäkinen & Scheinin*, 1975 a.

S: *1* (79), *6* (48), *14* (63), *16* (124), *38* (71), *41* (98), *57* (84), *58* (61), *76* (55), *79* (65), *80* (45), *86* (71), *107* (46), *119* (72), *120* (83) (n = 15).

F: *2* (50), *3* (41), *4* (47), *15* (126), *19* (92), *25* (92), *29**) (63), *33* (47), *34* (44), *35* (49), *49* (77), *50* (66), *56* (75), *61* (58), *67* (86), *96* (63), (n = 16).

X: *10* (53), *12* (40), *13* (54), *29**) (46), *31* (48), *40* (38), *45* (44), *47* (76), *70* (51), *73* (44), *75* (59), *91* (37), *93* (51), *94* (55), *101* (58), *123* (66), *124* (68) (n = 17).

The following results (Tables XI & XII) were considered important, the means of the total groups and those of the large-scale consumers being compared:

1. *Weight of subjects.* No significant differences were found.
2. *Reducing sugars in whole saliva.* There were no profound differences between the whole groups and the large-scale consuming subgroups. In the F-group the differences were very small, and in the S- and X-groups the differences observed during the course of the

*) Subject No 29 was one year in the fructose group and one year in the xylitol group. The quantities of sugars consumed in this case stand for one year intakes.

Table XII. *The means and standard deviations of certain chemical and other values calculated separately for the total (Tot) sugar groups and the subgroups (Sub) of large-scale consumers ($n_S = 15$, $n_F = 16$, $n_X = 17$). The determinations were carried out at all six main analysis periods indicated. Pregnant and adolescent subjects were excluded in the weight column. The enzyme and protein determinations were carried out with centrifuged oral fluid (paraffin stimulation). Ala = alanine; Arg = arginine; Pro = proline; 2-NA = 2-naphthylamine*

Analysis period	Sugar group	Total or subgroup	Weight kg		Reducing sugars in saliva μmol/ml × 10^3		Plaque wet weight mg		Plaque index		Invertase μmol/(min × mg) × 10^3	
			$\bar{x}$	S.D.	$\bar{x}$	S.D.	$\bar{x}$	S.D.	$\bar{x}$	S.D.	$\bar{x}$	S.D.
I (Initial stage)	S	Tot	67.1	13.9	2.13	1.34	23.4	14.13	1.08	0.37	14.28	15.19
		Sub	67.2	14.6	1.90	0.81	24.5	15.36	1.07	0.35	12.90	13.35
	F	Tot	65.0	15.4	2.13	1.19	23.5	16.82	0.97	0.45	16.55	18.86
		Sub	66.1	16.4	2.20	1.34	25.5	20.07	1.00	0.44	21.37	25.24
	X	Tot	67.6	14.4	1.91	1.16	25.1	22.99	1.12	0.37	15.19	20.08
		Sub	73.3	11.6	2.24	1.33	34.8	33.37	1.18	0.42	20.60	28.22
II (3.5 months)	S	Tot	68.5	14.2	3.49	2.10	18.3	11.92	0.99	0.44	21.25	35.87
		Sub	68.4	14.4	3.18	2.00	19.5	11.69	1.04	0.48	22.95	29.68
	F	Tot	66.1	15.8	3.20	1.67	23.8	20.62	1.01	0.51	10.41	9.84
		Sub	67.2	17.3	3.23	1.99	28.6	25.78	1.22	0.43	11.98	8.96
	X	Tot	67.9	14.8	3.36	1.95	9.5	8.63	0.74	0.43	6.41	4.59
		Sub	74.1	11.4	3.43	1.75	10.4	10.26	0.67	0.42	6.52	5.61
III (7.5 months)	S	Tot	68.2	14.7	2.21	1.36	23.1	13.8	1.15	0.34	19.14	32.07
		Sub	67.9	14.7	2.32	1.05	25.5	13.5	1.21	0.39	28.46	41.91
	F	Tot	65.6	16.2	2.41	1.32	20.6	14.58	0.90	0.38	6.91	7.54
		Sub	67.1	17.7	2.45	1.00	24.3	17.02	1.01	0.33	6.48	7.45
	X	Tot	67.1	14.6	2.18	1.27	10.7	12.04	0.72	0.42	4.56	5.29
		Sub	72.6	11.0	2.34	0.97	13.7	14.53	0.70	0.49	6.85	7.98
IV (13 months)	S	Tot	68.4	13.9	1.92	1.03	23.9	15.12	1.23	0.38	11.16	14.44
		Sub	68.4	13.9	2.12	1.05	26.1	15.08	1.40	0.24	16.02	19.12
	F	Tot	65.3	15.7	2.06	1.44	21.2	16.05	1.07	0.39	4.71	5.30
		Sub	66.7	17.2	2.02	1.46	26.7	17.68	1.16	0.31	4.21	2.98
	X	Tot	67.7	14.1	2.04	1.37	11.6	10.81	0.75	0.36	3.20	3.83
		Sub	72.7	10.7	2.13	1.13	10.4	10.08	0.68	0.36	3.01	2.28
V (20 months)	S	Tot	68.9	14.4	3.43	2.25	20.6	12.57	1.26	0.35	11.79	17.09
		Sub	69.0	14.7	3.85	1.86	24.9	13.27	1.44	0.33	15.00	19.88
	F	Tot	66.3	16.6	3.35	1.53	16.5	11.03	1.13	0.41	7.57	5.76
		Sub	68.4	17.7	3.43	1.57	18.8	13.41	1.13	0.37	6.22	4.85
	X	Tot	67.4	15.0	3.48	1.89	12.1	10.24	0.75	0.39	5.77	4.41
		Sub	71.6	12.5	3.79	2.05	12.0	13.28	0.76	0.47	6.36	5.37
VI (24 months)	S	Tot	68.8	13.6	3.00	1.47	20.6	13.28	1.14	0.39	13.37	21.25
		Sub	68.6	14.3	3.30	1.88	27.1	15.33	1.29	0.41	16.12	25.54
	F	Tot	67.3	16.5	3.14	1.59	21.1	14.30	1.04	0.37	6.97	5.71
		Sub	69.0	17.3	2.93	1.25	27.4	16.87	1.13	0.28	4.97	3.97
	X	Tot	68.5	14.5	3.23	2.06	11.9	9.10	0.53	0.38	5.24	5.74
		Sub	72.7	12.8	3.52	2.39	10.4	9.31	0.49	0.36	7.42	8.83

		Aminopeptidases											
Dextranase		Ala-2-NA		Arg-2-NA		Pro-2-NA		LDH				Proteins	
μmol/ (min × mg) × 10^3		μmol/ (min × mg) × 10^3		μmol/ (min × mg) × 10^3		μmol/ (min × mg) × 10^3		U/mg		U/ml		mg/ml	
$\bar{x}$	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.
3.18	1.12	0.43	0.24	0.56	0.37	0.21	0.12	200.84	81.67	294	111	1.53	0.45
2.99	0.95	0.37	0.24	0.47	0.33	0.20	0.12	180.48	91.42	286	121	1.67	0.55
3.66	1.59	0.49	0.29	0.59	0.39	0.29	0.20	224.06	95.55	297	114	1.40	0.40
4.00	1.27	0.59	0.30	0.56	0.41	0.31	0.22	237.09	104.80	319	125	1.42	0.42
3.04	0.79	0.53	0.27	0.67	0.45	0.32	0.27	250.03	108.67	346	166	1.44	0.40
2.89	0.69	0.56	0.22	0.63	0.35	0.31	0.20	277.70	101.06	425	217	1.51	0.42
5.33	1.98	0.70	0.35	0.86	0.45	0.39	0.21	277.18	114.28	298	137	1.09	0.31
4.87	1.09	0.71	0.46	0.76	0.50	0.42	0.28	268.06	132.95	316	175	1.16	0.31
5.37	1.42	0.72	0.40	0.81	0.42	0.47	0.29	264.68	127.91	273	147	1.04	0.22
5.56	1.86	0.82	0.43	0.82	0.42	0.49	0.26	285.84	139.44	287	156	1.01	0.25
5.19	1.28	0.81	0.53	0.87	0.47	0.51	0.46	307.93	143.32	297	124	1.02	0.26
5.25	1.78	0.80	0.53	0.81	0.43	0.47	0.24	303.67	124.79	316	152	1.07	0.28
3.91	1.21	0.57	0.45	0.74	0.94	0.34	0.39	282.11	173.14	372	188	1.42	0.38
4.05	1.21	0.60	0.43	0.90	1.33	0.42	0.55	332.41	206.83	436	210	1.43	0.41
3.75	0.84	0.58	0.33	0.80	0.85	0.42	0.29	277.67	154.48	354	169	1.34	0.31
4.13	1.40	0.62	0.26	0.73	0.47	0.46	0.33	315.70	157.41	393	164	1.30	0.26
3.90	1.12	0.62	0.42	0.80	0.57	0.48	0.35	282.37	145.54	338	148	1.26	0.39
3.95	0.88	0.72	0.59	0.85	0.52	0.56	0.41	320.15	138.27	409	163	1.29	0.30
3.05	0.82	0.44	0.24	0.58	0.36	0.27	0.17	201.69	81.39	308	130	1.57	0.38
3.01	0.92	0.51	0.28	0.65	0.41	0.31	0.17	217.74	105.78	337	163	1.61	0.39
2.99	0.74	0.44	0.30	0.62	0.42	0.29	0.20	189.92	93.19	277	103	1.59	0.39
3.00	0.68	0.47	0.25	0.62	0.35	0.29	0.20	182.79	109.14	277	132	1.63	0.36
3.06	0.75	0.46	0.23	0.70	0.36	0.33	0.18	221.32	84.67	318	124	1.46	0.3ç
3.04	0.82	0.46	0.19	0.82	0.40	0.42	0.20	229.82	82.52	346	125	1.55	0.43
3.91	1.32	0.58	0.53	0.80	0.69	0.33	0.43	223.32	152.94	254	181	1.22	0.41
3.68	1.50	0.67	0.65	0.86	0.88	0.43	0.58	231.64	199.42	261	226	1.23	0.37
3.93	0.96	0.59	0.39	0.71	0.52	0.39	0.40	187.92	106.45	198	126	1.13	0.29
3.82	0.88	0.57	0.45	0.56	0.48	0.32	0.25	187.22	78.67	195	87	1.20	0.27
3.78	1.27	0.59	0.41	0.90	0.66	0.37	0.31	190.49	119.67	200	106	1.14	0.39
4.05	1.57	0.59	0.48	0.96	0.61	0.44	0.37	238.79	136.57	260	104	1.22	0.39
3.71	3.11	0.58	0.40	0.72	0.52	0.32	0.21	251.25	113.16	323	132	1.33	0.28
3.42	1.56	0.61	0.52	0.73	0.66	0.36	0.28	267.72	154.19	345	168	1.36	0.31
3.69	1.14	0.57	0.37	0.71	0.83	0.36	0.29	246.63	119.20	314	135	1.33	0.28
3.57	1.50	0.54	0.30	0.53	0.35	0.29	0.21	240.23	106.93	319	136	1.38	0.30
3.72	1.56	0.57	0.40	0.75	0.59	0.37	0.29	251.03	114.07	311	144	1.27	0.31
3.58	1.40	0.68	0.55	0.94	0.58	0.48	0.32	287.59	155.54	355	178	1.28	0.24

study already existed in the base line analyses. The study naturally indicated that the higher the consumption of sugars was, the more reducing sugars were found in whole saliva.

3. *Plaque wet weight and plaque index.* It was obvious that high sugar consumption in the S- and F-groups resulted in high plaque values. The amount of plaque in relation to X-consumption was approximately the same in the high-intake subgroup as in the whole group.

4. *Invertase-like activity in whole saliva.* Table XII indicates that the high sucrose consumption was related to high invertase-like activity in the supernatant fluid of whole saliva. In the X- and F-groups there was no such repeatedly observable trend as in the S-group. The large-scale X-consumers also consumed higher quantities of other carbohydrate food (potatoes, rice, bread, etc.). This may explain the finding that the high-intake X-subjects displayed slightly higher activity of invertase-like enzymes at most analysis periods than the whole X-group. The present paper also indicates that in addition to transfering enzymes, plaque and whole saliva contain true invertase-like enzymes.

5. *Dextranase in whole saliva.* No clear or repeatedly detectable differences between the high-intake persons and the whole test groups were found.

6. *Aminopeptidases in whole saliva.* A high intake of xylitol seemed to induce a slightly higher aminopeptidase activity.

7. *Lactate dehydrogenase in whole saliva.* The differences found during the course of the study were already seen in the base line values.

8. *Proteins in whole saliva.* No differences were found.

9. *Ionized iodine, fluorine and SCN^- ions in whole saliva.* It was obvious that the high-intake X-group exerted lower concentrations of ionized iodine and fluorine in the centrifuged whole saliva than the whole group. For SCN^- ions the situation was reverse.

10. *Amylase in whole saliva.* The high X-intake subgroup exhibited decreased salivary amylase activity in comparison to the total X-group. It is, however, likely that the amylase method used for saliva is not specific. The reduction in the enzyme activity in whole saliva almost certainly partly results from the fact that related bacterial enzymes were also reduced.

11. *Sialic acids and immunoglobulins in whole saliva.* No clear differences were found.

Table XIII. *Significance levels of differences in certain plaque and whole saliva values between sugar groups at various stages of the two-year study. The statistical procedures were described earlier (Scheinin, Mäkinen & Ylitalo, 1974)*

Variable	Figure	Kruskal-Wallis	Wilcoxon S/F	Wilcoxon S/X	Wilcoxon F/X
Plaque wet weight (mg)	3				
0 Months		n.s.	n.s.	n.s.	n.s.
3.5 »		***	n.s.	***	***
7.5 »		***	n.s.	***	***
13 »		***	n.s.	***	**
20 »		***	n.s.	***	n.s.
24 »		***	n.s.	***	***
Plaque index	4				
0 Months		n.s.	n.s.	n.s.	n.s.
3.5 »		**	n.s.	**	•
7.5 »		***	*	***	*
13 »		***	n.s.	***	***
20 »		***	n.s.	***	***
24 »		***	n.s.	***	***
Whole saliva invertase-like activity	42				
0 Months		n.s.	n.s.	n.s.	n.s.
3.5 »		***	n.s.	***	•
7.5 »		•	n.s.	**	*
13 »		**	n.s.	**	n.s.
20 »		n.s.	n.s.	n.s.	n.s.
24 »		*	n.s.	*	*

$P \leq 0.05$ * n.s. = Not significant
$P \leq 0.01$ **
$P \leq 0.005$ ***

25. *Significance levels of certain plaque and whole saliva parameters between test groups*

Table XIII shows the significance levels of differences in certain plaque and whole saliva values between the sugar groups. No significant differences were found in the following cases at any stage of the two-year study: pH of centrifuged whole saliva, whole saliva lactate dehydrogenase activity, whole saliva dextranase activity and whole saliva reducing sugars.

DISCUSSION

1. *Nature and chemical characteristics of the test material*

Pooling of samples. A part of the biochemical studies definitely suffered from the pooling of individual samples. Similarly, the storage of the samples at —20°C for several weeks or months affected some chemical assays. Regardless of this, the commensurability of the samples was assumed to remain as a characteristic of the experimental groups. It may be understandable that a simultaneous assay of several enzymes and chemical compounds on fresh samples of saliva, serum, plaque and other subjects, produced practical problems.

Sonic treatment. The ultrasonic disintegration of plaque and salivary sediment samples requires special consideration. Various oral micro-organisms resist the sonic treatment in drastically different ways (*Robrish et al.*, 1975). Gram negative cells are generally more sensitive to sonic treatment than gram positive cells. Prolonged treatment may again affect certain enzymes to an unnecessarily high extent. The purpose of the sonication steps of the present study was to produce comparable biological samples in which minimum destruction of enzyme activities would have occurred. Consequently, the sonicated materials did not represent the entire metabolic or enzymic potential of plaque and salivary sediment.

Freezing and thawing. It is an equally well-known fact that freezing and thawing according to the »slow method» destroys more enzyme activity than the rapid one (*Chilson, Costello & Kaplan*, 1965). For numerous practical reasons the biological samples of the present studies were frozen and thawed in the first mentioned way. The commensurability between the groups was, however, assumed to remain unaffected.

Solubility of plaque ingredients. It is understandable that when various amounts of plaque are suspended in the same buffer volume and when the mixtures are centrifuged, the supernatant fluids may chemically differ from each other due to the differences in the original weight/volume ratios. As a consequence of several practical reasons (e.g., some subjects provided only a few mg or less plaque) no chemical assays on individual plaque samples were performed. In each sugar group the pooled plaque samples were suspended in 5.0 ml of cold 0.9 % NaCl solution. Because the amount of plaque in the X-group was approximately 50 % lower than in the other groups, these resulting plaque preparations may have exhibited slightly different rate of dissolution of certain water-soluble compounds during the suspension (3 min.). However, the number of subjects was approximately 45 % higher in the xylitol group than in the other groups. This automatically renders the three plaque pools fairly comparable.

The differences in the quantity of plaque were also taken into account when giving the results (paragraph No. 5 in the Materials and Methods section). Many of the compounds (e.g. lactate) were also expressed per mg protein and the enzyme activities were given as specific activities to obtain a better idea about the relative amounts and activities in the samples. In the present study mixing of X-plaque in 2.5 ml of NaCl solution instead of 5.0 ml would have led to more serious methodological consequences.

2. *Diet-dependent biochemical changes in plaque and saliva*

Despite the above limitations, it is obvious that the 50 % reduction of the plaque

wet weight in the X-group led to several consecutive changes which were more or less directly proportional to the plaque reduction. Such were, for example, the apparently lower concentration of proteins (Fig. 5), total nitrogen (Fig. 7), total sugars (Fig. 8), glucose (Fig. 11), RNA (Fig. 13), keto acids (Figs. 18—19), etc. in certain plaque analyses. All these changes indicated an advantageous development due to X-consumption.

It should be emphasized that diet displays a strongly selective effect on the chemical composition of salivary secretions. Such a conclusion is justified on the basis of earlier and present data. A classic example is amylase which is secreted selectively in high amounts on a predominant carbohydrate diet (*Walker*, 1925; *Squires*, 1953). Some 50 years after the original findings, an explanation of this effect in biochemical terms was provided (*Ferguson*, 1975). The lactoperoxidase effect (*Mäkinen, Tenovuo & Scheinin*, 1975) represents another example of the selectivity; X-consumption led to a pronounced elevation of the enzyme activity. It is possible that the differences in the amylase activities between the S- and X-groups (and between the F- and X-groups) reflect a similar phenomenon (Fig. 35), although the assays on whole saliva samples without fractionation do not entitle to firm conclusions.

The proposed selectivity was above illustrated by enzyme changes. As shown by *Shannon, Suddick & Dowd* (1974), the inorganic composition of the salivary secretions may also be affected by the chemical nature and concentration of acidic and sweet compounds. Sweet carbohydrates have been shown to cause clearly varying sensory stimuli in laboratory animals. The above examples were from studies of immediate sugar effects and thus greatly differ from the present trial in which long-term effects were elucidated.

Diet also influences the composition of animal plaque. The carbohydrate content of plaque declined, but calcium and phosphorus levels increased, when monkeys received no food by mouth or ingested casein orally. Feeding invert sugar had the opposite effects (*Bowen*, 1974). In the present study, relative to paraffin stimulation, no permanent or pronounced changes in the salivary flow rate, physicochemical characteristics or concentration of inorganic compounds were found between the sugar groups for Na, K, Ca, Mg, P_i, SCN^-, H^+, e^-, p_{H_2}. Ionized fluorine and iodine were somewhat reduced in the X-group (*Mäkinen, Tenovuo & Scheinin*, 1975). The low content of ionized fluorine in X-group saliva can be understood on the basis of lower caries incidence, lower plaque and salivary lactate, and reduced dissolution of mineral, observed in this group. Fluorine may thus remain to a greater extent where it should occur, i.e. in enamel. According to Table XI, the SCN^- ion was also affected when the X-intake was high.

It was expected that there was a great number of chemical agents which were not changed by the sugar diets to any noticeable extent (Table XI). While the diet affected certain enzyme proteins in saliva, it did not have any clear influence on immunoglobulins, for example. The levels of IgA, IgG, and IgM obtained in this study were comparable to those given by *Shillitoe* (1972) and *Brandtzaeg* (1972), except for serum IgM which was to a certain extent higher than reported by the two latter authors. *Taubman* (1974) showed IgA and IgG in human dental plaque. IgG concentration was greater in plaque than in whole saliva, suggesting that IgG

could be derived from gingival crevice fluid. Fig. 49 indicates that the basic protein pattern of saliva was not affected by the sugar diets, although certain enzyme changes were observed.

Another group of biochemical changes caused by various sugar diets is formed by variations in the oral flora. Although the present study did not separately elucidate the composition of pure secretions and oral fluid, a distinction between the actual salivary and other compounds was in some cases possible (*Mäkinen, Tenovuo & Scheinin*, 1975). Both type of shifts are included in Table XII, but the ones provoked by microbial alterations can also be examined separately. They comprised, for example, the following:

— Decrease of lactate in the X-group (Table III, Fig. 20).
— Decrease of invertase-like activity in the X-group (Figs. 42 & 44).
— Increase of proteinase activity in the saliva of the X-group (Fig. 39).
— Increase in the amount of amino acids (excluding hydroxyproline and a few others) in the saliva of the X- and F-groups (*Mäkinen, Lönnberg & Scheinin*, 1975).
— The slight increase in the aspartate transaminase activity in the plaque samples of the X-group (Fig. 28).

3. *Examination of certain chemical changes*

a. *Plaque aspartate transaminase.* As indicated in Fig. 28, the specific activity of plaque aspartate transaminase was to a certain extent higher in the X-group than in the other groups. This finding may be related to the elevated proteinase activity and amino acid concentration of oral fluid in the X-group. Micro-organisms deprived of a required nutrient, e.g. sucrose, generally elaborate increased amounts of extracellular proteinase-like and intracellular aminopeptidase-like enzymes (*Goldberg*, 1975; *Knuuttila & Mäkinen*, 1975a, b). It may be expected that an increased proteolysis and amino acid utilization also presupposes elevated transamination, in the present case involving increased aspartate transaminase activity.

b. *Ratio of proteins to total sugars.* Xylitol consumption seemed to increase the ratio of proteins to carbohydrates in plaque when compared to the use of sucrose. This may be expected on the basis of the known role of sucrose in plaque growth and polysaccharide formation. The present result is also in accordance with that of *Grenby, Powell & Gleeson* (1974). Sucrose was found to increase plaque, plaque carbohydrate level and the carbohydrate:protein ratio, when compared to the effect exerted by non-sucrose products (note that in the present study and in the study referred to, the ratios were expressed in opposite ways).

c. *Dehydrogenase activity.* A short-term study carried out in this laboratory indicated that human dental plaque does not exhibit reliably measurable xylitol dehydrogenase activity (*Mäkinen & Scheinin*, 1972). In the present study almost similar low activity was detected. Because this was manifested throughout the study, the data was included and expressed as U/ml and U/mg (Fig. 33). In this assay the reaction expected to take place was: xylitol + NAD → xylulose + NADH. It is not possible to conclude, without enzyme purification, whether specific or unspecific enzymes were involved. Because alcohol dehydrogenase activity was elevated to a slight extent in the X-group Table VIII), nonspecific enzymes attacking xylitol may be involved. No

induction of xylitol dehydrogenase activity was found.

d. *Glycosidohydrolases (acting on p- and o-nitrophenyl derivatives)*. Many earlier studies have suggested that the bulk of the oral fluid glycosidase activity stems from plaque (*Leach & Melville*, 1970; cf. also *Mäkinen et al.*, 1975). It can be concluded that at least the activity toward the glucoside, galactoside, and fucoside substrates used (Figs. 36 & 37; Table IX) has almost exclusively originated in plaque. X-consumption increased the overall specific glycosidase activity of plaque (this paper and *Mäkinen et al.*, 1975), but simultaneously reduced the same activity in gingival exudate of the same subjects (*Mäkinen et al.*, 1975). These findings may be explained in terms of a favourable effect of xylitol as regards the gingival pockets (less plaque and less inflammatory changes), and in terms of a metabolic or qualitative change in the plaque flora, resulting in the synthesis of more glycosidases in plaque. The possible selective effect of diet on the enzymic composition of exudate should, however, be separately studied.

The consumption of xylitol only slightly lowered the plaque dextranase activity. Several plaque bacteria produce dextranase and levanase (for brief reviews, see *Mäkinen*, 1972 b, 1974). Recently, a plaque strain of *Fusobacterium fusiforme* was reported to possess a cell-bound dextranase (*Da Costa, Bier & Gaida*, 1974).

The reduction of plaque invertase-like activity (or of activity related to the formation of reducing sugars from sucrose) by X-consumption has been demonstrated by now in several separate studies (*Mäkinen & Scheinin*, 1971, 1972; *Mouton, Scheinin & Mäkinen*, 1975a, b). The chromatographic findings (Figs. 44 & 45) provided more information about the enzymes involved. It can be concluded that any »invertase» assay of plaque with certainty also includes true invertase(s). The relative portion of transferases, for example, depends on diet, person, and microbiological traits of the mouth. It is obvious that a regular invertase assay on crude samples cannot take into account the simultaneous enzymic breakdown of formed reducing sugars, e.g. via oxidation.

e. *Peptidohydrolases*. Collagenase-like activity is readily demonstrated in oral tissues, including carious dentine (*Mäkinen*, 1970), although the latter study utilized a synthetic substrate which may be attacked by other proteinases as well. The use of this substrate reveals an activity which has been termed PZ-peptidase activity (*Hino et al.*, 1975). PZ-peptidase occurs in bovine and human tissues and in rat granuloma. The present paper was not able to show any measurable PZ-peptidase activity in plaque and saliva (no concentration was performed). The concentration of hydroxyproline in saliva was lowest in the X-group (paragraph 13 in the Results section). This may in part indicate insignificant collagenase-activity to occur in the material studied, although it is known that certain plaque bacteria may break down hydroxyproline.

The reason for the increased salivary proteinase activity of xylitol-consuming subjects when compared to the S-group (Fig. 39), was touched upon in paragraph 3.a. of the Discussion section. It is likely that the increased amino acid concentration (*Mäkinen, Lönnberg & Scheinin*, 1975), and the elevated activity of proteinase (*Knuuttila & Mäkinen*, 1975b), aminopeptidases (*Knuuttila & Mäkinen*, 1975a, b; *Mäkinen*, 1972a), and transaminase (Fig. 28) are all interrelated phenomena. In the absence of required

amounts of preferred hexoses, many plaque micro-organisms produce extracellular proteinases for the liberation of amino acids needed as a source of energy and growth.

The possible occurrence in oral fluid or tissues of enzymes resembling aminopeptidase B (a chloride-activated arginine aminopeptidase, EC 3.4.1.16; APB) has been connected to inflammatory reactions (*Mäkinen*, 1975b; *Paunio, Mäkinen & Scheinin*, 1971, 1973). The extent of the contribution of this type of enzyme to the overall arginine aminopeptidase pattern has been studied by testing the rate-enhancing effect of 0.2 M NaCl. According to the present results, there was no clear difference between the test groups as regards the NaCl-effect (Figs. 40 & 41). These findings support the earlier ideas about the nonexistence or low activity of chloride-activated APB in the oral fluid of healthy persons. During a prolonged negligence of all oral hygiene measures, in ulcerative gingivitis, in other pronounced inflammatory reactions and in mechanical trauma, detectable APB-like activity in whole saliva can be encountered. Xylitol diet reduced in gingival crevice fluid an enzyme activity which was dependent on 0.2 M NaCl (*Paunio, Mäkinen & Scheinin*, 1975).

4. *Survey of biochemical changes in plaque and saliva*

In view of the above considerations it is possible to group the findings of the present and related biochemical papers of this series as indicated below. The survey attempts to describe such changes only which involved real concentration or activity shifts. Notice is taken primarily of X-induced effects, the F-effects being mentioned when applicable.

a. *No changes for the following enzymes, compounds or properties*

— Salivary flow rate (determined at 16.5-month phase only.)
— Specific resistance of oral fluid (determined at 24-month phase only)
— Salivary and plaque protein concentration (throughout the study)
— Salivary and plaque total nitrogen (throughout the study)
— Salivary reducing sugars (throughout the study)
— Salivary RNA and DNA (throughout the study)
— Ratio of proteins to RNA in saliva and plaque (throughout the study)
— Ratio of RNA to DNA in saliva (throughout the study)
— Salivary keto acid and pyruvate concentration (throughout the study; for keto acids slightly lower values in the saliva of the X-group; Fig. 18)
— Ratio of keto acids to pyruvate in saliva and plaque (at most phases)
— Ratio of proteins to total keto acids or pyruvate in saliva and plaque (at most phases)
— Salivary K, Na, Ca, and Mg (determined at 13- and 24-month phases only)
— Salivary sialic acid (free and bound; at all phases tested; cf. Table VI)
— Salivary inorganic phosphorus (determined at 13-and 24-month phases only)
— Salivary and plaque alanine transaminase and salivary aspartate transaminase (at most phases)
— Salivary and plaque lactate dehydrogenase (at most phases)
— Salivary and plaque xylitol dehydrogenase (at most phases; cf. notice about this enzyme in paragraph b below)
— Salivary and plaque aldolase (at most phases)
— Salivary amylase (no change between sucrose and fructose groups; deter-

mined at 24-month phase only; opposite to xylitol effects; see below point c)
— Salivary glycosidase activity (throughout the study; opposite to plaque; see below point b)
— Salivary and plaque collagenase-like activity (no detectable activity was observed throughout the study)
— Salivary arginine aminopeptidase activity (determined at 24-month phase in the presence of 0.2 M NaCl and without added salt, at other phases only without added salt)
— Salivary and plaque dextranase (at most phases; in plaque extracellular fluid a slight tendency to yield lower activities in the xylitol group; see below point c)
— Salivary and plaque enzymes yielding keto acids from L-djencolic acid (throughout the study)
— Isoelectric focusing pattern of salivary proteins (determined at 16.5-month phase only)
— Salivary IgA, IgG, and IgM (determined at 24-month phase only)
— Salivary pH, rH, and Eh; rH and Eh determined at 16.5-month phase only)
— Salivary I^- and SCN^- (determined at 24-month phase only). See, however, comments in paragraph 24
— Salivary catalase (no activity detected in centrifuged saliva at 24-month phase; see *Mäkinen, Tenovuo & Scheinin*, 1975)
— Salivary urea (determined at later phases of the trial; (*Mäkinen, Lönnberg & Scheinin*, 1975)

b. *Increasing values for the following compounds, enzymes, or properties*

— Ratio of proteins to total sugars in plaque preparations (in three out of six analyses; Fig. 9)
— Ratio of proteins to lactate in saliva and plaque (determined at 13- and 20-month phases; Table III)
— Plaque aspartate transaminase (at several phases; Fig. 28)
— Plaque xylitol dehydrogenase (at most phases; given in U/mg; Fig. 33; the activity was low)
— Plaque and saliva alcohol dehydrogenase (determined at 13- and 20-month phases; slight increase in most cases; Table VIII)
— Plaque glycosidase activity (an increase in most cases with α-glucosidase, galactosidase, and fucosidase; Figs. 36 & 37; Table IX; opposite to saliva. Increased α-glucosidase activity was also found in plaque due to fructose consumption)
— Salivary proteinase activity (Fig. 39)
— Salivary amino acid concentration (concerns most ninhydrin-positive compounds; *Mäkinen, Lönnberg & Scheinin*, 1975; This was also observed to a certain extent in the fructose group)
— Salivary lactoperoxidase activity (*Mäkinen, Tenovuo & Scheinin*, 1975; this was also observed to a certain extent in the fructose group)

c. *Decreasing values for the following compounds, enzymes or properties*

— Ratio of glucose to proteins in plaque (at most phases; Fig. 11 E—F)
— Ratio of proteins to DNA in plaque extracellular phase (Fig. 16)
— Salivary and plaque lactate (Table III)
— Salivary amylase activity (Fig. 35)
— Plaque and salivary invertase-like activity (at most phases; Figs. 42—44; this was also observed in the fructose group)
— Plaque dextranase activity (at several phases; Fig. 46)
— Salivary ionized fluorine concentration (*Mäkinen, Tenovuo & Scheinin*, 1975)

— Salivary hydroxyproline (paragraph 13 in the Results section)
— Plaque wet weight (Fig. 3)

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