CHEMICAL ASPECTS OF HUMAN PLAQUE AND ENAMEL

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A THESIS SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (ODONTOLOGY) OF THE UNIVERSITY OF STELLENBOSCH

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ACKNOWLEDGEMENTS

The author wishes to record his sincere appreciation for the assistance rendered by all the organisations, departments and people in bringing this study to early fruition. I am specially indebted to the following persons:

- Professor G N Jenkins, Head, The Dental School, University of Newcastle-Upon-Tyne, England, for his advice and fruitful discussions.

- Professor G Rølla, Head, Department of Pedodontics and Caries Prophylaxis, University of Oslo, Norway, for his assistance and tangible support in permitting the use of his laboratory, where a part of this project was completed.

- The Statistical Section of the M R C, Tygerberg, for their advice and statistical treatment of the results.

- The M R C and C S I R of South Africa for their financial support. Without their assistance this research project would have been impossible.

- Mrs M A de Vries, U W C, who under difficult circumstances, cheerfully transformed my scrawl into beautiful typescript.

- Finally, a special word of thanks is due to my wife for her linguistic provision of this thesis.

- I give all the honour which might arise out of this thesis to GOD

CHAPTER I

A COMPARISON BETWEEN THREE METHODS FOR THE MICRODETERMINATION OF PHOSPHORUS IN DENTAL PLAQUE

A. SUMMARY

The ideal conditions for the use of hydrazinium sulphate as a reducing agent to determine phosphorus in dental plaque were investigated. When this method was compared to the tin (II) chloride (Kuttner and Cohen, 1927) and ascorbic acid method (Chen, Toribara and Warner, 1956), which is generally in use for the measurement of phosphorus in plaque, it was found to have certain advantages. Different ashing techniques were compared, as well as the influence of different acids on wet ashing. The hydrazinium sulphate method could determine as little as 1,8 µg phosphorus per 50 ml and is recommended for the routine analysis of phosphorus in dental plaque samples with wet ashing.

B. INTRODUCTION

Today phosphorus can be determined, among other methods gravimetrically, titrimetically and spectroscopically (Williams, 1979). Through spectroscopic methods phosphate might be analysed spectrophotometrically by 3 main methods, as molybdophosphoric acid, its reducing product molybdenum blue and the yellow vanadomolybdophosphoric complex (Stuart and Duff, 1980). In addition indirect methods derived from molybdophosphoric acid might also be used (Williams, 1979).

When phosphorus is spectrophotometrically determined during the reduction of a phosphomolybdate complex by means of different reducing agents (Kuttner and Cohen, 1927; Chen *et al*, 1956; Fiske and Subbarow, 1925 and 1929, Taylor and Miller, 1914; Martin and Doty, 1949; Eibl and

Lands, 1969; Vogel, 1961; Boltz and Mellong, 1947; Lazarus and Chou, 1972; Laws and Webley, 1959; Burton and Riley, 1955; Dickman and Bray, 1940), it is necessary to establish the optimum conditions under which the reducing agent, hydrazinium sulphate, can be used. In spite of this, many details about exact experimental procedures are not known (Taylor and Miller, 1914; Vogel, 1961; Boltz and Mellon, 1947). The present investigation is an attempt to establish the ideal conditions for the use of hydrazinium sulphate as a reducing agent during the formation of molybdenum blue (Schirmer *et al*, 1942) and the effectiveness of the method for the determination of phosphorus in dental plaque by different ashing techniques. The hydrazine method is also compared to that of Chen *et al* (1956) which is generally in use for plaque phosphate determinations by many authors (Ashley, 1975; Ashley and Wilson, 1976; Kleinberg *et al*, 1971; Zuniga *et al*, 1973), as well as to the sometimes used (Dawes and Jenkins, 1962) tin (II) chloride method of Kuttner and Cohen (1927). For the review of many other accepted methods, see Lindberg and Ernster (1956), and Williams (1979). The results are compared to the modified ascorbic acid method (Chen *et al*, 1956) as well as to the tin (II) chloride method (Kuttner and Cohen, 1927).

C. EXPERIMENTAL

The reagents were of analytical-reagent grade and were used without further purification. All solutions were prepared in double glass-distilled water and all acids including ascorbic acid (Grobler and Coetzee, 1977), were standardized by titration. Sodium molybdate solution was prepared by dissolving 12,500 g of salt in 4,000 N sulphuric acid and made up to 250 ml. Potassium dihydrogen phosphate solution was prepared by first heating the salt for one hour at 110°C.

Subsequently/....

0,3200 g of this salt was dissolved in water, and then made up to 1000 ml with water. Two to three drops of chloroform were added to the solution to prevent mold formation and thereby enabling it to be used as a standard for a long period. The hydrazinium sulphate solution was prepared by dissolving 1,500 g in water and made up to 1000 ml. The ascorbic acid and ammonium molybdate solutions were prepared according to the methods described by Chen *et al* (1956), and the tin (II) chloride solutions according to the methods described by Kuttner and Cohen (1927).
A Bausch and Lomb Spectronic 20 colorimeter with IP40 tube and red filter was used for all readings.

Precise weighing was done by the use of a Cahn Model 4100 Electrobalance and plaque mixing by the Soniprobe Type 530 A, Serial.

A maximum of 75 µg P/50 ml was used throughout the experimental work unless otherwise stated. This amount was chosen as it is seldom necessary to determine more than this amount of phosphorus at one time in dental plaque.

Calibration Curves. With a burette the required quantities of phosphorus per 50 ml were measured from the standard potassium dihydrogen phosphate solution (quantities ranged from 1 µg - 75 µg P/50 ml) and made up to a volume of ~ 40 ml each. To each, standardised sulphuric acid was added to obtain a final concentration of 1,05 N, followed by 4,00 ml sodium molybdate and 1,60 ml hydrazinium sulphate solution. The mixtures were then diluted to 50 ml with water, shaken well, heated for 15 minutes at 75°C in a water bath, cooled to room temperature and the percentage transmission (T) measured at a wave length (λ) of ~ 810 nm, against a blank solution containing the

same constituents as above, but without potassium dihydrogen phosphate. A calibration curve was obtained by plotting log. percentage transmission (T) against µg P/50 ml (Fig. 1 (a)

For the investigation of the possible influence of fluoride ions in plaque in high fluoride water areas on the phosphorus determination, different calibration curves containing 0,950; 2660 and 3775 μgF^-/50 ml, respectively were plotted.

A calibration curve with ascorbic acid as the reducing agent was plotted, using as a blank solution 50 per cent of solution C and a wave length of ~ 810 nm (Fig. 1 (b) : Chen *et al*, 1956).

A calibration curve with tin (II) chloride as the reducing agent was also plotted (Fig. 1(c)) (Kuttner and Cohen, 1927).

The reproducibility of the hydrazinium, ascorbic acid and tin (II) chloride methods was tested by means of the phosphorus determinations in a standard potassium dihydrogen phosphate (Table I) solution, as well as in a dry homogenous plaque sample (Table II).

This homogeneous plaque sample was obtained by collecting plaque from an isolated population group; then water added and mixed to a homogeneous sample by the use of the Soniprobe (5 ampere). The water of this mixture was evaporated at 70°C and the sample dried at 105°C for 2 hours. The dry mixture was ground and tested for homogeneity by various phosphorus determinations of different separate samples of it (Table II).

The following ashing techniques were tested by the use of the homogeneous plaque sample and with hydrazinium sulphate as reducing

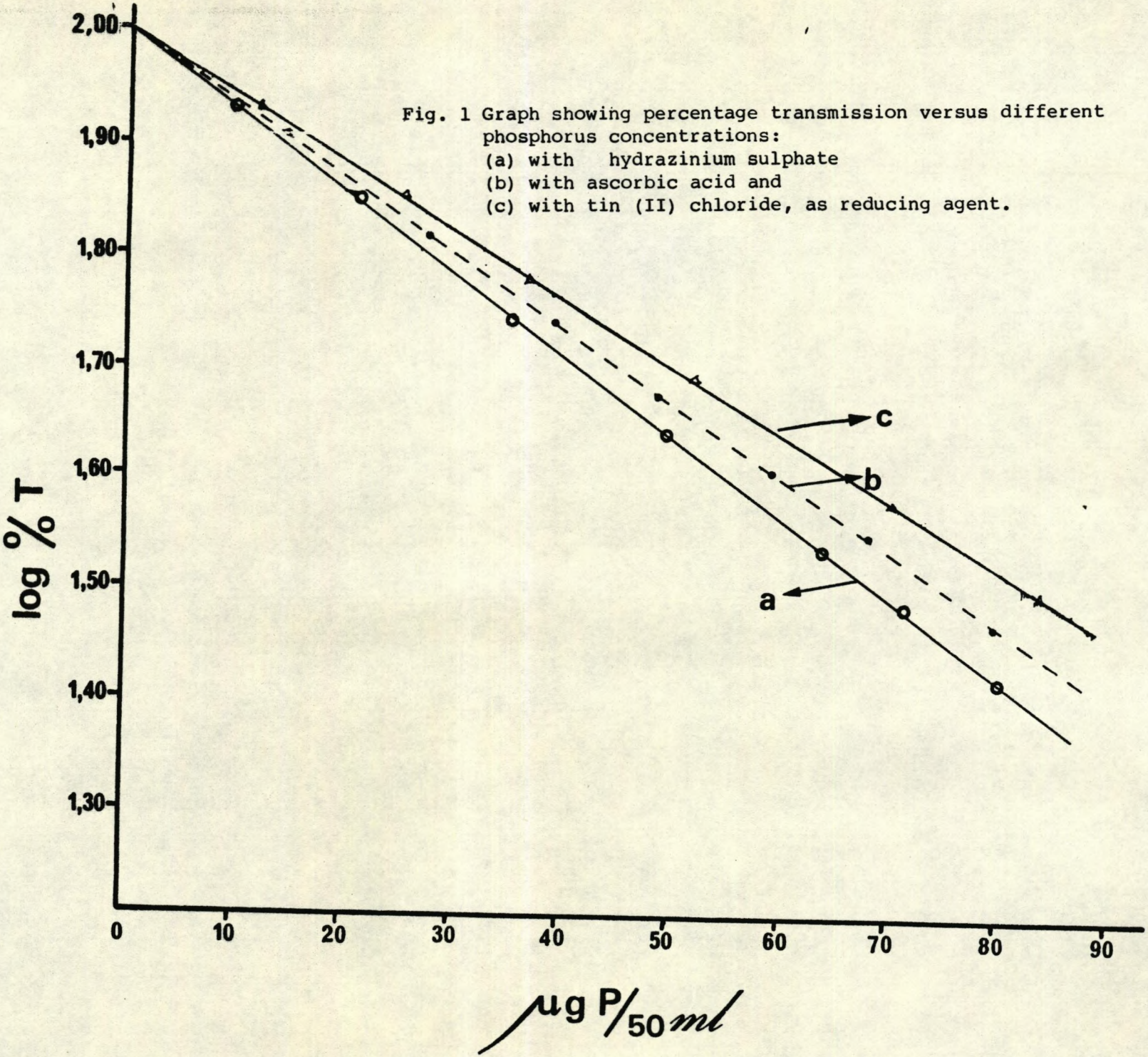


Fig. 1 Graph showing percentage transmission versus different phosphorus concentrations:
(a) with hydrazinium sulphate
(b) with ascorbic acid and
(c) with tin (II) chloride, as reducing agent.

agent (Table III). Of the dry homogeneous plaque 1,00 mg was weighed out on the Cahn balance in a self-made small platinum crucible and (1)ashed in a borosilicate glass test tube by heating in concentrated (conc.) H_2SO_4 solution until the apppearance of white fumes of sulfur trioxide; 2 drops of conc. perchloric acid were added to the warm solution to get the liquid clear; (2) ashed at 600°C for 4 hours and the ash dissolved by dropping the platinum in a glass tube in conc. H_2SO_4. This mixture was then shaken well to dissolve all of the ash; (3) ashed at 1000°C for 20 minutes and the product hydrolysed by heating at 100°C in 50 per cent sulphuric acid, while shaking occassionally on a "Rotamixer" for an hour.

Estimation of Phosphorus in Dental Plaque

The determination of phosphorus by the ascorbic acid and tin (II) chloride method was done as described by Chen *et al* (1956) and Kuttner and Cohen (1927) respectively by means of hydraxinium sulphate as the reducing agent: The ~ 1,00 mg homogeneous plaque sample was ashed at 600°C for 4 hours in a self-made small platinum crucible, the boat was dropped in 0,30 ml conc. sulphuric acid, shaken by the use of the "Rotamixer" until the ash was dissolved. An amount of 0,70 ml water was added and again shaken well. Of this solution 0,60 ml was pipetted into a 50 ml volumetric flask and ~ 25 ml water, 0,84 ml conc. H_2SO_4 (N = 35,8583) and 4,0 ml sodium. molybdate solution added and shaken well. To this mixture 1,60 ml hydrazinium sulphate solution was added, made up to the 50 ml with water, shaken well, heated for 15 minutes at 75°C, cooled to room temperature and the percentage transmission read. The amount of phosphorus in the plaque sample was calculated by means of the calibration curve (Fig. 1(a)).

As the addition of nitric acid, hydrochloric acid, trichloroacetic acid and perchloric acid have been advocated for wet ashing, it was decided to evaluate the influence of various concentrations on colour developments.

Table 1 Comparison of values obtained in the analysis for potassium dihydrogen phosphate standards using (a) hydrazinium sulphate, (b) ascorbic acid and (c) tin (II) chloride methods. An amount of 14,98 µg P/50 ml has been used in each case.

	(a)	(b)	(c)
No of Samples	30	30	30
Mean ($\bar{x}$)	14,99	15,00	14,96
SD[a]	0,15	0,17	0,32
%SD	1,00	1,13	2,14

[a]SD represents standard deviation

Table II Comparison of values obtained in the analysis for phosphorus in the test of the homogeneity of the dry plaque sample by the (a) hydrazinium sulphate, (b) ascorbic acid and (c) tin (II) chloride methods.

	(a)	(b)	(c)
No. of Samples	35	35	35
Mean ($\bar{x}$)	26,03	26,01	26,02
SD[a]	0,32	0,38	0,58
%SD	1,23	1,46	2,23

[a]SD represents standard deviation

Table III Comparison of values obtained in the analysis for using different ashing techniques. (a) Wet ashing, (b) ashing at 600°C for 4 hours and (c) ashing at 1000°C for 20 minutes.

	(a)	(b)	(c)
No of Samples	25	25	25
Mean ($\bar{x}$)	30,15	30,01	29,69
SD[a]	0,33	0,36	0,38

[a]SD represents standard deviation

D. RESULTS

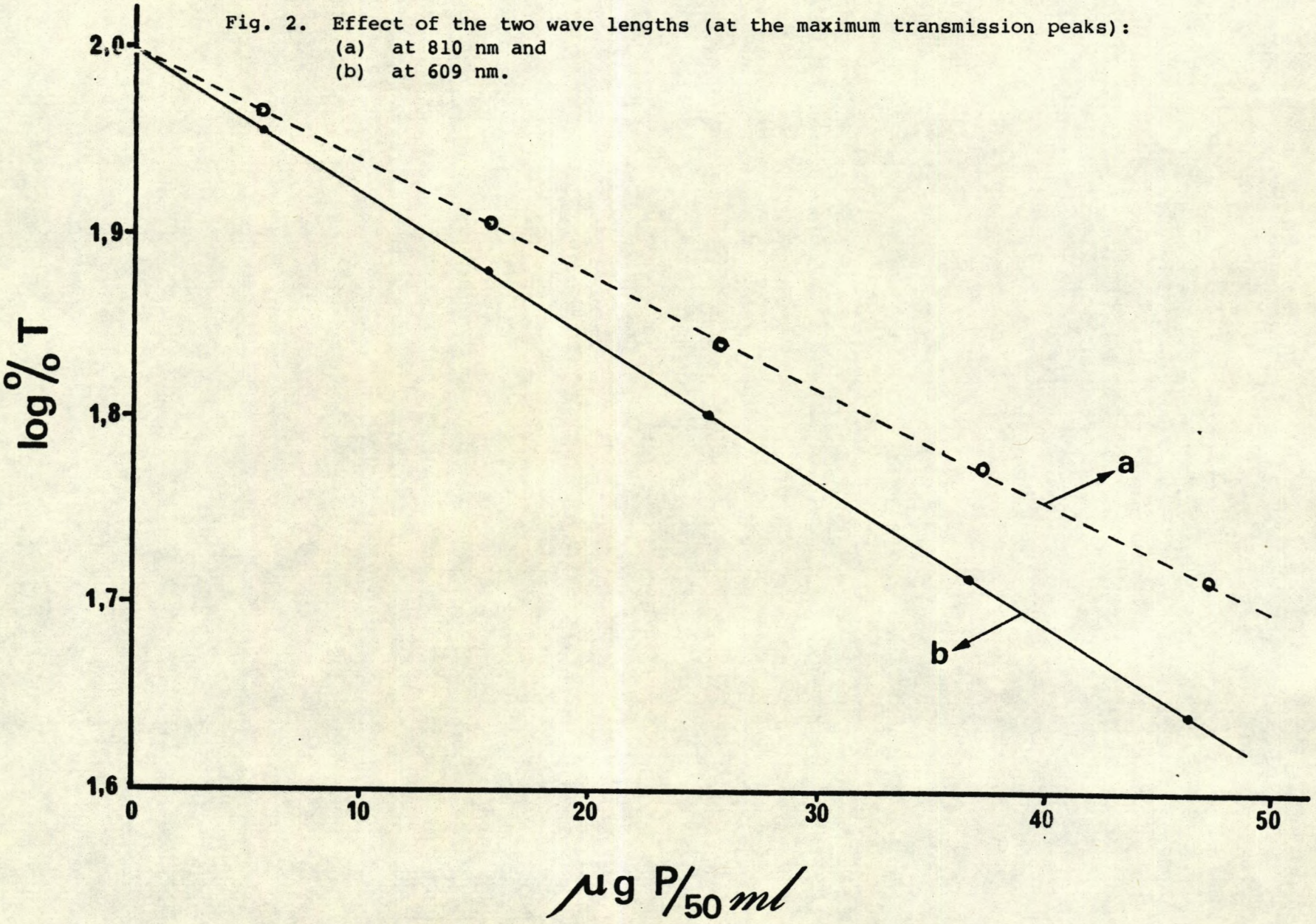
The transmission spectra of a coloured phosphomolybdate reduction product showed two peaks, namely at ~ 810 nm and at ~ 610 nm. The calibration curves at both wave lengths follow Beer's Law (Fig. 2). They are relatively close to each other and anyone of them can therefore be used for analysis. With the blank solution the calibration curves passed exactly through the 100 per cent transmission mark. The latter was not possible with water as the blank solution (Chen *et al*, 1956). The wave length used in this method is ~810 nm, while the one used for example in the ascorbic acid method is 820 nm. This difference is because a relatively simple inexpensive colorimeter (tested with Varian Techtron Model 635 spectrophotometer) was used.

Stability of the Reagent. The stability of the solutions at room temperature, as indicated by a setting of calibration curves, remained stable for a minimum of one week. In comparison some of the solutions used in the method of Chen *et al* (1956) had to be prepared fresh each day.

It was found that the plateau of absorbance (the ideal conditions) was obtained at hydrazinium sulphate concentrations of 0,0035 to 0,0140 per cent (Fig. 3), at sodium molybdate concentrations of 0,20 to 0,53 per cent (Fig. 4), and at acid concentrations of 0,82 to 1,35N (Fig. 5) (for phosphorus determinations tested up to 75 µg P/50 ml).

Effect of time and temperature. The colour intensity with the hydrazine sulphate method at room temperature (20°C) was 50 per cent complete after 3 hours. At 53°C it took 1 hour for maximum colour development. At 75°C the colour development was complete after 15 minutes with the hydrazinium sulphate method (according

Fig. 2. Effect of the two wave lengths (at the maximum transmission peaks):
(a) at 810 nm and
(b) at 609 nm.



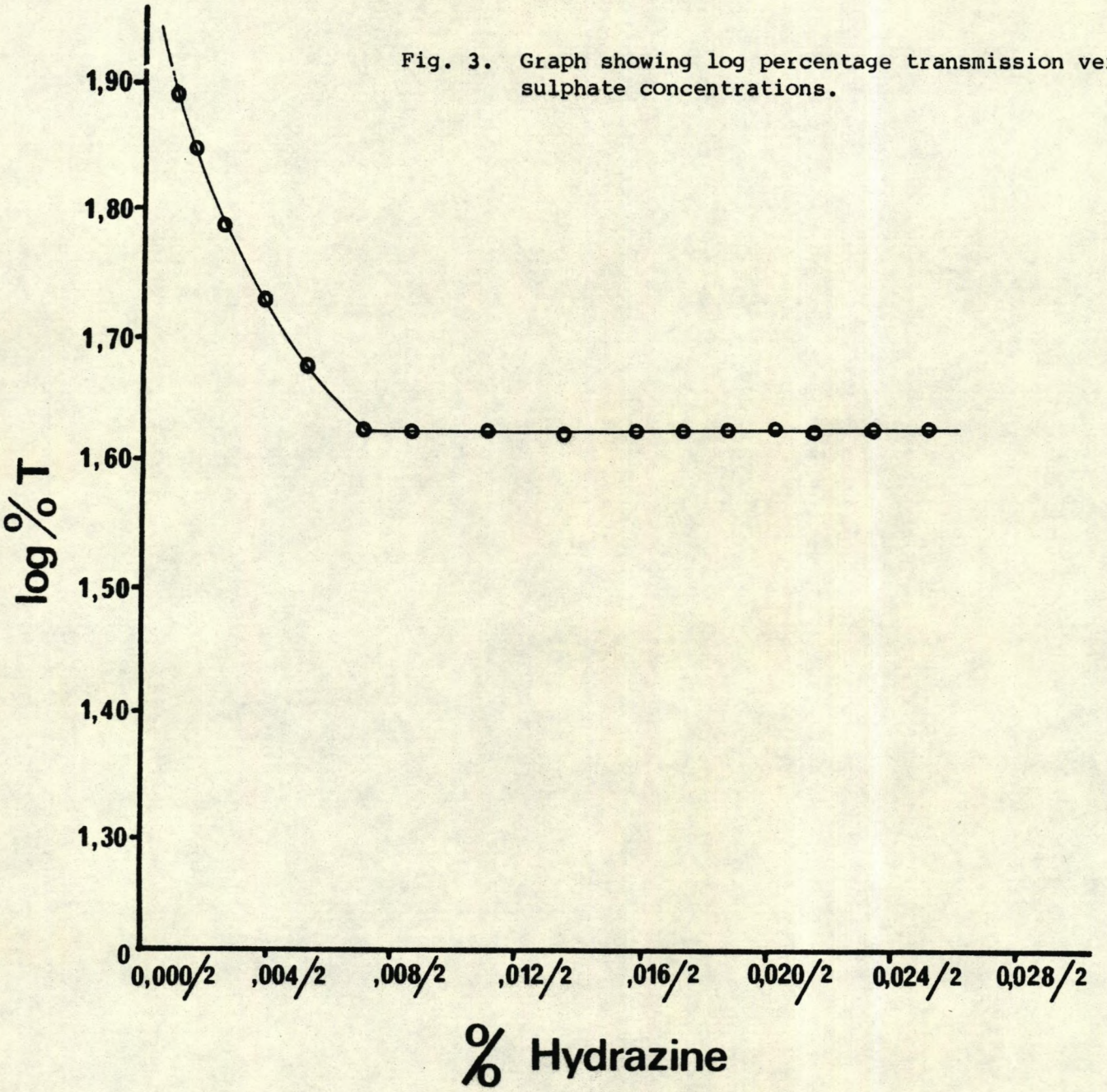


Fig. 3. Graph showing log percentage transmission versus different hydrazinium sulphate concentrations.

Fig. 4 Graph showing different sodium molybdate concentrations versus log percentage transmission.

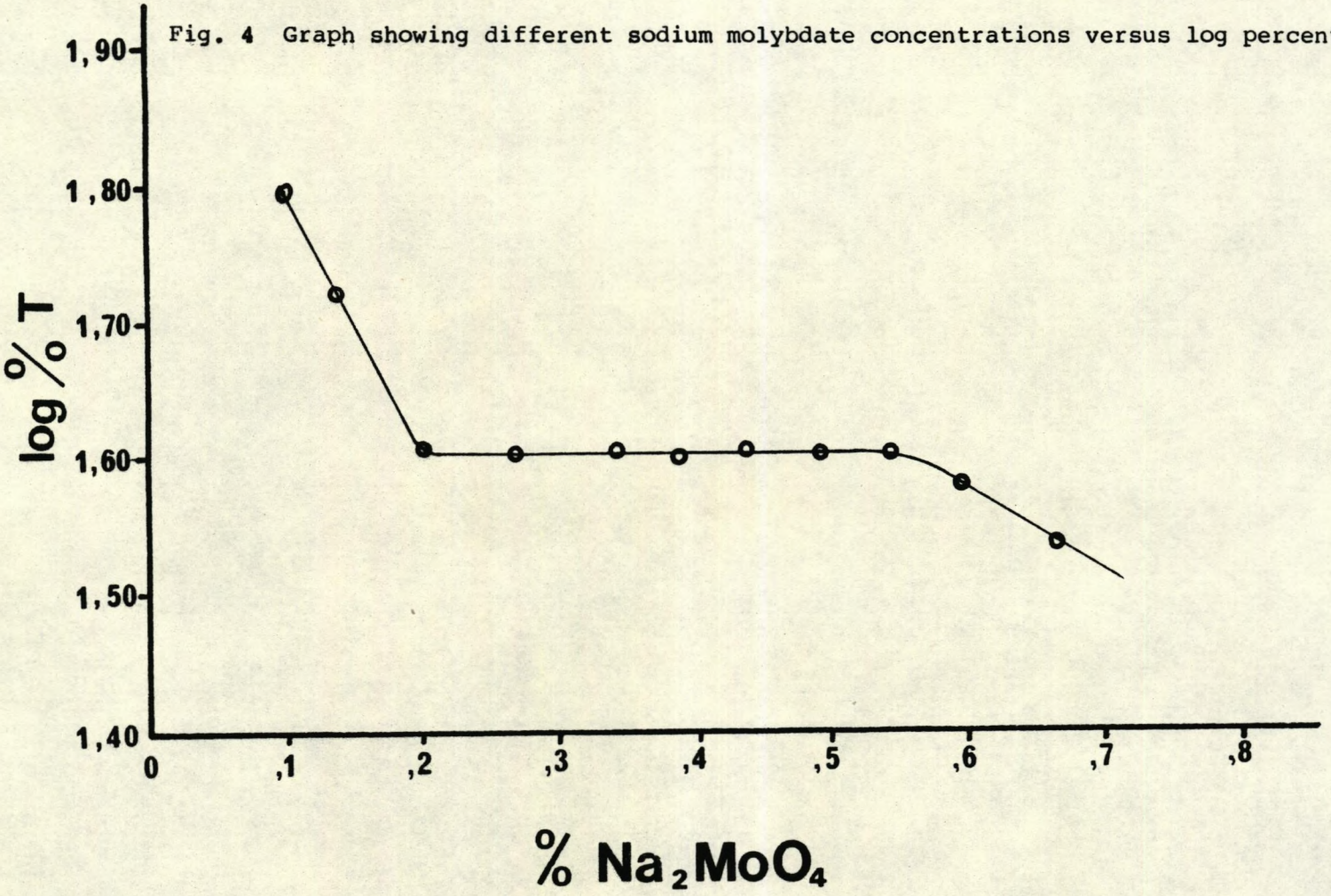
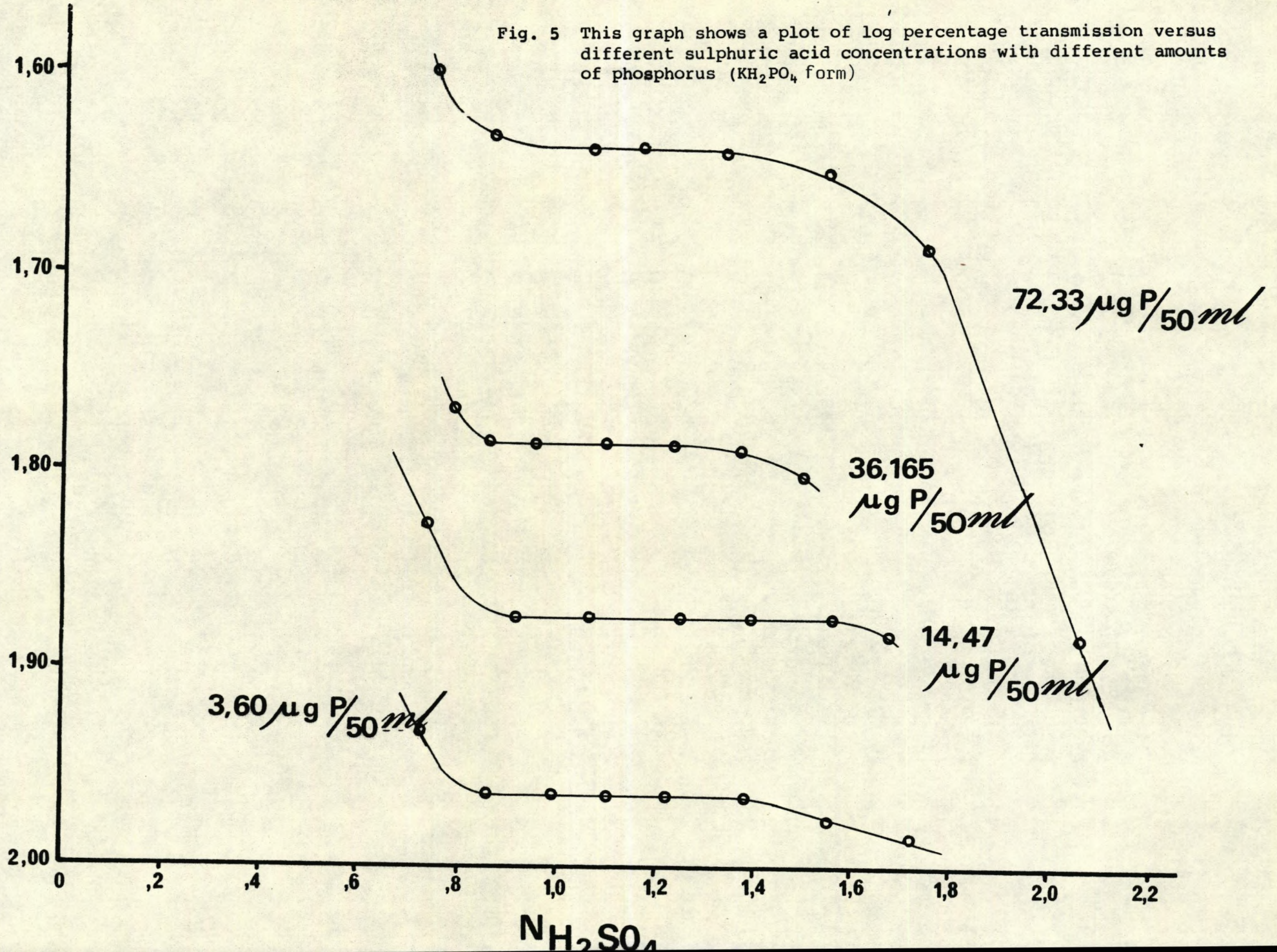


Fig. 5 This graph shows a plot of log percentage transmission versus different sulphuric acid concentrations with different amounts of phosphorus (KH_2PO_4 form)



to the authors this is the ideal time and temperature). Chen *et al* (1956) recommended 1 - 2 hours at 37°C for the ascorbic acid method.

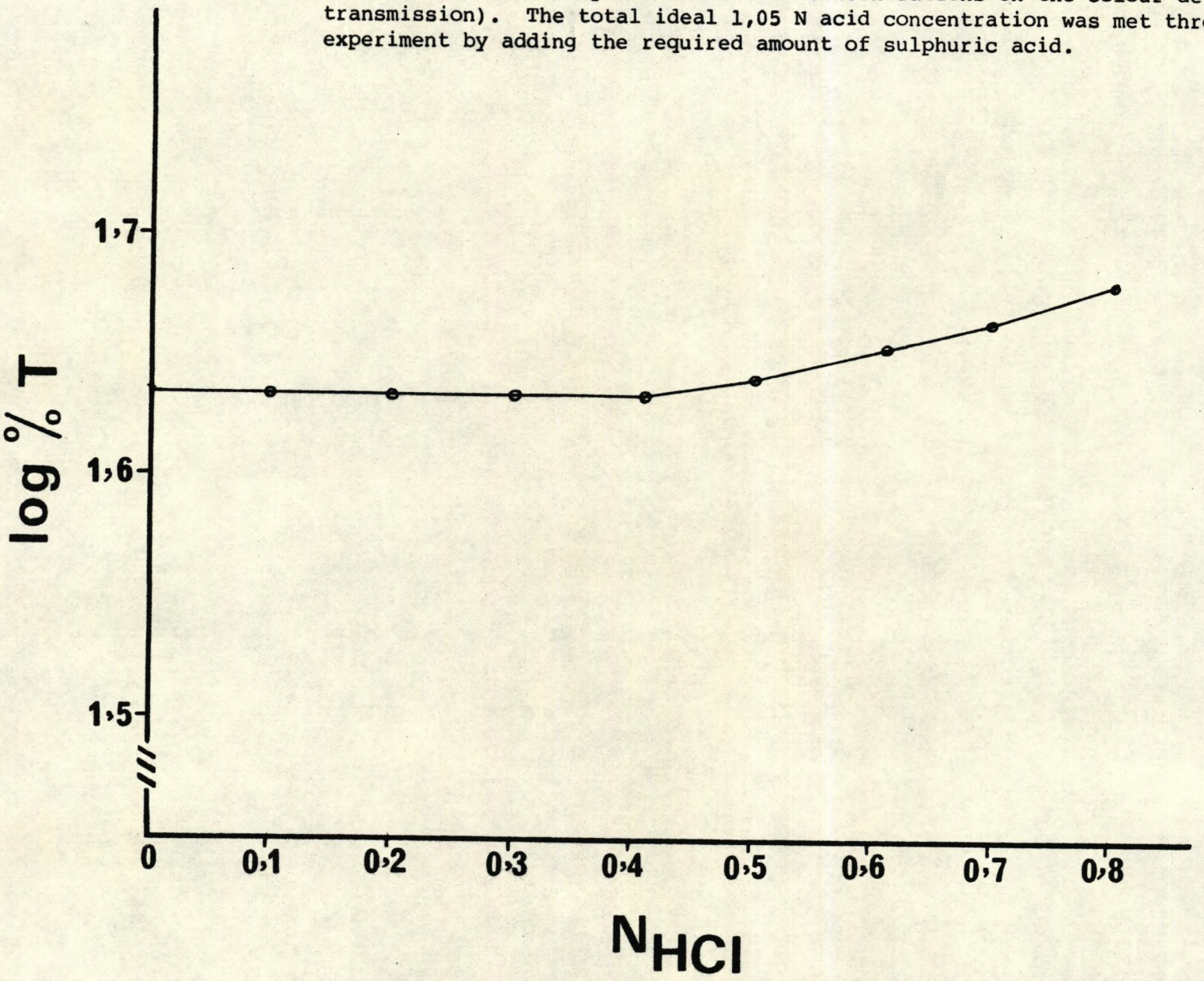
The Addition of Acids. Maximum colour development was inhibited by as little as $1,6 \times 10^{-4}$ M nitric acid and this became increasingly evident at higher concentrations. Similarly 0,5 M and stronger solutions of hydrochloric acid also affected colour development (Fig. 6).

Stability of the Molybdenum Blue. The colour intensity of the molybdenum blue solution formed by the reduction with hydrazinium sulphate, was stable for a minimum of 2 days at room temperature which was not the case with the reducing reagents of the tin (II) chloride and ascorbic acid methods. It was therefore unnecessary to read the percentage transmission immediately or after a precise time of reduction, as suggested by Kuttner and Cohen (1927), where the colour intensity decreased after 60 minutes. In the case of the ascorbic acid method the colour intensity increased only slightly after 1,5 hours (Chen *et al*, 1956), while Boltz and Mellon (1947) claimed a colour stability for only 12 hours in their hydrazine method.

Reproducibility. From Table I it is clear that the highest percentage standard deviation (%SD) is found with the tin (II) chloride method (2,14%) and the lowest with the hydrazinium sulphate method (1,00%).

Homogeneity of the Plaque Sample. From Table II it can be seen that the test for the homogeneity of the plaque sample was found to be good.

Fig. 6 Effect of different hydrochloric acid concentrations on the colour development (log % transmission). The total ideal 1,05 N acid concentration was met throughout this experiment by adding the required amount of sulphuric acid.



E. DISCUSSION AND CONCLUSION

As maximum colour development occurs between the concentrations of 0,82 and 1,35 N acid (Fig. 5), it would seem reasonable to assume that the midpoint between these concentrations (1,05 N) will allow for small variations in the acid concentration of the solution without affecting the results. This principle is important, because acids are usually used to dissolve biological material, as well as for the conversion of phosphates such as meta- and pyro-phosphates to orthophosphates. This value of 1,05 N is in good agreement with the value of 1,00 N (Vogel, 1961) and in reasonable agreement within the limits 0,75 to 1,25 N (Boltz and Mellon, 1947). The disagreement in the limits between the above values and my values (0,82 - 1,35 N) is possibly an acid standardization error in their case.

An advantage of the use of hydrazinium sulphate is that the salt contains crystal sulphuric acid, which does not affect colour development. My findings indicate that the concentrations of nitric acid and hydrochloric acid (Fig 6) should be appreciably less than that suggested by Kuttner and Cohen (1927). In fact, it would seem preferable not to use nitric acid at all. On the other hand, it appears that concentrations of up to 10 per cent trichloroacetic and 1,276 M perchloric acid can be used safely. When testing the influence of different acids it is important not to exceed the upper acid concentration limit of 1,35 N (Fig. 5). In order to achieve this the sulphuric acid concentration must be changed accordingly.

My ideal time for heating (15 minutes) at a temperature of 75°C in order to obtain maximum colour development is in relatively good agreement with the suggested time of 10 minutes at boiling point

(Vogel, 1961; Boltz and Mellon, 1947). The temperature of 75°C has been chosen because the high evaporation rate at boiling point will cause a greater volume decrease (practical problem). Furthermore, Stuart and Duff (1980) found a higher fluoride interference at higher temperatures. This effect may be due to the attack by hydrogen fluoride on the walls of the glass calibrated flasks used in the experiment when the acidic reagent is heated in the presence of high fluoride levels (0,02 M). The elimination of the effect is possible by the use of plastic instead of glass containers and by working at lower temperatures.

My recommended wavelength of 820 mμ is in good agreement with 830 mμ (Boltz and Mellon, 1947) and with 820 - 830 mμ (Vogel, 1961).

Beer's law (Fig. 2) applies for our system as tested from 0 - 75 μg P/50 ml, while Boltz and Mellon (1947) also recommended a phosphorus maximum of 75 μg P/50 ml (1,5 ppm).

The influence of 0,12, 0,50 and 0,75 per cent sodium molybdate (Boltz and Mellon, 1947) on the final solution has been investigated. I feel they should have used a smaller increment variation (Fig. 4) and disagree with their limits, especially with the use of 0,50 per cent molybdate which will fall outside the ideal range and will not be an ideal concentration. They also gave no figures as they did for some other influences. However, their suggested 2,5 per cent (0,25 per cent in final solution) amount of molybdate for general analysis also fall in the ideal range I recommend.

As calculated from the given amounts, Boltz and Mellon (1947) found 0,003 and 0,006 per cent hydrazinium sulphate in the final solution as ideal. Again they did not give the exact limits or any figures

and should have used smaller hydrazine concentration increments. According to my results (0,0035- 0,0140 per cent, Figure 3) their amount of 0,003 per cent is too low, while the suggested amount of 0,006 per cent falls well in my ideal condition range.

Although certain authors might believe that fluoride influences phosphate determinations, it was found that the tested fluoride levels of 0,95, 2660 and 3775 µg F^-/50 ml had no influence on my results, which is in good agreement with 3800 µg F^-/50 (0,004M) of Stuart and Duff (1980), while amounts of 0,02 M fluoride exhibited a pronounced positive effect according to the above authors. Fluoride levels need never occur in higher amounts during determinations of phosphorous in dental plaque.

The higher standard deviation with the Tin (II) chloride method (Table I and II) could be expected for three reasons.

1. The diluted tin (II) chloride solution should be prepared fresh every few minutes and therefore could cause a variation in the results. The same argument applies to the reagent in the ascorbic acid method but to a less extent, because this solution should be prepared fresh each day.

2. Reduction of the phosphomolybdic acid starts as soon as the tin (II) chloride solution is added and could therefore cause a variation in the results because the optimum conditions apply on the moment of reduction. Furthermore, the optimum conditions in the direct surroundings of the tin (II) chloride are changing while adding it and are different from the real optimum conditions. This is not the case with the other two methods, because reduction starts after complete mixing of the solutions.

3. The optimum conditions of some of the solutions during the tin (II) chloride reduction are very limited; that is, 0,73 - 0,75 per cent for tin (II) chloride in comparison to the hydrazimium sulphate and ascorbic acid methods. It is therefore easy to exceed the optimum conditions in the tin (II) chloride method when preparing solutions and during pipetting.

It was found in general that the standard deviation is slightly higher during the comparative application (Table II) on the homogeneous plaque sample in contrast with that done on the standard potassium dihydrogen phosphate solutions (Table I). Two reasons may be advanced for this.

1. A weighing error of 0,01 mg on 1,00 mg dry plaque containing 0,26 µg P/mg will cause a final error of 1 per cent.

2. The ashing techniques are more prone to experimental errors.

The finding of slightly more phosphorus by wet ashing (Table III (a)) in comparison to the dry ashings (Table III (b)) and (c) is possibly the result of the problem of solubilizing ash and/or ashing product. It is a problem to hydrolyse the ashing product of 1000°C, but good agitating and warming gave the results of Table III (c).
Therefore as a whole any one of the ashing techniques can be used, but the methods used must be strictly obeyed.

A further advantage of the hydrazinium sulphate method is that the time necessary to complete a phosphorus analysis is short, compared

to the other two methods. In addition as little as 1,81 µg P/50 ml can be determined reasonably accurately by both the ascorbic acid and tin (II) chloride methods, while Kuttner and Cohen (1927) gave a value of 10 µg P/50 ml.

As a result of the many advantages demonstrated in this paper, the hydrazinium sulphate method is recommended for the determination of phosphorus in dental plaque with wet ashing.

Acknowledgement

The author is grateful to the M.R.C. of the Republic of South Africa for financial assistance in undertaking this research.

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CHAPTER II

CALCIUM, PHOSPHORUS, FLUORIDE AND pH LEVELS OF HUMAN DENTAL PLAQUE FROM AREAS OF VARYING FLUORIDE LEVELS IN THE SOUTH WESTERN CAPE, REPUBLIC OF SOUTH AFRICA

A. SUMMARY

The total contents of calcium, phosphorus, fluoride and the pH of plaque in the 6 - 7 and 12 - 13 year age groups were determined in individual samples, of lifelong resident scholars from the natural fluoridated areas: Klipfontein (F = 2,5 ppm), Garies (F = 1,06 ppm) and Elim (F = 0,02 ppm). The mean concentrations were: fluoride = 125;31 and 2 ppm; phosphorus = 24; 23 and 17µg/mg;calcium = 7,3; 8,8; and 9,6µg/mg and the pH = 7,0; 6,4 and 5,9 for Klipfontein, Garies and Elim, respectively.

Very significant overall differences in means of the three areas were observed for pH, P, F and Ca/P. The differences in means for the two separate age groups for all three areas vary from very significant to significant except for Ca and P in the 6 - 7 year age group. A strong positive interdependence overall (three areas) correlation coefficient between elements is shown for most elemental correlations.

B. INTRODUCTION

Numerous studies have clearly established that dental plaque plays a significant role in the aetiology of the major dental disease of caries and periodontal disease (Loe, Theilaede and Jensen, 1965; Lindhe and Axelsson, 1978; Lovdal, Arno and Waerhaug, 1958).

Dental plaque which is largely a collection of bacteria has the ability to concentrate calcium, phosphorus (Dawes and Jenkins, 1962;

Schroeder, 1963; Grøn, Yao and Spinelli, 1969; Ashley and Wilson, 1978; Schamschula et al, 1977; Swift, 1967; Puttman, Bradshaw and Platt, 1966; Kleinberg et al, 1971, and 1977 ; Ashley, 1975) and fluoride (Hardwick and Leach, 1963; Dawes et al, 1965). It is postulated that during the carious process these elements would diffuse out from the tooth surface and dental plaque when the pH reaches a critical level of between 4,0 and 5,0. On the other hand, when these elements accumulate in dental plaque calculus formation is the result.

Several investigators have reported variation in calcium and phosphorus concentrations according to site of collection (Dawes and Jenkins, 1962; Schroeder, 1963; Ashley, 1975; Kaminsky and Kleinberg, 1967; Zuniga et al, 1973). The reports generally agree that plaque from lower incisor teeth have higher calcium and phosphorus levels than plaque from maxillary incisor teeth. It has been further found that the maxillary anterior plaque have a lower pH than mandibular anterior plaque. Plaque calcium and phosphorus also vary with the age of the patient, with plaque from 12 - 13 year old subjects having a lower level than 6 - 7 year olds (Kleinberg et al, 1971).

Since pH is an important factor in determining calcium-phosphate solubility it is conceivable that it would also be an important variable in affecting plaque calcium and phosphate levels.

Schamschula et al (1978) found that these elements in plaque are inversely related to dental caries and that there are probably common fundamental mechanisms regulating their concentrations in diverse populations.

The use of fluoride in combatting dental caries is well documented (Dean, 1938; Pameijer, Brudevold and Hunt, 1963; Birkeland, Jorkjend and Von der Fehr, 1971). Studies have found significant concentrations of fluoride in plaque and calculus and that these concentrations were related to the water fluoride levels (Dawes et al, 1965). Moreover, the affinity of fluoride for the calcium phosphates, particularly hydroxyapatite, is well established (McGrath, Featherstone and Smith, 1978). Birkeland (1973) observed that fluoride in plaque may be bound to a calcium phosphate precipitate. On the other hand Jenkins et al (1969) concluded that the bacterial cells within dental plaque may be an important source of fluoride storage. It has been found that about 2 - 5% of the total plaque fluoride is present as free fluoride ions (Jenkins, Edgar and Ferguson, 1969) and that these ions are released by a decreasing pH. A number of studies have demonstrated that the fluoride in plaque inhibits pH fall in the presence of sugar fermentation (Jenkins et al, 1969; Woolley and Richles, 1971).

I decided to study the concentrations of calcium, phosphorus, fluoride and the pH of dental plaque because of their well established relevance to enamel dissolution and remineralisation reactions in the oral cavity. The three areas chosen for study are unique in that they have differing levels of fluoride in the water and the subjects are life-long residents. The respective fluoride levels are Klipfontein (2,5 ppm), Garies (1,06 ppm) and Elim (0,02 ppm).

C. EXPERIMENTAL

This investigation was undertaken among scholars of Elim, Garies and Klipfontein situated in the south western part

of the Republic of South Africa. The subjects included 6 - 7 year old children and 12 - 13 year old children of both sexes. As far as can be ascertained these children were life-long residents of these areas.

On the first visit each subject was given a thorough dental prophylaxis so as to remove all visible plaque deposits. For the next 3 days the subjects were asked to refrain from all oral hygiene procedures. Plaque was collected on the morning of the 3rd day preceded by a 12 hour starvation period. A polyethylene spatula was used to remove plaque from all accessible surfaces of teeth. The plaque was then transferred immediately to preweighed polyethylene tubes, sealed stored at -7°C until analysis could be done. Normally it was analysed 2 - 3 days after collection.

pH Determination

The pH of the plaque samples was determined according to the methods of Luoma and Luoma (1968). Each of the polyethylene tubes was weighed for plaque mass deleminations. The suspension was agitated on a rotamixer for 10 minutes at 4°C. Thereafter the pH was determined using a pH glass combination electrode. (Beckman 39030 B3).

Fluoride Determination

Fluoride in the plaque samples was determined according to the methods of Grøn, Yao and Spinelli (1969), and McCann (1968). Plaque samples were homogenized by sonication and heated at 60°C in the polyethylene tubes to evaporate the water, before drying at 105°C for two hours. The plaque of half the number of subjects

was weighed and suspended in 1ml 0,5N perchloric acid for 3 hours by vigorous stirring on the rotamixser and the F^- was analysed in polyethylene tubes after addition of 4ml of 0,5M sodium citrate solution. The Orion fluoride ion selective electrode (F1052F) was used to measure the fluoride levels after calibration with a perchloric-sodium citrate buffer (same strength as in the plaque F^- measurement).

Phosphorus and Calcium Determinations

The dried plaque samples of the other half of the number of subjects were weighed in a platinum crucible, ashed at 600°C for 4 hours and the phosphorus determined by the hydrazine method (Grobler, Reddy and Van Wyk, 1980).

Calcium was determined by atomic absorption spectrophotometry (Pye Unican SP 90). This was done by diluting 0,20 ml of the above diluted ashed solution (Grobler et al, 1980) to 4,00 ml with 16% lanthanum nitrate. Calcium standards were used containing lanthanum nitrate and sulphuric acid in concentrations matching those of the samples.

Statistical Analysis

The mean values and standard deviations for the different levels of calcium, phosphorus and fluoride in plaque were calculated for (1) each of the two age groups in each area (2) for the two age groups combined in each area (Table 1). Secondly, analysis of variance was used to test for significant differences between means of all three areas (Table 2). Thirdly, the product-moment correlation coefficients were determined between pairs of mineral levels in plaque for each age group in each area and overall.

D. RESULTS

Since analysis of the data showed no sex differences in the concentrations of calcium, phosphorus or fluoride or in pH, the results were combined. Results for the three areas of Klipfontein, Garies and Elim are expressed first separately for the 6 - 7 year age group and 12 - 13 year age group and secondly together.

Table 1: The mean values (and s.d.) for the amounts of different elements in plaque in the age groups separately, as well as combined, for the three areas.

		Klipfontein (F = 2,5 ppm)			Garies (F = 1,06 ppm)			Elim (F = 0.02 ppm)		
		6-7	12-13	Overall	6-7	12-13	Overall	6-7	12-13	Overall
pH	n*	14	31	45	30	35	65	18	21	35
	x̄	7,039	6,797	7,010	6,293	6,525	6,418	5,790	6,064	5,938
	s	0,267	0,298	0,287	0,236	0,263	0,275	0,595	0,364	0,497
Ca µg/mg	n*	8	16	24	14	15	32	17	20	37
	x̄	3,184	6,920	7,341	10,779	7,259	8,816	12,006	7,575	9,611
	s	1,971	2,630	2,463	5,403	2,562	4,351	5,315	3,367	4,853
P µg/mg	n*	8	15	23	14	18	32	17	19	36
	x̄	27,520	22,462	24,221	21,314	24,400	23,050	18,012	16,416	17,169
	s	11,085	10,391	10,659	9,899	6,354	8,103	7,183	8,332	7,743
F ppm	n*	11	15	26	15	11	26	18	18	36
	x̄	144,546	111,693	125,592	38,400	20,155	30,681	2,606	1,461	2,033
	s	106,000	94,923	99,020	38,691	17,975	32,435	2,466	1,068	1,961
Ca/F	n*	3	15	23	14	18	32	17	19	36
	x̄	0,322	0,333	0,329	0,564	0,312	0,423	0,753	0,561	0,652
	s	0,078	0,100	0,091	0,266	0,119	0,231	0,509	0,321	0,425

Where : * - number of subjects; x̄ - mean; s - standard deviation

The pH, P and F values within each age group in each area, as well as for the combined age group, decrease with decreasing fluoride levels of the drinking water with the exception of the 12 - 13 year age group for Garies. On the other hand the Ca and Ca/F levels increase with decreasing fluoride in the water, again except for the 12 - 13 year age group in Garies.

Analysis of variance to test for difference between the means in the 6 - 7 and 12 - 13 year age group separately, as well as combined, is shown in Table 2.

Table 2: Analysis of the variance to test for differences between the means of all three areas.

	ANOVA		
	F-TEST FOR DIFFERENCE IN MEANS		
	6-7 years	12-13 years	Overall
pH	$2^{a}59^{b}$ $42,44^{c}$ $p \leq 0,0000^{d}$	$2^{a}84^{b}$ $60,64^{c}$ $p \leq 0,0000^{d}$	$2^{a}146^{b}$ $99,58^{c}$ $p \leq 0,0000^{d}$
Ca µg/mg	$2^{a}36^{b}$ $1,67^{c}$ $p \leq 0,2032^{d}$	$2^{a}51^{b}$ $0,23^{c}$ $p \leq 0,7987^{d}$	$2^{a}90^{b}$ $2,15^{c}$ $p \leq 0,12$
P µg/mg	$2^{a}36^{b}$ $2,99^{c}$ $p \leq 0,0626^{d}$	$2^{a}49^{b}$ $4,55^{c}$ $p \leq 0,0154^{d}$	$2^{a}55^{b}$ $5,98^{c}$ $p \leq 0,005^{d}$
F ppm	$2^{a}41^{b}$ $21,62^{c}$ $p \leq 0.0358^{d}$	$2^{a}41^{b}$ $17,04^{c}$ $p \leq 0,0013^{d}$	$2^{a}85^{b}$ $37,58^{c}$ $p \leq 0,0000^{d}$
Ca/P	$2^{a}36^{b}$ $3,66^{c}$ $p \leq 0,0358^{d}$	$2^{a}49^{b}$ $7,61^{c}$ $p \leq 0,0013^{d}$	$2^{a}88^{b}$ $9,86^{c}$ $p \leq 0,0000^{d}$

Where: a - df_1; b - df_2; c - F; d - p value

This table is therefore representative for the values df_1, df_2, F and p of all the areas in the two age groups separately and combined. Very significant overall differences in means of the three areas are observed for pH (p = 0,0000), P (p = 0,0000), F (p = 0,0000) and Ca/P (p = 0,0000), while no significant value could be obtained for Ca^{++}(p = 0,12). The difference in means for the 2 separate age groups for all three areas vary from very significant to significant except for Ca and for P in the 6 - 7 year age group (p = 0,06).

Table 3 shows interdependence between elements, in the two age groups and when combined for the three areas at various levels for clusters of correlation coefficients for the concentrations of pairs of elements in plaque. The amount of plaque per subject was too small in determining Ca, P and F with the methods employed. Therefore, no correlation coefficient could be obtained for calcium versus F or phosphorus versus F in individual subjects. For calcium versus pH, except for the 1% correlation found in the 6 - 7 year group in Garies (F^- = 1,06 ppm), no significant further correlations, even for all the areas,exist. However, a strong positive (three area) overall correlation coefficient based on one percent is shown for all the other elemental correlations, except for Ca/P versus pH, and Ca/P versus P with a significant negative correlation (p<o,01) coefficient.

The following is a summary of further correlation results with pairs of elements:

1) <u>P versus pH</u>.

A significant value (p<0,05) exists for both age groups of Garies, but there is also a significant correlation for the separate combined two age groups of the three areas.

Table 3: Product-moment correlation coefficients between minerals in plaque: calcium, total phosphorus, fluoride and pH.

	pH	Ca	P
Ca	0,5166(8)[a] 0,0806(16)[b] 0,6998(14)**[c] 0,3515(18)[d] 0,2393(17)[e] 0,0896(20)[f] 0,0030(39)[g] 0,0207(54)[h] -0,0837(93)[i]		
P	0,4002(8)[a] 0,2292(15)[b] 0,6311(14)*[c] 0,5004(18)*[d] 0,4308(17)[e] 0,3867(19)[f] 0,5351(39)**[g] 0,4108(52)**[h] 0,4562(91)*[i]	0,7089(8)*[a] 0,8198(15)**[b] 0,5873(14)*[c] 0,3103(18)[d] 0,1660(17)[e] 0,7835(19)**[f] 0,2336(39)[g] 0,5794(52)**[h] 0,3435(19)**[i]	
F	0,6506(11)*[a] 0,1379(15)[b] 0,5513(15)*[c] -0,1345(10)[d] 0,2469(18)[e] 0,3352(18)[f] 0,6600(44)**[g] 0,5114(43)**[h] 0,5763(87)**[i]		
Ca/P	-0,3491(8)[a] -0,3059(15)[b] 0,4227(14)[c] -0,1130(18)[d] -0,1275(17)[e] -0,5003(19)*[f] -0,3742(39)*[g] -0,4970(54)**[h] -0,4549(91)**[i]	-0,3738(8)[a] 0,0432(15)[b] 0,3009(14)[c] 0,6697(18)x*[d] 0,7809(17)**[e] -0,0456(19)[f] 0,5979(39)**[g] 0,1210(52)[h] 0,5054(91)x*[i]	-0,8815(8)**[a] -0,5094(15)[b] -0,5101(19)[c] -0,4610(18)[d] -0,4547(17)[e] -0,5916(19)**[f] -0,4936(39)**[g] -0,5727(52)**[h] -0,4900(91)**[i]

Where: *p <0,05 a - Klipfontein 6 - 7 years e - Elim 6 - 7 years
**p<0,01 b - Klipfontein 12-13 years f - Elim 12-13 years
c - Garies 6 - 7 years g - Average 6 - 7 years
d - Garies 12-13 years h - Average 12-13 years

2) P versus Ca.

Significant correlation coefficients exist for the Klipfontein 6 - 7 ($p<0,05$) and 12 - 13 ($p<0,01$) years, for the Garies 6 - 7 years ($p<0,05$), for the Elim 12 - 13 years ($p<0,01$), as well as for the combined 12 - 13 years ($p 0,01$) of all areas.

3) F versus pH.

A significant correlation value of $p<0,05$ is found for both Klipfontein and Garies 6 - 7 years and also for the separate two age groups ($p<0,01$) combined for all the areas.

4) Ca/P versus pH.

All the age groups in the different areas show a significant correlation for the separate combination of the two age groups of the three areas, but also for the 12 - 13 years ($p<0,05$) of Elim.

5) Ca/P versus Ca.

These correlation values are not consistent, but there is a significant positive correlation of $p<0,01$ for the 12 - 13 years of Garies, the 6 - 7 years of Elim and for the combined 6 - 7 years of Elim and for the combined 6 - 7 years for all the areas. A negative correlation is found for both the 6 - 7 years of Klipfontein and for the 12 - 13 years of Elim.

6) Ca/P versus P.

The correlation value is significant for the 12 - 13 years ($p<0,01$) for Elim, the 6 - 7 years ($p<0,01$) for Klipfontein, as well as for the combined 6 - 7 years ($p<0,01$) of all the areas and also for the combined 12 - 13 years ($p<0,01$).

The present analysed elements have also been studied by some other authors. For comparison with my results, the results in Table 4 were selected and summarized from other relevant studies.

E. DISCUSSION AND CONCLUSION

As it has been reported that the Ca, P and H^+ concentrations in plaque vary with time since the last nutrition and beverage (Ashley, 1976; Edgar, Jenkins and Tatevossian, 1970; Kleinberg and Jenkins, 1964) the plaque was collected 12 hours prior to starvation. After this starvation-time it was shown that association between dietary sugar experience and plaque H^+, Ca and P concentrations was very limited (Ashley and Wilson, 1977 ; Kleinberg and Jenkins, 1964).

That dental plaque can store significant amounts of Ca and P is confirmed in this study. The source of these two elements, however, is not clear. Gingival crevicular fluid which has higher levels of Ca than saliva is a possible source (Kaslick et al, 1980).

A second possibility is that these elements are the result of acid decalcification of the tooth. The third possible source is that Ca and P are derived from saliva and is deposited during plaque formation as a Ca-P carbohydrate-protein complex (Kleinberg et al, 1971; Kaufman and Kleinberg, 1973). The levels of Ca and P found in this study are in good agreement with that of a number of other published studies (Table 4). Kleinberg (1970) has postulated that this reservoir of calcium phosphate at the plaque enamel interface would protect the tooth from demineralization should the pH fall to a critical level. This may explain Schamschula's (1978) findings of an inverse correlation between these two elements in dental plaque and caries. On the other hand, an alkaline pH would favour calcification of these deposits into calculus.

Several interesting findings of this study need to be explained. Firstly, the Ca concentration in plaque is inversely correlated with the fluoride level of the water. This is in agreement with the results of Kleinberg et al (1977). The latter authors attribute this unusual finding to the

Table 4: Mean concentrations of some elements in human dental plaque compared with published data.

	Age (years)	n	F[1] (ppm)	Ca (µg/mg)[x]	Total p[x] (µg/mg)	F(ppm) in plaque	Fasting pH
Present Study: 1)Klipfontein	6-7	See Table 1	2,5	8,2	27,5	144,5	7,04
	11-13		2,5	6,9	22,5	111,7	7,00
2)Garies	6-7		1,06	10,8	21,3	38,4	6,29
	11-13		1,06	7,3	24,4	20,2	6,53
3)Elim	6-7		<0,02	12,0	18,0	2,6	5,79
	11-13		<0,02	7,6	16,4	1,5	6,06
Ashley and Wilson (1978)	young adults	43		5,27			
Schamschula et al (1977)							
1) Katoomba	9,7-13	26	<0,1	7,1	15,0		
2) Sydney	9,7-13	21	1	7,2	13,2		
3) Yass	9,7-13	25	1	7,4	14,3		
Schamschula et al (1977)	12-24	301	0,052	24,4	16,9	37	
Ashley (1975)	11-14	33	probably low	5,5	13,8		
Ashley (1975)	19,-21	68		14,8-5,43	15,1-10,8		
	11-12	39		12,4-5,7	14,5-10,2		
Kleinberg et al (1971)	12-14	7		~30 to ~5	~32 to ~14		
Grøn et al (1969)	adults	8	low	16,8	12,6+	25,2	
Swift (1967)				~30	~30		
Puttman et al (1966)	adults	5(11)*		2,2	4,5		
Dawes et al (1965)							
1) North Shield	11	53(25)*	low			125(25[2])	
2) West Hartlepool	11	57(22)*	~2			235(47[2])	
Schroeder (1963)	students	5		14,0	13,9		
Dawes and Jenkins (1962)	students	41-85		4,5	16,5		
	11	19-21		3,8	18,0		
Jenkins et al (1969)							
1) Tyneside	11		low				6,29
2) Watford	11		1				6,38
3) West Hartlepool	11		1,8				6,32

Where:

1. Fluoride level in drinking water supply. n-the number of subjects. x-dry weight. *-No of samples.
2. Determined on assumption that 80% of wet plaque is water.

systematic effects of fluoride. High fluoride levels favour the formation of a less soluble fluorapatite in the calcified tissues of the body. This in turn would mean that less Ca and more P are being lost through secretions such as urine, saliva, sweat. Since saliva appears to be the principal source of plaque, Ca and P, the lower salivary levels of Ca would be reflected in the plaque.

Recently, Ingram and Nash (1979) have shown that one possible anti-cariogenic mechanism of fluoride is that it reverses the outflow of Ca-P from the tooth surface; in other words, during the mineralization phase, in the presence of F at the plaque-enamel interface, Ca is preferentially deposited into the tooth - this would deplete Ca-relative to P in plaque, thus explaining the findings in my study.

Another interesting finding is the significant decrease of Ca plaque-levels in the 12 - 13 year age group compared to the 6 - 7 year olds. The level of fluoride in plaque shows a similar decrease - one possible explanation is that the 12 year olds are in a relative growth phase, particularly with respect to the calcified tissues. Consequently these elements are utilised, causing at first a depletion in the body fluids and subsequently in the plaque levels.

The plaque F levels decrease with decreasing levels of F in the water. There is, however, considerably higher levels of fluoride in the 6 - 7 year olds as compared to the 12 - 13 year olds. This positive correlation between plaque F levels and F levels in the water has been well established (Dawes et al, 1965; Hardwick and Leach, 1963). Although most of the plaque fluoride is in bound form (Jenkins, Edgar and Ferguson, 1969), it appears that some, if not a large part of it, is present within the

bacteria. Another possibility is that it is complexed to the Ca-P salts in the form $Ca_{10}(PO_4)_6(OH)_2$, $CaPO_3F$ or $Ca(PO_2F_2)$ (Birkeland, 1973). Jenkins et al (1969) also found with chromatography studies some indication of fluoro-phosphate in plaque.

The inhibition of acid production in dental plaque and oral bacteria by low levels of F is well established (Wright and Jenkins, 1954; Weiss et al, 1965; Hamilton, 1969 ; Jenkins, 1967). Kanapka et al (1971) demonstrated that 2,4M fluoride inhibited glucose uptake, enolase activity and glucose-6-P formation in Streptococcus salivarius. It is possible that either fluoride interferes with the transport of glucose through the bacterial cell wall or it inhibits one or more enzymes along the glycolytic pathway.

Schamschula et al (1978) reported a high consistency in the magnitude of the correlation coefficients between the towns Katoomba (F in water 0,1 ppm), Sydney (F in water 1 ppm) and Yass (F in water 1 ppm), as well as over the whole sample, while the consistency is weakened by values of Katoomba with its low fluoride level in the drinking water supply. Their overall correlation coefficient value of 0,51 for Ca versus F is in good agreement with my overall value of 0,57 (Table 3). The strength of correlation between Ca and P indicates (Schamschula et al, 1978) that the concentration ratios of these elements are nearly constant for all areas. This is directly in contrast with my results, where the Ca/P ration increases from about 0,30 through 0,38 to about 0,56 as the fluoride concentrations decrease from Klipfontein through Garies to Elim, respectively.

I regard the values of Swift (1967) and Puttman, Bradshaw and Platt

(1966) as approximations and not representative of populations. (Table 4). The published Ca and F levels of plaque are subject to more variations than the phosphorus levels. However, my mean Ca levels of all areas are in relatively good agreement to that of New South Wales with fluoride levels in the drinking water supply varying between 0,1 to 1 ppm. The mean Ca level of New Guinea (24,4) is more than twice as high my results (Table 4), while the mean plaque Ca levels of the English school children (Ashley, 1975) and students (Ashley, 1975) are also in good agreement with my results.

The mean total phosphorus value for Elim (~17) with 0,02 ppm F in the drinking water is in very good agreement with the New Guinea phosphorus value (16,9) where the F level of New Guinea falls between those found in the largest samples drawn from Western communities (Agus et al, 1976 ; Birkeland, Jorkjend and Von der Fehr, 1971), but is about 18 times higher than my comparable areas (Elim). As a matter of fact, such small plaque fluoride levels of 1,5 to 2,6 ppm (Table 4) were never reported before.

Dawes et al (1965) found 125 ppm F for children in the North Shield area, with low F in the drinking water which is more than 50 times the F levels of my comparable area, Elim (Table 4). However, the plaque F level of 235 ppm of children in the West Hartlepool area is nearly twice as high as the overall average of the two age groups (~128 ppm) of Klipfontein.

The Ca results (Table 2) of this study show no significant differences among the means of all three areas. However, the means of both age groups (Table 4) increase with decreasing F in the drinking water. In comparing the Ca results (Table 4) of the two age groups within each area, more Ca is found in the 6 - 7 year age group than in the 12 - 13 year group. In contrast, the P levels decrease with decreasing F, while the

6 - 7 year age group of all three areas contains more P than the corresponding area and age group. The present study also shows a general trend in the plaque F levels. These levels decrease with decreasing F in the drinking water, but with more plaque F in the 6 - 7 year age groups than in the 11 - 13 year age groups. This effect is found in all three areas.

The resting pH values of the three areas decrease with decreasing F in the plaque, while the mean pH values of the 6 - 7 year age groups of both Garies and Elim areas are lower than that of the 11 - 13 year age groups. Jenkins et al (1969) also reported a mean lower resting plaque pH value for the low water fluoride Tyneside area, than for the Watford and West Hartlepool areas (Table 4).

A possible explanation for the Ca, P and F plaque level trend in the three areas (Table 1) could be the formation of certain compounds in the following sequence: Ca_3PO_4; $CaPO_3F$ and $Ca(PO_2F_2)$ with increasing F level in the drinking water supply. Furthermore, Birkeland's observations indicated that fluoride in plaque may be bound to a calcium phosphate precipitate and that the binding is affected by the pH. Jenkins et al (1969) also found with chromatography studies some indication of pure fluorophosphate in plaque.

The very high variations of results stated in Table 4 might be due to many factors, such as population selection, age and dietary habits of subjects, sites of plaque collections, plaque age and oral hygiene practices.

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CHAPTER III

CARBOHYDRATE FERMENTATION BY HUMAN DENTAL PLAQUE

A. SUMMARY

The degree of fermentation of very small concentrations of pentoses, hexoses and various sugar analogues by human dental plaque was investigated. It was found that glucose, fructose, mannose, galactose, sucrose, lactose and maltose are fermented to a pH value of less than 5,5. Plaque also breaks down xylulose, trehalose and melibiose but to a less extent.

B. INTRODUCTION

It is widely accepted that dental caries can be controlled by a reduction in the frequency of intake of fermentable carbohydrates. Dental plaque is a community of micro-organisms of which some will fermentate certain carbohydrates,which will cause a rapid production of large quantities of acid; thus the pH of the plaque is lowered and consequently that of the tooth surface (Stephan, 1940; Kleinberg and Jenkins, 1964). Fermentation of dietary carbohydrate results in a decrease in the plaque-pH; a condition conductive to enamel dissolution and the initiation of the dental caries lesion (Stephan, 1944; Fosdick and Starke, 1939). The association of fermentable sugars with dental caries had led to attempts to reduce the consumption of sucrose or increase of substitute sweeteners in diets. The cariogenic nature of sucrose (Gustaffson et al, 1954) had led to an intensive search for alternative compounds possessing sweetness, but no cariogenicity (Drucker, 1979). A number of sweeteners have been tested in vitro for their effects upon acidogenicity of plaque or suspensions of S mutans (Druker and Verran, 1980; Edwardson et al, 1977). Higher pH values were obtained with sorbitol (Cornick and Bowen, 1972), trichlorosucrose, and xylitol (Drucker and Verran, 1980), while inhibition of acid production occured on the addition of 2-deoxy-D-glucose, 5-thio-D-glucose, cellobiose (Roberts and Hayes, 1980) and xylitol (Mäkinen, 1976; Gehring et al, 1976; Gallagher and Fussel, 1979).

Sugars such as sucrose, glucose, maltose and fructose are readily fermented by plaque to acid, both in vitro (Frostell, 1964) and in vivo (Frostell, 1973;

Neff, 1967). However, the fermentation of some sugar alcohols by plaque, in vivo (Ahlden and Frostell, 1975; Frostell, 1973), in suspension (Birkhed, 1978; Frostell, 1964; Hayes and Roberts, 1978) and to cultures (Edwardsson et al , 1977; Gehring et al , 1976) gives little or no acid production.

Various attempts have been made to study the effect of acids on decalcification of tooth enamel (Jenkins, 1966; Fosdick, 1938; McCann, 1968; Leach, 1959). A compound can only dissolve if its solubility product value is exceeded, while factors like variations in Ca, P, F, pH, ionic strength and temperature affect the solubility of compounds.

The present investigation was carried out to determine the in vitro fermentability of the pentoses, hexoses and other sugar analogues in pooled human dental plaque bacteria. This was done by measuring the potential change (pH drop) in the absence of CO_2, from as low as 10^{-6}M of different sugar analogous, during fermentation. Freshly collected human plaque samples, containing a mixed collection of bacteria, was used as it is believed that the joint action of different bacteria types is important in the carious process.

C. EXPERIMENTAL

Apparatus

Potential measurements were made with a Corning Model 12 Research pH-meter in conjunction with a Heath-Schlumberger strip-chart recorder, Model SR-255-B. A Corning flat-surface pH combination electrode (cat no. 476216) was used and measurements were made in a sealed cell kept at 37° ± 0,2°C.

Reagents

Normal 24hr-old human dental plaque in a non-fluoridated area was used as the source of the test fermenting bacteria. Such plaque was collected from volunteers as described in the procedure.

The carbohydrates or carbohydrate analogues: β-D(+)glucose, β-D(-)fructose, D(+)mannose, D(+)galactose, L(-)mannose, β-D-allose, D-altrose, D-gulose, D-idose, D-talose, L(-)sorbose, D(+)tagatose, D(-)arabinose, L(+)arabinose D(+)xylose, L(-)xylose, D-ribose, L(-)ribose, D-lyxose, L-lyxose, D-ribulose, D-xylulose, sucrose, lactose, maltose, D(+)trehalose, β-D(+)cellobiose, α-D(+)melibiose, α-L-rhamnose, dulcitol, meso-inositol, α- L (-) fucose, -D(+)fucose, adonitol, D(+)arabitol, L(-)arabitol, D-erythrose, and i-erythritol in purest form available were used without further purification.

All other chemicals were of analytical grade. Glass distilled water was used in all the experimental work.

Procedure

The 24 hour human dental plaque was collected from 5 participants, whose teeth had previously been cleaned and who had fasted for 10 hrs prior to each collection. The plaque was collected under sterile conditions by scraping it from all avail= able tooth surfaces, immediately suspendend in sterilized Ringer's solution (consisting of 0,015 M sodium chloride; $3{,}1 \times 10^{-4}$M potassium chloride and $2{,}2 \times 10^{-4}$M calcium chloride) and cooled to 4°C. The pH of the Ringer solution was set at 6,95 with a diluted sodium hydroxide solution prior to use. The cold pooled plaque was homogenized by light ultrasonics and washed once with Ringer's solution (pH = 6,95).

Before each plaque collection, a dialysis membrane (molecular weight cut-off 10^{-4}) was soaked for 1 hr in Ringer's solution and put over the sensitive surface of the glass electrode, where it was held by an elastic band. This assembly was soaked for another 2 hrs in Ringer's solution to mould the membrane. The glass electrode was then inverted, the membrane removed and ~2mg of the washed plaque was placed on the flat sensitive part of the electrode, where it was kept in position by replacement of the dialysis membrane. Only plaque stored for not longer than 1 hour in Ringer's solution at 4°C was used for experimental work.

Measurements were made in 1,50 ml of Ringer's solution (pH = 6,95), contained in a cell sealed from the atmosphere. The space above the solution was flushed with nitrogen gas. Potential (mV) versus molar carbohydrate (or carbohydrate analogues) solution curves were constructed by adding successive portions of a carbohydrate solutions and by measuring the potential of the cell by the use of the pH meter-glass electrode combination. After each carbohydrate solution addition of known concentration (~0,0050M) time was allowed for the potential reading to stabilize and the mV(or pH) reading was noted.

D. RESULTS

Figure 1 representative of a pH flat surface combination glass electrode-plaque assembly.

Table 1 gives the hexoses (monosaccharides) which will be fermented by the 24 hr plaque, as well as the possibility of the specific carbohydrate to occur in nature in a free form. (Brimacombe, 1976).

The degree of fermentation of the pentoses by human plaque is given in Table 2,

Fig. 1

Schematic diagram of the plaque electrode assembly.

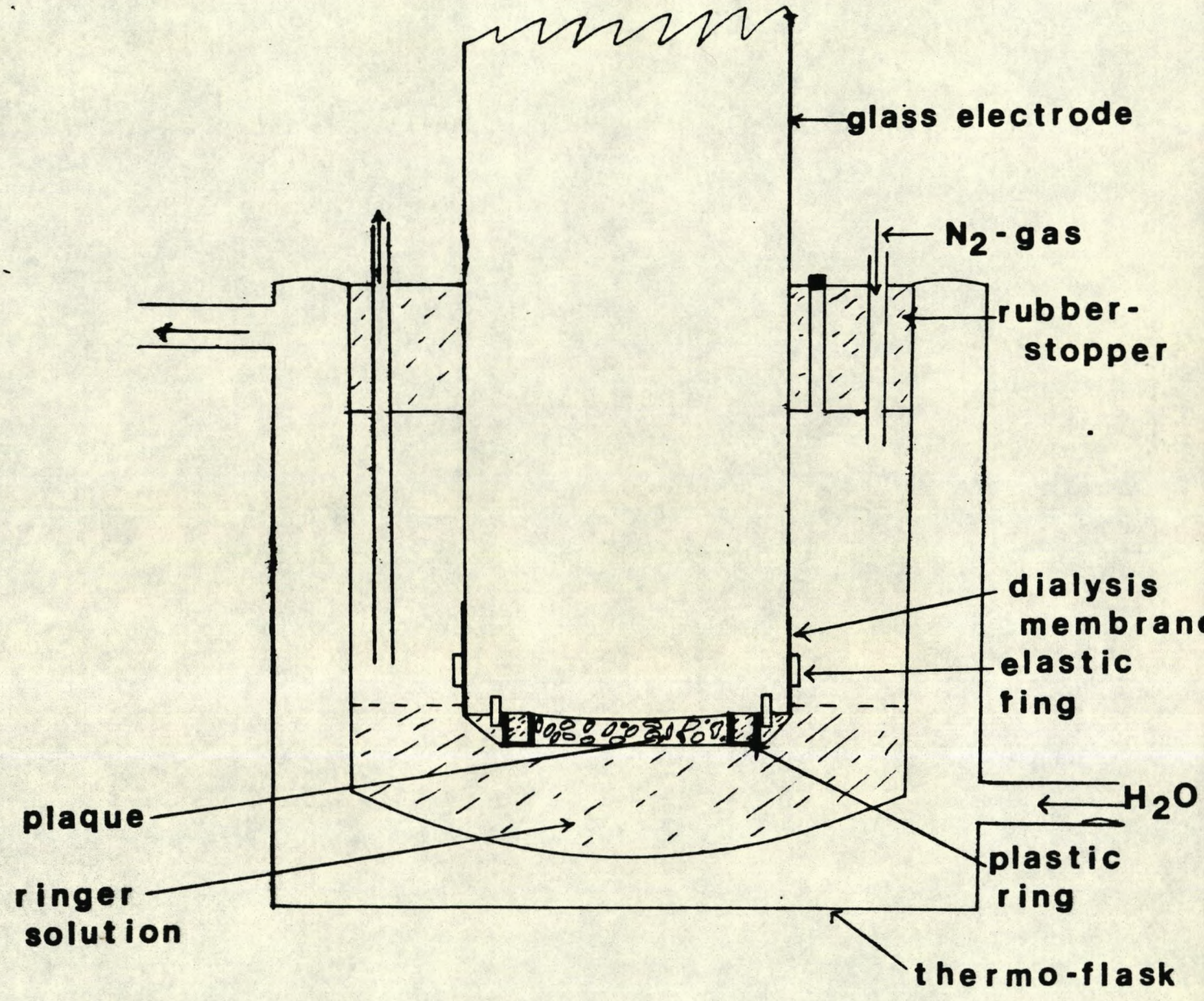


TABLE 1

The degree of fermentation of the hexoses, by plaque.

Carbohydrate	Fermented by plaque	Free Natural Occurrence
β-D(+)glucose	yes	yes
β-D(-)fructose	yes	yes
D(+)mannose	yes	rarely
D(+)galactose	yes	rarely
L(-)mannose	negligible - no *	not
β-D-allose	negligible - no *	rarely
D-altrose	negligible - no *	rarely
D-gulose	negligible - no *	not
D-idose	negligible - no *	not
D-talose	negligible - no *	not
L(-)sorbose	negligible - no *	rarely
D(+)tagatose	negligible - no *	yes

*pH drop not exceeding 6,4 when present in a solution up to 3×10^{-3}M

Table 2

The degree of fermentation of the pentoses, by plaque.

Carbohydrate	Fermented by plaque	Free Natural Occurrence
D(-)arabinose	negligible - no*	rarely
L(+)arabinose	negligible - no*	yes
D(+)xylose	negligible - no*	yes
L(-)xylose	negligible - no*	not
D-ribose	negligible - no*	yes
L(-)ribose	negligible - no	not
D-lyxose	negligible - no*	not
L-lyxose	negligible - no*	not
D-ribulose	negligible - no*	rarely
D-xylulose	yes (very slow)	rarely

* pH drop not exceeding 6,4 when present in a solution up to 3×10^{-3}M

while Table 3 represents the extent of fermentation of other carbohydrate ana= logous.

Tables 1, 2 and 3 also specified whether the compounds under question occur free in nature, rarely free in nature or not at all.

A plot of pM carbohydrate concentration versus potential reading (mV) for the monosaccharides (marked glucose) and for the disaccharides (marked sucrose) is given in Fig. 2. This plot may in many ways be compared with the well-known Stephan's curve (Stephan, 1944).

The potential change (mV) observed by plaque fermentation of melibiose, trehalose and glucose solutions is given in Fig. 3.

Scheme 1 represents the metabolism of monosaccharides by plaque (Tanzer, 1973) and Scheme 2 the four systems of particular interest in the metabolism of sucrose (disaccharide) by Streptococcus mutans (Tanzer, 1978). Two of these systems are extra-cellular or associated with the cell surface and two of the systems are intracellular.

E. DISCUSSION AND CONCLUSION

The degree of carbohydrate fermentation, i.e. the amount of acid production, was measured in mV reading in stead of pH reading. This approach enabled the observa= tion of a very small change in the acid production, which could not be accurately measured in terms of the pH value. Furthermore, the fermentation process took place under a nitrogen atmosphere as dissolution of CO_2 from the air could lower the pH value of the plaque containing solution to a reading <5,6 within 15 minutes at 37°C. The dialysis membrane (molecular weight cut-off 10^4) served to hold the plaque in position on the electrode, while allowing the escape of any plaque

Table 3

The degree of fermentation of various carbohydrate analogues, by plaque.

Carbohydrate	Fermented by plaque	Free natural occurrence
sucrose	yes	yes
lactose	yes	yes
maltose	yes	yes
D(+)trehalose	yes (very slow)	yes
β-D(+)cellobiose	negligible - no*	not
α-D(+)melibiose	yes(very slow)	yes
α-L-rhamnose	negligible - no*	rarely
starch (plant)	yes	yes
dulcitol	negligible - no*	yes
meso-inositol	negligible - no*	yes
α-L(-)fucose	negligible - no*	yes
α-D(+)fucose	negligible - no*	rarely
adonitol	negligible - no*	yes
D(+)arabitol	negligible - no*	yes
L(-)arabitol	negligible - no*	not
D-erythrose	negligible - no*	not
i-erythritol	negligible - no*	yes

*pH drop not exceeding 6,4 when present in a solution up to 3×10^{-3}M

Fig. 2. A plot of pM carbohydrate versus the potential value (E) for the monosaccharides (like glucose) and the disaccharides (like sucrose).

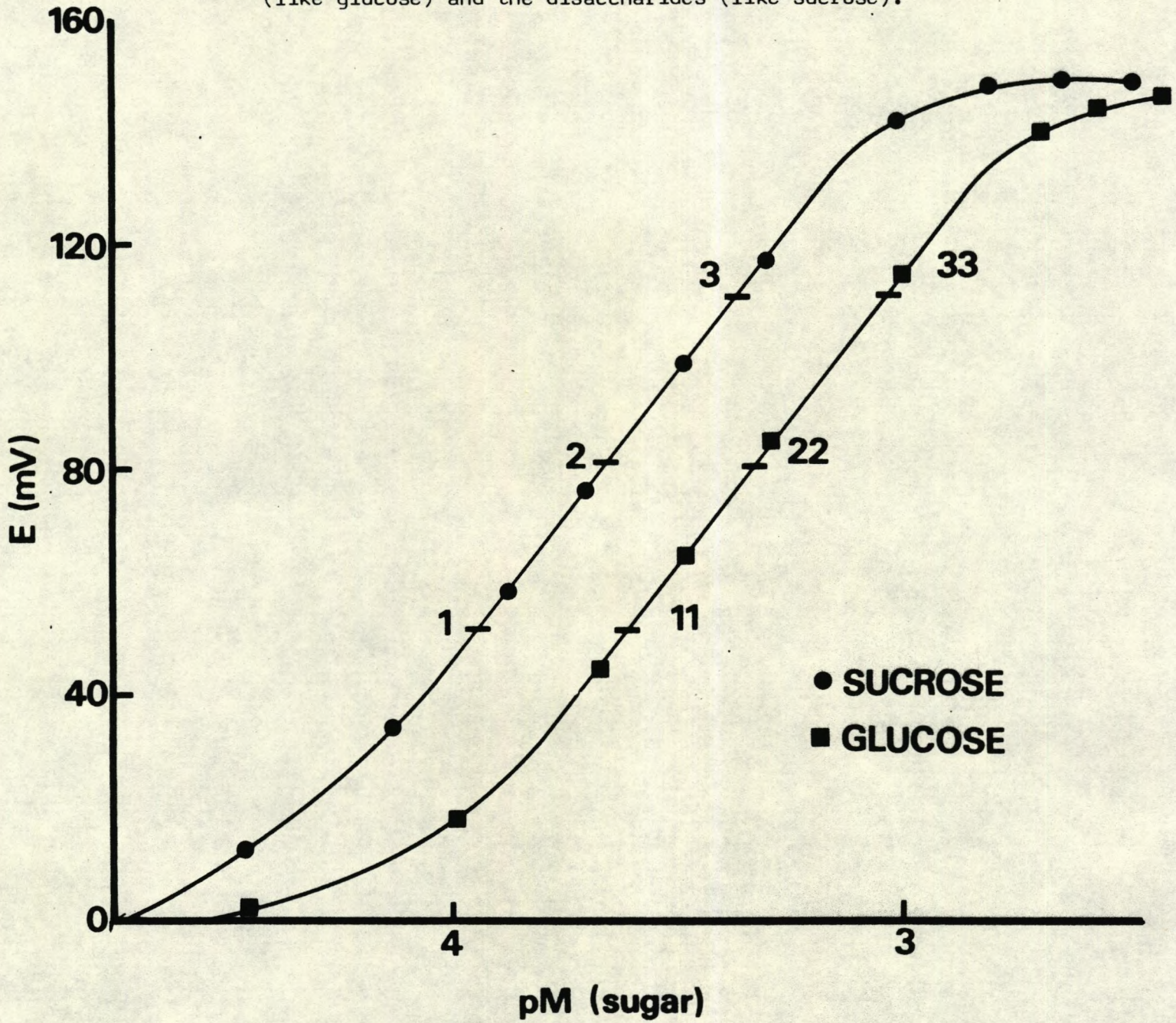
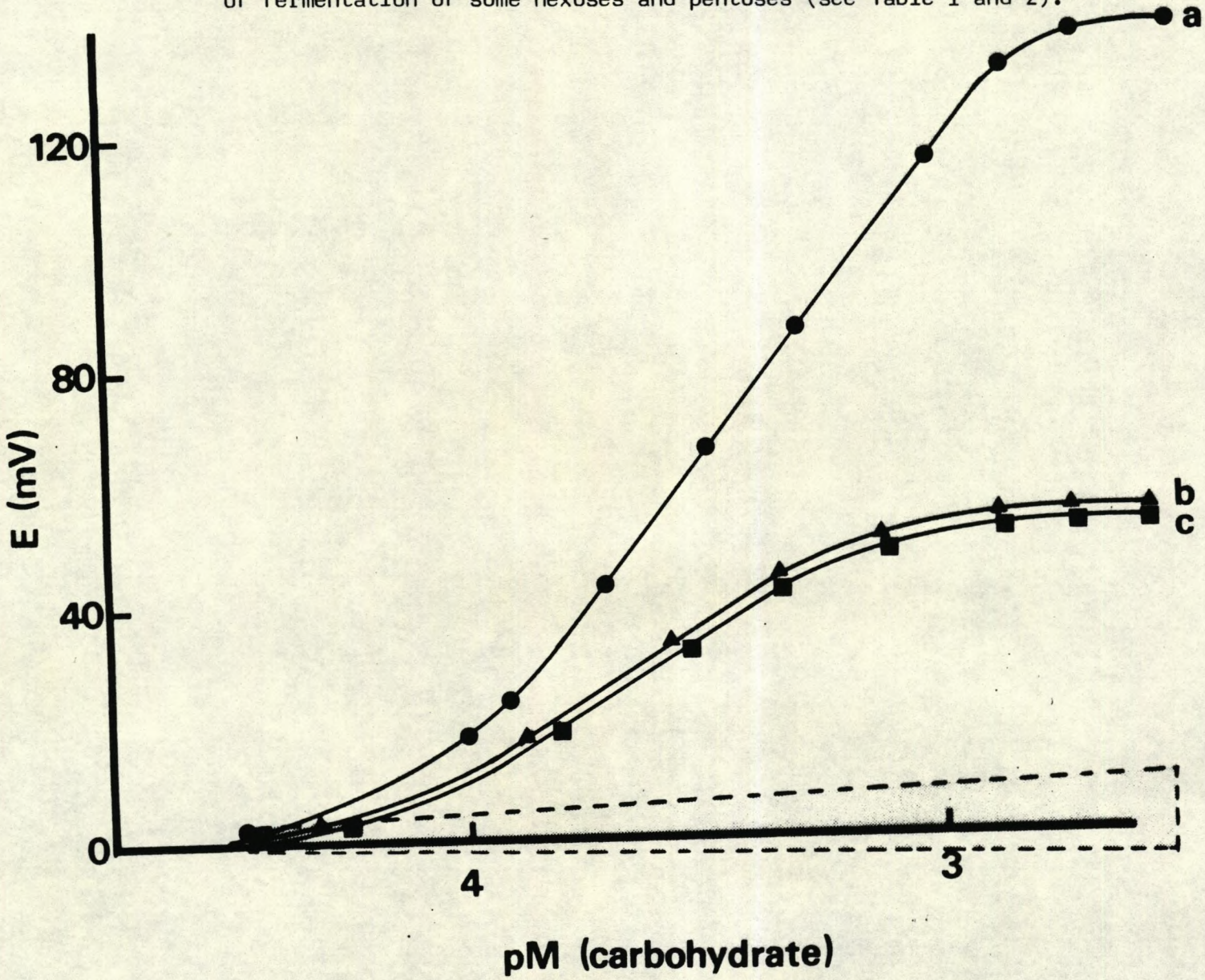
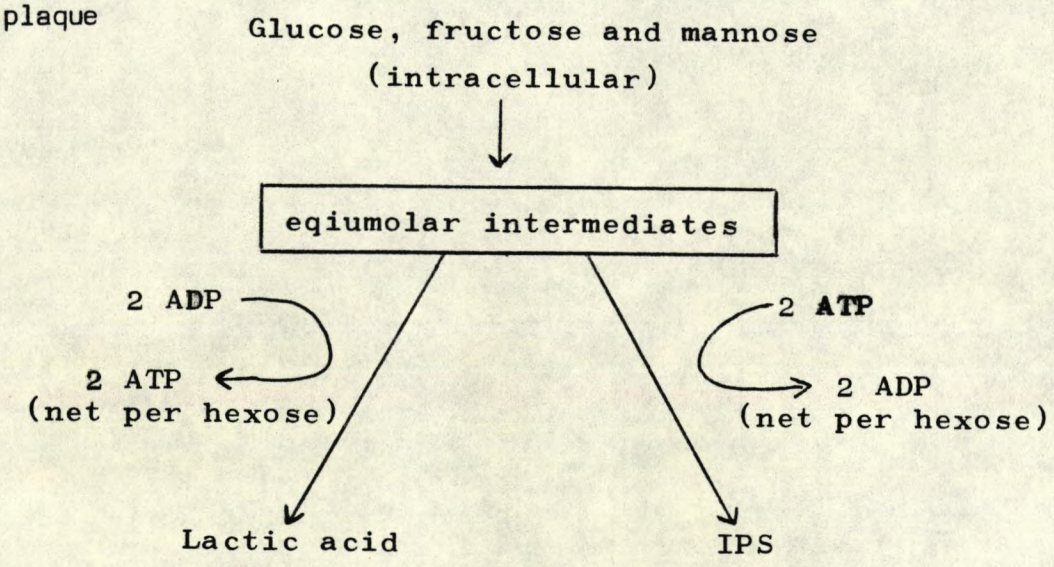


Fig. 3. A plot of pM carbohydrate versus the potential value (E). Curves a, b and c are representative for glucose, melibiose and trehalose, respectively. The dotted region represents the degree of fermentation of some hexoses and pentoses (see Table 1 and 2).



Scheme 1

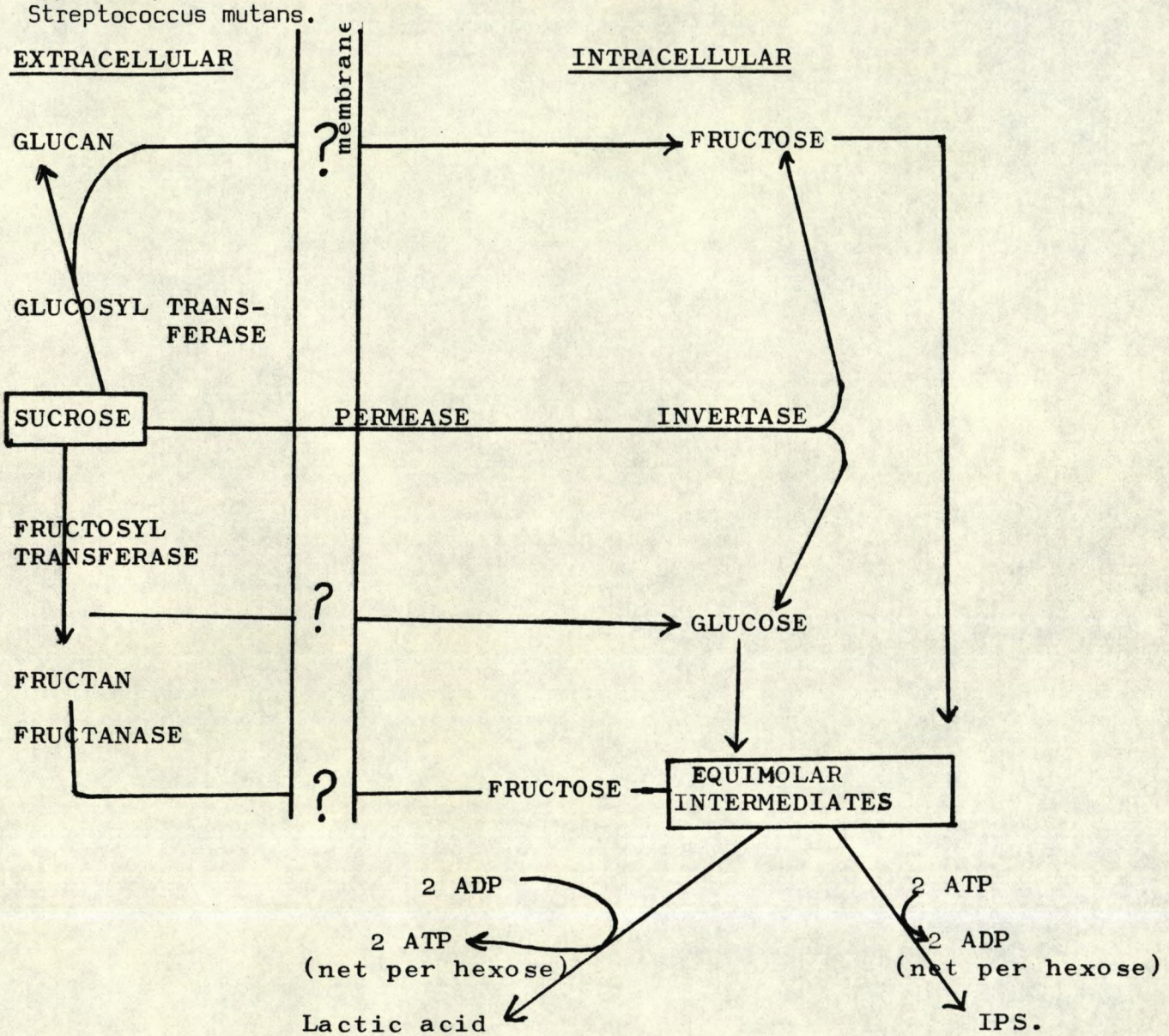
Types of processes involved in the fermentation of monosaccharides to acid by plaque



where: ADP stands for adenosine diphosphate,
ATP for adenosine triphosphate and
IPS for intracellular polysaccharides

Scheme 2

Types of processes involved in the fermentation of sucrose to acid by Streptococcus mutans.



where; ADP stands for adenosine diphosphate
ATP for adenosine triphosphate
IPS for intracellular polysaccharides.

waste products. With the plaque kept on the glass electrode surface (Fig. 1) smaller potential changes and faster electrode response time (i.e. the time required for the potential reading to come within one mV of the steady-state value) could be obtained than with plaque in suspension.

From Table 1 it is clear that of the tested hexoses only D-glucose, D-fructose, D-mannose and D-galactose are fermented by plaque. Roberts and Hayes (1980) also found that plaque breaks down glucose and fructose, but mannose and galactose to a less extent. Neff (1967) also found a reduced decrease in the pH with mannose and galactose in compasison to glucose. However, in this study the eventual drop of the pH to a value of at least 5,5 was found to be the same for glucose, fructose, mannose and galactose, after the addition of equimolar sugar amounts. [It is generally considered that rapid demineralisation of tooth surface occurs below pH 5,5 (Bowen, 1975; Gehring et al, 1976)] It seems, however, possible (Neff, 1967) that the pH did not drop to such low levels (~4,4) as during glucose fermentation. The reason for this might be because certain bacteria, like S.mutans does not fermentate galactose (Grobler and Van Wyk, 1980), even when being about 8% (Bowen, 1979) of the plaque bacteria and thus possibly not all the added galactose was fermented. Of the above four fermentable hexoses only β-D(+)glucose and β-D(-) fructose have a significant free natural occurrence (Brimacombe, 1976).

Of all the pentoses (Table 2) tested for fermentation only D-xylulose is very slowly fermented and then to a low degree. Xylulose occurs seldom free in nature (Brimacombe, 1976). According to this author its decalcification effect can be neglected as the pH did not fall below 6,0 within 2 hours (in ~10^{-3}M xylulose). No detectable acid production from D-ribose in plaque suspension at a concentration of 21 μM was reported (Roberts and Hayes, 1980). This is in agreement with the results of Table 2.

Of all the other carbohydrate analogues tested for plaque fermentation (Table 3) only sucrose, lactose, maltose, trehalose and melibiose are fermented to a pH value of below 6,0 when added to a final concentration of ~2 x 10^{-3}M. Of these only trehalose and melibiose could not cause a pH drop of below 5,5 (82 mV). The degree of acid production of melibiose and trehalose was found to be nearly the same over the same concentration range (Fig. 3b and 3c). As found by Roberts and Hayes (1980) no significant difference could be detected between lactose or sucrose fermentation, although other authors (Frostell, 1973; Neff, 1967; Frostell, 1964; Miller, Muntz and Bradel, 1940) reported a less readily fermentation of lactose by plaque. Plant starch is fermented to a high degree (Table 3) and is of general natural free occurrence (Brimacombe, 1976). It is believed that enzymes produced by the plaque bacteria (Aksnes, 1976) are responsible for the break down of maltose, sucrose and lactose in dental plaque, whereas salivary derived amylases might be more important for the fermentation of starch in plaque (Birkhed and Skude, 1978).

From the Nernst equation (Eisenman et al, 1972)

$E = Eo - S \log M$

or $E = Eo + S \times pH$ (glass electrode)

where: E is the measured electrode potential, Eo the reference potential (a constant), M the hydronium ion concentration (or the carbohydrate concentration) and S the electrode slope, a straight line is obtained from the plot of E vs pM carbohydrate concentration. When freshly prepared the glass electrode-plaque system exhibited a response slope of 104 mV/log decade ($\pm$ 4 mV) in the linear range from 1,2 x 10^{-4}M to 1,1 x 10^{-4}M (Fig. 3a). The doted region (Fig. 3) represents the degree of fermentation of all the pentoses (Table 1) and hexoses (Table 2) other than D-glucose, D-fructose, D-mannose and D-galactose. Therefore, it is found that D(+)glucose, D(-)fructose, D(+)mannose and D(+)galactose fermentation by plaque eventually gives the same potential reading ($\pm$ 4 mV/Log decade; Fig. 3a) i.e. the same pH.

The points marked 11,22 and 33 (Fig. 2) on the glucose curve correspond to a pH value of 6,0 (52mV); 5,5 (82mV) and 5,0 (112mV), respectively. These points are equivalent to a glucose, fructose, mannose and galactose concentration of $2{,}51 \times 10^{-4}$M($\pm 0{,}10 \times 10^{-4}$M), $4{,}73 \times 10^{-4}$M ($\pm 0{,}22 \times 10^{-4}$M) and $9{,}44 \times 10^{-4}$M ($\pm 0{,}47 \times 10^{-4}$M), respectively.

On the other hand represent points 1($1{,}19 \times 10^{-4}$M $\pm$ $0{,}07 \times 10^{-4}$M), 2($2{,}24 \times 10^{-4}$M $\pm$ $0{,}11 \times 10^{-4}$M) and 3($4{,}47 \times 10^{-4}$M $\pm 0{,}22 \times 10^{-4}$M) on the curve marked sucrose (Fig. 2) (which is also representative for maltose and lactose) the same pH values as points 11,22 and 33 respectively. However, these pH values were obtained through fermentation of a smaller molar disaccharide concentration. This effect might be explained by the types of processes involved in the fermentation of monosaccharides, like glucose (Scheme 1; Tanzer, 1973) and disaccharides, like sucrose (Scheme 2; Tanzer, 1978) to acid. From these two schemes it becomes clear that from each molecule of sucrose (maltose or lactose) two molecules of monosaccharides are formed, namely fructose and glucose. Thus, theoretically twice the amount of acid must be released from a disaccharide than from an equimolar amount of monosaccharide. This effect is clearly confirmed by the curves of Fig. 2 where less molar sucrose is necessary for the same pH drop obtained with a monosaccharide. However, this effect is only of interest at molar concentrations of $<10^{-3}$M (Fig. 2) and is negligible at higher concentrations. In both cases (Fig. 2) the final pH drop was already obtained at concentrations of $2{,}5 \times 10^{-3}$M carbohydrate. If that is the case, it is obvious that a disaccharide could be responsible for a higher tooth demineralization effect especially when present in small amounts. Sucrose has probably been blamed as the main dietary culprit in caries causation simply because it is the sugar which is most frequently ingested. There is no evidence that its substitution by glucose or fructose could lead to a significant reduction in dental decay in human beings, although there could be a difference when low carbohydrate concentrations are used.

ACKNOWLEDGEMENT

The author is grateful to the M R C of South Africa for financial assistance in undertaking this research.

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CHAPTER IV

DETERMINATION OF D(+)GLUCOSE, D(+)MANNOSE, D(+)GALACTOSE OR D(-)FRUCTOSE IN A MIXTURE OF HEXOSES AND PENTOSES BY USE OF DENTAL PLAQUE COUPLED WITH A GLASS ELECTRODE

A. SUMMARY

A potentiometric sensor has been developed by coupling dental plaque with a flat-surface glass electrode. Selectivity of this electrode for hexoses and pentoses has been tested. The electrode responds linearly to β-D(+)glucose, D(+)galactose and β-D(-)fructose over a narrow concentration range between 10^{-4} - 10^{-3}M, but has negligible response to the other hexoses and pentoses. This "plaque" electrode, using live bacterial cells, may serve as a model for the development of other selective electrodes for carbohydrates.

B. INTRODUCTION

There are many methods for the individual determination of reducing sugars. The Sichert-Bleyer method (Whistler, Hough and Hylin, 1953; Sichert and Bleyer, 1936) is widely used, and reducing hexoses and pentoses can be determined by measurement of the amount of formic acid produced after sodium periodate oxidation (Hirst and Jones, 1949). Aldoses may be titrated with iodine and alkali (Vogel, 1958) and individual polyhydric alcohols can be determined iodometrically (Vogel, 1958). The Lane and Eynon (1923) copper reduction method has been recently modified by Khan (1979).

A mixture of sugars is normally analysed by first separating the sugars by partition chromatography (Koch, Geddes and Smith, 1951; Somogyi, 1945) and then determining the individual sugars by oxidation.

Fermentation methods (Whistler, Hough and Hylin, 1953; McLachlan, 1928) provide a fairly reliable quantitive measure of D-glucose in a mixture of sugars, and it was shown as early as 1928 that the enzyme glucose dehydrogenase could be successfully used for manometric estimations of glucose in biological material in the presence of other carbohydrates and proteins (Keilin and Hartree, 1948).

I now describe the development and use of a selective sensor in which human dental plaque is employed at the surface of a pH-type glass electrode to prepare a sugar-sensing membrane electrode.

In this study I describe a bacterial electrode with response and selectivity to β-D(+)glucose, D(+)galactose, D(+)mannose and β-D(-)fructose, but with negligible response to the other hexoses and pentoses. The response of this plaque electrode is based on the measurement of local pH changes produced during the bacterial fermentation of some carbohydrates.

It has been shown that lactic acid is the main product during the fermentation of carbohydrates (Tanzer, 1973; Platt and Niven, 1942; Gunsalus and Niven, 1942). The acid produced in this manner is sensed by the internal pH element of the electrode and a potentiometric steady-state response proportional to the sugar concentration is obtained.

C. EXPERIMENTAL

Apparatus

A Corning flat-surface pH combination electrode (cat. no. 476216) was used in the construction of the bacterial electrode. Potential

measurements were made with a Corning Model 12 Research pH-meter in conjunction with a Heath-Schlumberger strip-chart recorder, model SR-255-B. Measurements were made in a cell kept at 37° ± 0,2°C.

Reagents

Normal 24-hr old human dental plaque in a non-fluoridated area was used as the source of the fermenting bacteria. Such plaque was collected from laboratory volunteers as described below.

The carbohydrates β-D(+)glucose, D(+)galactose, β-D-allose, L-mannose D(+)mannose, D-idose, D-gulose, D-talose, D-altrose, β-D(-)fructose, L(-)sorbose, D(+)tagatose, L(+)arabinose, D(-)arabinose, L(-)xylose, D(+)xylose, D-ribose, D-lyxose, L-lyxose, D-ribulose and D-xylulose in purest form available were used without further purification. DL-Dithiothreitol was also obtained.

All other chemicals used were of analytical grade. Distilled water was used in all the experimental work.

Procedure

Day old dental plaque was collected from 25 members of the laboratory staff, whose teeth had previously been cleaned and who had fasted for 10 hr before plaque collection. The plaque was collected under sterile conditions by scraping from tooth surfaces, immediately suspended in sterilized reduced transport fluid (RTF) (Syed and Loesche, 1972) and cooled to 4°C. The suspension was homogenized by ultrasonics and stored at 4°C.

A fraction of the plaque (~ 2 mg air-dried weight) was washed three

times with Ringer's solution (0,015M sodium chloride; $3,1 \times 10^{-4}$M potassium chloride and $2,2 \times 10^{-4}$M calcium chloride), and before use was adjusted to pH 6,95 with a diluted sodium hydroxide solution.

A dialysis membrane (molecular weight cut-off 10^4) was soaked for 1 hr in Ringer's solution and put over the flat surface of the glass electrode, where it was held by an elastic band. This assembly was soaked for another 2 hrs in Ringer's solution to mould the membrane. The glass electrode was then inverted, the membrane removed, and a plastic ring of ~0,3 mm thickness and ~3,2 diameter placed between the two reference junctions and the pH-sensing portion of the glass electrode (Fig. 1). The washed plaque, suspended in Ringer's solution, was placed in the plastic ring and kept in position by replacement of the dialysis membrane. Care had to be taken not to trap air bubbles under the membrane.

Measurements were made in 1,50 ml of Ringer's solution (pH 6,95) contained in a cell sealed from the atmosphere. The space above the solution was flushed with nitrogen. Calibration curves were constructed by adding successive portions of carbohydrates solutions, previously standardized by conventional methods (Vogel, 1958; Khan, 1979) and measuring the emf of the cell.

When not in use, plaque electrodes were stored in RTF medium at 10-12°C. In comparison 10 separate plaque electrodes were constructed and tested for response to glucose.

D. RESULTS AND DISCUSSION

Figure 2 represents the calibration curves for the plaque electrode at

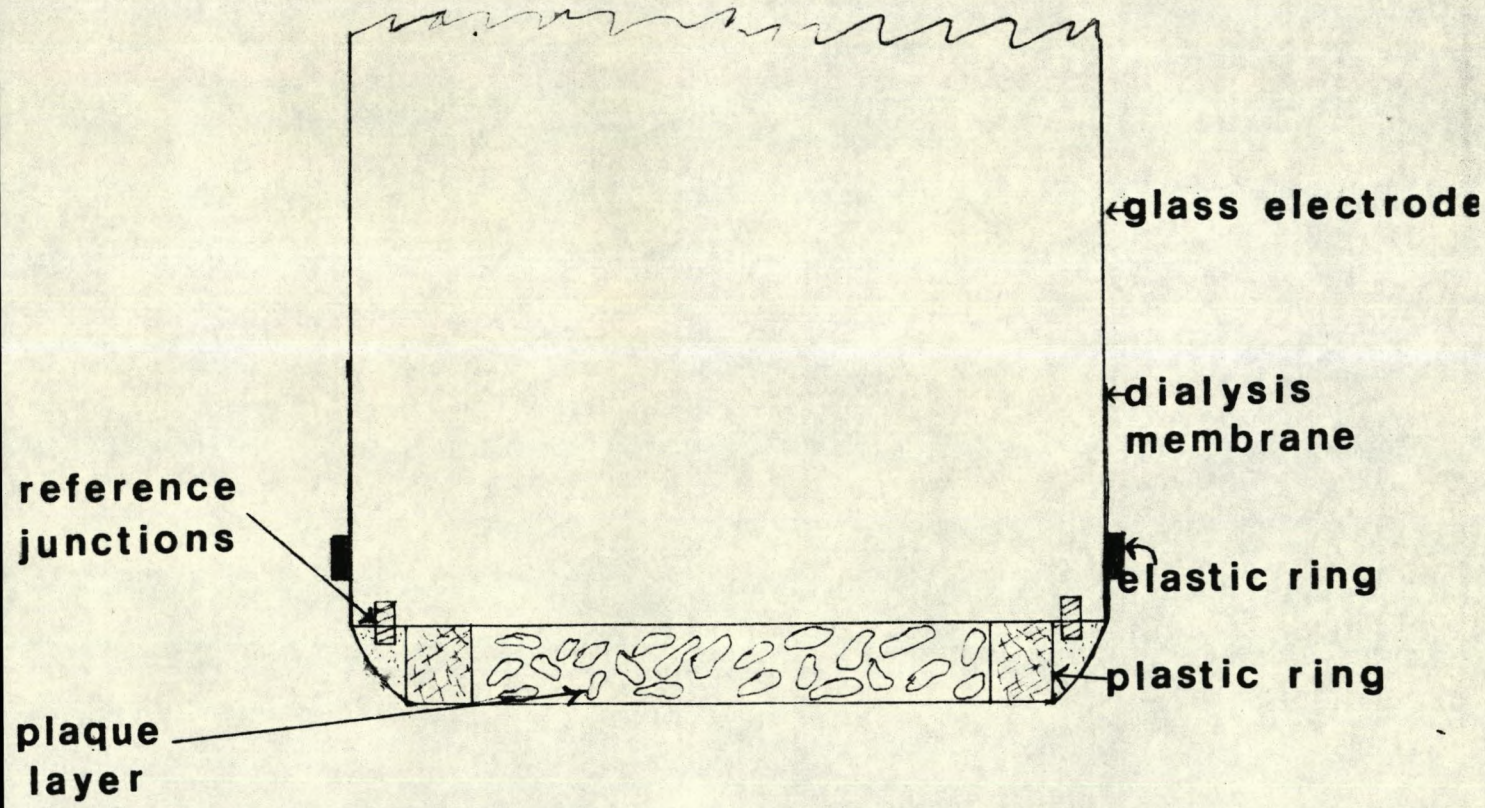
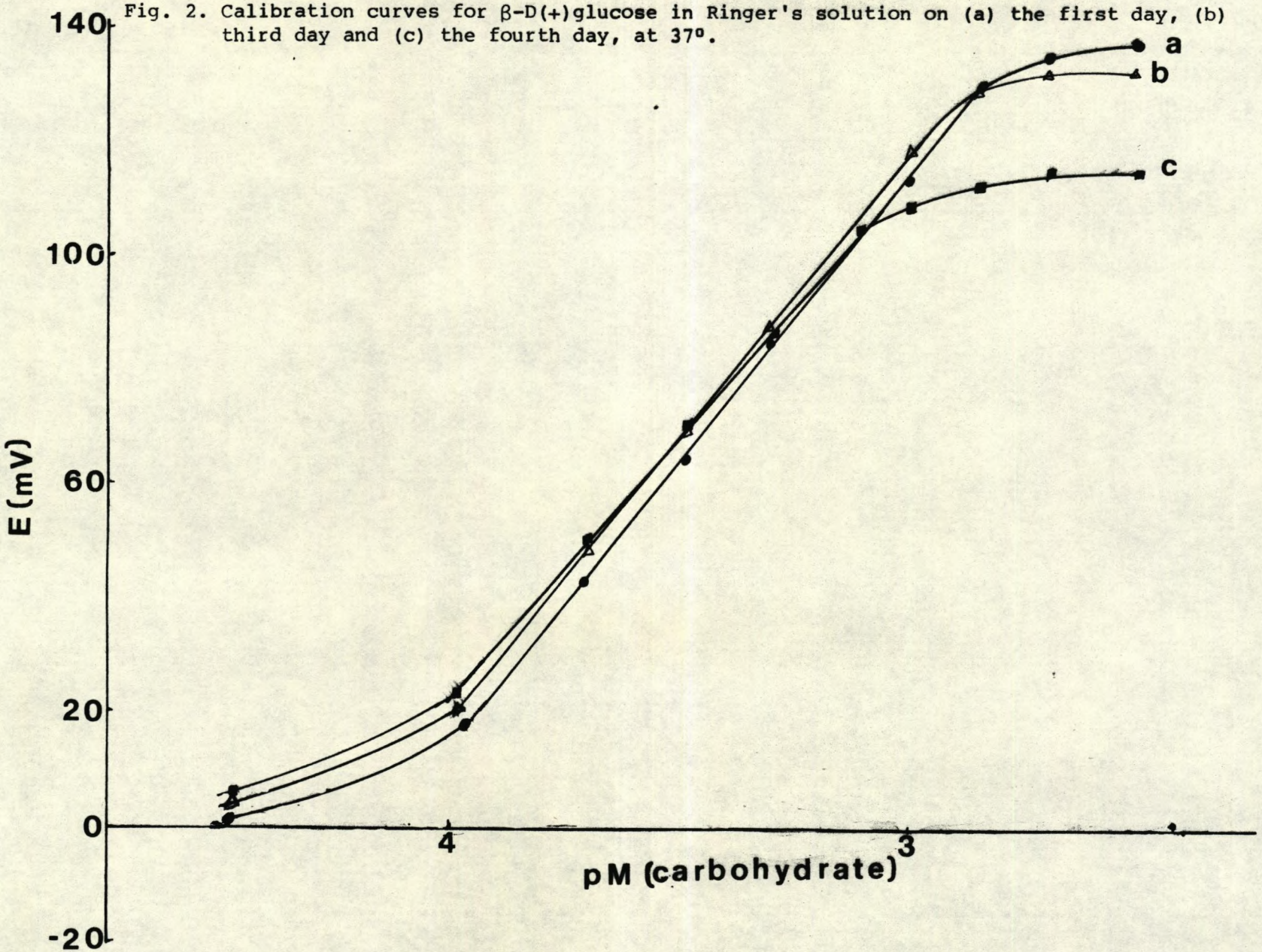


Fig. 1. Schematic diagram of the plaque electrode assembly.

Fig. 2. Calibration curves for β-D(+)glucose in Ringer's solution on (a) the first day, (b) third day and (c) the fourth day, at 37°.



different ages, with β-D(+)glucose at 37°. When freshly prepared, the electrodes exhibited a response slope of 104 mV/log decade (±4 mV), in the linear range from $1,2 \times 10^{-4}$M to $1,1 \times 10^{-3}$M (Fig. 2, curve a). By the third day the slope had dropped slightly to 101 mV/log decade (curve *b)* over a linear range of $1,3 \times 10^{-4}$- $1,0 \times 10^{-3}$M. By the 4th day it was 95 mV/log decade (curve *c)* over a linear range of $1,3 \times 10^{-4}$-$7,5 \times 10^{-4}$M; electrodes were disassembled on the fifth day. I believe that the decrease in the slope and range is due to inactivation of the plaque bacteria on the electrode on exposure to various media (Syed and Loesche, 1972).

The response slopes of the plaque electrode to D(+)galactose, D(+)mannose and β-D(-)fructose were also 104 ± mV/log decade in the linear range $1,2 \times 10^{-4}$-$1,1 \times 10^{-3}$M, but the plaque electrode has negligible response to the other hexoses and pentoses. Plaque will also ferment some disaccharides (Guggenheim, 1968; McCabe, Keyes and Howell, 1967; Tanzer and McCabe, 1968; Edwardson, 1968).

The non-Nernstian response slopes obtained throughout (104 mV/log decade) result from the fact that the yield of lactic acid increases with decreasing pH (Tanzer, 1973; Platt and Foster, 1958; Gunsalus and Niven, 1942) during the fermentation process and the amounts of volatile acids and alcohols decrease (Friedman, 1938; Friedman 1939; Foster, 1921; Langwell, 1929; Barron and Jacobs, 1938). Similar results were obtained when the plaque was mixed with carbohydrate solution, the glass electrode inserted and the pH change monitored, showing that the effect is not attributable to the electrode.

It is evident that the linear range of the response slope starts at

~30 mV (pH ≃ 6.5), possibly because the increase in volatile acids and alcohol product is negligible (Gunsalus and Niven, 1942) at lower pH values.

Optimum results were obtained when 1,5-2,5 mg of air-dried plaque was used to prepare the electrode. Smaller amounts resulted in decreased sensitivity and larger amounts gave longer response times.

When the plaque electrode was new, the response time, i.e. the time required for the emf reading to come within 2 mV of the steady-state value, increased from 20 to 40 min as the carbohydrates concentration increased from 5×10^{-5}M to 3×10^{-3}M, and increased as the plaque electrode became older.

The response of the plaque electrode to four hexoses and pentoses is due to the presence of more than one fermenting strain found in plaque (Guggenheim, 1968). I believe that the selectivity of the electrode can be increased by using single strains.

The plaque electrode, although neither selective for a single carbohydrate (enzyme) nor effective for longer periods than 4 days, offers a very sensitive electrode for four carbohydrates in a mixture with their isomers and pentoses.

I thus believe that this biological electrode is only the beginning of biological carbohydrates electrodes and that more selective electrodes with extensive effectiveness may be constructed.

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CHAPTER V

POTENTIOMETRIC DETERMINATION OF D(+)GLUCOSE, D(+)MANNOSE OR D(-)FRUCTOSE IN A MIXTURE OF HEXOSES AND PENTOSES, BY USING *STREPTOCOCCUS MUTANS* FERMENTATION

A. SUMMARY

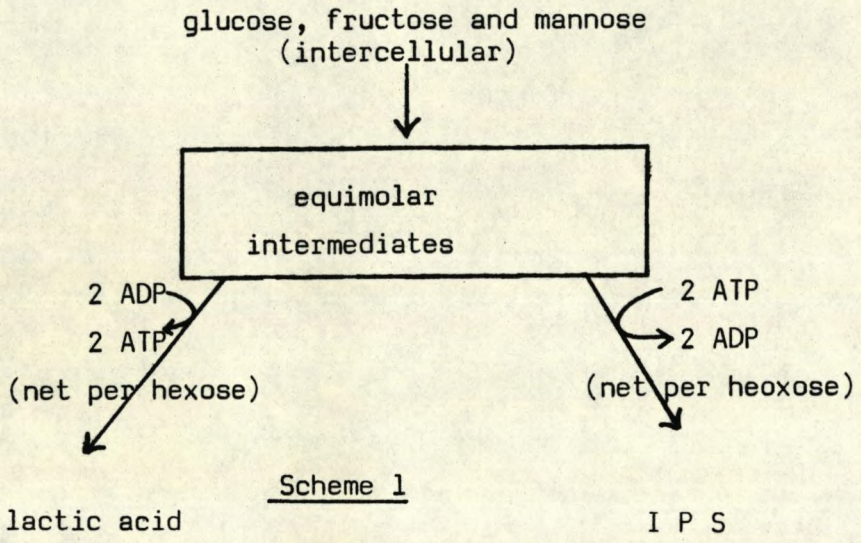
A potentiometric sensor has been developed, based on selective fermentation of carbohydrates by *Streptococcus mutans*. This combination of bacterial action and a glass electrode responds to β-D(+)glucose, D(+)mannose and β-D(-)fructose over a narrow concentration range, with a response time of 4 min, and has negligible response to other hexoses and pentoses.

B. INTRODUCTION

Determination of carbohydrates in the presence of other reducing sugars is a frequent laboratory problem. The normal procedure is to separate the carbohydrates by partition chromatography (Koch, Geddes & Smith, 1951; Somogyi, 1945) and then determine the individual sugars (Whistler, Hough and Hylin, 1953; Sichert and Bleyer, 1936; Hirst and Jones, 1949; Vogel, 1958; Lane and Eynon, 1923; Khan, 1979). However, fermentation methods (Whistler, Hough and Hylin, 1953; McLachlan, 1928) for the determination of D-glucose in a mixture of sugars or of D(+)glucose, D(+)mannose, D(+)galactose and D(-)fructose in a mixture of hexoses and pentoses, are available. The enzyme glucose dehydrogenase can be used for manometric estimations of glucose in the presence of carbohydrates and proteins (Keilin and Hartree, 1948).

I now describe the development and use of a selective carbohydrate-sensing sensor in which living micro-organisms, such as S.*mutans*, are employed in suspension. The sensor gives a selective response to β-D(+)glucose, D(+)mannose and β-D(-)fructose, but negligible response

to other hexoses and pentoses. The response is based on the measurement of hydronium ions produced during the bacterial fermentation of certain carbohydrates. The type of processes involved in the fermentation of glucose, fructose or mannose, resulting in the formation of lactic acid (Tanzer, 1973), is shown in Scheme 1, where ADP stands for adenosine diphosphate, ATP for adenosine triphosphate and IPS for intracellular polysaccharides.



Scheme 1

It has been shown that lactic acid is the main product during the fermentation of carbohydrates (Platt and Foster, 1958; Gunsalus and Niven, 1942). The acids produced in this matter cause a change in pH which is sensed by the glass electrode and a potentiometric steady-state response, proportional to the sugar concentration, is obtained.

C. EXPERIMENTAL

Apparatus

All potentiometric measurements were taken on a Corning model 12 pH-meter connected to a Heath-Schlumberger strip-chart recorder model SR-225-B. Measurements were made with a Corning pH combination electrode (cat. no. 476216), in a cell controlled at 37,0 ± 0,2°C. Cells were grown in a Torbal model AJ-3 jar.

Reagents

Streptococcus mutans No. 25175 was obtained from the American Type Culture Collection, Rockville, Maryland. Todd-Hewitt broth was purchased from Difco Lab., Michigan. The carbohydrates β-D(+)glucose, D(+)galactose, β-D-allose, L-mannose, D(+)mannose, D-idose, D-glucose, D-talose, D-altrose, β-D(-)fructose, L(-)sorbose, D(+)tagatose, L(+)arabinose, D(-)arabinose, L(-)xylose, D(+)xylose, D-ribose, L(-)ribose, D-lyxose, L-lyxose, D-ribulose and D-xylulose in purest form available, were obtained from Sigma Chemicals Co. and were used without further purification. All other chemicals used were of analytical grade. Distilled water was used in all the experimental work.

Procedure

The S.*mutans* strain was maintained (at 4°C in Todd-Hewitt broth with excess of calcium carbonate added (Tanzer, Krichevsky and Keyes, 1969). Transfer to fresh media was made every 2 weeks. For experimental purposes, the organisms were cultivated in a medium of the following composition: trypticase, 2%; NaCl, 0,2%; KH_2PO_4, 0,4%; Na_2HPO_4,0,2%; K_2CO_3,0,1%; $MgSO_4$, 0,012%; $MnSO_4$, 0,0015%; D(+)glucose, 0,2%. The D(+)glucose was autoclaved separately and added aseptically to complete the medium. The culture was incubated anaerobically at 37°C for 8 hours in a Torbal jar filled with a mixture of 95% nitrogen and 5% carbon dioxide. Cocci were harvested by centrifugation (4500 rpm) at room temperature, washed 3 times with 0,004M potassium phosphate buffer (pH 6,8) prepared in 0,050M potassium chloride, and stored at 4°C for 24hrs in the phosphate buffer solution (Tanzer, Krichevsky and Keyes, 1969).

A fraction of the cells (~ 2mg air-dry weight) was washed three times with Ringer's solution, consisting of 0,015M NaCl, 3,1 x 10^{-4}M KCl and 2,2 x 10^{-4}M $CaCl_2$, and before use the pH was set at 6,95 with diluted sodium hydroxide solution. The washed cocci were suspended in 1,50 ml of Ringer's solution (pH = 6,95) in a temperature-controlled cell sealed from the atmosphere, and were kept in suspension by magnetic stirring. The glass electrode was immersed in this solution and the head-space was flushed with nitrogen. Calibration curves were then constructed by adding successive portions of the standard carbohydrate solutions, which had been standardized by conventional methods (Vogel, 1958; Khan, 1979). The largest volume added was 120 µl and a volume-correction was used in the calculations. After the cells had been used, they were harvested, washed and stored in the potassium chloride-phosphate buffer solution at 4°C. Eight separate calibration curves for each of D(+)glucose, D(+)mannose and β-D(-)fructose were constructed. The selective response of S. *mutans* to all three carbohydrates was tested by the use of more or less equal numbers of cells (~ 1,5 mg air-dry weight).

D. RESULTS AND DISCUSSION

Typical calibration curves are shown in Fig. 1 for the response of the same cell crop at different ages to β-D(+)glucose. Similar calibration curves (i.e. within ±5 mV) were obtained with D(+)mannose or D(-)fructose. Freshly cultivated cell crops exhibited a response slope of 148 mV/decade (±5 mV) in the linear range from 4,6 x 10^{-5} to 2,5 x 10^{-4}M glucose (Fig. 1a). By the fourth day the response slope had dropped slightly to 124 mV/decade (Fig. 1c), for the same cell crop. S. *mutans* has less than 4 mV/decade response to other hexoses and pentoses, which I consider negligible, but high concentrations (>10^{-3}M) of D-xylulose and D(+)galactose will interfere. The linear response is over a narrower range than that of the plaque electrode, (ChapterIV ; Grobler and Rechnitz, 1980), but starts at a lower

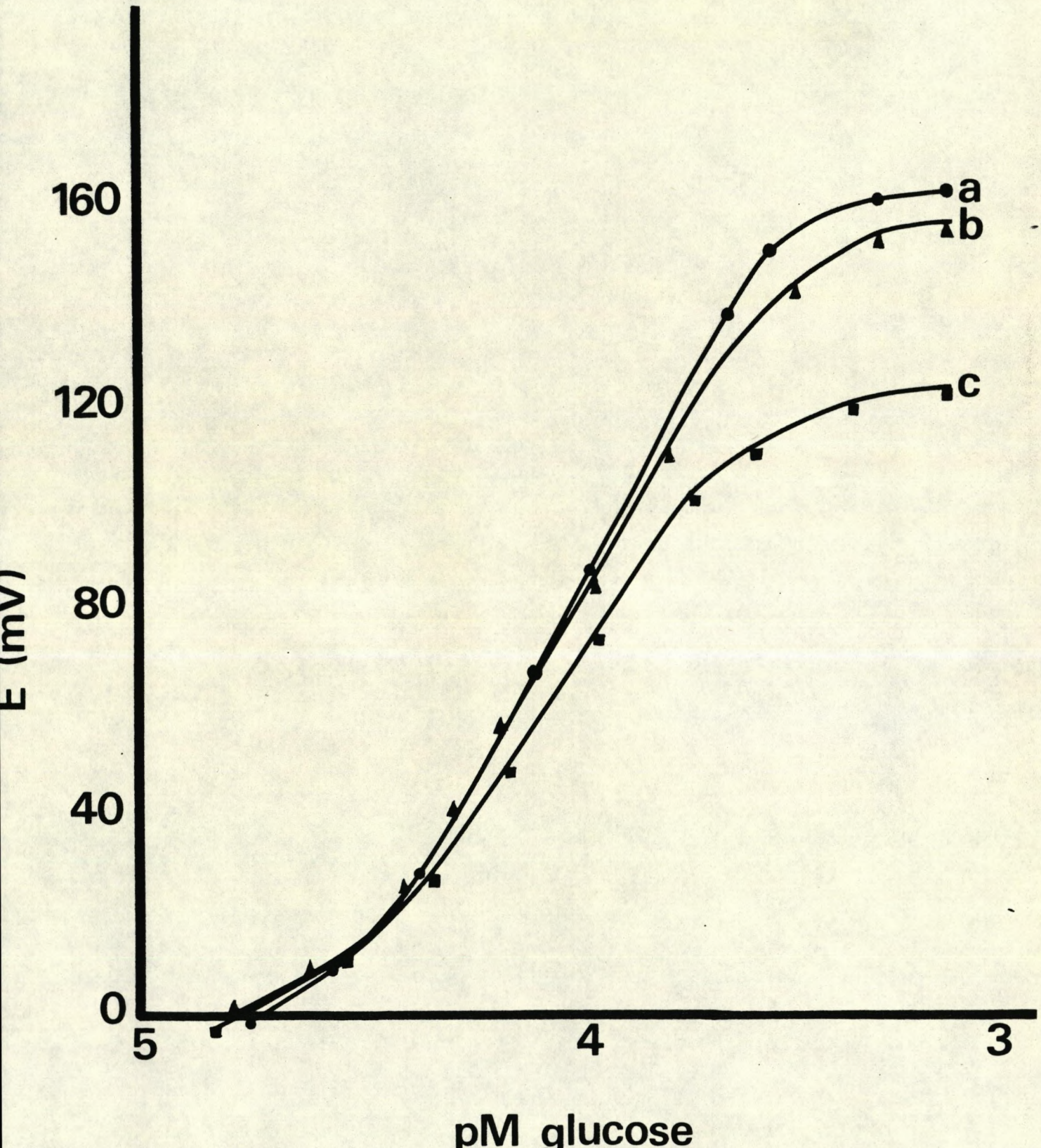


Fig. 1. Calibration curves for S. *mutans*-glass electrode combination for β-D(+)glucose in Ringer's solution on (a) the first day, (b) the fourth day and (c) the fifth day, at 37°C.

concentration, and the slope is higher by ~ 45 mV/decade. The reason for the higher response may be the inhibitive effect of various elements on the plaque fermentation process (Kleinberg et al, 1977; Hamilton, 1977; Fallagher and Curtress, 1977), because the plaque cells are not cultures. The response slope is greater than the theoretical Nernstian value, because the yield of lactic acid increases while that of the volatile acids and alcohols decreases with decreasing pH, during the fermentation process (Chapter IV; Grobler and Rechnitz, 1980). The effective lifetime of the S. *mutans* electrode is also about a day longer than that of the plaque electrode, which contains some fermentation species which deactivate faster (Syed and Loesche, 1972; Guggenheim, 1968) than S. *mutans*.

The ideal amount of S. *mutans* cells to be used in 1,50 ml of solution is 1,0 - 2,0 mg (air-dry weight). Smaller amounts result in decrease of the linear range, showing there is an optimum fermentation limit for each amount of cells, but larger amounts make no difference.

The time required for the potential reading to come within 2 mV of the steady-state value increased from 4 - 6 min as the carbohydrate concentration increased from $4,0 \times 10^{-5}$M to $1,0 \times 10^{-3}$. It also increased as the cells became older, showing a drop in the cell activity, possibly on exposure to various media (Syed and Loesche, 1972). The longer response time (20 min) of the plaque electrode (Chapter IV; Grobler and Rechnitz, 1980) could have been due to the decrease of the cell contact area while the cells were being slightly squeezed between the membrane and the glass membrane of the electrode. The same long response time was observed if S.*mutans* was similarly held on the glass electrode.

Table 1 Analysis of a known amount of (a) β-D(+)glucose, (b) D(+)mannose and (c) β-D(-)fructose in Ringer solution in the presence of $1,7 \times 10^{-4}$M of each of the following: β-D-allose, L-mannose, D-idose D-gulose, D-talose, D-altrose, L(-)sorbose, D(+)tagatose, L(+)arabinose D(-)arabinose, L(-)lyxose ,D(+)lyxose ,D-ribose, L(-)ribose, D-lyxose L-lyxose and D-ribulose. Five samples of each taken amount of each carbohydrate were analysed

Amount of carbohydrate taken (µg)	Mean absolute difference and SD	Mean percentage difference
(a) β-D(+) glucose		
20,21	1,11 (0,60)	-0,23
36,39	1,91 (0,56)	-0,50
(b) D(+)mannose		
18,75	0,96 (0,53)	-0,41
37,82	1,79 (0,49)	-0,11
(c) β-D(-)fructose		
20,61	1,20 (0,43)	-0,66
35,90	1,56 (0,59)	-0,23

Table 1 shows little or no interactions between the different carbohydrates during the determination of β-D(+)glucose or D(+)mannose or β-D(-)fructose.

As expected, S. *mutans* is slightly more selective than plaque for carbohydrate fermentation (S. *mutans* is a minor plaque component of which contains a number of other species; Guggenheim, 1968).

It is clear that the use of S. *mutans* offers some advantages over the use of plaque for the determination of the three carbohydrates in a mixture of their isomers and pentoses.

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CHAPTER VI

ALKALINE AND NON-ALKALINE SOLUBLE FLUORIDE UPTAKE AND RETENTION BY SOUND ENAMEL AFTER A SINGLE 4 MINUTE NEUTRAL 2% SODIUM FLUORIDE TREATMENT, IN VIVO.

A. SUMMARY

Alkaline (CaF_2) and non-alkaline (F-apatite) soluble fluoride uptake and retention *in vivo* by sound enamel, after a single 4 minute neutral 2% Na F treatment, was investigated. Three layers of ~7µm each were etched off. The F treatment gave only a fluoride increase within a ~7µm depth. Subjects in the age 10 - 12 years responded to a total fluoride increase of 33% (significant) of which 27% (significant) was CaF_2 and 6% (significant) fluorapatite. No significant fluoride difference could be detected between 1 or 2 or 3 weeks in vivo exposure of the premolars.

B. INTRODUCTION

The use of fluoride in the control of dental caries is based on the observation made by Dean (1938), that less caries was observed in communities with high fluoride in their drinking water supply. From the report of Cheyne (1942) the caries-reducing effect of topical potassium or sodium fluoride applications on teeth has been demonstrated and depends on several factors (Adler, Straub and Popovics, 1950).

Brudevold, McCann, Nilsson, Richardson and Coklica (1967) have postulated that the effect of a topical agent is related to the formation of fluorapatite in the enamel. This concept is based on findings of fluoride content in caries resistant teeth in relation to fluoride in the drinking water (Isaac, Brudevold, Smith and Gardner, 1958).

High fluoride concentrations (0,1 M $\bar{F}$) and low solution to solid ratios favored CaF_2 formation (McCann, 1953; McCann and Bullock,

1955), while fluoride concentrations of 0,005 M or less favored fluorapatite formation (Grøn, 1977). The chemical conditions for CaF_2 formation and its transformation into a fluoride-containing apatite had been investigated (Larsen, Jensen and Thorsen, 1977). Gerould (1945) demonstrated the formation of a CaF_2 layer on enamel when exposed to a 4% sodium fluoride solution. Calcium fluoride was the major reaction product when treating powdered enamel with acid fluoride solutions containing phosphate (Frazier and Engen, 1966; Wei and Forbes, 1968 ; Baud and Bang, 1970; Caslavska, Brudevold, Vrbic and Moreno, 1971), while the use of acid fluoride solutions caused greater fluoride deposition than neutral solutions. (Aasenden, Brudevold and McCann, 1968; Englander, Carlos, Senning and Mellberg, 1969). The above authors also suggested that the caries protection effect of fluoride is related to the form in which this element is incorporated, while only a small fraction of the formed fluoride did not leach away after 24 hours or more (Brudevold et al, 1967; Aasenden et al, 1968; Sundvall-Hagland, Brudevold, Armstrong, Gardner and Smith, 1959).

Grøn (1977) supposed that dicalcium phosphate dihydrate might be formed under the conditions of topical treatment, which may dissolve and precipitate as CaF_2 or fluorapatite. According to McCann (1968) the oral environment is not saturated with respect to CaF_2 and the formation of fluorapatite or dicalcium phosphate will be favoured.

No fluoride was dissolved from blocks of intact enamel treated with KOH for 24 hours, but most of the fluoride deposited from topical treatments was lost by KOH treatment (Caslavska, Moreno and Brudevold, 1975). Fluoride absorped to calcium ions in the hydration layer will also be removed by KOH treatment (Rölla and Bowen, 1978).

The total increase in fluoride on sound enamel in teeth of 10 to 11 year old children after a single 4 minute neutral 2% NaF treatment was analysed by Bruun (1973), but his results were not significant.

According to the knowledge of this author no attempts have yet been made to determine the types of fluorides formed during a single neutral 2% NaF application on sound enamel. Therefore, the aim of the present study has been to determine the uptake and retention of calcium fluoride and fluorapatite in sound enamel after a single 4 minute neutral 2% NaF treatment, in vivo. Some useful relevant in vitro studies have also been done:

1. possible differences in fluoride levels in biopsies taken from buccal and lingual sides of the same tooth;
2. the amount of alkaline soluble fluoride formed after tooth crown exposure to neutral 2% sodium fluoride solutions, for different periods of time, as well as the effect of saliva proteins on fluoride treatment; and
3. the influence of pure water on the fluoride retention after a single 4 minute 2% sodium fluoride treatment of sound enamel.

C. EXPERIMENTAL

The main study included 10 participants in the age group 10 - 12 years, where two first or two second maxillar premolars had to be removed for periodontal reasons. Both the buccal and lingual sides of the selected pairs of teeth were without caries or demineralised areas appearing as white or brown spots. All participants were permanent residents from the Raufoss area and did not come into contact with fluoride except that in food. The fluoride level in the drinking water supply of Raufoss is negligibly low (>0,01 ppm).

The selected pairs of teeth were cleaned by light pumicing with polyethylene microspheres (Microthene FN 510 U.S.I. Chemicals). The pumiced teeth were rinsed with tap water and dried with compressed air. One of the above two premolars was treated with 2% neutral sodium fluoride for a period of 4 minutes and the participant starved for 6 hours, where relevant. After this treatment both premolars (untreated one serving as control) were left in vivo for a period of 1 or 2 or 3 weeks and extracted for analysis. Until analysis could be done the teeth were kept in a moistened atmosphere at 4°C. The pairs of premolars were given one biopsy each, either on the buccal or lingual side, their roots were coated with blue inlay casting wax (Kerr. Co.) each tooth dipped into 2 ml 1 M KOH solution for 24 hours. This treatment removes all the alkaline soluble fluoride such as CaF_2 (Caslavska et al, 1975). The pairs of teeth were rinsed (for 10 seconds each) in distilled water, dried with compressed air and given 3 independent biopsies on the buccal or lingual side that had not been etched yet. In this way 3 separate layers were etched from each area.

In comparison to the above in vivo experiment, selected premolars were extracted, etched on one side (buccal or lingual), soaked for 4 minutes in 2% neutral NaF, immediately rinsed in distilled water (as above), each tooth exposed to 1 litre distilled water, which was renewed every day for 1 week and biopsy taken on the other side (buccal or lingual).

All experiments were done on selected premolars by alternative etchings on the buccal and lingual sides and all fluoride treated teeth were exposed for 4 minutes to a neutral 2% sodium fluoride solution, unless otherwise stated.

By taking biopsies from both the buccal and lingual sides of the same

tooth, possible significant fluoride enamel differences between the two sides could also be determined. Either the buccal or lingual side of each of 10 premolars was cleaned by giving it a light pumice, as described above and biopsies were taken from both sides. By comparing the fluoride levels of the buccal and lingual sides of the same tooth, it was possible to determine the effect of pumicing through biopsies.

The following experiment was done to determine the effect of enamel adsorped protein on fluoride treatment: The roots of selected pumiced premolars were coated with the above mentioned blue wax and the whole tooth was dipped into 2% neutral NaF for different periods: 1, 4, 10, 30, 120 or 1380 minutes. This experiment was repeated, but prior to fluoride treatment the teeth were exposed to saliva for 2 hours. Afterwards the teeth were rinsed in distilled water and each was soaked for 24 hours in 2 ml. 1 M KOH solution. The alkaline soluble fluoride in each 2 ml KOH solution was determined by first neutralising it with 6 M HCl and then buffering it to the same final compound concentration for fluoride potential neasurements as stated by Brudevold, Reda, Aasenden and Bakhos (1975).

The amount of free unbound, umcomplexed fluoride in each etched sample was measured by means of a fluoride selective electrode (Brudevold et al, 1975). An amount of 40 µl of this sample was diluted to a 4,00 ml solution containing 0,25% $La(NO_3)_3$, $6H_2O$ and the calcium was determined by means of atomic absorption spectrometry. Biopsies were taken as outlined by Brudevold et al (1975), but the etched area was 12,50 mm^2, the etching time 60 seconds and the total sample volume 1,20 ml.

ANALYTICAL

In fluoride determinations potential readings (mV) were taken on a Radiometer PHM 64 pH Meter and the fluoride concentration calculated from a calibration curve set between 10^{-1} and 10^{-7}M fluoride by means of the Orion sodium fluoride standard, using the buffer solution as described by Brudevold et al (1975). Potential measurements were made with an Orion Research, Inc., model 94-09 combination fluoride ion selective electrode.

Calcium was determined by an atomic absorption spectrophotometer, by comparing sample readings with a series of standard solutions of an analogous composition.

By assuming 38% of calcium in sound enamel and an enamel density of 2,95 (Brudevold, Aasenden and McCann, 1959; McCann, 1969), the con= centration of enamel fluoride is calculated from the equation given by Bruun (1973).

All chemicals used were of analytical grade, while the same supplies as outlined by Brudevold et al (1975) were used.

D. RESULTS

The results based on the above methodology gave no significant F difference between the buccal and lingual sides of the same tooth (Table 1).

Table 1 Fluoride concentrations in the sound enamel of the buccal and lingual sides of premolars.

BUCCUL SIDE F (ppm) and s.d.	LINGUAL SIDE F (ppm) and s.d.
303,9 (75,2)	294;1 (69,7)
n = 12	n = 12

Tooth crowns soaked for two hours in saliva prior to fluoride treatment took up less fluoride than unsoaked ones. (Figure 1; Table 2)

Table 2 Uptake of alkaline soluble F by premolar sound enamel crowns, in vitro:

(a) without saliva treatment and

(b) soaked for two hours in saliva

TIME (MIN.)	(a) µg F/crown and s.d.	(b) µg F/crown and s.d.
1	4,4 (1,9)	4,2 (1,9)
4	10,5 (6,3)	8,3 (3,9)
10	26,3 (9,6)	22,6 (12,5)
30	43,4 (24,3)	31,9 (11,7)
120	60,6 (26,5)	57,9 (52,0)
60 X 23	235,6 (82,4)	209,3 (58,9)

Each set of values represents the mean of 7 crowns.

After a one week exposure of the fluoride treated teeth to distilled water an increase in the total fluoride level, as a result of fluoride treatment, could still be found (Table 3)

Table 3 The effect of pure water (1 week) on the F formed in/on sound enamel after a single 4 minute 2% NaF treatment, in vitro.

Control side F (ppm) and s.d.	Test side F (ppm) and s.d.	%F increase and s.d.
577 (260)	626 (258)	8,5 (6,3)

where the etching depth of the control side and test side are 7,5 µm (0,63) and 7,1 µm (0,56), respectively.

The total percentage fluoride uptake and retention by sound enamel having been in vivo for 2 weeks are shown in Table 4, as well as the amounts of CaF_2 and F-apatite formed.

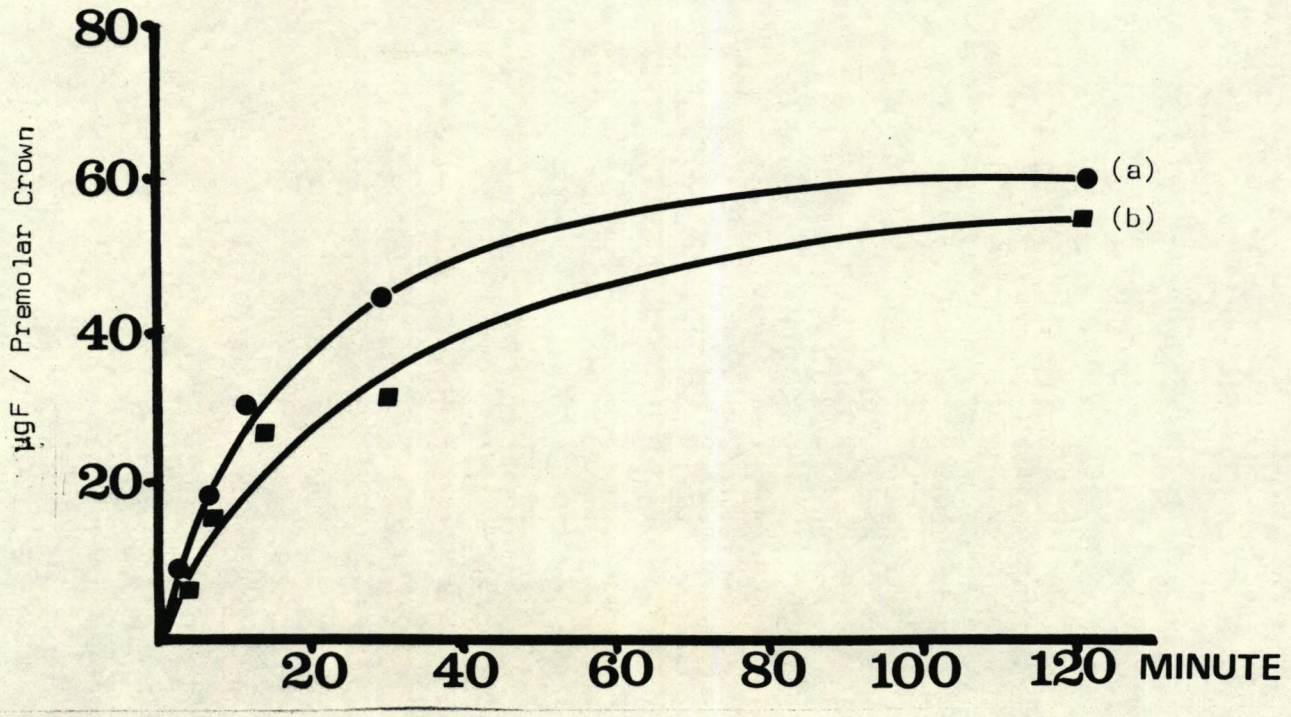

Fig.1

Uptake of alkaline soluble F by premolar sound enamel crowns, in vitro:

(a) without saliva treatment

(b) soaked for two hours in saliva.

Each point represents the mean of 7 crowns.

Table 4 The in vivo alkaline soluble F in sound enamel of premolars before and two weeks after 2% NaF treatment

% F increase	% CaF_2	% F-apatite
33% (significant)	27% (significant)	6% (significant)

n = 10 pairs

The statistical treatment of the above results are represented in Table 5.

Table 5 Statiscally treatment of the results obtained during NaF treatment

NaF treatment

Before O^-H treatment	After O^-H treatment
^{1}C mean = 751,64 ppm	^{2}C mean = 679,81 ppm
std. dev. = 157,62 ppm	std. dev. = 147,94 ppm
^{3}T mean = 900,65 ppm	^{4}T mean = 718,10 ppm
std. dev. = 193,31 ppm	std. dev. = 146,24 ppm

Matched pairs in T-test for differences

1 vs 2 :	t = -6,67	sign. decrease
	df = 9	due to $O\bar{H}$
1 vs 3 :	t = 9,48	sign. increase
	df = 9	due to NaF.
1 vs 4 :	t = -3,51	sign. decrease
	df = 9	after both treatments.
3 vs 4 :	t = -8,44	sign. decrease
	df = 9	

C stands for control teeth, T for NaF teeth, std. dev. for standard deviation and sign. for significant.

The mean biopsy depth for the 40 biopsies of Table 5 is 7,22 µm (s.d. = 0,63).

E. DISCUSSION AND CONCLUSION

As debris on the teeth surface interferes with the efficiency of topical fluoride, while ommittance of a cleaning procedure prior to application reduced the caries inhibitive effect (Knutson, Armstrong and Feldman, 1947), a cleaning process with polyethylene microspheres was included in this study. A too heavy pumice could remove some of the fluoride in the outer enamel where the concentration could be high (Brudevold, McCann and Grøn, 1968), but no significant pumice effect is observed in this study.

Although the research of Aasenden, Allukian, Brudevold and Wellock (1971) indicated that differences of fluoride in enamel within an oral cavity between various tooth types were fairly small, Weatherell, Deutsch, Robinson and Hallsworth (1977) determined the fluoride level of enamel at several places on the same tooth and stated clearly that it would be a real problem in finding a suitable control area, especially in measuring small amounts of fluoride uptake of sound enamel. However, I could not estimate a significant difference between the buccal and lingual side of the same tooth, when etching an area of 12,6 mm^2 and 7,2 µm deep from the middle part of each side (Table 1). Furthermore, this possibility was completely eliminated by doing etching alternatively on the two sides, i.e. using the buccal and lingual sides alternatively as controls.

Although the standard deviation of the results shown in Table 2; (Fig. 1) might be high, a protein saliva effect on the fluoride - hydroxyapatite interaction is observed, as stated by Birkeland and Rölla (1971). The high standard deviation could be expected because of differences in exposed crown areas between various tooth crowns.

Figure 1 also proves that an optimum alkaline soluble fluoride formation is probably reached after 23 hours, while Fisher and Mühler (1952) could not observe transformation of basic apatite structure to CaF_2 after 24 hours.

Scanning electron microscopical studies of Larsen and Fejerskov (1978) showed that the enamel surface was covered by a layer of CaF_2 after exposure for 2 hours to a fluoride solution which could be removed by an alkaline solution. The greater part of fluoride acquisition tends to leach away within one day in both in vivo and in vitro studies. (Mellberg, Laakso and Richardson, 1966; Brudevold et al, 1967; Aasenden, Brudevold and Richardson, 1968) and all acquired fluoride was lost after 1 week (Mellberg, Laakso and Nicholson, 1966). However, the results in Table 3 show a mean total increase of 8,5% (50 ppm) in the fluoride level of enamel after having being soaked in water for one week after fluoride treatment.

The results on teeth left in vivo for 15 minutes after fluoride treatment, show a fluoride increase of 150% (S.D. = 60%) indicating that a very big amount leaches away within 1 week (Brudevold et al, 1967; Aasenden et al, 1968). The in vivo studies are showing a significant average total fluoride increase of 33% (220 ppm) (Table 4 and 5) after 1 - 3 weeks, while Bruun (1973) found a ~259 ppm (not significant) increase for 10 - 11 year old children. These values are in fairly good agreement, if the etching depth of 7,22 µm in comparison to 2,9 µm of Bruun (1973) is taken in consideration, as deeper etchings tend to lower the fluoride level, and the highest fluoride level is in the outermost enamel (1-2 µm) (Brudevold, McCann and Grøn, 1968; Grøn, Brudevold and Aasenden,, 1971; Bruun, 1973).

As was found by Bruun (1973), no mean fluoride difference in enamel could be found after fluoride treatment when left in vivo for 1 or 2 or 3 weeks. The difference found in the total enamel fluoride level between the in vivo (33%, Table 4 and 5) in vitro (8,5; Table 3) studies, may be attributable to a few factors:

1) The in vitro teeth were immediately exposed to water (after fluoride treatment), while the in vivo teeth were exposed only to the normal saliva flow for the first 6 hours prior to fluoride treatment and

2) Richardson (1967) stated that washing immediately after fluoride treatment removed all the applied fluoride.

It is interesting to note from Table 5 (compare ^{1}C and ^{2}C) that the in vivo control teeth contained also alkaline soluble fluoride (72 ppm), although not directly treated with a sodium fluoride solution. As the control teeth were not removed from the oral cavity before or after fluoride treatment, the fluoride that leached from the fluoride treated tooth could have had an effect on these results (Shannon, 1977; Margalit and Gedalia, 1969). The fluoride on the controls might also come through nutrition fluoride adsorption (Rölla and Bowen, 1978) or from the suggestion of Duff (1973) and Grøn et al (1971) that monofluorophosphate was released from apatite during acid dissolution and would undergo hydrolysis in the presence of hydroxapatite to orthophosphate and fluoride. Later Rölla and Grobler proved that most of the above effect is the result of the NaF treatment (results will soon be in press). Thus the fluoride levels of the control teeth after $O\bar{H}$ treatment were taken as the real fluoride concentration of the control teeth during the discussion of my results (Table 5, ^{2}C).

Therefore, the actual overall fluoride increase due to 2% NaF treatment is equal to 220,8 ppm (Table 5; $^3T - ^2C$; ~33%) of which 182,6 ppm (~27%) is alkaline soluble (CaF_2)

However, after alkaline treatment a fluoride level difference between the control (Table 5, ^2C) and fluoride treated tooth (^3T) was still found ($^4T - ^2C$ = 38,29 ppm = 5,6%) which is attributed to fluorapatite formation (Caslavska et al, 1975). This finding is in agreement with suggestions of other authors that only modest amounts of fluoride are retained permanently (Grøn, 1977; Richardson, 1967; Brudevold et al, 1967; Sundvall-Hagland et al, 1959).

As fluoride is incorporated into the crystal lattice of enamel through systematic ingestion (Fowler, 1967) forming fluorapatite which would be stable in saliva (Mc Cann, 1968 ; Grøn, 1973), fluorapatite formation is a desirable goal for topical fluoride therapy. However, extensive fluorapatite formation is not readily achieved from topical applications under clinical conditions, but caries reduction effects were obtained as a result of topical treatments. As CaF_2 was the major fluoride formed (27%) during a single fluoride treatment and is not lost to the oral environment within at least 3 weeks (8 weeks for Bruun, 1973) it must persist within the enamel surfaces for a long period possibly because of a lack of access for the oral fluid and/or the effect of different ions in saliva surrounding the solid F-compounds. The CaF_2 within the enamel therefore serves as a fluoride reservoir, leaching slowly into the oral environment for a long time period and reducing caries.

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CHAPTER VII

ALKALINE AND NON-ALKALINE SOLUBLE FLUORIDE UPTAKE AND RETENTION BY SOUND ENAMEL AFTER DURAPHAT (2,26% F) TREATMENT, IN VIVO

A. SUMMARY

Acid biopsies showed sound enamel fluoride increase after a single Duraphat (2,26% F)* treatment. Results indicated the same fluoride increase over a period of 1 - 4 weeks. In the teeth of 10 - 12 year olds, it was found that a single duraphat treatment is responsible for a total fluoride increase of 77%, in comparison to the 33% found after a single 2% neutral NaF treatment. Of this 77% a amount of 59% was mainly CaF_2 and 18% fluorapatite.

B. INTRODUCTION

The caries reducing effect of natural or artificial fluoride applications have been demonstrated a long time ago (Dean, 1938; Cheyne, 1942; Adler, Straub and Popovics, 1950) and also quite recently (Retief, Bradley, Barbakow, Friedman, Van der Merwe and Bischoff, 1979; Thylstrup, Fejerskov, Bruun and Kann, 1979).

Depending on the fluoride application concentration on tooth enamel it favoured the formation of CaF_2 (McCann, 1953; McCann and Bullock, 1955; Larsen, Jensen and Thorsen, 1977) or fluorapatite (Grøn, 1977; McCann, 1968), while acid fluoride solutions containing phosphate favoured CaF_2 as the major reaction product (Frazier and Engen, 1966; Wei and Forbes, 1968 ; Baud and Bang, 1970; Aasenden, Brudevold and Mc Cann, 1968; Englander, Carlos, Senning and Mellberg, 1969). Only a fraction of the enamel incorporated fluoride as a result of F treatment did not leach away after 24 hours or more (Brudevold, Mc Cann, Nilsson, Richardson and Coklica, 1967; Aasenden, Brudevold and McCann, 1968).

*Woelm Pharma,Eschwege, West Germany.

The CaF_2 deposited on enamel from topical treatment dissolved within 24 hours when exposed to a potassium hydroxide solution without altering the remaining enamel (Caslavska, Moreno and Brudevold, 1975), while fluoride adsorption to calcium ions in the hydration layer will also be removed by KOH treatment (Rölla and Bowen, 1978).

The increase in fluoride on sound enamel in teeth of 10 to 11 year old children after a single 4 minute neutral 2% NaF treatment was analysed by Bruun (1973), while the types of fluoride formed during a similar treatment of sound enamel was investigated (Chapter VI).

According to the knowledge of this author no attempts have yet been made to determine the types of fluorides formed during a fluoride sealer treatment (duraphat in this case). The aim of the present study has been to determine the uptake and retention of alkaline and non-alkaline soluble fluoride in sound enamel after a single duraphat treatment of teeth which did not come into contact with fluoride, except for that present in food.

C. EXPERIMENTAL

The investigation included 10 selected participants aged 10 - 12 years, whose two first or two second maxillar premolars had to be removed for periodontal reasons. None of the participants had been in contact with any fluoride except for that in nutrition. All subjects were permanent residents in an area with less than 0,01 ppm fluoride in the drinking water supply.

Both the buccal and lingual sides of the teeth were without detectable

caries or demineralized areas appearing as white or brown spots. The selected pairs of teeth of each person were cleaned with a polyethylene microsphere slurry. (Microthene FN U.S.I. Chemicals). The teeth were washed and dried well. One of the above two permolars was coated with duraphat (Woelm Pharma GmbH & Co Eschwege West-Germany) sealer (2,26% F) and the participant fasted for 6 hours. After this treatment both premolars (untreated one serving as control) were left in vivo for 2 weeks, extracted and kept in a moistened atmosphere at 4°C until analysis could be done.

The pairs of premolars were given one biopsy each alternatively on the buccal and lingual sides. This biopsy approach eliminates the possible influence of fluoride level differences between the two tooth sides. Biopsies were taken as outlined by Brudevold, Reda, Aasenden and Bakhos (1975), but the etched area was 12,60 mm^2, the etching time 60 seconds and the total sample volume 1,20 ml. Afterwards the teeth were rinsed in water and each one was soaked separately for 24 hours in 3 ml 1 M KOH solution. This treatment removes all the alkaline soluble fluoride, like CaF_2 (Caslavska et al, 1975).

The pairs of teeth were rinsed twice (for 10 seconds each) in distilled water, dried with compressed air and given 3 independent biopsies on the buccal or lingual side that had not been etched yet. In this way 3 separate layers were etched from each area.

The test solutions were analysed for fluoride and calcium. The fluoride content was determined with an Orion Research, Inc., model 94-09 combination fluoride ion selective electrode coupled to an Orion Model 801A pH/mV meter as described by Brudevold et al (1975). The calcium content was

determined in aliquots of etching solutions diluted 100 times with a $La(NO_3)_3,6H_2O$ solution (to a final 0,25% concentration) to prevent interference from phosphorus and aluminium (Willis, 1961). Calcium was determined by flame absorption spectrophotometry. Assuming 38% Ca in the enamel (Brudevold, Aasenden, McCann and McCann, 1969) and an enamel density of 2,95 ml^{-1} (Manly and Hodge, 1939) the concentration of fluoride is calculated.

The depth of etch was calculated by the following formula:

$$\text{Depth of etch } (\mu m) = \frac{\text{Weight of dissolved enamel}}{\text{Density of enamel X Biopsy surface area}}$$

The mean and s.d. of the enamel fluoride levels for all 10 pairs of premolars before and after alkaline treatment were determined (Table 1).

The fluoride concentrations were calculated from a calibration curve set between 10^{-1} and 10^{-7} M fluoride by means of the Orion sodium fluoride standard, using the buffer solution as described by Brudevold et al (1975).

All chemicals used were of analytical grade, and the same supplies as outlined by Brudevold et al (1975) were used.

D. RESULTS

The fluoride levels in the enamel of the control (C) (mean = 950,4 ppm; s.d.=179,1) and duraphat treated (T) (mean = 1697,4 ppm; s.d. = 444,5) premolars are given in Table 1 column 2, while the fluoride levels of the sound enamel (C = 948,3 ppm; s.d. = 183,5; T = 1127,1 ppm; s.d. = 279,9) after alkaline treatment are given in column 3 i.e. mainly without CaF_2 (Caslavska et al, 1975) and adsorbed fluoride (Rölla et al, 1978).

Table 1: The determination of the mean and standard deviation of the enamel fluoride levels for all 10 pairs of premolars

Etching before OH treatment		Etching after OH treatment	% Fluoride increase (Duraphat treatment)	% Fluoride decrease (OH treatment)	Increase of F-apatite (% Non-alkaline soluble fluoride)
No of Tooth	ppm.	ppm.			
1 C - 5 T 5-	1032,2 1975,9	1024,0 1178,0	91,4	0,8 77,3	15,0
2 C + 4 T 4+	960,2 1633,1	934,3 1153,1	70,1	2,7 50,0	23,4
3 C - 4 T 4-	1031,8 1863,3	1037,3 1396,9	80,6	0,5(increase) 45,2	34,7
4 C - 4 T 4-	787,4 1281,1	798,2 943,3	62,7	1,4(increase) 42,9	18,2
5 C + 4 T 4+	659,3 996,9	650,7 691,6	51,2	1,3 46,3	6,3
6 C + 5 T 5+	1142,7 2579,1	1167,9 1597,5	125,7	2,2(increase) 85,9	36,8
7 C - 5 T 5-	1213,4 1908,7	1200,7 1361,6	57,3	1,1 45,1	13,4
8 C 5- T - 5	971,2 1790,1	947,2 1070,3	84,3	2,5 74,1	13,0
9 C 5+ T + 5	991,6 1664,9	1021,3 1106,1	67,9	3,0(increase) 56,4	8,3
10 C 4+ T + 4	714,3 1280,7	701,6 772,8	79,3	1,8 71,1	10,2
179,1	Abs. mean C = 950,4 (79,1) Abs. mean T = 1697,4 (444,5)*	Abs. mean C = 948,3 (183,5)* Abs. mean T = 1127,1 (279,9)	Abs. mean = 77,1%(21,2)*	Mean C =-0,31 (1,98)* Mean T = 59,4(16,1)*	Mean 17,9 (10,6)*

*Standard deviation

The total percentage fluoride increase as a result of duraphat-sealer treatment is given in column 4 (mean = 77,1%; s.d. = 21,2%) and is calculated as an increase on the control of each participant. Column 5 represents the percentage fluoride decrease, as a result of alkaline treatment, expressed as a percentage decrease on the control tooth, for each pair of premolars (mean T = 59,4%; s.d. = 16,1%). Column 5 also represents a decrease in fluoride (sometimes an increase) of the controls as a result of alkaline treatment (Mean C = 0,31%; s.d. = 1,98%). The last column represents the percentage fluoride increase (mean = 17,9%; s.d. = 10,6%) on sound enamel that is not alkaline soluble i.e. mainly fluorapatite. The statistical treatment of the results of Table 1 are represented in Table 2.

The mean biopsy depth for the 40 biopsies of Table 1 is 6,95 µm (s.d. = 0,67).

E. DISCUSSION AND CONCLUSION

No significant pumice effect was observed when the teeth were given a light pumice with polyethylene micropheres (Chapter VI). It was therefore decided to clean the teeth with polyethylene before topical fluoride treatment, as debris on the teeth surface interferes with the efficiency of topical treatment (Knutson, Armstrong and Feldman, 1947).

Although Weatherwell, Deutsch, Robinson and Hallsworth (1977) observed differences in fluoride levels between different teeth in the same mouth as well as from area to area on the same tooth, small differences were estimated by Aasenden, Allukian, Brudevold and Wellock (1971). No significant differences were observed when etching a area 12,60 mm^2 from the buccal and lingual side of the same tooth (Chapter VI). However,

control etchings were done alternatively on the buccal and lingual sides of one of the premolar pair of each participant.

As is the case with a single neutral 4 minute 2% NaF treatment (Bruun, 1973; Chapter VI) the duraphat treatment resulted the same fluoride level increase over a period of 1 - 4 weeks. In comparison with the results obtained during a 2% NaF treatment the premolars were extracted after having been in vivo for two weeks. In the case of duraphat treated teeth a total sound enamel fluoride increase of 77,1% was observed in comparison to an average total fluoride increase of 33% (Chapter VI) and 15,2% (Bruun, 1973) after a single neutral 2% NaF treatment. This higher fluoride increase can be expected as the concentration of the fluoride in the duraphat complex (2,26%) is higher than the 2% F^- in the NaF solution. Secondly and more important might be the limitation of the influence of environment fluid because the duraphat sealer is partially resistant to this. Therefore, the fluoride-enamel contact time has increased, giving the chemical reaction more time to take place and thus more fluoride could be formed during duraphat treatment.

An amount of 59,4% of the total sound enamel fluoride increase was alkaline soluble in comparison to the 27% when treated with 2% NaF. Thus, the duraphat treatment gave about two times more CaF_2 than 2% NaF, as high fluoride concentrations and low solution to solid ratios favoured CaF_2 formation (McCann, 1953; McCann et al, 1955). Furthermore the enamel-fluoride contact time was increased by duraphat treatment while the fluoride concentration was kept high for a longer period. This high amount of CaF_2 formed is supported by scanning electron microscopy findings where the enamel surface was covered by

a fine-granular layer of CaF_2 after a 2 hour exposure to a fluoride solution (Larsen and Fejerskov, 1978).

No significant decrease of the fluoride content of the controls after alkaline treatment was observed (Table 1, Column 5). In comparison to this there was a 72 ppm fluoride decrease on the controls during the NaF experiment (Chapter VI). This might be due to the fact that the leaching of fluoride ions from the duraphat on the teeth is very slow, at least much slower than that of fluoride ions from NaF. Thus it is also possible that the CaF_2 formed on the controls during 2% NaF treatment is a result of the NaF leaching effect just after treatment and not because of the CaF_2 reservoir formed on the teeth. Otherwise this effect should as well have been discovered during duraphat treatment.

As was expected duraphat treatment also increased the percentage of fluorapatite formation (17,9%) as fluoride concentrations of 0,005 M or less favoured fluorapatite formation (Grøn, 1977). In the case of 2% NaF treatment the fluorapatite formation was only 6% of the enamel-fluoride increase, giving a 3 times lower fluorapatite formation.

All the results discussed above (Table 1) are found to be significant to a positive extent as can be seen from Table 2.

Table 2: Statistical treatment of the results obtained during duraphat treatment.

Duraphat treatment

Before OH treatment		After OH treatment	
^{1}C mean	= 950,42 ppm	^{2}C mean	= 948,32 ppm
std. dev.	= 179,12 ppm	std. dev.	= 183,50 ppm
^{3}T mean	= 1697,38 ppm	^{4}T mean	= 1127,12 ppm
std. dev.	= 444,54 ppm	std.dev.	= 279,87 ppm

Matched pairs T-test for differences

1 vs 2	t = 1,03 df = 9	no sign. change due to $\overline{OH}$ treatment
1 vs 3	t = +7,9 df = 9	sign. increase due to duraphat
1 vs 4	t = 4,19 df = 9	sign. increase after duraphat and $\overline{OH}$ treatments.
3 vs 4	t =-8,65 df = 9	sign. decrease.

The symbols C, T and std. dev. define control teeth, treated teeth and standard deviation, respectively.

As continuous fluoride treatment (in natural high fluoride areas) might be compared with duraphat treatment the high total enamel-fluoride increase (77,1%) might confirm the findings, in accordance with current theories, that topical effects of fluoride rather than systematic effects prevent dental caries (Thylstrup et al, 1979).

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