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THE RELATIONSHIP BETWEEN SUGAR METABOLISM AND POTASSIUM TRANSLOCATION BY CARIES-INDUCING STREPTOCOCCI AND THE INHIBITORY ROLE OF FLUORIDE

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Summary—The caries-conducive strain FA-1 of *Streptococcus mutans* removed more glucose than sucrose during anaerobic incubation, mainly at pH 5·8, in a medium that did not promote growth. The addition of fluoride reduced the sugar disappearance but this reduction was partly alleviated by extra potassium. The uptake of potassium by the cells was also greater in the presence of glucose than sucrose. Potassium was extruded from both fermenting and non-fermenting cells on addition of about 50 ppm fluoride. This was accompanied by a rapid uptake of fluoride by fermenting cells followed by an immediate release back into the fluid phase.

Simultaneously, the concentration of intracellular sugar was higher in the presence of glucose than of sucrose. The intracellular sugar increased during the initial disappearance of the external sugar but diminished during the period of reduced sugar utilisation. Fluoride or potassium modified the intracellular sugar changes only slightly or negligibly. Acid production from the cells was greater from glucose than sucrose and both were slightly enhanced by potassium. Acid production was completely inhibited by about 50 ppm fluoride concentration.

The results suggest a close association of potassium transport of this streptococcus to sugar uptake and metabolism. The inhibition by fluoride of sugar uptake and metabolism seems to be largely due to its interferring action of the cation translocation and vice versa.

INTRODUCTION

The association of the metabolism of sugar by microogranisms with accumulation of potassium and extrusion of sodium has been established in numerous investigations (reviewed by Rothstein, 1972; Armstrong, 1972) concerning different species. Some observations on the effect on caries incidence in animals of dietary mineral additives having different cations (Nizel and Harris, 1964; Luoma *et al.*, 1968) have stimulated studies on the relationship of the alkali cations to sucrose fermentation and phosphate accumulation by caries-inducing streptococci (Luoma, 1969, 1971, 1972a, b, 1973; Knuuttila, 1973). In these studies, the dependence of the bacteria on the available potassium was demonstrated as well as changes in its accumulation by these cells through factors, such as pH-variations, fluoride, alcohols, sodium and chlorhexidine. Little is known about the relationship of many other aspects of the carbohydrate metabolism of caries-conducive bacteria such as unlimiting the concentration of available potassium, the nature of the sugar, more exact nature of the action of fluoride and the intracellular sugar concentration, to the cation transport of these organisms. The present studies were therefore conducted in order to elucidate these aspects in some detail.

Skinner and Naylor (1972) found that during the incubation of *Streptococcus mutans* with glucose or fructose, acid production was rapid, while in the presence of sucrose it was considerably delayed. Glucose and fructose together caused a rapid acid production. This was attributed to the existence of a substrate-controlled transport mechanism in the cell membrane. Robrish and Krichewsky (1972), while studying acid production by several strains of caries-conducive streptococci from different substrates at limiting concentrations, found that the yield of acid per mole of sucrose was the same as that produced from both hexose units. Extracellular polymer was formed from sucrose but only to a small extent.

Experiments in this study were designed to compare the influence of potassium and fluoride on four main parameters in sugar metabolism:

(1) The utilisation of exogenous sugar, i.e. the loss of glucose or sucrose from the medium during incubation.

(2) Change in intracellular glycogen content of cells incubated in the presence of glucose or sucrose.

(3) The degradation of intracellular glycogen in the absence of exogenous substrate, i.e. the endogenous energy metabolism.

(4) The possible uptake and release of fluoride by bacteria in the presence of some factors that modify energy metabolism.

In ionic transport studies, special attention was paid to the uptake of potassium by cells and the effects of potassium on sugar uptake, acid production and the cellular sodium content in the presence or absence of fluoride.

MATERIAL AND METHODS

The bacteria

The FA-1 strain of *Streptococcus mutans*, cariogenic in gnotobiotic rats (Fitzgerald *et al.*, 1960), was grown

in NIH-thioglycollate broth. Between the growth phases, the culture was transferred into a new medium twice a week. For growing the cells in large amounts, two successive transfers into new broth were made during the preceding day and 20 ml from the last "maintenance" growth of 16–17 hr was added as inoculum per 500 ml of the thioglycollate broth. The growth was performed at 37°C under continuous agitation and neutralization with 20 per cent NaOH and under N_2 atmosphere. The cells were harvested by centrifugation at the exponential growth phase, the cells were washed at least twice with cold solution of 0·2 per cent $CaCl_2$ and 0·1 per cent $MgCl_2$. It was found earlier that cells grown by this procedure were able to accumulate potassium and also were sensitive to fluoride as indicated by a leakage of bacterial potassium extracellularly. For overnight storage, the cells were suspended at +4°C in fresh thioglycollate medium and centrifuged (225 *g* at +4°C). The cell suspension in 50 ml $CaCl_2$–$MgCl_2$ solution had an absorbance of 0·060 at 660 nm when diluted 100-fold. The undiluted suspension contained 525 μg of cellular nitrogen per ml. Volumes (1 ml) of this suspension were then employed.

Solutions

For the sugar and potassium uptake studies, with or without fluoride, 2 ml volumes of 0·2 M tris-acid maleate-NaOH buffer pH 5·8 were used as the main solution. Luoma (1972a) found that at this pH, potassium and phosphorus accumulation by cells took place, although suboptimally, and that the inhibition of these accumulations by fluoride, for example, became clearly detectable. For the acid production studies, the same buffer was used in 2 ml volumes and in final concentration of 0·05 M (pH 6·8) or 0·02 M (pH 5·8). Sucrose or glucose were added in 1 ml volume of 0·146 M concentration. In the sugar and the respective control solutions, 100 mM Na_2HPO_4 and 0·3 M NaCl were added, the former for the satisfaction of bacterial phosphate need, because bacterial potassium and phosphate metabolism are coupled with each other, (Rothstein, 1972) and the latter for decreasing the "basal" potassium in cells thus rendering them more susceptible for potassium uptake (Zarlengo and Schultz, 1966). The volume of bacterial cell suspension was 1 ml. In potassium uptake studies, 1 ml of 31·5 mM potassium chloride was added and 1 ml of distilled water in the controls. In inhibition studies, 1 ml volume of 237·5 ppm fluoride with or without 31·5 mM potassium was added as sodium fluoride. Compared with the concentration of sodium in the other salts used, the sodium in NaF had no modifying significance on the metabolic changes observed.

Procedures

Incubations were performed in a waterbath at 37°C under an atmosphere of 95 per cent N_2 and 5 per cent CO_2. The buffer and sugar solutions were preheated for 10 min and the bacterial suspension for 5 min before mixing together at zero time. Fifteen minutes later, potassium or fluoride ion additions were made. After incubations for 10–75 min, the samples were cooled in ice and the cells were separated with Millipore® filtration (pore size 0·45 μM) and washed twice with cold solution of 0·2 per cent $CaCl_2$ and 0·1 per cent $MgCl_2$ to give 10 ml of filtered liquid. The pH of the incubation mixtures did not fall more than 0·1 unit.

In the pH measurements, a Leeds-Northrup pH-meter with a miniature electrode kit was employed. Fluoride was determined using a specific F^- ion electrode (Orion Research Inc.) with Orion Research Ionanalyzer model 407. Potassium and sodium were determined by flame photometry after wet digestion in 0·5 ml concentrated nitric acid or perchloric acid. All sugar determinations were carried out by the phenol-sulphuric acid method (Dubois *et al.*, 1956). Before sugar determinations, cellular glycogen was extracted by the method of Northcote (1953) with 0·5 M perchloric acid. Cellular nitrogen was determined by an adaptation of the method of Conway (1962) and cellular protein by the biuret method (Robinson and Hogden, 1940). The detailed arrangements of each experiment are given with the results.

Experiment 1. *The use of exogenous sugar, i.e. the loss of glucose or sucrose from the media during incubation and its modification by potassium and fluoride.* Two series, one containing glucose and the other sucrose as external energy source were incubated with the FA-1 streptococcus cells, final sugar concentration in the incubation medium being 29·2 mM. Comparison of the effects of glucose and sucrose may seem difficult but since the range of concentrations of sugars used here apparently were far above limiting values (Skinner and Naylor, 1972) the comparison was thought justified. Samples were taken at intervals and the remaining sugar was determined using separate glucose and sucrose standards. At zero time, sugar was added to the medium and 15 min later potassium,

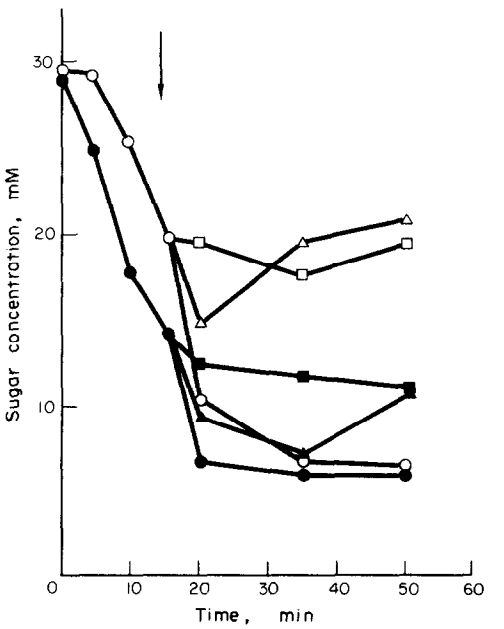

Fig. 1. The disappearance of glucose or sucrose from the cell suspension media. Open points, sucrose; closed points, glucose; circles, incubated with sugar and 6·3 mM potassium; squares, 47·5 ppm fluoride added; triangles, both potassium and fluoride added. All potassium and fluoride additions were made after 15 min preincubation with sugar (arrow).

with or without fluoride or fluoride alone were added, the final concentrations being 6·3 mM, 6·3 mM with 47·5 ppm or 47·5 ppm, respectively, in the incubation fluid.

Results. During the first 20 min, the disappearance of glucose and sucrose from the media was rapid, especially after the addition of potassium glucose decreasing faster. During the next 30 min appreciably less glucose than sucrose was taken up by the cells (Fig. 1).

When fluoride was added to the medium after 15 min incubation, the removal of both sugars was almost completely inhibited. When both potassium and fluoride were added after 15 min, the inhibitory effect of fluoride was partly alleviated in both cases during the following 20 min. However, both the sucrose and glucose were later partly extruded from the cells (Fig. 1).

Experiment 2. *The synthesis or breakdown of intracellular glycogen by cells in the presence of glucose or sucrose in the fermenting media.* The incubation conditions were the same as above. The cells were harvested and washed twice and the last wash shown to contain no sugar. The cells were extracted with 0·5 M perchloric acid for 90 min at 38°C with shaking and centrifuged; the supernatant contains all the ribonucleic acid but neglible carbohydrate, and it was discarded. Then the cells were extracted with 0·25 M Na_2CO_3 for 45 min at 100°C and centrifuged; the extract contains all the alkali-soluble glycogen and it was combined with the cell residue extract obtained with 0·5 M perchloric acid incubation for 30 min at 100°C; so-called acid-soluble glycogen. The sugar was determined by the phenolic–sulphuric acid method, glucose being used as a standard.

Protein determinations by the biuret method were made from the supernatant obtained by the alkali extraction procedure and the cell residues dispersed in 1 M NaOH after the acid extraction procedure. The values obtained were combined. The protein estimations were converted to cellular nitrogen values with the aid of control cell samples whose nitrogen was determined.

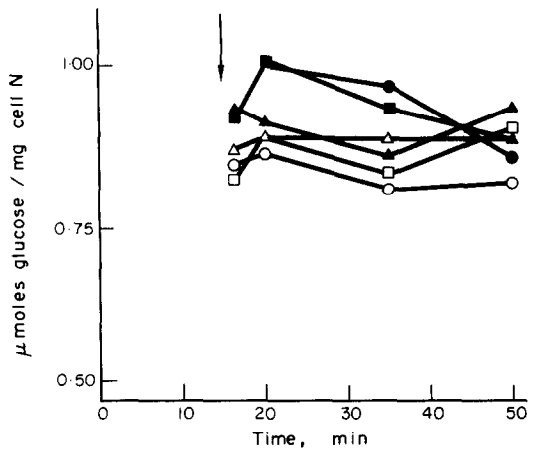

Fig. 2. Intracellular sugar content of the cells during incubation with glucose (solid points), or with sucrose (open points). At 15 min, additions were made of 6·3 mM potassium (circles); 47·5 mM fluoride (squares); potassium plus fluoride (triangles).

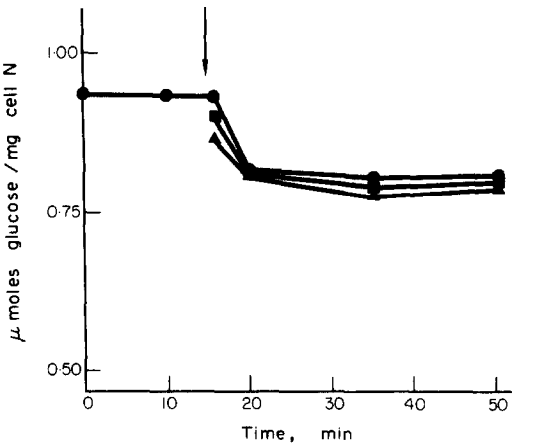

Fig. 3. Intracellular sugar concentration of the cells in the absence of external energy. At 15 min, the same additions were made as in Fig. 2.

Results. When the rate of sugar uptake was highest during the first 20 min incubation, the cellular glycogen content was slightly increasing in both media, but afterwards, during the time of reduced sugar utilization, the cellular glycogen, expressed as glucose, was decreasing (Fig. 2) in glucose medium. In sucrose medium, the cellular glycogen content varied slightly without a definite trend in the presence of added 6·3 mM potassium. When 47·5 ppm of fluoride was added to the sucrose medium, the cellular glycogen first rose and then decreased. When both 47·5 ppm fluoride and 6·3 mM potassium were added, the cellular glycogen did not show any definite changes. Altogether, the cellular glycogen content remained generally higher in glucose than in sucrose medium (Fig. 2).

Experiment 3. *The degradation of cellular glycogen in the absence of external energy* i.e. *endogenous metabolism.* The incubation conditions were the same as above except that sugar was omitted from the fermenting medium and both the glycogen and protein content of the cells were determined.

Results. Intracellular sugar content decreased in each case after the addition of potassium, fluoride or both. No distinction can be drawn between incubation media and no inhibition in the use of glycogen by fluoride was observed (Fig. 3).

Experiment 4. *Changes of* pH *during incubation of* FA-1 *streptococcus cells with sucrose or glucose in the presence of potassium with or without fluoride in the medium.* The only difference from the previous experiments was that the molarity of the buffer was 0·05 or 0·02 M to allow the pH drop to occur.

Results. The fall in pH was rapid in glucose media and delayed in sucrose media. When potassium was added, the pH fall was slightly enhanced (Fig. 4a).

When fluoride was added simultaneously with potassium after 15 min preincubation with sucrose or glucose, an inhibition of the pH fall was observable (Fig. 4b). In fact, an elevation of pH occurred followed by a stable period in the presence of each of the sugars.

Experiment 5. *Cellular uptake of potassium and fluoride during sucrose or glucose fermentation or in the absence of sugar. The effects on cellular sodium*

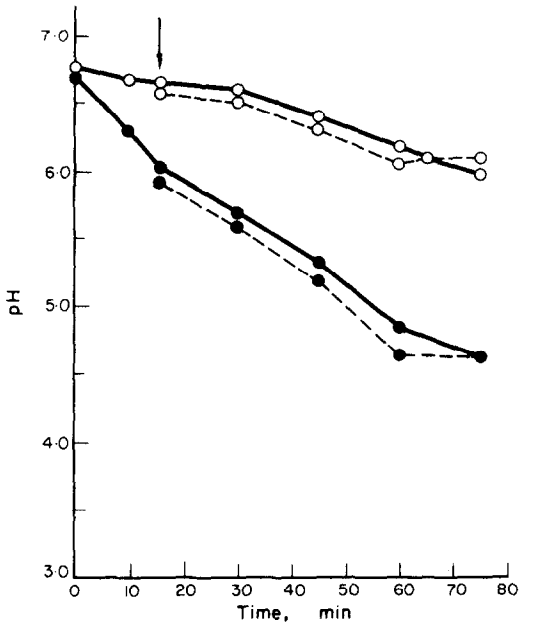

Fig. 4a. Change in extracellular pH, when streptococcus cells are incubated in 0·05 m TAM-buffer, initial pH 6·8, with glucose (solid points), or sucrose (open points). At 15 min 6·3 mM potassium was added (dotted lines).

content in the presence of absence of fluoride. Incubation conditions were the same as in experiment 1.

Results. The changes in cellular potassium are shown in Fig. 5(a, b, c). The results of comparisons of the cellular sodium content at 10 and at 50 min of incubation are given in Table 1. When the incubation medium contained no sugar and potassium was

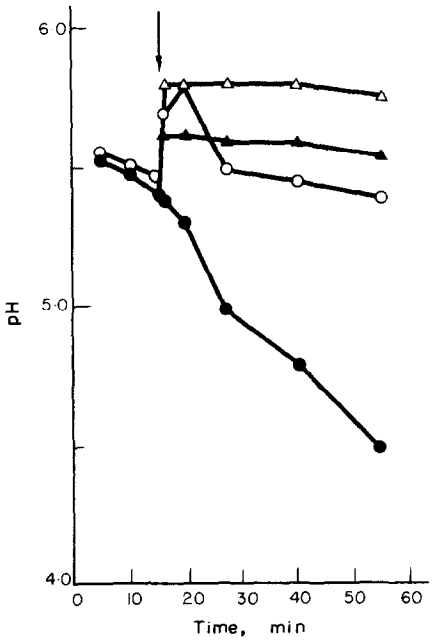

Fig. 4b. Change in extracellular pH when streptococcus cells are incubated in 0·02 M TAM-buffer, initial pH 5·8, and in glucose media (solid points) or in sucrose media (open points). At 15 min addition of 6·3 mM potassium (circles); 47·5 ppm fluoride plus potassium (triangles).

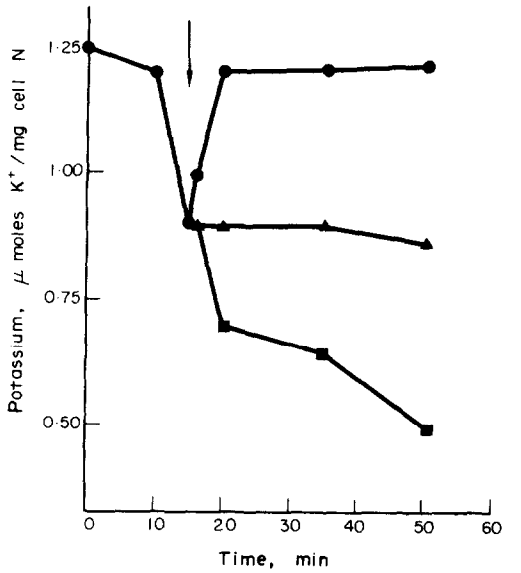

Fig. 5a. Change in cellular potassium content when cells are incubated without external energy. After 15 min preincubation of the cells without sugars, the following additions were made: 6·3 mM potassium (circles); 47·5 ppm fluoride (squares); potassium and fluoride (triangles).

added, the cellular potassium content increased (Fig. 5a). When sucrose or glucose was present, the increase was much more pronounced (Fig. 5b).

When fluoride was added, a significant loss of cellular potassium was observed in all incubation media. When fluoride and potassium were added simultaneously, the cellular potassium loss was not as prominent as in the presence of fluoride alone. In the glucose system, there was an initial accumulation of potassium by the cells in the presence of both potassium and fluoride (Fig. 5b).

When comparing the net transport (values in the presence of substrate minus values in the absence of

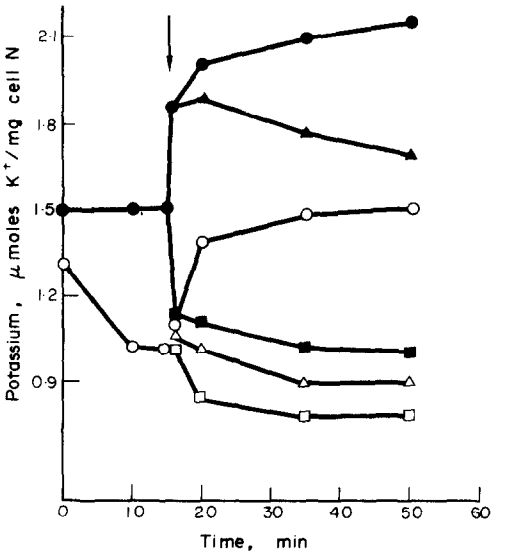

Fig. 5b. Changes in intracellular potassium when incubated in glucose (solid points) or in sucrose (open points) medium. At 15 min, the same additions were made as in Fig. 5a.

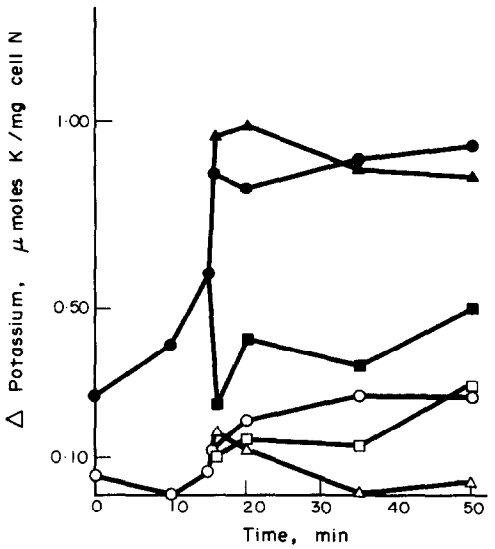

Fig. 5c. Net uptake of potassium by cells when incubated in glucose media (solid points) or in sucrose media (open points). At 15 min were added potassium 6·3 mM (circles), fluoride 47·5 ppm (squares) and potassium plus fluoride (triangles).

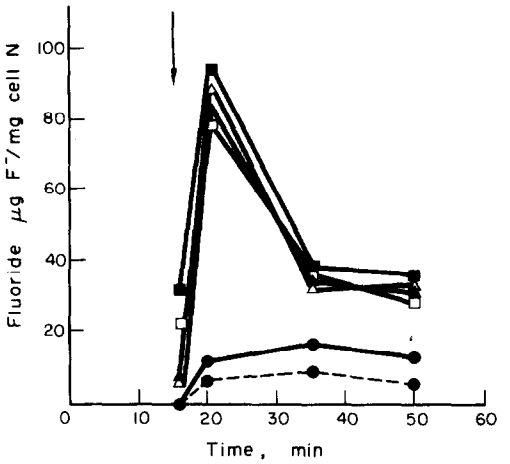

Fig. 6. Temporary uptake of fluoride into cells when incubated in glucose medium (solid points) or in sucrose medium (open points) or controls without sugar (two lower curves) but potassium being present (solid line) or absent (broken line). At 15 min were added: fluoride (squares), potassium with fluoride (triangles).

substrate) of potassium into the cells during the incubation (Fig. 5c), more potassium was taken up after it was added during glucose fermentation. During sucrose fermentation, the net uptake of potassium was less than during glucose fermentation. When fluoride and potassium were added simultaneously to glucose medium, the antagonism between potassium and fluoride was again observable.

The changes in cellular sodium content were generally moderate or small (Table 1). The fluoride tended to extrude the sodium as it did with potassium but to a smaller extent. Potassium, when added with fluoride, also here counteracted the effect of fluoride, especially in the glucose medium.

Figure 6 shows that in the presence of both sucrose and glucose, the fluoride was rapidly taken up by the cells from the medium but then almost completely restored. This was not modified by potassium that was added simultaneously. In the non-fermenting system, fluoride disappearance from the medium was very slight. However, it was more prominent in the presence of extracellular potassium than in its absence.

Table 1. Cellular sodium content during glucose or sucrose fermentation or without sugar, when potassium or fluoride or both are added to the incubation media.

Incubation conditions	Addition at 15 min	Cellular sodium (μM/mg cell N) 10 min	50 min
Buffer only	K^+ 6·3 mM	1·333	
	F^- 47·5 ppm		
	K^+ 6·3 mM, F^- 47·5 ppm		
Buffer and glucose	K^+ 6·3 mM	1·266	1·300
	F^- 47·5 ppm		0·967
	K^+ 6·3 mM, F^- 47·5 ppm		1·533
Buffer and sucrose	K^+ 6·3 mM	1·420	1·367
	F^- 47·5 ppm		1·233
	K^+ 6·3 mM, F^- 47·5 ppm		1·300

DISCUSSION

Sugar fermentation and acid production by the dental plaque cells in relation to caries

Caries-active plaques have shown lower average pH-values than the caries-inactive plaques after sucrose ingestion (Stephan, 1944; Rosen and Weisenstein, 1965) and even during the ingestion (Turtola and Luoma, 1972a). For this reason, acid production and other functions of cariogenic plaque organisms (Turtola and Luoma, 1972b) deserve continuing interest.

The fall in pH caused by *Strep. mutans* FA-1 in the presence of sucrose was delayed compared with the rapid fall in the presence of glucose. This has also been observed with *Strep. mutans* Ingbritt strain by Skinner and Naylor (1972). According to them, different rates of sugar utilization could be explained if sugar transport across the cell membrane is considered as the rate limiting step. However, when measuring the disappearance of glucose or sucrose from the extracellular media during the first 20 min, glucose transport across the cell membrane is more rapid but already at 50 min, the extracellular sugars are nearly equal. Probably, the invertase synthesis is activated for the hydrolysis of sucrose into glucose and fructose, after the initial energy utilization, thus facilitating the utilization of sucrose as its monosaccharide components. The generally noted high cariogenicity of sucrose found in most animal experiments has been attributed to its role as a substrate for the formation of the relatively stable extracellular polysaccharides. It has been speculated that this may slow down diffusion of acids out of the plaque though there is no direct evidence to substantiate this.

Cellular glycogen and effect of fluoride on the sugar uptake by bacterial cells

The polysaccharide content of the cells, determined as glycogen, was higher in all cases when different ions were added to the glucose medium. No significant increase was observed in cells incubated in the sucrose medium. Sucrose was probably used in part for extracellular polysaccharide synthesis and the sugar components were not extractable by the glycogen method used. When fluoride was added to the media after 15 min preincubation with substrates, both the sucrose and glucose uptake were inhibited, like the pH-fall. Glucose is transported to *Strep. mutans*, *Strep. salivarius* and *Strep. sanquis* cells via the phosphoenol-puruyvate-dependent (PEP-) transferase system (Kanapka and Hamilton, 1971; Schachtele and Mayo, 1973). It has been suggested that in *Strep. salivarius* cells, sodium fluoride alters the process of sugar translocation across the membrane and not the phosphorylation of sugar itself (Kanapka and Hamilton, 1971). Schachtele and Mayo (1973) found, indeed, that PEP-dependent transferase system was not sensitive to fluoride. However, the transport study of a glucose analogue showed that the transport was sensitive to as low as 0·1 mM fluoride. A fluoride concentration of 1 mM (19 ppm) reduced the transport by 37–48 per cent and a complete blocking occurred at 10 mM fluoride. Our results are thus not in disagreement with these findings but our results on cation translocation indicate that the inhibition of bacteria by fluoride may be in considerable part mediated through altered potassium transport.

Weiss *et al.* (1965) and Sandham and Kleinberg (1969) obtained a definite inhibition by fluoride of intracellular carbohydrate storage by *Strep. mitis* and by cells of salivary sediment, respectively. These groups considered the glucose uptake as the prime object of the inhibition.

When measuring the endogenous glycogen degradation, no inhibition was observed with fluoride. The apparent absence of glycogen degradation in the absence of added potassium must signify that potassium is present in limiting concentrations intracellularly. The slight activation of the glycogen degradation by fluoride addition remains unexplained. Perhaps the cell uses some energy to adapt itself by some way in the disturbing environment containing fluoride. In *Strep. salivarius*, only a slight inhibition by fluoride was observed (Hamilton, 1969).

Cation transport and its relation to sugar transport

The rapid fall in cellular potassium between 10 and 15 min incubation may be due to the release of potassium from capsular polysaccharides (Rorem, 1955). The present results clearly demonstrate, in accordance with earlier data on functions of the K-1 streptococcus (Luoma, 1971, 1972a, b; Luoma *et al.*, 1971) the necessity of potassium and its cellular accumulation for the maximum sugar uptake and the ensuing acid production by a cariogenic microorganism, i.e. in a "cariogenic" situation. Also, the rapid extrusion of potassium by the same cells was shown with decreased sugar uptake and acid production in the presence of fluoride, i.e. in a "caries-inhibitory" situation. The above earlier data, obtained with another organism and with sucrose, were confirmed here by using a cariogenic FA-1 streptococcus and both sucrose and glucose. A part of the preliminary observations on the relations of cations to acid production, done with the FA-1 streptococcus (Luoma, 1968, 1969), were also confirmed.

The cation transport systems in all organisms tested do require metabolic support and they are repressed by the absence of appropriate substrates, by metabolic inhibitors, or by any other conditions that markedly reduce the rate of metabolism (Rothstein, 1972). When potassium was taken up by our cells, a transient increase in the rate of glycolysis occurred, suggesting a metabolic coupling of cation transport and metabolism as observed earlier with *Strep. faecalis* cells (Zarlengo and Schultz, 1966). It is difficult, perhaps impossible, to say which one of the two functions, the potassium transport or the sugar uptake, is the more "basal" or central one. Each of these functions needs the support of the other for the activation. Indeed, fluoride could exert its inhibitory function on both the ionic transport by inhibiting the sugar translocation process and by inhibiting potassium uptake by fermenting *Strep. mutans* cells. It even extruded potassium ions, especially when no energy and potassium were available. Furthermore, some potassium was taken up by cells when no external energy was available, probably mainly as a result of passive diffusion or adsorption on cell surface layers. Some potassium could, however, be taken up as a result of the utilization of endogenous energy.

Fluoride and energy metabolism

The fact that no inhibition of the use of the endogenous energy occurred in the presence of fluoride suggests that this function occurs entirely inside the membrane.

The inhibitory effects of 25–50 ppm fluoride on non-fermenting bacteria are reversible because after washing of the cells, pretreated with fluoride but without sugar, the rate of acid production was near the rate of untreated control cells (Luoma, 1972).

Our present findings concerning cellular fluoride in the absence of energy (Fig. 6, lower curves) are partly in line with results of Birkeland (1973) and of Birkeland and Rølla (1972) who found that fluoride is not concentrated by oral bacteria. Our present results (Fig. 6, upper curves) concerning fluoride in fermenting cells are partly in agreement with the opposite results of Williams, (1968) and Jenkins, *et al.* (1969), who found that fluoride was accumulated *in vitro* by growing streptococci originating from dental plaque. They added fluoride to the medium of growing microbial cells while ours were not growing during the ordinary experiments. Our results on uptake and release of fluoride by fermenting, non-growing cells, described by Fig. 6, therefore provide a possible explanation for the above conflicting results. It is conceivable that fluoride was trapped and transported into cells through the phosphate-accumulating system that is activated by glycolysis (Rothstein, 1972) and then extruded through some other way.

Apparently, the mere presence of fluoride outside the cells was sufficient to promote a decrease in cellular potassium content (Fig. 5a,) whereas the inhibition of the strong tendency of cellular potassium accumu-

lation in the presence of sugar needed a stronger action. This appeared to include the short and reversible intracellular accumulation of fluoride. The concentrations of cellular fluoride, even at the stable level (Fig. 6) were higher than values of Jenkins *et al.* (1969). This may be most simply explained by different shape and composition of the cell exterior that apparently may adsorb or bind widely differing amounts of certain ions in different organisms. The pH conditions of Fig. 4a, suggest that the release of fluoride in experiment 5 back into the fluid phase was not a result of a decrease in the medium pH. The consequences of the short inhibitory presence of fluoride within the fermenting cells may also be fully reversible because earlier (Luoma, 1973) a 25 ppm "pretreatment fluoride" concentration, pH 5·8, around fermenting K-1 cocci for 1 hr, which reduced both cell potassium and phosphorus, had no influence on subsequent acid production from sucrose after the cells had been washed.

Our findings support the idea that fluoride exerts its effect on ionic transport and sugar metabolism by interacting with the membrane itself. Furthermore, the impairment of cation transport may decrease the rate of sugar metabolism and vice versa.

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