THE *IN VIVO* EFFECTS OF FLUORIDE ON GLUCOSE METABOLISM BY *STREPTOCOCCUS SALIVARIUS*

by

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ABSTRACT

The research presented in this thesis was designed to elucidate the mechanism by which fluoride inhibits glycolysis and glycogen synthesis in whole cells of the homofermentative oral microbe, Streptococcus salivarius. A review of the literature revealed that enolase in the glycolytic pathway and phosphoglucomutase in the glycogen synthesis pathway were sensitive to fluoride in vitro. However, previous studies with S. salivarius had provided evidence which indicated that glycolysis and glycogen synthesis were inhibited by fluoride at some unknown site prior to glucose-6-P formation. As a result of these considerations, experiments were designed to determine the in vivo effect of fluoride on the activity of enolase, phosphoglucomutase and the reactions leading to glucose-6-P formation.

The concentrations of glucose-6-P and ATP in intact cells were analyzed by enzymatic-fluorometric techniques during anaerobic glucose metabolism in the presence and absence of fluoride. The addition of 2.4 mM NaF to cells actively degrading glucose resulted in an immediate decrease in the cellular content of glucose-6-P and ATP, concomitant with the complete inhibition of glucose uptake and glycogen synthesis. This effect was a normally sustained one since the glucose-6-P and ATP concentrations in cells incubated with fluoride usually remained at depressed levels as long as the inhibitor was present. A noticeable decrease in the cellular glucose-6-P concentration was produced by NaF as low as 0.06 mM, regardless of whether the fluoride was added before or after the substrate. After the initial decline in glucose-6-P content, the intracellular concentration of this intermediate often increased as a result of the

degradation of endogenous glycogen. ATP, on the other hand, always remained at a low level in the presence of the inhibitor.

Experiments with crude enzyme preparations of the organism demonstrated that the glycogen synthetic enzymes, phosphoglucomutase, ADP-glucose pyrophosphorylase, and ADP-glucose : glucan transferase, as well as phosphorylase in the degradative pathway, were fluoride insensitive at NaF concentrations inhibiting in vivo synthesis. The inability of NaF (2.4 mM) to inhibit glucose-6-P formation from glycogen in vivo in the absence of exogenous glucose confirmed that phosphoglucomutase and phosphorylase in this organism were not inhibited by fluoride. Hence, the inhibition of glycogen synthesis was probably due simply to the unavailability of glucose-6-P and/or ATP for synthesis.

The effect of fluoride on intracellular enolase activity was determined by measuring the cellular content of 2-P-glycerate, the substrate for the enzyme, and P-enolpyruvate, the product. The addition of 2.4 mM NaF to cells metabolizing glucose at a constant pH of 7.2 resulted in a marked increase in the cellular 2-P-glycerate concentration and a decline in the amount of P-enolpyruvate demonstrating the inhibition of enolase activity in vivo. In addition, the cellular concentration of glucose-6-P decreased markedly. These two apparently independent fluoride effects could not be separated regardless of the pH at which the cells were incubated (pH 7.2, 8.0, and 5.8) or the concentration of NaF added to the cells (final concentration: 2.4, 0.36 and 0.12 mM). Since the addition of 2.4 mM NaF to cells metabolizing intracellular glycogen at pH 7.2 had no effect on the cellular content of glucose-6-P but resulted only in the inhibition of enolase activity, it was apparent that fluoride inhibition of glucose-6-P formation was associated solely

with exogenous glucose metabolism. The relationship between the inhibition of enolase activity and the inhibition of glucose-6-P formation was clarified when it was demonstrated that the P-enolpyruvate phosphotransferase system was involved in the transport of glucose into cells of S. salivarius. Fluoride did not inhibit the cellular production of 2-deoxyglucose-6-P-^{14}C from 2-deoxyglucose-^{14}C and P-enolpyruvate, but it did inhibit the formation of this phosphorylated hexose when 2-P-glycerate was the phosphorylated substrate. These results strongly suggest that fluoride inhibition of intracellular enolase activity results in the inhibition of glucose transport by reducing the amount of P-enolpyruvate available for phosphorylation. As a consequence of this, the cellular concentrations of glucose-6-P and ATP would decrease and the inhibition of glycogen synthesis would occur.

In an attempt to determine the mechanism of fluoride-resistance, additional studies were performed to provide a comparison between fluoride-treated and untreated cells of strains of S. salivarius resistant to the inhibitor. The addition of 2.4 mM NaF to fluoride-resistant cells metabolizing glucose at a constant pH of 7.2 resulted in only a slight inhibition of glucose metabolism. The cells behaved as if little, or no fluoride had reached the sensitive enolase site. However, when the same experiments were carried out at pH 5.8, marked inhibition of enolase activity and glucose-6-P formation was observed. In the presence of fluoride, the metabolic patterns of the resistant cells resembled that of the wild-type fluoride-sensitive cells. These results are inconsistent with the hypotheses of other workers who proposed that resistance to fluoride was conferred either by the development of a

fluoride-insensitive alternate glycolytic pathway or by the development of fluoride resistant enolase. They are, however, consistent with the hypothesis that fluoride-resistant cells are impermeable to the inhibitor at neutral pH.

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CHAPTER 1

INTRODUCTION

Previous studies with the oral microbe Streptococcus salivarius have characterized the fluoride sensitivity of exogenous glucose catabolism, glycogen synthesis, and glycogen degradation by intact cells of this organism. The synthesis of glycogen, completely inhibited by NaF as low as 0.48 mM, was the most sensitive of these metabolic parameters. The uptake of glucose from the medium was next in sensitivity and was completely inhibited by 0.96 mM NaF. In contrast to this, however, the endogenous degradation of glycogen continued at appreciable rates at seven times this sodium fluoride concentration.

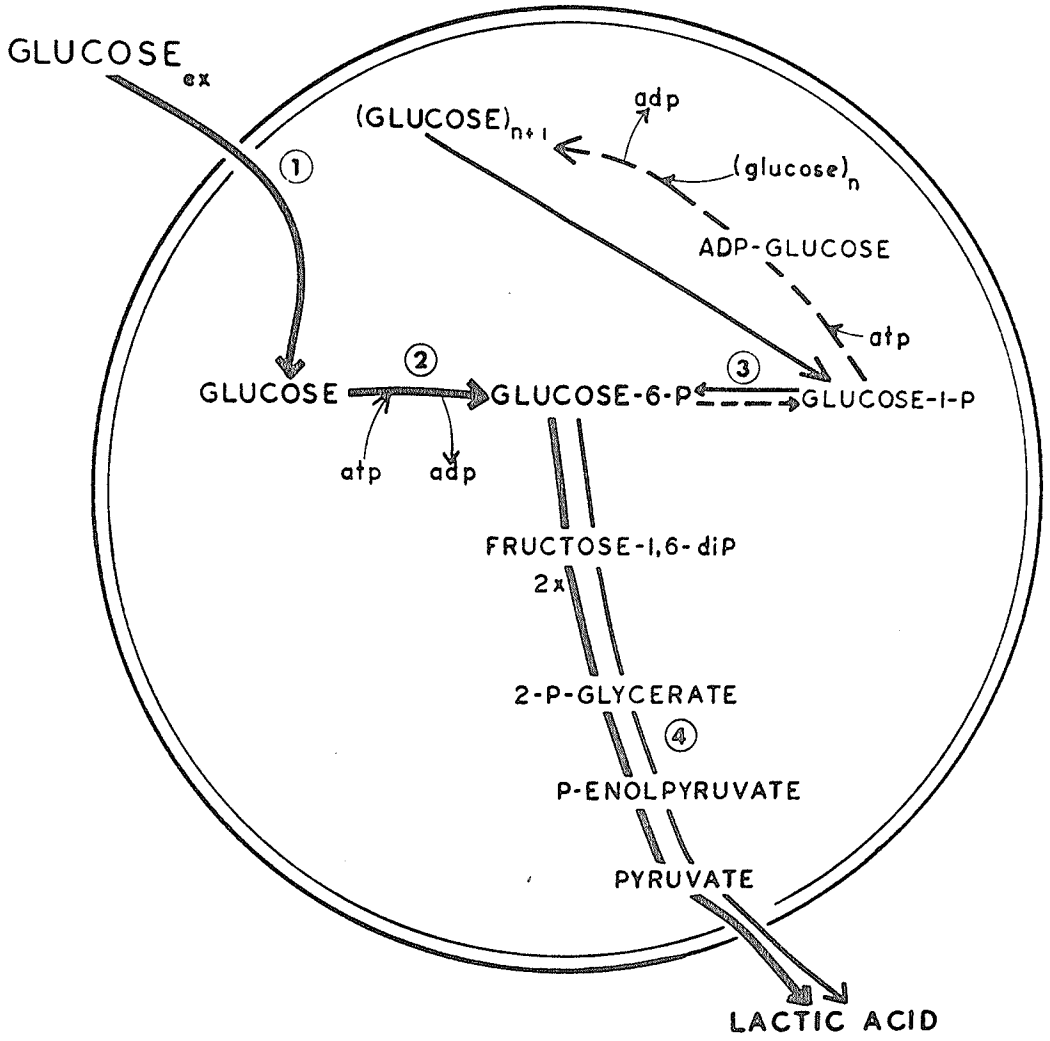
Since both exogenous and endogenous glucose degradation utilized the same reactions in glycolysis from glucose-6-P to lactate (Fig. 1), and yet have such widely differing fluoride sensitivities, it was concluded that the reactions in the shared pathway were not as sensitive to the inhibitor as were the non-shared reactions in the glycolytic scheme. Since the only reactions outside the common pathway are those leading to glucose-6-P formation, it was further concluded that the primary site of fluoride inhibition of glycolysis was at some point prior to glucose-6-P formation. The nature of these reactions was unknown, but presumably they would include the glucose transport process

Fig. 1. Diagrammatic representation of the pathways involved in glucose metabolism in S. salivarius:

exogenous glucose metabolism

glycogen synthesis

glycogen degradation


GLUCOSE ex
(GLUCOSE)n+1
adp
(glucose)n
ADP-GLUCOSE
atp
1
2
3
GLUCOSE
GLUCOSE-6-P
GLUCOSE-1-P
atp
adp
FRUCTOSE-1,6-diP
2x
2-P-GLYCERATE
4
P-ENOLPYRUVATE
PYRUVATE
LACTIC ACID

(reaction 1, Fig. 1) and associated processes, such as glucose phophorylation (reaction 2).

These results were somewhat surprising since they indicated that enolase (reaction 4), which has been known for many years to be extremely sensitive to fluoride, was not the prime site of fluoride action in whole cells of S. salivarius. Furthermore, the relative fluoride-insensitivity of glycogen degradation as compared to glycogen synthesis demonstrated that the phosphoglucomutase reaction (reaction 3), common to both of these processes, was not inhibited in vivo, even though the in vitro activity of this enzyme from various sources has often been shown to be inhibited by fluoride. Thus, it is clear that one cannot extrapolate the results of in vitro enzyme studies to explain the inhibition of metabolic processes occurring within intact cells.

With these considerations in mind, experiments were designed to determine the in vivo effect of fluoride on the reactions prior to glucose-6-P formation and on the intracellular activity of both enolase and phosphoglucomutase in cells of S. salivarius metabolizing glucose. It was hoped that this study would help to elucidate the mechanisms by which fluoride inhibits glycolysis, glycogen synthesis and glycogen degradation in cells of this organism.

CHAPTER 2

LITERATURE REVIEW

A. EARLIEST REPORTS

One of the earliest demonstrations of the inhibitory effect of fluoride compounds on microbes was provided by Tappeiner (1), who in 1890 found that sodium fluoride inhibited the growth of various cultures of bacteria, including the "cholera bacilli" and the "lactic acid bacilli". Effront (2), in the same year, also demonstrated the fluoride inhibition of microbial metabolism, noting that hydrofluoric acid was much more effective than either hydrochloric acid or sulfuric acid in inhibiting the lactic and butyric acid fermentations. Later, Effront was the first to demonstrate that the degree of fluoride inhibition of these microbial fermentations increased as the acidity of the external medium increased and that the presence or absence of inorganic phosphates had an important effect on the severity of the resulting inhibition (3). He also observed that the growth of some types of microorganisms, inhibited at high fluoride concentrations, could be stimulated by low levels of the inhibitor (4). These early descriptive studies with microorganisms, coupled with reports demonstrating the inhibitory effect of fluoride on blood glycolysis (5, 6, 7), on muscle pulp metabolism (8) and on plant cells (9), clearly established the toxic effect of fluoride on living matter.

B. FLUORIDE INHIBITION OF GLYCOLYSIS: EARLY STUDIES

Early observations on the mechanism of fluoride inhibition of glycolysis were a by-product of the now classical studies of Embden, Meyerhof and others which led to the elucidation of the glycolytic pathway. In some of these studies, fluoride and other metabolic inhibitors were used to disrupt the normal glycolytic pathway so that a comparison could be made between normal and abnormal metabolism. In other studies, the accumulation of glycolytic intermediates in the presence of fluoride was used to facilitate their isolation.

The earliest biochemical studies on glycolysis in muscle homogenates demonstrated that inorganic fluoride compounds inhibited lactate formation both from endogenous carbohydrate reserves (8) and from added substrates, such as starch or glycogen (10). Furthermore, the addition of fluoride to muscle homogenates resulted in the "disappearance" of free phosphate from the system, a process which was stimulated by excess glycogen (11, 12). Beattie and Milroy (13) confirmed and extended these results by demonstrating that the loss of inorganic phosphate from muscle homogenates incubated with fluoride was coupled with the disappearance of glycogen and a reduction in lactate production. Later, quantitative data (14) showed that this decrease in the inorganic phosphate content was approximately equal to the decrease in total carbohydrate thereby suggesting that, in the presence of fluoride, the phosphate was bound to the carbohydrate. Thus, these early studies demonstrated that fluoride inhibited the degradation of carbohydrates to lactate, but also indicated that phosphorylated compounds accumulated in the presence of fluoride.

With these results in mind, Lipmann (15) studied the effect of fluoride on the hydrolysis of a variety of glycolytic intermediates in muscle homogenates and in acetone-dried yeast preparations. He found that lactate production from hexose diphosphate, hexosemonophosphate or glycerophosphate was inhibited by fluoride. Furthermore, inorganic phosphate analyses revealed that the hydrolysis of phosphate from these esters was inhibited to the same degree as was lactate production. Since lactate is an unphosphorylated compound the conclusion was drawn, and generally held by other workers (16), that fluoride inhibited glycolysis simply by preventing dephosphorylation.

Contrary to this conclusion, however, was evidence that fluoride-treated muscle homogenates were capable of converting phosphorylated glycolytic intermediates into different phosphorylated intermediates. For example, in the presence of fluoride, fructose-1,6-diphosphate (17) glucose-6-P and fructose-6-P (18) were all converted to a "difficultly hydrolyzable" phosphate ester, which was later shown to be phospho-glycerate (2-P-glycerate) (19). The fact that these phosphorylated compounds were converted to 2-P-glycerate, but not to lactate, coupled with the knowledge that pyruvate could be converted to lactate in the presence of fluoride (20), indicated that some unknown enzyme involved in the conversion of 2-P-glycerate to pyruvate was fluoride-sensitive. This enzyme, enolase, was discovered in 1934 by Lohmann and Meyerhof (21) simultaneously with the discovery of its product: phosphoenolpyruvate (P-enolpyruvate). This compound could be converted into pyruvate in the presence and absence of fluoride but could not be formed from 2-P-glycerate if fluoride was present. Thus, the enzyme responsible for

the formation of P-enolpyruvate, i.e., enolase, was the primary site of fluoride action on muscle homogenate glycolysis.

Three years later, parallel studies of a different nature demonstrated the sensitivity of bacterial enolase to fluoride (22, 23). Cells, treated with toluene or chloroform to disrupt the membrane and incubated overnight with the inhibitor, produced abnormal amounts of 2-P-glycerate which accumulated in the medium. This abnormal accumulation did not occur in the control cells which were treated with solvents but not with fluoride. Thus, the accumulation of 2-P-glycerate was fluoride-induced, thereby demonstrating that the enzyme responsible for its subsequent conversion to P-enolpyruvate, enolase, was sensitive to the inhibitor.

From these early studies, it was apparent that the inhibition of cellular metabolism by fluoride was due to its effect on enzymes. It is now known that a wide variety of enzymes from various sources are sensitive to fluoride (see reviews, 16, 24). Two of these, enolase and phosphoglucomutase, are of central importance to the present study.

C. EFFECT OF FLUORIDE ON ENOLASE AND PHOSPHOGLUCOMUTASE

1. Enolase. Enolase was first isolated from crude extracts of yeast by Warburg and Christian (25) and subsequently purified and crystallized by these workers. The enzyme required a divalent cation such as Mg^{++}, Zn^{++}, or Mn^{++} for activity; all of these metal-enzyme complexes were inhibited by fluoride. Kinetic studies (26) revealed that divalent cations, phosphate and fluoride were all required to inhibit enolase;

for the purified magnesium enzyme, the product (K) of reaction (1) was approximately 3.2×10^{-12}.

$$[Mg] \cdot [PO_4] \cdot [F]^2 \times \text{residual activity/inhibition} = K \qquad (1)$$

As a consequence of this relationship, the fluoride concentration required to produce a given degree of inhibition actually decreased as the magnesium and/or phosphate concentration was increased. From these observations, the following mechanism of inhibition was predicted:

$$\text{Mg - fluorophosphate} + \text{Mg - enolase} \rightleftharpoons \text{Mg-fluorophospho-enolase} + \text{Mg-salt} \qquad (2)$$

However, at a high fluoride concentrations ($F^- > 10^{-2}$ M) phosphate was not required for inhibition and for this condition the following mechanism was proposed:

$$Mg_2F + \text{Mg - enolase} \rightleftharpoons \text{Mg - fluoro-enolase} + \text{Mg - salt} \qquad (3)$$

The validity of equation (1) has been known for some time and recent data with the enolases from trout (27), coho salmon and chum salmon (28), reporting K values of 1.4×10^{-12}, 1.2×10^{-12} and 1.3×10^{-12}, respectively, have confirmed this point. However, the validity of mechanism (2), which requires the reaction of Mg-fluorophosphate with Mg-enolase is in doubt. This arose, in part, from the work of Peters, Shorthouse and Murray (29) who demonstrated that while a mixture of fluoride plus phosphate (both 5 mM) almost completely inhibited the activity of purified rabbit muscle enolase, an equal concentration of fluorophosphate ($F \cdot PO_3$) did not produce any inhibition. In both of these

determinations, 1 mM $MgSO_4$ was present. More recently, Cimasoni (30) demonstrated that fluoride as low as 0.026 mM inhibited purified rabbit muscle enolase in the presence of 5 mM phosphate and 1 mM magnesium, yet 5 mM fluorophosphate was not inhibitory. Thus, the inhibition of enolase by fluoride in the presence of phosphate and magnesium does not require the initial formation of an inorganic Mg-fluorophosphate complex as Warburg and Christian had proposed in equation (2). As did Warburg and Christian, Cimasoni also found that muscle enolase could be inhibited by fluoride in the absence of phosphate, however, inhibition in this case required twenty times as much fluoride (0.52 mM). Kinetic analysis of these results (30) revealed that competitive inhibition occurred in the presence of phosphate, while uncompetitive inhibition was observed in the absence of this compound. Uncompetitive kinetics indicate a binding of the inhibitor to the enzyme-substrate complex, a mechanism which has been postulated previously to explain the fluoride inhibition of certain non-metaloenzymes (31, 32).

2. Phosphoglucomutase. Phosphoglucomutase is a Mg^{++}-activated enzyme involved in both glycogen synthesis and degradation:

$$\text{Glucose-6-Phosphate} \rightleftharpoons \underset{\text{(Glucose-1,6-Diphosphate)}}{\text{Glucose-1-Phosphate}} \qquad (4)$$

Najjar (33) demonstrated that the purified phosphoglucomutase from muscle was sensitive to fluoride and showed that the kinetic data for inhibition was analagous to that of enolase. The product (K) of equation (5) was 1.7×10^{-12}.

$$[\text{Mg} \cdot \text{organic phosphate}] \cdot [\text{F}]^2 \times \text{residual activity/inhibition} = \text{K} \qquad (5)$$

This equation is similar to the one proposed by Warburg and Christian for enolase (equation 1) except that "organic phosphate", rather than inorganic phosphate, was involved in the inhibition. Either glucose-6-P or glucose-1-P satisfied the "organic phosphate" requirement. As a consequence of these results, Najjar proposed that an inhibitory complex composed of Mg-fluoro-"organic phosphate" displaced enzyme-bound magnesium, resulting in an inactive enzyme.

In contrast to this study, however, experiments with partially purified phosphoglucomutase from Jack-bean meal indicated that inhibition of this plant enzyme did not follow the above mechanism. Yang and Miller (34) found that the degree of inhibition was proportional to both the fluoride and the glucose-1-P concentration, but not to the extent predicted by equation (5). In contrast to Najjar's findings, the degree of inhibition of the plant enzyme was independent of the magnesium concentration. Also, kinetic data predicted that only one molecule of fluoride would be present in the "inhibitory complex" for the plant phosphoglucomutase, as compared to the two molecules of fluoride predicted for the muscle enzyme. Various kinetic mechanisms were examined to account for these observations, but as was the case with muscle phosphoglucomutase, each mechanism predicted that the inhibited enzyme form was an enzyme-metal-fluoride-substrate complex.

Recent studies have provided evidence that chemical interaction does occur among Mg^{++}, F^-, and glucose-1-P in the absence of protein (35). Measurement of the activity of Mg^{++} and F^- with specific-ion electrodes demonstrated that there was interaction between Mg^{++} and F^-, between Mg^{++}

and glucose-1-P, and amongst all three of these components when mixed together. These results are consistent with the formation of a magnesium-fluoride-substrate complex in the absence of phosphoglucomutase, but whether this complex would react with the enzyme was not established.

D. FLUORIDE INHIBITION OF CELLULAR METABOLISM

The effects of fluoride on isolated enzyme preparations may not reflect the effects of the inhibitor on enzymes in the complex metabolic systems of intact cells (24). The studies of Runnstrom and associates with yeast during the late 1930's and early 1940's demonstrated that the fluoride sensitivity of a cell is dependent upon the metabolic processes taking place at the time the inhibitor is introduced into the system (36). In this section, the effect of fluoride on the metabolism of intact cells, especially oral bacteria, will be discussed in relation to the factors which regulate the degree of inhibition of cellular metabolism. As will be shown, the *in vivo* effects of fluoride are regulated by the concentration of the inhibitor added to the cells, the pH of the medium and the permeability of the cells to fluoride.

1. *Effect of fluoride concentration and pH.* Low concentrations of fluoride stimulated glycolysis in *Lactobacillus casei* (37) and in yeast (38), while higher fluoride concentrations inhibited this metabolism in these same organisms. Similar findings have been reported for the aerobic degradation of carbohydrate by *Propionibacterium sp.* (39) and *Mycobacterium* BCG (40). Runnstrom, Borei and Sperber (41) postulated that the stimulation of aerobic and anaerobic glycolysis in yeast cells

incubated with fluoride was due to the inhibition of glycogen synthesis, thereby leaving more substrate available for degradation.

Glycolysis by oral bacteria, on the other hand, is generally inhibited by low concentrations of fluoride. Using acid production as a measure of metabolism, Bibby and van Kesteren (42) found that glycolysis by pure cultures of oral streptococci and lactobacilli was inhibited by 0.024 mM NaF. Clapper (43) confirmed these results by demonstrating that 0.024 mM NaF inhibited acid production by pure cultures of salivary lactobacilli. In addition, Wright and Jenkins (44) showed that acid production by the mixed organisms present in saliva-glucose mixtures was reduced by NaF as low as 0.012 mM. In contrast to this latter finding, however, Lilienthal (45) reported that 0.012 mM NaF did not inhibit acid production from glucose and sucrose in a salivary sediment system; concentrations as high as 1.66 mM were required for the consistent production of slight inhibition. These apparently contradictory findings were resolved by Jenkins (46) who showed that the degree of inhibition of acid production by mixed oral bacteria increased as the pH of the incubation medium decreased. When 0.048 mM NaF was added to oral microbes, the degree of inhibition was greater at pH 5.0 than at pH 7.6. As a consequence of these observations, Jenkins ascribed the negative results of Lilienthal to the use of an incubation medium strongly buffered by carbonate at pH 6.8.

Later studies with pure cultures of oral streptococci (47, 48) and the mixed microbial flora of salivary sediment (49) confirmed that the degree of fluoride inhibition of oral microbial metabolism increased as the pH decreased. The occurrence of the pH effect had been noted

earlier in yeast (50, 51, 52), in Mycobacterium BCG (40) and in Staphlococcus aureus (53). In Mycobacterium BCG, the complete spectrum of inhibition occurred over a narrow pH range (40). The oxidation of glucose and fructose by this organism was strongly inhibited by fluoride at pH 6.0, while the oxidation of these sugars at pH 6.7 was actually stimulated by the same concentration of the inhibitor. While NaF had no effect on respiration by yeast at pH 5.5, metabolism was completely inhibited at pH 4.5 (51).

Runnstrom and Sperber (50) calculated the concentration of fluoride present as HF at various pH values and found that the effect of pH on inhibition could be explained if the fluoride entered the cell as undissociated HF. Malm (51) tested this hypothesis and found that the pH of the external medium increased as the inhibitor was being assimilated into the cells. This was presumably the result of the reaction of NaF with H_2O, forming HF and NaOH. The HF would then enter the cells, while the NaOH remained in the exogenous medium thereby raising the pH. Malm also noted that diffusion of the inhibitor from yeast cells also occurred, but at least twenty-five per cent of the fluoride was strongly retained by the cells.

Evidence that HF is the ionic form of fluoride which enters bacteria was recently provided by Sims (54), who demonstrated that acid production by various strains of oral Lactobacilli and Streptococci incubated with glucose and fluoride ceased when a characteristic concentration of HF was reached. As a general rule, inhibition by salts of weak acids or bases such as fluoride, cyanide or azide is pH dependent, with maximum inhibition occurring at or near the pK of the inhibitor (55). The pK for HF is 3.14 (16).

2. Substrate effect and cell permeability. Early studies with yeast (56) demonstrated that the addition of the glucose substrate to the cells before the addition of fluoride always protected the cells from the inhibitor for as long as the glucose was present in the medium. If the inhibitor was added before the glucose, or if both were added simultaneously, then inhibition occurred. Thus, the presence of substrate, and/or a metabolite produced from it, protected the cells from the inhibitor: a phenomenon termed the "substrate-protective effect". This effect occurred under both aerobic and anaerobic conditions and was associated only with intact cells, since it was not observed in inhibition studies with yeast homogenates. Impermeability to fluoride in the presence of the substrate could not explain this phenomenon since Malm (51) had demonstrated previously that glucose did not affect the fluoride permeability of intact yeast cells. An explanation for this protective effect was proposed by Runnstrom, Gurney and Sperber (38) who suggested that a "protected unit" and fluoride competed for the "primary" fluoride-sensitive enzyme. The "protected unit" was thought to be a metabolite produced during the degradation of the substrate, but its existence was never demonstrated. After the fluoride sensitivity of purified enolase had been established (26), a later theory (52, 57) proposed that the phosphate necessary for enolase inhibition would not be available during glucose metabolism since the phosphate content of the cells would be lowered by phosphate ester formation.

Although the "substrate-protective effect" in yeast was confirmed in later studies (58), a similar effect in bacteria has not been observed in the few cases where it has been studied. Thus, anaerobic glucose

metabolism by the oral microbe S. salivarius was strongly inhibited by fluoride regardless of whether the inhibitor was added before or after the substrate (48, 59). Aubel, Rosenberg and Szulmajster (60) found that fluoride did not inhibit respiration of E. coli when glucose was present, but unlike the earlier results with yeast, fermentation of this substrate was inhibited. Since NaF inhibited the oxidation of glucose in homogenates of E. coli (61), these workers concluded that intact E. coli cells incubated aerobically with glucose were impermeable to fluoride.

These latter results also serve to indicate that anaerobic metabolism by intact microbial cells is generally more sensitive to fluoride than is aerobic metabolism. This observation originated with the studies of Runnstrom and co-workers who demonstrated that intact yeast incubated anaerobically were more sensitive to fluoride than were aerobically incubated yeast. "Brewer's" yeast, which metabolizes carbohydrates anaerobically, was more fluoride-sensitive than was aerobic "Baker's" yeast (see review, 16). Also, Molan and Hartles (62) found that glucose metabolism by salivary microorganisms was inhibited to a much greater extent anaerobically than aerobically.

The differences in sensitivity may be a permeability phenomenon, since Malm (51) showed that fluoride penetrated into yeast cells more rapidly under anaerobic conditions than under aerobic conditions. This is supported by the fact that dried yeast, i.e., cells which have an altered permeability barrier, and yeast homogenates, were found to be more sensitive to fluoride than were intact cells (52, 56).

E. FLUORIDE INHIBITION OF GLYCOGEN METABOLISM

The organisms of dental plaque (63) and salivary sediment (64) are able to synthesize large quantities of intracellular polysaccharide, presumably glycogen, from exogenous carbohydrate sources. The presence of this endogenous substrate in the plaque bacterium S. mitis has recently been shown to enhance its survival under starvation conditions (65), probably because endogenous metabolism of this polymer produces both energy and carbon precursors for the synthesis of vital cellular components (66). Of particular interest is the finding by Gibbons and Socransky (63) that there is a positive correlation between the number of microbes capable of synthesizing intracellular glycogen in plaque and the incidence of dental caries. Although many mechanisms probably contribute to the cariogenicity of dental plaque (see reviews, 67), Gibbons and Socransky pointed out that these cells could metabolize intracellular glycogen when exogenous carbohydrate was depleted, thereby producing metabolic acids over a long period of time and thus furthering enamel decomposition.

In this connection, the finding of Hardwick and Leach (68) that dental plaque contains relatively high (mean = 3.5 mM) amounts of fluoride is of interest, since glycogen synthesis by cells of S. mitis (47) and S. salivarius (59) was almost completely inhibited by 0.95 mM fluoride. In fact, fluoride as low as 0.12 mM significantly inhibited carbohydrate storage by the microorganisms of salivary sediment (49). However, Jenkins and Edgar (69) have demonstrated that only 2 - 3% of the total fluoride in plaque is present as free ions as measured by the fluoride

ion electrode. In spite of this low fluoride ion concentration, acid production, as measured by pH change, was lower in dental plaque taken from subjects living in areas with fluoridated water than in plaque taken from subjects living in areas with little or no water-borne fluoride (69, 70). Hamilton (48, 59) has found that glycogen synthesis by cells of S. salivarius was more sensitive to inhibition by fluoride than was acid production by glycolysis. If this is the case in other oral bacteria, then glycogen synthesis by these organisms could be significantly inhibited by the water-borne fluoride concentrated in dental plaque.

Although glycogen synthesis by intact cells is very sensitive to fluoride, the mechanism of this inhibition is unknown. Preiss and co-workers (see review, 71) have shown that bacteria synthesize and degrade glycogen by the following reactions:

$$\text{glucose-6-P} \longrightarrow \text{glucose-1-P} \quad (5)$$

$$\text{glucose-1-P} + \text{ATP} \longrightarrow \text{ADP-glucose} + PP_i \quad (6)$$

$$\text{glycogen}_N + \text{ADP-glucose} \longrightarrow \text{glycogen}_{N+1} + \text{ADP} \quad (7)$$

$$\text{glycogen}_N + \text{orthophosphate} \longrightarrow \text{glycogen}_{N-1} + \text{glucose-1-P} \quad (8)$$

Reactions (5) to (8) are catalyzed by phosphoglucomutase, ADP-glucose pyrophosphorylase, ADP-glucose: glucan transferase and phosphorylase, respectively. Of these enzymes, purified mammalian phosphoglucomutase is known to be sensitive to fluoride (33) and thus may be the site where intracellular polysaccharide synthesis is inhibited by fluoride (72, 73).

Results from early experiments with liver homogenates, however,

indicated that glycogen synthesis and degradation were relatively insensitive to 5 mM NaF. Ostern, Herbert and Holmes (74, 75) found that homogenates treated with fluoride degraded glycogen to glucose-6-P, thereby demonstrating the insensitivity of phosphorylase and phosphoglucomutase to the inhibitor. Also, glucose-1-P could be converted into glycogen by fluoride-treated homogenates, thereby demonstrating the insensitivity of the enzymes involved in glycogen synthesis.

Recent studies with intact oral streptococci have also indicated that intracellular phosphoglucomutase activity is insensitive to fluoride (47, 59). Hamilton (59) found that glycogen synthesis in intact cells of S. salivarius was almost completely inhibited by low fluoride concentrations (0.48 mM NaF), but degradation of this polymer was relatively fluoride insensitive since the degradative process was only partly inhibited by 2.4 mM NaF. If phosphoglucomutase was the primary site of fluoride action in the glycogen synthetic pathway, then both the synthetic and degradative processes would have been equally susceptible to fluoride. The results of these studies with the oral streptococci, as well as those with salivary sediment microorganisms (49) further revealed that glucose uptake was inhibited by low concentrations of fluoride. Hence, the inhibition of glycogen synthesis in whole bacterial cells may be due simply to the inhibition of glucose uptake (47, 48).

F. FLUORIDE RESISTANCE

In view of the well-known ability of microorganisms to adapt to adverse conditions, it is not surprising to find that many species of bacteria, including oral lactobacilli (76, 77) and oral streptococci

(78, 79), can adapt to the presence of fluoride by producing resistant strains. These strains are characterized by their ability to grow at fluoride concentrations which inhibit the growth of the sensitive parental strains. One of the first reports of such resistance was provided by Effront (80), who found that yeast could be adapted to growth at fluoride concentrations as high as 15.8 mM. The resistant cells differed from the parental strains since they produced more alcohol and had slower growth rates. Other early studies confirmed that fluoride resistance could be achieved readily with yeast (81), bacteria (82, 83) and fungi (84).

Early studies by Euler and Cramer (85) pointed to fluoride resistance as being phenotypic in nature. Resistant yeast contained an abnormally high calcium concentration, the level of which decreased upon cultivation of the resistant strains in a fluoride-free medium. Because resistance to fluoride decreased as the cellular calcium was depleted, Borei (16) suggested that resistance may have been due to the removal of free cellular fluoride by the formation of insoluble calcium fluoride. Phenotypic expression of resistance was also observed with oral Lancefield Group D streptococci (86). Cells acquired resistance to fluoride after serial passage through increasingly higher fluoride levels but lost this characteristic when cultured in the absence of the inhibitor for two to three days.

In contrast to these studies, genotypic resistance to fluoride has been observed with strains of E. coli and S. aureus (87). Cultures grown for 52 generations in media containing increasing amounts of fluoride developed 3 - 4 fold resistance toward the inhibitor, which was not lost

by continued cultivation in fluoride-free media for fifty generations. Genotypic resistance was also observed with mutants of Saccharomyces cerevisiae, resistant to arsenate or azide, or to both of these inhibitors (88). Mutation was spontaneous, occurring prior to exposure of the cells to the inhibitors. Two mutant strains, which had been isolated by selection against azide and arsenate were also found to be resistant to iodoacetate and fluoride. The resistant characteristics of the mutant strains were maintained even when they were grown in the absence of the inhibitors. Genotypic resistance to fluoride was also observed in strains of S. salivarius (77). Resistant strains were obtained both by a step-wise adaptation procedure and by the selection of resistant strains following ultra-violet irradiation. Although slight differences were observed in the growth and metabolic characteristics of the adapted and UV-induced strains, both cell types retained their resistant characteristics despite passage through fluoride-free media for 300 generations.

Wiggert and Werkman (89) found that in the presence of fluoride, sensitive cells of Propionibacterium pentosaceum could not ferment glucose while resistant cells were able to ferment this substrate. Control cells were able to ferment 2-P-glycerate in the absence of fluoride, while resistant cells which had been grown in NaF could not. Since the resistant strain was able to utilize glucose, but not 2-P-glycerate, these workers concluded that the resistant cells fermented glucose through a different "glycolytic" pathway than the one used by wild-type fluoride-sensitive cells. The resistant pathway would not include 2-P-glycerate as an intermediate.

A later study by Volk (90), however, produced a new interpretation of these earlier results. While glucose metabolism by the fluoride-resistant strain of P. pentosaceum was not inhibited by fluoride as before, inhibition could be achieved after the cell membrane had been altered by lyophilization. Volk concluded that the mechanism of resistance was associated with the inability of fluoride to penetrate the cell membrane. The inability of the adapted strains to ferment 2-P-glycerate in the Wiggert and Werkman study was considered to be due to fluoride inhibition of phosphatase activity. In this case, control cells were able to metabolize the non-permeable 2-P-glycerate after dephosphorylation and transport. On the other hand, the fluoride-grown resistant cells were unable to dephosphorylate 2-P-glycerate, since their phosphatase activity was inhibited by fluoride.

Acquired impermeability to fluoride may be a common mechanism of resistance since Williams (91) has demonstrated that "fluoride-trained" cells of S. faecalis were less permeable to the inhibitor than were the parental strains.

In a latter study, Williams (92) suggested that an unusual form of enolase might be produced by resistant strains of S. faecalis. Analysis of the content of 3-P-glycerate, 2-P-glycerate and P-enolpyruvate in neutralized extracts of control and "fluoride-trained" cells revealed significant differences in the ratios of 3-P-glycerate and 2-P-glycerate to P-enolpyruvate. In all cases, the ratios P-enolpyruvate/2-P-glycerate and P-enolpyruvate/3-P-glycerate were higher in control cells than in the "fluoride-trained" cells, while there were no significant differences between the cells in the ratio of 2-P-glycerate/3-P-glycerate. This was

indicative of a reduced apparent equilibrium constant for enolase in the "fluoride-trained" cells suggesting that enolase had become a rate-limiting enzyme. Williams suggested that one way in which this change could occur would be through the formation of a low activity, fluoride-resistant enolase in the trained cells. However, such an enzyme has yet to be isolated.

Recent studies have demonstrated that mammalian cells also can acquire resistance to fluoride. Carlson and Suttie (93) developed fluoride-resistant HeLa cells by a step-wise adaptation process. These cells were able to grow in the presence of 4.9 mM F, a concentration of inhibitor which completely prevented the growth of the parental strain. In a more recent study, Hongsle and Jonsen (94) demonstrated that the growth rate of mouse fibroblast cultures was reduced by 1.31 mM F, while complete growth inhibition and cell death occurred at 2.37 mM F. However, growth in the presence of 5.95 mM NaF could be observed following a step-wise adaptation procedure. Resistance appeared to be a genetic phenomenon since adapted cells cultured in the absence of the inhibitor for sixty generations immediately reassumed their normal growth rate when fluoride was added.

A recent detailed analysis of resistance in L cells (mouse fibroblasts) was performed by Quissell and Suttie (95). A step-wise adapted resistant strain of L cells could grow in the presence of 3.68 mM F, a concentration which completely prevented the growth of the parental strain. The adapted cells did not lose their resistant characteristics after growth without fluoride for eighty generations, thereby demonstrating the genetic nature of the resistance phenomenon. Quissell

and Suttie further found that the ratio of intracellular to extracellular fluoride was 0.27 in the controls and only 0.03 in the resistant cells. When incubated at 0 C, fluoride entered the resistant cells but the inhibitor was removed against a concentration gradient after the temperature was increased to 37 C suggesting that a pump mechanism acted to remove the fluoride. This hypothesis was further supported by the finding that the intracellular fluoride concentration was maintained at an insignificant level in adapted cells until an extracellular concentration of 5.3 mM F was reached after which the intracellular fluoride concentration rapidly increased. This is consistent with the hypothesis that a 'fluoride pump' acts to remove intracellular fluoride until the system is saturated at high extracellular fluoride concentrations. Although the nature of the pump was not examined in this study, the authors speculate that resistance to fluoride may involve a mutation resulting in an alteration of a pre-existing carrier such that fluoride is carried from the cell.

CHAPTER 3

METHODS AND MATERIALS

A. BACTERIOLOGICAL

1. Wild-type cells.

The wild-type strains of Streptococcus salivarius (ATCC 25975) used in this study was originally isolated from whole human saliva by methods previously described (96). Some of the major taxonomic characteristics of this strain are listed in Table 1. This strain was maintained aerobically by daily transfer in 5 ml of a 0.1% glucose-tryptone broth which had the following composition: tryptone, 10 g; yeast extract, 5 g; K_2HPO_4, 3 g; glucose, 1 g; and deionized water, 1000 ml. Hamilton (96) had previously demonstrated that sufficient growth occurred in this broth for daily maintenance and transfer, but not enough lactate was produced from the 0.1% glucose to affect cell viability. The fluoride content of this medium was 0.65 µg F/ml (79).

Stock cultures were grown for six to eight hours in 0.1% glucose-tryptone broth and then stored at -10 C, where they remained viable for at least one year. Every four to six weeks, the viability and purity of these cultures was checked, and the resulting fresh cultures were used to augment the stock collection and to replace the culture maintained by daily transfer. Before these new cultures were used experimentally, they were transferred daily in 0.1% glucose-tryptone broth for at least three days.

The purity of the cultures was assessed by Gram staining procedures

TABLE 1

Taxonomic characteristics of Streptococcus salivarius

Morphology	Coccus, occurring singly and in short or long chains
Gram reaction	Positive
Growth on 5% sucrose medium and "Mitis-salivarius" agar	Large mucoid colonies
Growth on 5% glucose medium	Small, non-mucoid colonies
Hemolytic reaction on blood agar	Gamma
Catalase	Negative
Growth in 5% NaCl	Slight
Growth at 45 C	Negative
Growth at pH 9.5	Negative
Survival at 60 C for 30 min.	Negative
Fermentation reactions	
glucose, sucrose, inulin, raffinose, mannose, fructose, maltose, lactose, galactose	Acid, no gas
xylose, arabinose, rhamnose, glycerol, mannitol, sorbitol, pyruvate, citrate, succinate, ribose	No reaction

and by growth on solid media. Typical strains of S. salivarius developed "pin-point" colonies when grown on 5% glucose-tryptone agar, while a characteristic large mucoid colony developed on both 5% sucrose-tryptone agar and Mitis-Salivarius agar (Difco). Before every experiment, the purity of the culture was examined microscopically after Gram-staining.

2. Fluoride-resistant cells.

Strains of S. salivarius resistant to fluoride were derived from the original parent by the following procedures (79). Overnight cultures of wild-type cells were heavily inoculated into 0.1% glucose-tryptone broth containing 1.2 mM NaF. After growth had occurred at this fluoride concentration (usually within twenty-four hours), the resulting cells were transferred into media containing 2.4 mM NaF ("A-100" strain). By continued transfer in a step-wise manner, strains of S. salivarius resistant to 14.3 mM NaF were obtained. Mutants resistant to fluoride were also obtained by ultra-violet irradiation of wild-type cells spread on plates of 5% glucose-tryptone agar containing 2.4 mM NaF ("UV-100" strains).

The fluoride-resistant strains used in these experiments (A-100 and UV-100) were maintained aerobically by daily transfer in 0.1% glucose-tryptone broth containing 2.4 mM NaF. Stock cultures were maintained in fluoride-containing media at - 10 C and periodically used to replace the daily-transferred cultures by the same procedures described for the wild-type parental strain. Three days prior to an experiment, A-100 and UV-100 were grown in 0.1% glucose-tryptone broth containing 4.8 mM NaF so that a high degree of resistance to the inhibitor would be exhibited.

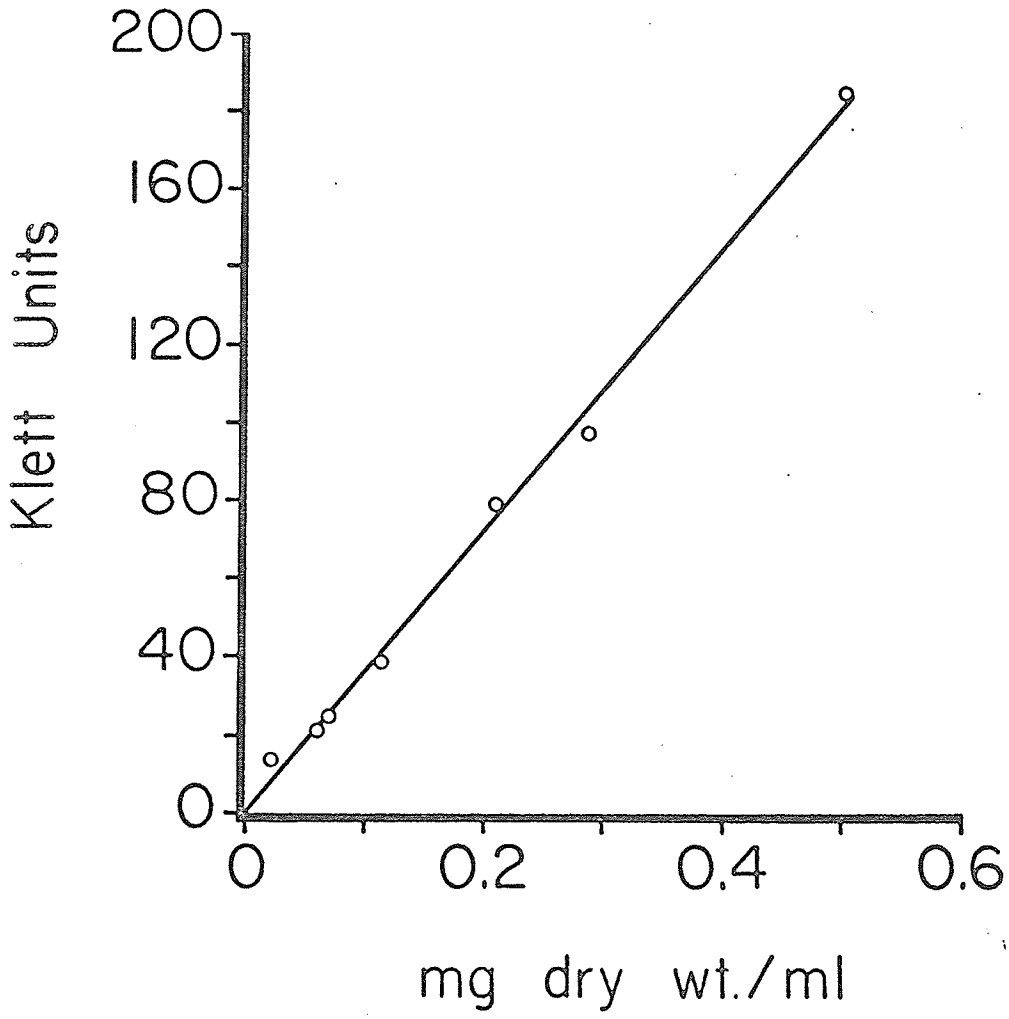
3. Production of Cells.

For experimental use, both the wild-type and fluoride-resistant cells were grown anaerobically at 37 C in 0.1% glucose-tryptone broth except that the media for the latter cells always contained 4.8 mM NaF. Anaerobic conditions were produced by evacuating the growth flask with suction and refilling (5 - 7 times) with 5% CO_2 in nitrogen.

Large quantities of cells were obtained by inoculating an overnight anaerobic culture (5 - 10% of the final volume) into 1.5 - 3.0 liters of medium. The subsequent increase in cell numbers was followed turbimetrically in a Klett-Summerson Colorimeter (Klett Manufacturing Co., New York) containing a red filter (640 to 700 mμ). The cells were always harvested in the late exponential phase of growth (95 - 100 Klett units) by centrifugation at 10,000 g for 15 min at 4 C in a Sorvall RC2-B centrifuge (Ivan Sorvall Inc., Norwalk, Connecticut). Wild-type cells were washed once and finally suspended in ice-cold 50 mM potassium phosphate buffer containing: NaCl, 2.0 g; Mg SO_4-$7H_2O$, 80 mg; $FeSO_4 \cdot 7H_2O$, 4.0 mg; $MnCl_2$, 1.2 mg; and deionized water, 1 liter. The pH of the buffer was either 5.8, 7.2 or 8.0 depending on the experiment. Fluoride-resistant cells were washed twice with this buffer in order to remove exogenous fluoride.

The cell concentration of the final suspension was determined by turbidity measurements in a Klett-Summerson colorimeter. Standard curves had been established previously for Klett units vs dry weight (Fig. 2) and for dry weight vs cell numbers. One mg dry wt cell material contained 1.27×10^9 cells (96).

Fig. 2. Standard dry weight curve for Streptococcus salivarius suspended in 50 mM potassium phosphate buffer (pH 7.2).


200
160
120
80
40
0
Klett Units
0
0.2
0.4
0.6
mg dry wt./ml

B. IN VIVO EXPERIMENTS

The majority of experiments to be reported in subsequent chapters were designed to test the fluoride-susceptibility of (1) the reactions prior to glucose-6-P formation, (2) phosphoglucomutase, and (3) enolase in whole cells of S. salivarius metabolizing glucose or intracellular glycogen. This section will present all of the experimental and analytical procedures used in these studies.

1. Experimental rationale.

While classical studies with cell-free enzyme preparations had previously demonstrated the fluoride inhibition of enolase and phosphoglucomutase, the in vivo sensitivity of these enzymes to this inhibitor had not been established. One method by which a qualitative determination of the effect of an inhibitor in vivo can be ascertained is based on the hypothesis that inhibition of a cellular enzyme involved in a functioning pathway will result in an increase in the concentration of the substrate of the inhibited reaction and a decrease in its product. A statement of this principle and its applicability to inhibition studies has been described by Chance (97).

Thus, the fluoride inhibition of glycolysis, glycogen synthesis or glycogen degradation in S. salivarius should result in an alteration in the steady-state concentration of various intracellular glycolytic intermediates so that the substrates of the inhibited reactions accumulate while the products are depleted. A comparison of the concentrations of the substrate and product of a particular reaction

in control and in fluoride-treated cells should determine the sensitive enzymes in the metabolic pathways. The fluoride sensitivity of intracellular enolase can be determined by monitoring the levels of 2-P-glycerate and P-enolpyruvate in control and in fluoride-treated cells, while intracellular phosphoglucomutase activity can be monitored by assaying glucose-6-P and glucose-1-P. Although the cellular concentration of glycolytic intermediates is very low at any one time (98, 99), the recent development of sensitive enzymatic-fluorometric techniques has made possible the quantitative analysis of these compounds.

2. Cell concentration.

Since initial experiments were designed to determine the effect of NaF on cellular glucose-6-P formation, a preliminary experiment was performed in order to determine the concentration of cells that was required to produce sufficient levels of this intermediate for accurate analysis. The results (Table 2) demonstrate that during exogenous glucose metabolism, the total amount of glucose-6-P in the cells at various times increased as the cell concentration was increased to 10 mg dry wt cells per ml. Since 10 mg dry weight of cells gave sufficient glucose-6-P for accurate analysis, the cell concentration was used in all *in vivo* experiments. Later experiments demonstrated that this amount of cell material was also adequate for the assay of the other glycolytic intermediates of interest in this study.

3. Experimental procedure.

Washed resting cells (10 mg dry wt/ml) were suspended in

TABLE 2

The effect of cell concentration on glucose-6-P production by *Streptococcus salivarius*[a]

Cell concentration	Cellular glucose-6-P content, minutes after glucose addition			
(mg dry wt/ml)	1.0	2.0	4.0	6.0
2	5.1[b]	4.8	4.8	5.2
4	17.7	15.9	13.7	15.4
8	41.2	-	-	72.9
10	43.7	-	-	71.7

[a] Cells, suspended in pH 7.2 phosphate buffer at the concentration noted, were preincubated anaerobically for 10 min at 37 C. Glucose (3.0 μmoles/mg dry wt cells) was then added to the incubation chamber and samples were withdrawn at the times noted. The subsequent sample treatment and analyses for glucose-6-P are described in the text.

[b] nmoles/ml of cell suspension.

potassium phosphate buffer (50 mM, pH 7.2) unless otherwise indicated. After a 10 minute equilibration period at 37 C, a zero time sample was taken and immediately thereafter glucose (usually 1.5 μmoles/mg cells) was added to start the reaction. This amount of substrate was normally metabolized within 2 to 4 min by cells incubated at pH 7.2. With the exception of one experiment where cells were exposed to various NaF concentrations during the equilibration period, the cells were exposed to the inhibitor after the onset of glucose metabolism. Several samples were always removed for analysis during glucose metabolism prior to NaF addition in order to establish the normal concentration of the various components of the system under study. All concentrations of NaF referred to in this thesis are final concentrations of NaF in the reaction mixture.

Reactions were carried out in one of two ways: (a) In early experiments, the cells were pipetted into 50 ml Erlenmeyer flasks and sealed with tight-fitting serum caps. The flasks were then evacuated by suction, refilled (5 - 7 times) with 5% CO_2 in nitrogen and incubated in a shaking water bath. Glucose and fluoride were added by syringe and samples were withdrawn in the same mammer (b) In the majority of experiments, the washed cells were incubated in buffer in a 40 ml water-jacketed chamber at 37 C under conditions of constant stirring in an atmosphere of flowing 5% CO_2 nitrogen. The pH of the reaction mixture during glucose metabolism was kept constant by the addition of NaOH which was added by means of a magnetic valve coupled to a Radiometer-PHM 26 pH meter with a TTTII Titrator assembly (Radiometer A/S, Copenhagen, Denmark). Preliminary experiments demonstrated that the controlled

addition of NaOH (1.0 N) kept the pH of the cell-glucose incubation medium relatively constant (±0.2 pH), while the amount of base used for this purpose represented only 1 - 2% of the total incubation volume by the end of the experiment. At a predetermined point during glucose degradation, an appropriate quantity of cells was rapidly transferred to a similar reaction vessel which contained NaF at the appropriate concentration.

4. Preparation of samples for assay.

Samples (0.5 ml) were removed from each reaction mixture periodically and the cells extracted with an equal volume of 1.0 N perchloric acid for 25 - 30 min , at room temperature. The extracts were then adjusted to pH 7.6 with KOH in triethanolamine buffer (0.5 M) (100) and immediately frozen. After rapid thawing, cell debris and precipitated $KClO_4$ were removed by centrifugation at 3000 g (4 C) for 20 minutes in an International Clinical Centrifuge (International Equipment Co., Boston, Massachusetts). The samples were stored frozen until analyzed with all of the assays for glycolytic intermediates being completed within 48 hours after sampling. Preliminary experiments (Table 3) demonstrated that the glycolytic intermediates measured in these experiments were unaffected by perchloric acid extraction. In addition, the mean recovery of standard amounts of glycolytic intermediates added to neutralized cell extracts ranged from 96 - 106% (Table 4). The concentration of all of these metabolites, except pyruvate, was unaffected by storage at -10 C for 72 hours. The pyruvate concentration in neutralized cell extracts decreased progressively to

TABLE 3

The effect of incubation with perchloric acid on the stability of the glycolytic metabolites assayed in this study[a]

Metabolite	Minutes in acid			
	0	10	20	30
Glucose-6-P	19.5[b]	19.0	18.0	19.5
Glucose-1-P	17.0	18.5	17.0	17.0
ATP	11.5	11.5	11.0	11.5
2-P-glycerate	10.5	-	-	10.5
P-enolpyruvate	10.5	-	-	11.0
Pyruvate	11.0	-	-	12.0

[a] The various intermediates were incubated in 50 mM potassium phosphate buffer (pH 7.2) at room temperature with an equal volume of 1.0 N $HClO_4$. Aliquots were withdrawn at the times noted and neutralized with KOH-triethanolamine buffer. The subsequent sample treatment and analyses are described in the text.

[b] nmoles/ml of neutralized solution.

TABLE 4

Percent recovery of glycolytic intermediates from neutralized perchloric acid cell extracts[a]

Metabolite	Recovery range	Mean
Glucose-6-P	93-103	96
Glucose-1-P	104-109	106
ATP	96-104	101
2-P-glycerate	100-105	103
P-enolpyruvate	98-105	102
Pyruvate	96-100	98

[a] Each neutralized cell extract contained (in nmoles/ml): glucose-6-P, 19.5; glucose-1-P, 17.0; ATP, 11.5; 2-P-glycerate, 10.5; P-enolpyruvate, 10.5; and pyruvate, 11.0, in addition to the endogenous metabolites. Three samples and one endogenous control were analyzed in duplicate by the methods described in the text and the values for the endogenous control were subtracted from those of the experimental samples.

42% of the original value in 72 hours. For this reason, pyruvate assays were always performed immediately after completion of the experiment.

5. Determination of glycolytic intermediates.

The concentration of glucose-6-P, glucose-1-P, 2-P-glycerate, P-enolpyruvate, pyruvate, and ATP in the cell extracts was determined by the direct enzymatic-fluorometric methods outlined by Maitra and Estabrook (99). In this procedure, specific purified enzymes are used to couple specific metabolites to reactions which involve the oxidation or reduction of pyridine nucleotides. The quantity of the particular metabolite being assayed was determined from the extent of this oxidation or reduction which was directly proportional to the concentration of the specific metabolite. Fluorometric analysis was employed in this study because this detection system is at least 100-fold more sensitive than the commonly used spectrophotometric methods (98, 99). This high degree of sensitivity permitted the accurate determination of the low concentrations of glycolytic metabolites extracted from whole cells.

Fluorescence was measured in a Farrand Ratio Fluorometer equipped with a modified Heath Model EU-20B Servo Recorder and with a General Electric mercury vapor lamp as the radiation source (Farrand Optical Co., Mt. Vernon, New York). The light for sample excitation was isolated with a Farrand interference filter with peak transmission at 342 nm, while the fluorescent light emitted from the sample was isolated with a 435 nm filter.

All enzyme reactions and fluorescence determinations were carried

out in unmarked 10 x 75 glass test tubes. In order to keep the fluorescence of the blank to a minimum, the reaction tubes were cleaned by boiling for 30 minutes, first in 7 M HNO_3 and then in deionized water, followed by extensive rinsing (101).

The concentration of glucose-6-P, ATP, and glucose-1-P were determined sequentially by their enzymatic conversion to 6-P-gluconate according to the following reactions:

$$\text{Glucose-6-P} + \text{NADP}^+ \longrightarrow \text{6-P-gluconate} + \text{NADPH} + \text{H}^+ \quad (9)$$

$$\text{ATP} + \text{Glucose} \longrightarrow \text{Glucose-6-P} \quad (10)$$

$$\text{Glucose-1-P} \longrightarrow \text{Glucose-6-P} \quad (11)$$

Reactions (9) to (11) were catalyzed by commercial glucose-6-phosphate dehydrogenase, hexokinase, phosphoglucomutase, respectively. The basic assay mixture contained: triethanolamine-HCl (20 mM, pH 7.6), KCl, 10 mM; $MgCl_2$, 2 mM; and $NADP^+$, 0.25 mM, in a volume of 1.0 to 1.5 ml.

A typical determination of these intermediates is given in Fig. 3. The background fluorescence was established and adjusted to zero upon the addition of 0.1 - 0.2 ml sample to the basic assay mixture. Glucose-6-phosphate dehydrogenase (2 µg) was then added to the mixture and the increase in fluorescence was noted. This change in fluorescence represented the glucose-6-P content of the samples. When ATP was to be determined, hexokinase (10 µg) was then added and the subsequent increase in fluorescence again noted. Similarly, phosphoglucomutase (10 µg) was added when glucose-1-P was to be determined. The fluorescence scale was then standardized by the addition of an internal standard of glucose-6-P

Fig. 3. Recorder tracing of the fluorometric determination of glucose-6-P, ATP and glucose-1-P.

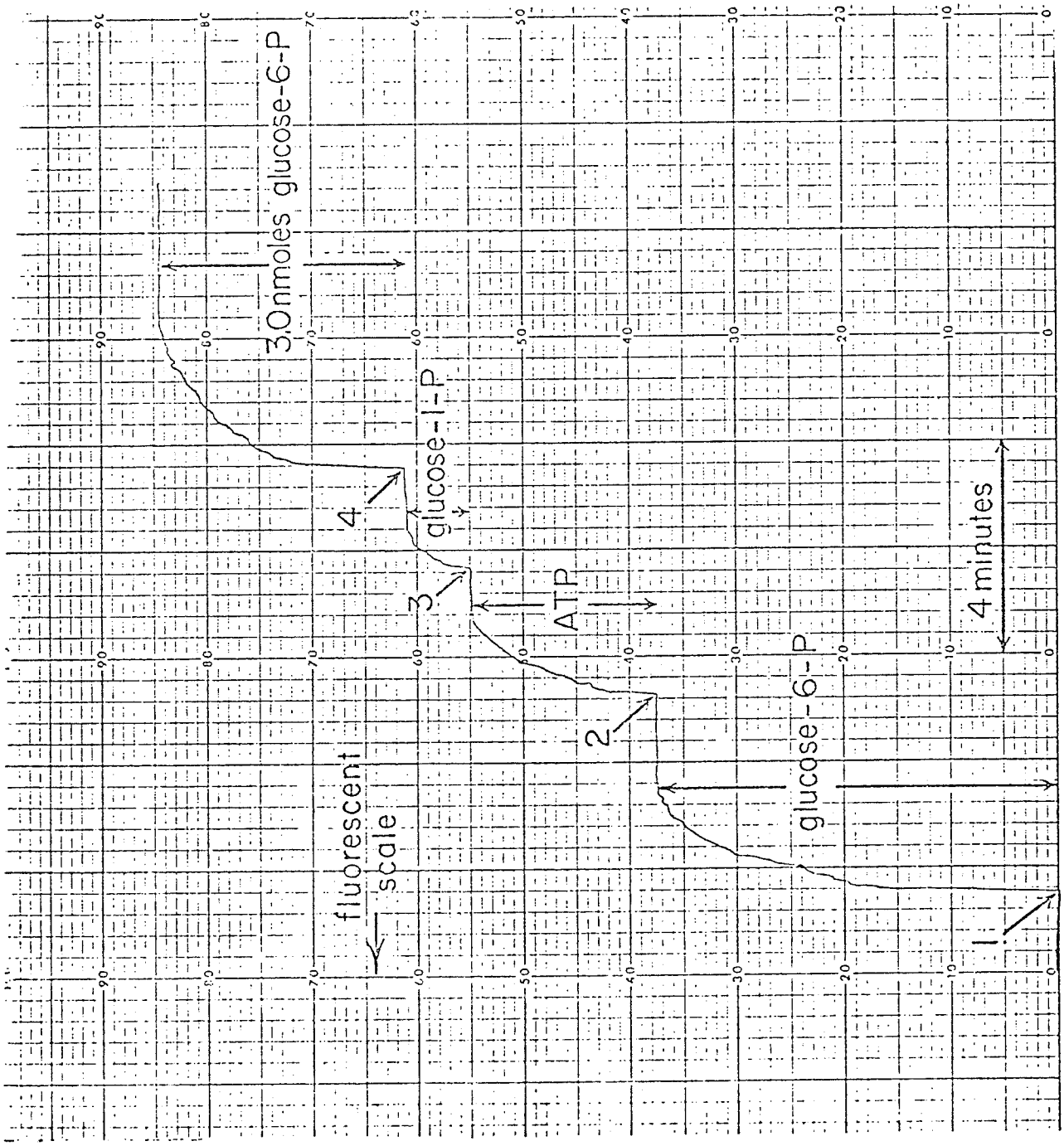
A sample (0.1 ml) of a neutralized perchloric acid cell extract was added to 1.0 ml of the basic assay mixture described in the text. The fluorescence of this mixture was set at zero. Further additions:

(1) glucose-6-P dehydrogenase (2 µg protein in 10 µl H_2O)

(2) hexokinase (10 µg protein in 10 µl of a 2% glucose solution)

(3) phosphoglucomutase (10 µg protein in 10 µl H_2O) and

(4) glucose-6-P (3.0 nmoles in 10 µl of triethanolamine-HCl buffer (20 mM, pH 7.6)).


3.0nmoles glucose-6-P
glucose-1-P
ATP
glucose-6-P
4 minutes
fluorescent scale
1
2
3
4

(10 µl) (usually, 1.5 - 3.0 nmoles in 20 mM, pH 7.6 triethanolamine-HCl buffer).

A plot of the change in fluorescence against the NADPH concentration was linear to at least 13.2 nmoles NADPH per ml of the reaction mixture and the system was sensitive enough to detect NADPH as low as 0.1 nmole/ml (Fig. 4). As would be expected from the above results, these relationships also applied to the determination of glucose-6-P (Fig. 5).

The glucose-6-P content of the internal standard was determined both gravimetrically and by fluorometric analysis according to the procedure already described. The increase in fluorescence due to NADPH production (reaction 9) was compared to the increase in fluorescence upon addition of a known amount of NADPH. In turn, standardized NADPH solutions were determined spectrophotometrically (Unicam SP-800, Pye-Unicam Limited, Cambridge, England) by dividing their absorbance at 340 mu by the molar extinction coefficient for NADPH (6.22×10^6 $CM^2 \times mole^{-1}$) (102). The gravimetric and fluorometric procedures gave 98% agreement with each other. A later restandardization by the fluorometric procedure just described demonstrated that the glucose-6-P standard was stable for at least six months when stored at - 10 C.

The concentration of pyruvate, P-enolpyruvate and 2-P-glycerate were determined sequentially by their enzymatic conversion to lactate by the following reactions:

$$\text{Pyruvate} + \text{NADH} + H^+ \longrightarrow \text{lactate} + NAD^+ \qquad (12)$$

$$\text{P-enolpyruvate} + \text{ADP} + H^+ \longrightarrow \text{pyruvate} + \text{ATP} \qquad (13)$$

$$\text{2-P-glycerate} \longrightarrow \text{P-enolpyruvate} \qquad (14)$$

Fig. 4. Standard curve comparing the change in fluorescence as a function of the NADPH concentration.

Inset: A demonstration of the sensitivity of NADPH detection. The inset determination was performed at a higher instrument sensitivity setting.

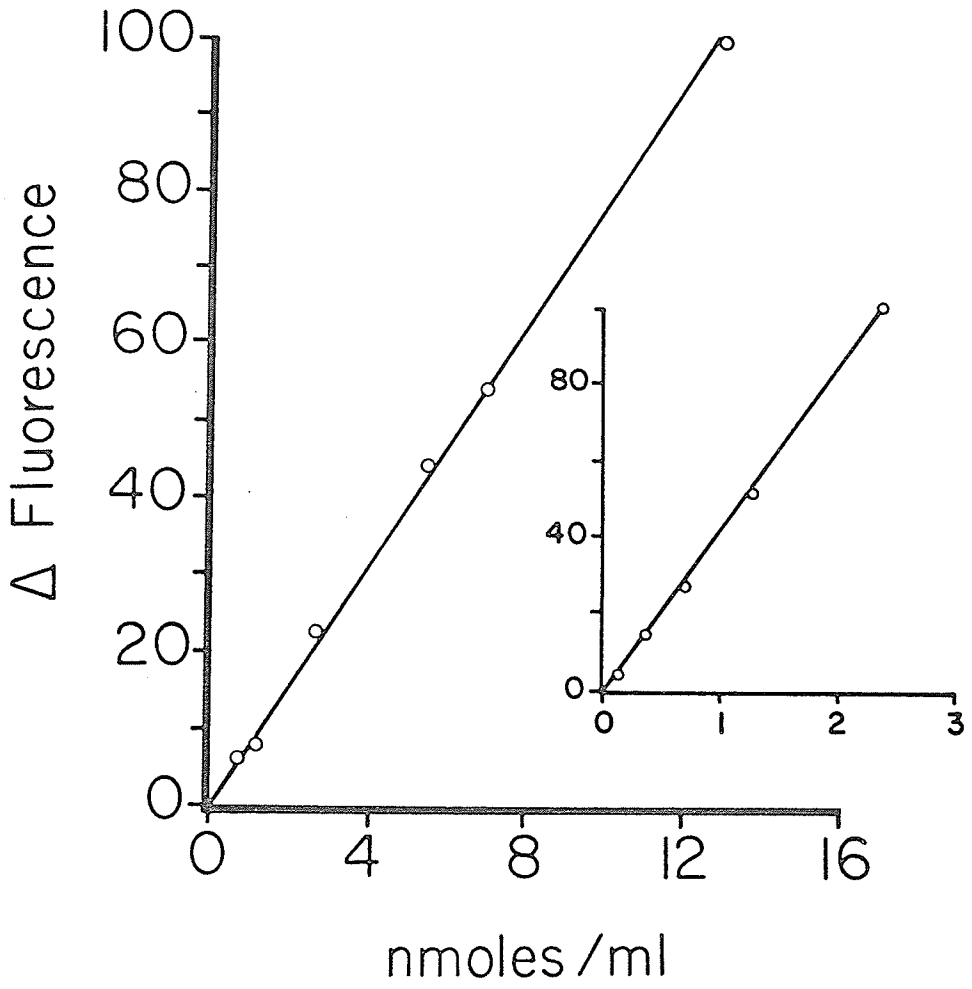
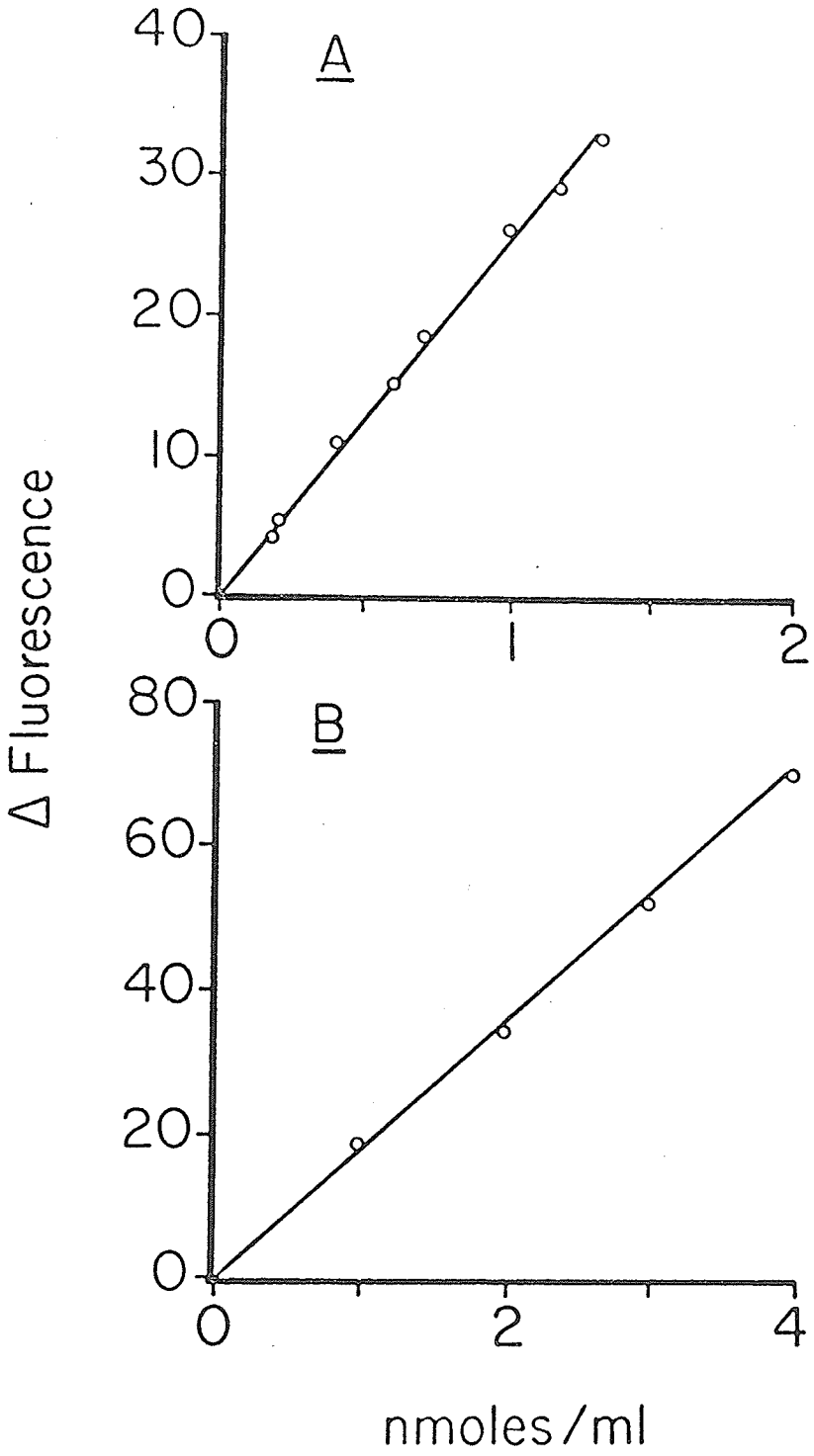

100
80
60
40
20
0
Δ Fluorescence
0
4
8
12
16
nmoles/ml
80
40
0
0
1
2
3

Fig. 5. Fluorescence change as a function of the concentration of (A) glucose-6-P, (B) pyruvate.


A
B
Δ Fluorescence
nmoles/ml
0
10
20
30
40
60
80
1
2
4

Reactions (12) to (14) were catalyzed by commercial lactic dehydrogenase, pyruvatekinase and enolase, respectively. The basic reaction mixture contained: triethanolamine-HCl (20 mM, pH 7.8), KCl, 20 mM; $MgCl_2$, 10 mM; and neutralized ADP, 2.5 mM, in a volume of 2.0 ml. A typical quantitative assay for pyruvate, P-enolpyruvate and 2-P-glycerate is given in Fig. 6. The background fluorescence was established and adjusted to zero after the addition of a 0.05 - 0.2 ml sample to the basic reaction mixture. Sufficient NADH (usually 10 - 20 nmoles) was then added to produce a fluorescence scale reading of 90 - 100. Pyruvate was then assayed by the addition of 10 µg lactate dehydrogenase. Since various batches of commercial lactic dehydrogenase possessed inherent fluorescence, the fluorescence of each lot was determined prior to each experiments and the appropriate corrections made when necessary. Furthermore, since pyruvate was often found in high concentrations in the samples, a significant fraction of the NADH was utilized in the pyruvate assay. When this occurred additional NADH was added to the reaction tube after the pyruvate assay in order to adjust the fluorescence scale to a higher level. Following this procedure, P-enolpyruvate was determined by the addition of enolase (10 µg). The concentration of these intermediates was calculated from the decrease in fluorescence obtained upon addition of an internal standard of 2-P-glycerate (2.7 - 5.4 nmoles in triethanolamine-HCl buffer).

In this assay system, the relationship between the NADH concentration and the change in fluorescence was linear to at least 15 nmoles NADH/ml of the assay mix while concentrations as low as 0.1 nmole/ml could be detected. A standard curve for a pyruvate determination is given in Fig. 5.

Fig. 6. Recorder tracing of the fluorometric determination of pyruvate, P-enolpyruvate and 2-P-glycerate.

A sample (0.2 ml) of a neutralized perchloric acid cell extract was added to 2.0 ml of the basic assay mixture described in the text and the fluorescence of this mixture was set at zero. Further additions:

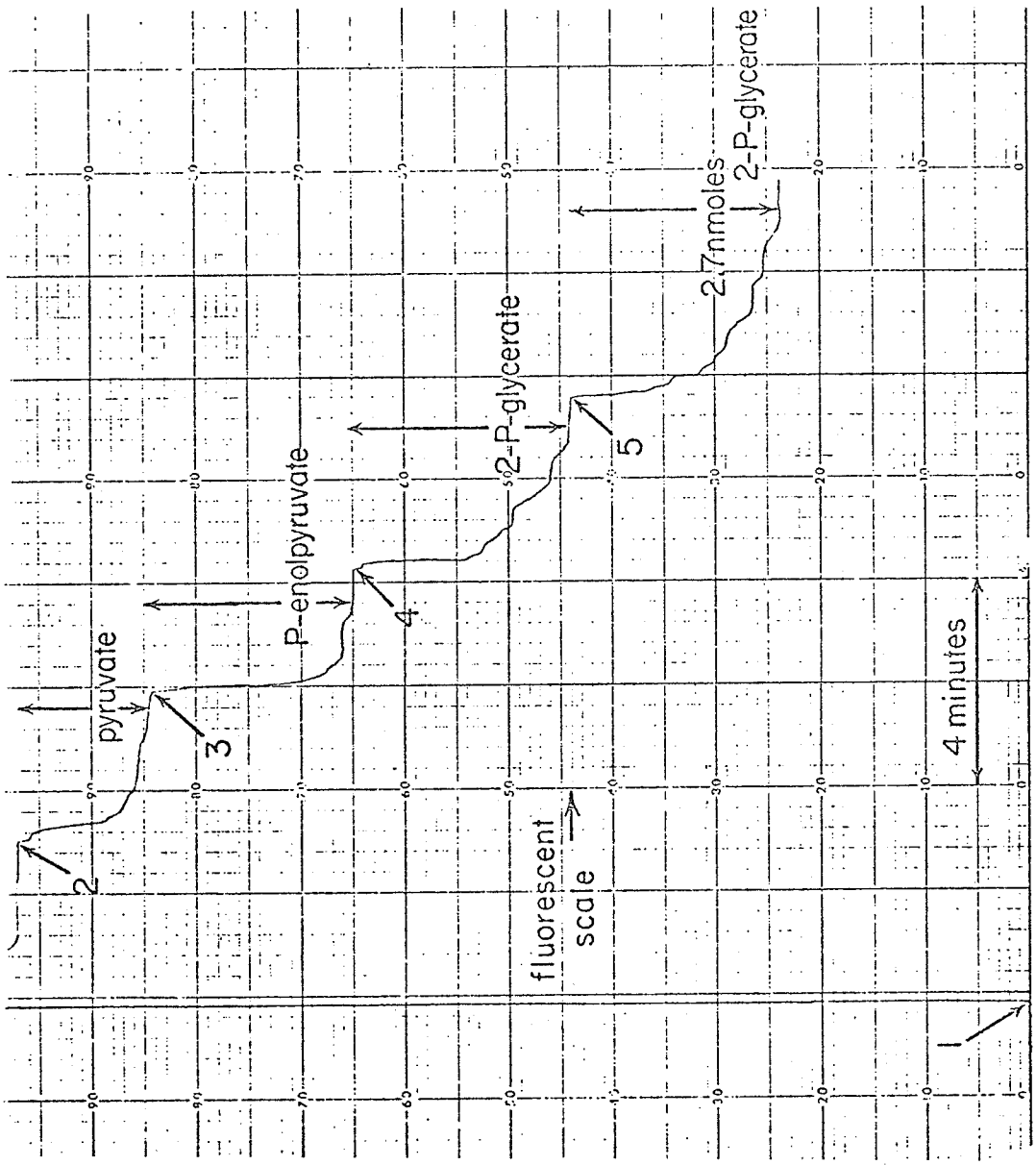
(1) NADH (12 nmoles in 10 µl of triethanolamine-HCl buffer (20 mM, pH 7.8)),

(2) lactic dehydrogenase (10 µg protein in 10 µl H_2O),

(3) pyruvate kinase (10 µg protein in 10 µl H_2O),

(4) enolase (10 µg protein in 10 µl H_2O) and

(5) 2.7 nmoles 2-P-glycerate in 10 µl of triethanolamine(HCl buffer.


2
pyruvate
3
P-enolpyruvate
4
2-P-glycerate
5
2.7nmoles
2-P-glycerate
fluorescent
scale
4 minutes
1

The 2-P-glycerate content of the internal standard was determined both gravimetrically and by the fluorometric procedure just described; these procedures gave 99% agreement with each other. In the latter procedure, the change in fluorescence due to the oxidation of NADH by reactions (12) to (14) was compared to the change produced by known amounts of standard NADH. The NADH concentration in the standard solution was determined as described previously for NADPH. The 2-P-glycerate standard solution was stable for at least six months if stored frozen.

Preliminary study demonstrated that with the exception of enolase, the commercial enzymes used in all of the fluorometric determinations were insensitive to 0.96 mM NaF. Enolase, however, was unaffected by NaF lower than 0.24 mM, the maximum concentration present in any assay tube.

6. Precision of the fluorometric determinations.

Since the concentration of glycolytic intermediates extracted from the cells was very low, it was necessary to determine the precision of their determination by the enzymatic-fluorometric analyses. Therefore, analyses for each metabolite assayed in the experiments reported in Chapters 4 , 5 and 6 were selected at random and grouped according to the concentration of the particular metabolite in nmoles/mg dry wt cells (Table 5). The mean and standard deviation of each duplicate determination was calculated. In order to normalize the results for subsequent comparison, the coefficient of variance (standard deviation x 100% divided by the mean) was calculated and the average of these for every concentration grouping is reported. As would be expected, the precision of the analyses increased as the metabolite concentration increased.

TABLE 5

Average coefficient of variance for duplicate fluorometric determinations of randomly selected experimental samples[a]

Metabolite	Concentration (nmoles mg/dry wt cells)			
	below 1.0	1.0-2.0	2.0-4.0	4.0 and above
Glucose-6-P	17.0	6.5	5.3	2.8
Glucose-1-P	16.4	-	-	-
ATP	14.5	6.1	4.5	-
2-P-glycerate	18.4	7.7	7.4	5.1
P-enolpyruvate	24.4	9.2	6.7	5.4
Pyruvate	-	-	-	3.0

[a] The average of the coefficient of variance for at least ten duplicate determinations is reported. (-) signifies that the particular intermediate was rarely present at this concentration in the experiments reported in this thesis.

Since the precision was low at metabolite concentrations below 1.0 nmole/mg dry wt cells, comparisons were not made between the concentration of a metabolite in control cells and in fluoride-treated cells at this level.

7. Determination of glucose.

The glucose content of the neutralized cell extracts was determined enzymatically by the glucose oxidase method (103) according to the following reactions:

$$\text{Glucose} + O_2 + H_2O \longrightarrow \text{gluconic acid} + H_2O_2 \qquad (15)$$

$$H_2O_2 + \text{o-dianisidine} \longrightarrow \text{color} \qquad (16)$$

Reactions (15) and (16) are catalyzed by glucose oxidase and peroxidase, respectively.

The assay mixture contained 0.2 ml of sample or standard (0 - 10 µg), 0.1 ml of glucose oxidase solution (1 mg protein) and 0.8 ml of a buffer reagent containing the following: Na_2HPO_4, 1.68 g; KH_2PO_4, 2.24 g; peroxidase, 11.3 mg; o-dianisidine, 113 mg, in one liter of deionized water. After the addition of the reagents, the tubes were mixed and incubated in a shaking water bath (37 C, 60 min). After incubation, 0.4 ml of 37.4% H_2SO_4 was added to each tube to stop the reaction and to facilitate further color development. The absorption of the samples was determined at 530 nm in a Unicam SP-500 spectrophotometer. All assays were conducted with duplicate samples and triplicate glucose standards.

8. Determination of glycogen synthesis and degradation.

Glycogen synthesis was determined by measuring the incorporation of glucose-U-^{14}C into intact cells, while glycogen degradation was determined by following the loss of this label from the cells. Hamilton (96) had previously demonstrated that 90% of the incorporated ^{14}C was present in the cell as a degradable glucose polymer. Cells were incubated as previously described (section on *in vivo* experiment) except that glucose-U-^{14}C was the substrate. Samples (0.1 ml containing 1.0 mg dry wt cells) were removed periodically from the incubation mixture and added to 2 ml of 50% ethanol in 0.1 N HCl. The cells were collected on 0.45 μ Millipore membrane filters (Millipore Corporation, Bedford, Massachusetts) and the filters washed three times with ethanol (2 ml, 50% solution) to remove the exogenous radioactive glucose. Washed filters were carefully placed on stainless steel planchets which were held in place by expandable brass rings. After drying at room temperature, the planchets were counted in a low background gas flow counter (Nuclear Chicago, Des Plaines, Illinois). The specific activity of the radioactive glucose was determined by counting aliquots of the original glucose-U-^{14}C solution on aluminum foil cups having the same geometry as the experimental system. This permitted the calculation of the amount of glucose incorporated into glycogen per mg dry wt of cell material.

C. IN VITRO ENZYME STUDIES

The *in vivo* studies referred to in preceding sections were designed to determine the sites of fluoride action within intact cells of

S. salivarius. These studies were supplemented by in vitro assays to determine the fluoride sensitivity of enzymes involved in the production of glucose-6-P from glucose and the enzymes involved in glycogen synthesis and degradation.

1. P-enolpyruvate phosphotransferase activity.

Studies with various bacterial species have indicated that the P-enolpyruvate phosphotransferase system is involved in the cellular transport of carbohydrates (104, 105). According to Kundig, Ghosh, and Roseman (106) the following reactions occur:

$$\text{P-enolpyruvate} + \text{HPr} \xrightarrow{\text{Enzyme I, } Mg^{++}} \text{pyruvate} + \text{HPr-P} \qquad (17)$$

$$\text{HPr-P} + \text{sugar} \xrightarrow{\text{Enzyme II, } Mg^{+}} \text{sugar-P} + \text{HPr} \qquad (18)$$

In this system, enzyme I and the heat stable protein, HPr, are normally found in the cytoplasm, while enzyme II is membrane-bound and specific for the particular carbohydrate being transported.

Cellular P-enolpyruvate phosphotransferase activity was assayed in toluene-treated cells of S. salivarius by the method of Gachelin (107). In this procedure, 2-deoxyglucose-U-^{14}C is used as the substrate since the product of the phosphotransferase system reactions, 2-deoxyglucose-6-P-^{14}C, cannot be metabolized by the cells. Although phosphorylated compounds normally do not cross bacterial membranes, treatment with toluene allows the entry of P-enolpyruvate into the cells and the exit of 2-deoxy-glucose-6-P-^{14}C into the medium so that this product can be isolated.

Washed cells (2 mg dry wt/ml) were suspended in potassium phosphate buffer (50 mM, pH 7.2) and toluene (5 µl/ml of cell suspension) added to the cells. The reaction flask was then tightly sealed with a serum cap and incubated at 37 C for 30 minutes with gentle shaking. Immediately thereafter, the toluene-treated cells (final concentration 1.6 mg dry wt/ml) were incubated with 2-deoxy-D-glucose-U-^{14}C (1.0 mM; 17,800 dpm/µmole) and with either P-enolpyruvate, 2-P-glycerate or ATP (all 2.0 mM) in a total volume of 1.0 ml. After incubation at 37 C for 30 min, the cells were removed by centrifugation at 3,000 g for 30 min.

The isolation of 2-deoxyglucose-6-P-^{14}C formed by the cells was carried out essentially by the method of Romano et al., (108). Duplicate 0.4 ml aliquots of each sample were applied to columns (4 by 0.6 cm) of Dowex-1-formate form (x8, 200-400 mesh) and the remaining 2-deoxyglucose-U-^{14}C eluted with deionized water (3 ml). 2-deoxyglucose-6-P-^{14}C was eluted by addition of 0.5 M ammonium formate in 0.2 M formic acid (3.5 ml) and the eluates collected in glass vials to which Bray's (109) scintillation fluid was later added. Samples were counted in a Nuclear Chicago 720 Series Liquid Scintillation Counter and the absolute counts (dpm) determined by the "channels ratio" method (110). The results were expressed as nmoles 2-deoxy-D-glucose-6-P formed/min/mg dry wt cell material as calculated from the specific activity measurements of the substrate 2-deoxy-D-glucose-U-^{14}C.

2. Other enzymes.

The activity of hexokinase and the enzymes involved in glycogen synthesis and degradation was determined in crude extracts of S. salivarius.

Cell-free extracts were prepared by disrupting cells (30-40 mg dry wt/ml) suspended in Tris-HCl buffer (50 mM, pH 7.5) in a Branson Sonifier (Heat Systems Ultrasonics, Plainview, New York) for 3 minutes at 0 C. The supernatant, obtained after centrifugation (30,000 g, 0 C, 30 minutes) and dialysis against 100 volumes of the Tris-HCl buffer, was used in the enzyme assays. The protein concentration of the extracts was determined by the method of Lowry et al., (111).

Phosphoglucomutase was assayed by a modification of a procedure for the determination of glucose-1-P (112). Activity was measured in a coupled enzyme assay wherein glucose-1-P was converted to glucose-6-P in the presence of catalytic amounts of glucose-1,6-diphosphate. The glucose-6-P was then further converted to 6-phosphogluconic acid with commercial glucose-6-phosphate dehydrogenase and $NADP^+$. The resulting NADPH was detected in a Unicam SP-800 spectrophotometer at 340 nm and the concentration was determined from the molar extinction coefficient. The reaction mixture consisted of Tris-HCl buffer (10 mM, pH 7.5); $MgCl_2$, 0.5 mM; glucose-1-P, 0.5 mM; glucose-1,6-diphosphate, 4 mM; $NADP^+$, 0.2 mg; commercial glucose-6-phosphate dehydrogenase, 20 µg; and extract protein, 5.3 µg, in a total volume of 1.0 ml.

Hexokinase activity in crude extracts was determined by the conversion of glucose and ATP to glucose-6-P. The rate of glucose-6-P formation was again detected with the $NADP^+$-glucose-6-P dehydrogenase assay system. The reaction mixture contained (50 mM, pH 7.6) triethanolamine-HCl buffer; glucose, 10 mM; ATP, 0.5 mM; $MgCl_2$, 0.5 mM; $NADP^+$, 0.2 mg; commercial glucose-6-P dehydrogenase, 20 µg; extract protein, 23 µg, in a total volume of 1.0 ml.

Adenosine diphosphate-glucose pyrophosphorylase (ADP-glucose pyrophosphorylase) activity in extracts was determined by the incorporation of glucose-U-^{14}C from glucose-^{14}C-1-P into ADP-glucose (113). The reaction mixture consisted of Tris-HCl buffer (50 mM, pH 7.8); glucose-^{14}C-1-P (1×10^6 dpm/μmole), 0.5 mM; ATP, 1 mM; $MgCl_2$, 10 mM; inorganic pyrophosphatase, 0.9 μg; and extract protein, 250 μg, in a total volume of 0.2 ml. After incubation at 37 C for 20 minutes, the reaction was stopped by the addition of 10% trichloroacetic acid (1.0 ml) and activated charcoal (Norit A, 7.5 mg/0.1 ml) was added to absorb the ADP-glucose-U-^{14}C. This mixture was centrifuged at 3,000 g for 15 min and the supernatant discarded. The sedimented charcoal was washed twice with 0.02 N HCl (1.0 ml) and then suspended in 1.0 ml of this solution and boiled for 10 minutes to release the labelled ADP-glucose. Following centrifugation, duplicate aliquots (0.4 ml) of the clear supernatant were added to Bray's solution (10 ml) and counted in a liquid scintillation counter.

ADP-glucose: glucan transferase was determined by the incorporation of glucose-U-^{14}C from ADP-glucose-^{14}C into glycogen (114). The reaction mixture contained 50 mM Tris-HCl buffer (pH 7.8); ADP-glucose-^{14}C (2×10^5 dpm/μmole), 0.5 mM; KCl, 25 mM; mercaptoethanol, 10 mM; glycogen, 0.5 mg; bovine serum albumin, 0.2 mg; and extract protein, 25 μg, in a total volume of 0.2 ml.

Phosphorylase activity was determined by the incorporation of glucose-U-^{14}C from glucose-^{14}C-1-P into glycogen in the absence of ATP (115). The reaction mixture contained 100 mM β-glycerophosphate buffer (pH 6.8), glucose-^{14}C-1-P (1×10^6 dpm/μmole), 10 mM; glycogen, 0.5 mg; and extract protein, 89 mg, in a total volume of 0.1 ml.

In the analyses for ADP-glucose:glucan transferase and phosphorylase, the reactions were terminated after a 20 min incubation period at 37 C by the addition of 75% methanol-1% KCl solution (2.0 ml). The mixtures were centrifuged at 3,000 g for 15 min and the pellet was washed twice with 2.0 ml of the methanol-KCl solution. The final radioactive glycogen pellet was dissolved in 1.0 ml of water and duplicate 0.4 ml aliquots dissolved in Bray's solution (10 ml) and counted in a liquid scintillation counter.

D. MATERIALS

Tryptone, yeast extract and dehydrated Mitis-Salivarius agar were purchased from Difco Laboratories Inc., (Detroit, Michigan). All enzymes, co-factors and glycolytic intermediates used in the studies to be reported were obtained from the Boehringer Mannheim Corporation (New York, New York). Glucose oxidase and peroxidase were purchased from Sigma Chemical Co., (St. Louis, Missouri). 2-deoxy-D-glucose-^{14}C was obtained from the International Chemical and Nuclear Corporation (City of Industry, California). All other radioactive chemicals were obtained from the New England Nuclear Corporation (Boston, Massachusetts). All other chemicals used in these studies were of reagent grade.

CHAPTER IV

FLUORIDE INHIBITION OF GLUCOSE-6-P FORMATION: RELATION TO GLYCOGEN SYNTHESIS AND DEGRADATION

A. INTRODUCTION

Anaerobic glucose metabolism by resting cells of S. salivarius results in the formation of lactic acid and large quantities of intracellular glycogen (96). Fluoride has been shown to inhibit exogenous glucose metabolism, intracellular glycogen synthesis and the degradation of this polymer in the absence of an external carbon source (59, 79). Of these three parameters, glycogen synthesis was by far the most sensitive, being completely inhibited by low levels of fluoride under conditions of appreciable glucose degradation. On the other hand, the degradation of this endogenous energy source was inhibited to a much lesser degree than was exogenous glucose metabolism. From these studies it was concluded that since the exogenous and endogenous pathways had widely differing fluoride sensitivities, and yet shared a common glycolytic pathway from glucose-6-P to lactate, the prime site of fluoride action in intact cells of S. salivarius was at some undetermined point in the glycolytic scheme prior to glucose-6-P formation. A similar conclusion was reached in studies with S. mitis (47) and mixed oral flora (49).

Evidence will be presented in this chapter supporting the above supposition by demonstrating that low concentrations of fluoride had a pronounced and rapid inhibitory effect on the synthesis of glucose-6-P and ATP in whole cells of S. salivarius metabolizing exogenous glucose. Further results with crude enzyme preparations will demonstrate that the enzymes in the glycogen synthetic pathway are insensitive to fluoride at inhibitor concentrations above those required to completely inhibit in vivo synthesis.

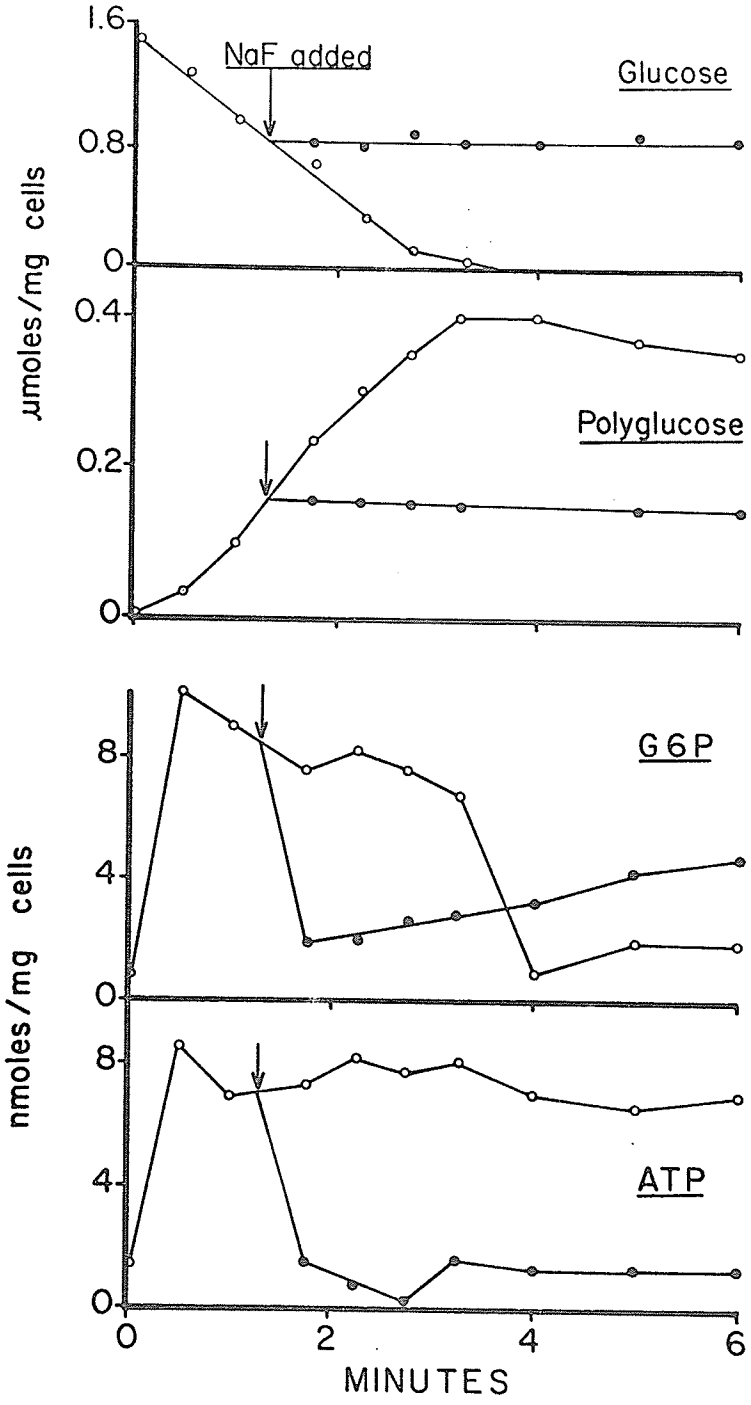
B. RESULTS

1. Effects of fluoride on in vivo glucose metabolism.

As shown previously (59, 79), the addition of exogenous glucose to the non-proliferating, intact cells of S. salivarius resulted in the immediate uptake of this substrate with concomitant glycogen synthesis. Confirmation of these results is shown in Fig. 7, along with the cellular concentrations of glucose-6-P and ATP. Upon glucose addition, the intracellular content of glucose-6-P and ATP rose rapidly from low endogenous levels to higher levels characteristic of exogenous glucose metabolism. At the point of glucose exhaustion (3.6 min), the intracellular glycogen content was maximum and the glucose-6-P concentration began to decline dramatically to a low level characteristic of endogenous metabolism. The ATP content at this point, however, continued at a relatively constant level probably because the cells had started to degrade the newly formed cellular polysaccharide for energy.

Fig. 7. The effect of 2.4 mM NaF on exogenous glucose uptake, glycogen metabolism and the intracellular content of glucose-6-P and ATP during anaerobic glucose metabolism by washed cells of S. salivarius.

Control cells - O , fluoride-treated cells- ● .


1.6
0.8
0
NaF added
Glucose
μmoles/mg cells
0.4
0.2
0
Polyglucose
8
4
0
G6P
nmoles/mg cells
8
4
0
ATP
0
2
4
6
MINUTES

The addition of 2.4 mM NaF (final concentration) to the cells 1.5 minutes after the addition of the glucose profoundly altered this metabolic pattern. Exogenous glucose uptake from the medium was immediately stopped, as was glycogen synthesis, confirming previous reports. Furthermore, the addition of fluoride also resulted in a rapid decrease in the cellular concentrations of ATP and glucose-6-P concomitant with the effects on glucose uptake and glycogen synthesis. Although glucose-6-P initially dropped to 15% of the control value 15 seconds after the fluoride addition, the concentration increased to 50% of this value before the end of the experimental period. ATP, on the other hand, dropped to 20% of the control value initially and remained at about this level in the remaining samples. Thus, the increase in glucose-6-P in the fluoride-treated cells was not associated with an increase in the cellular concentration of ATP. The continued low synthesis of both ATP and glucose-6-P in the presence of fluoride during the experimental period was the result of the degradation of small quantities of cellular glycogen (see Fig. 16, Chap. 5).

The fluoride effect on the cellular levels of ATP and glucose-6-P was a sustained one since prolonged incubation of the cells demonstrated that as long as fluoride was present in the medium the cellular content of both ATP and glucose-6-P remained at greatly reduced levels (Fig. 8). Unlike the ATP concentration which remained low throughout the experimental period, the cellular content of glucose-6-P in these cases often rose to levels slightly above the steady state concentration maintained in the control cells in the absence of exogenous glucose. In the control cells, on the other hand, the glucose-6-P concentration dropped immediately

Fig. 8. The effect of 2.4 mM NaF on exogenous glucose uptake and the intracellular content of glucose-6-P and ATP in intact cells of S. salivarius metabolizing glucose.

Control cells- ○ , fluoride-treated cells- ● .

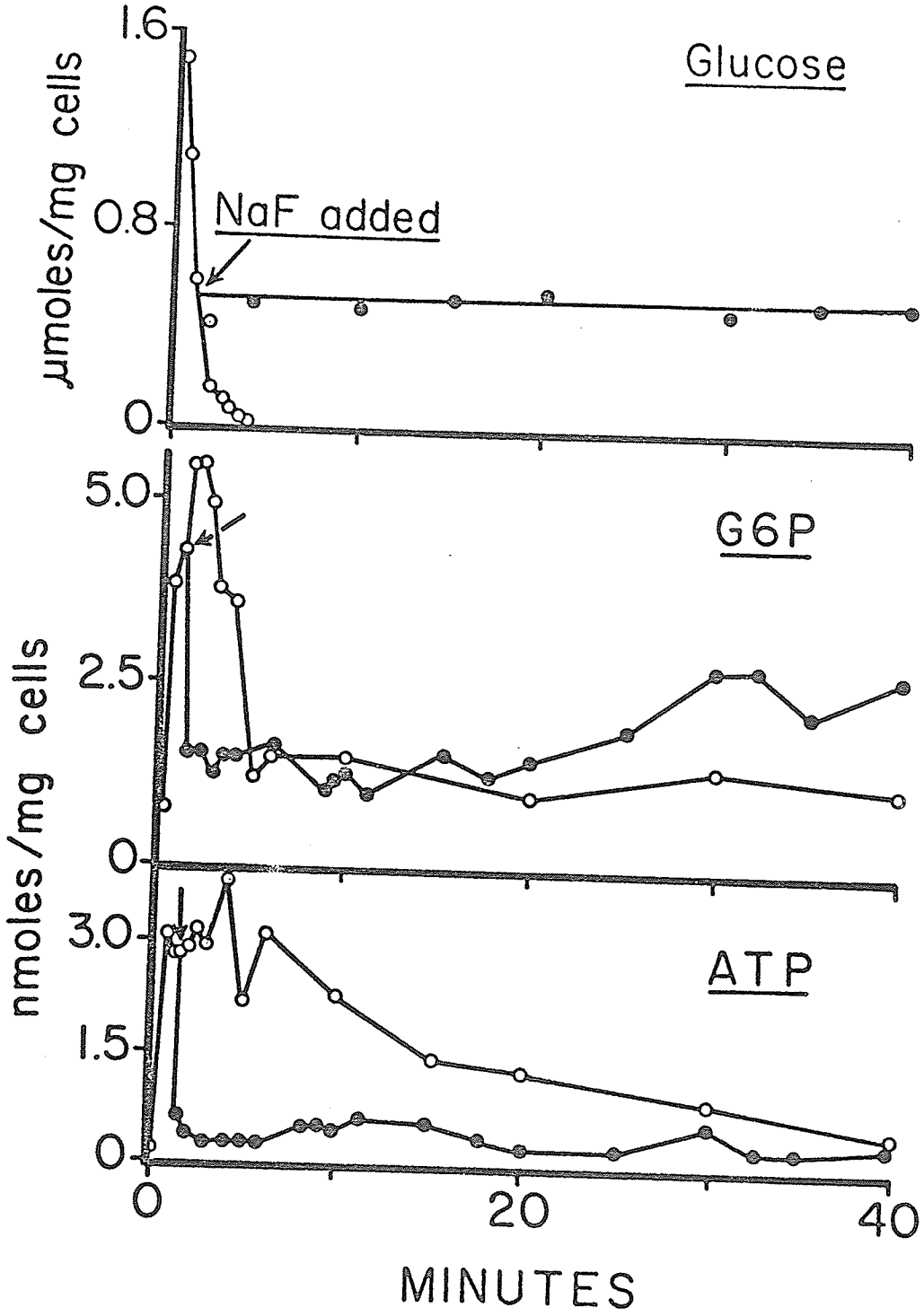
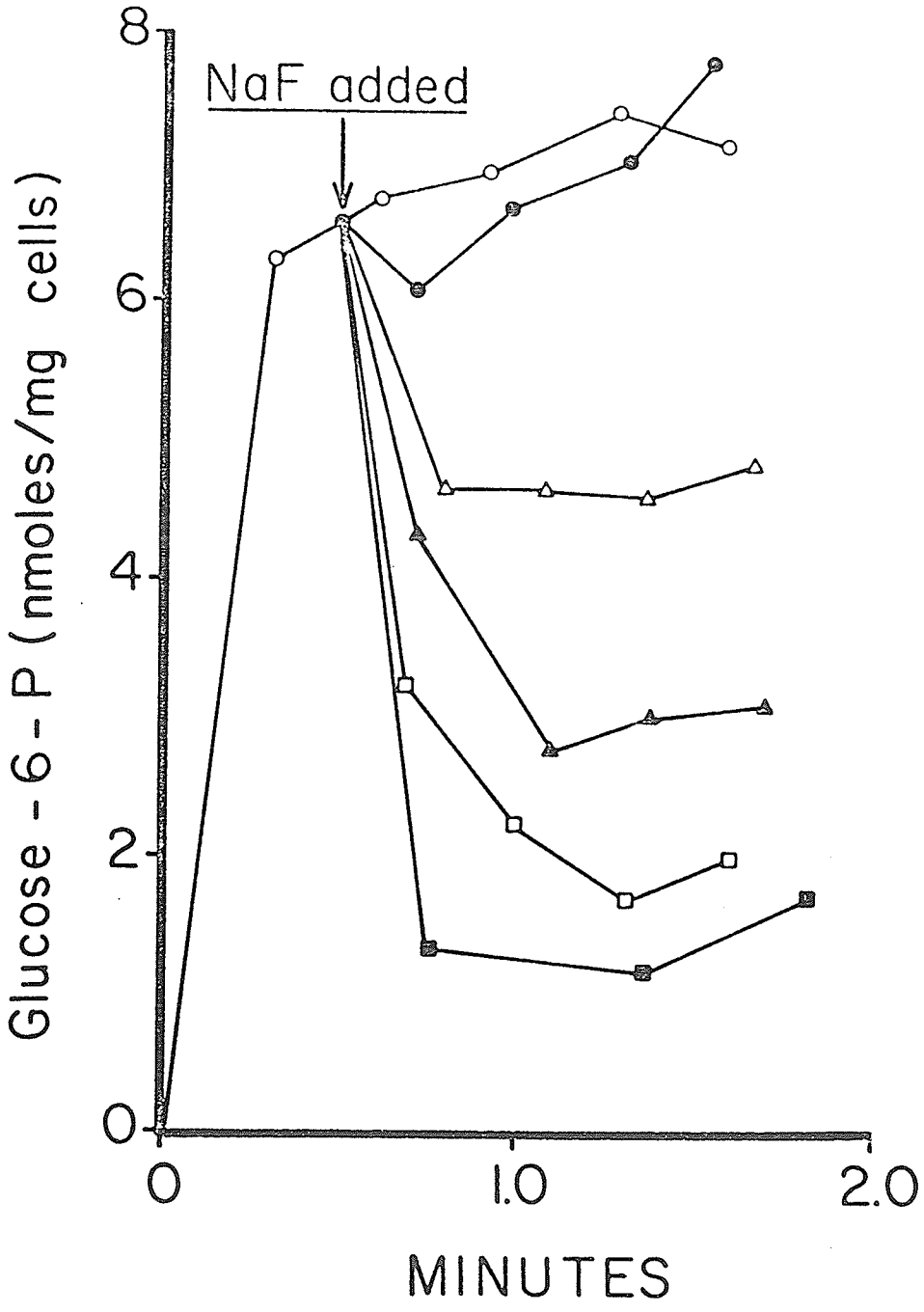

Glucose
NaF added
G6P
ATP
μmoles/mg cells
nmoles/mg cells
1.6
0.8
0
5.0
2.5
0
3.0
1.5
0
0
20
40
MINUTES

Fig. 9. The effect of varying concentrations of NaF on glucose-6-P levels in cells of S. salivarius metabolizing glucose.

NaF was added at 30 seconds to give the following final concentrations (mM):
0- ○ , 0.06- ● , 0.12- △ , 0.25- ▲ , 0.60- □ , 1.2- ■ .


NaF added
Glucose - 6 - P (nmoles/mg cells)
8
6
4
2
0
0
1.0
2.0
MINUTES

upon depletion of the exogenous glucose, while the ATP concentration declined very gradually, requiring almost 35 minutes to reach the ATP level observed in the fluoride-treated cells. This indicates that the endogenous degradation of intracellular glycogen in this organism can maintain the ATP content of the cells at a relatively high level for a considerable amount of time after the depletion of the exogenous carbon source.

2. Effect of fluoride concentration on intracellular glucose-6-P concentration.

The previous studies with S. salivarius (48, 59) demonstrated that 0.96 mM NaF completely inhibited the metabolism of exogenous glucose. Glycogen synthesis, on the other hand, was completely inhibited by fluoride at only half of this concentration, while a level as low as 0.06 mM produced a significant inhibitory effect. Therefore, an experiment was designed to investigate the effect of low fluoride levels on the intracellular concentration of glucose-6-P, the first apparent intracellular precursor in the glycogen synthetic pathway. Furthermore, in order to test for the so-called "substrate-protective" effect (38), fluoride was added to the cells before and after the substrate.

When the cells were preincubated for ten minutes at 37 C with NaF (0-1.2 mM) followed by the addition of glucose (1.5 μmoles/mg dry wt cells), a progressive decrease in the maximum intracellular glucose-6-P concentration was observed (Table 6). As the NaF concentration increased to 1.2 mM, the maximum glucose-6-P level decreased from 7.1 to 1.0 nmoles/mg dry wt cells.

TABLE 6

Effect of preincubation with NaF on the maximum cellular concentration of glucose-6-P in washed cells of S. salivarius during subsequent glucose metabolism[a]

NaF	Glucose-6-P	Percent of control
mM		
0	7.1[b]	100
0.06	4.3	61
0.12	3.1	44
0.25	2.4	34
0.60	1.6	23
1.20	1.0	14

[a] Cells (10 mg dry wt/ml) were preincubated (10 min) with NaF and glucose (1.5 µmoles/mg dry wt cells) was added to start the reaction. Samples were withdrawn periodically and treated as described in Methods.

[b] nmoles per mg dry weight of cells.

A more dramatic effect was observed under the same conditions when these NaF concentrations were added after the onset of glucose metabolism. In this experiment, the inhibitor was added 30 seconds after the addition of the glucose and again resulted in an immediate, rapid decrease in the intracellular glucose-6-P concentration (Fig. 9). NaF, as low as 0.06 mM, produced an initial slight drop in the glucose-6-P content followed by a rapid recovery to levels similar to those in the control cells. However, at higher NaF concentrations (0.12 - 1.2 mM) a much more pronounced initial drop in glucose-6-P levels was observed without a subsequent increase during the remainder of the experimental period. As this figure indicates, the decrease in the cellular glucose-6-P concentration was very rapid, occurring within 15 seconds of the fluoride addition. This represented the minimum time necessary for accurate sampling in the system employed.

3. Effect of fluoride on the enzymes involved in glycogen metabolism.

Two possibilities exist to explain the in vivo inhibition of glycogen synthesis in S. salivarius by fluoride: (a) fluoride interacts directly with one or more of the enzymes involved in the synthetic pathway, or (b) insufficient glucose-6-P is available for synthesis. Phosphoglucomutase, in the glycogen synthetic pathway, generally has been cited as the point of fluoride inhibition in microbes (72, 73), while in vitro fluoride inhibition of this enzyme from rabbit muscle (33) and higher plants (34) has been observed.

To test for the first possibility, dialyzed crude extracts of S. salivarius were prepared and the activities of phosphoglucomutase,

ADPG pyrophosphorylase and ADPG glucan transferase in the synthetic pathway, as well as phosphorylase in the degradative pathway, were assayed in the presence and absence of fluoride. The results (Table 7) indicate that these enzymes were insensitive to fluoride *in vitro* at concentrations equal to, or higher than, those which completely inhibited *in vivo* glycogen synthesis. In fact, phosphoglucomutase was resistant to NaF up to 9.6 mM regardless of whether the enzyme was preincubated with fluoride, or whether the inhibitor was added after the reaction had started. ADP

4. *Effect of fluoride on glycogen degradation in vivo.*

Since small intracellular glucose-1-P concentrations exist in cells of *S. salivarius* during exogenous glucose metabolism, it was difficult to measure accurately the amount of this compound in the presence of the normally high levels of glucose-6-P. Therefore, evidence that phosphoglucomutase was fluoride-insensitive in the direction of glycogen synthesis was difficult to confirm *in vivo* in the presence of exogenous glucose. However, the effect of fluoride on this enzyme and on phosphorylase could be measured accurately by monitoring the cellular content of glucose-6-P during glycogen degradation in the absence of external glucose. This was tested in an experiment where whole cells were incubated with glucose to permit the synthesis of intracellular glycogen. Approximately one minute after the exogenous glucose had been

TABLE 7

Influence of NaF on the enzymes involved in the synthesis and degradation of glycogen in crude extracts of Streptococcus salivarius

Enzyme	NaF (mM)					
	0	1.2	2.4	4.8	7.2	9.6
Phosphoglucomutase						
Preincubation	147[a]	159	171	147	-	171
No preincubation	147	159	147	147	-	147
ADPG Pyrophosphorylase	87[b]	90	97	85	-	-
ADPG glucan transferase	106[c]	107	111	108	-	-
Phosphorylase	235[d]	363	407	-	450	-

[a] nmoles glucose-6-P formed/mg protein/min.

[b] pmoles ADP-glucose formed/mg protein/min.

[c] nmoles glycogen formed/mg protein/min.

[d] pmoles glucose-1-P incorporated into glycogen/mg protein/min.

depleted, 2.4 mM NaF was added to a portion of the cells. The glucose-6-P level in the control cells rose upon the addition of the substrate immediately in the characteristic manner and dropped again once the exogenous substrate was exhausted (Fig. 10). Under these conditions of glycogen degradation, glucose-6-P formation was initially stimulated by the addition of the fluoride resulting in concentrations higher than those in the controls but which then decreased to the control level by the end of the experimental period. As inhibition of phosphoglucomutase and/or glycogen phosphorylase would have resulted in a decrease in the glucose-6-P concentration, it is clear that these enzymes were not fluoride-sensitive under *in vivo* conditions thereby confirming the *in vitro* enzyme assays.

C. DISCUSSION

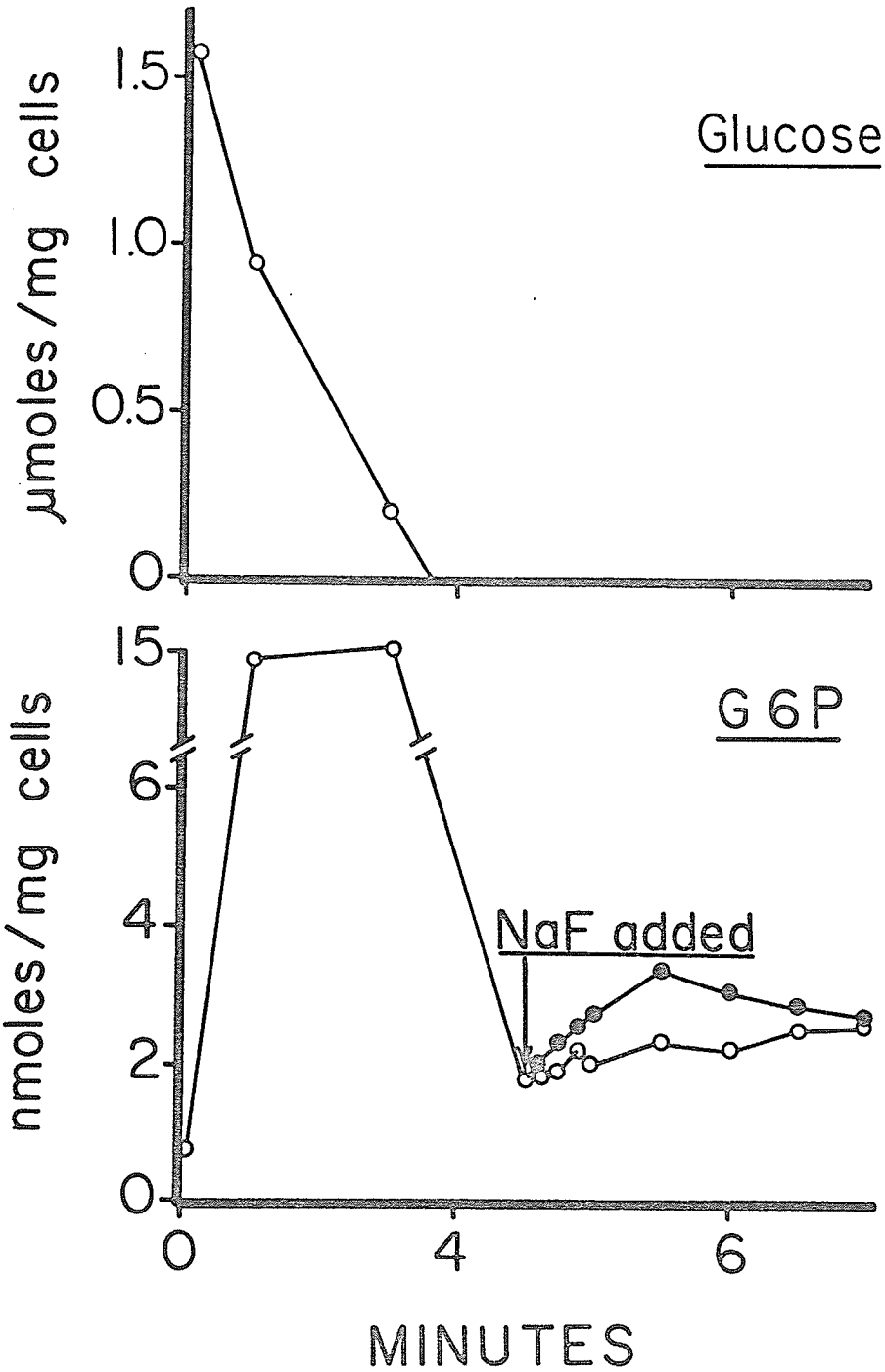
1. Effect of fluoride on glucose-6-P formation.

The fluoride inhibition of glucose-6-P formation in whole cells of *S. salivarius* confirms the indirect evidence obtained in previous studies which indicated that the site of inhibition was at some point in glycolysis prior to glucose-6-P formation. Although this fluoride-sensitive component is unknown, possible sites of inhibition would include the glucose phosphorylating enzyme, hexokinase, the reactions involved in the glucose transport process, or associated processes, such as the availability of ATP for phosphorylation.

In order to delineate the site of fluoride action in *S. salivarius*, hexokinase activity in crude extracts was assayed by the formation of glucose-6-P from glucose and ATP in the presence and absence of fluoride.

Fig. 10. The effect of 2.4 mM NaF on glucose-6-P levels in cells of S. salivarius metabolizing glycogen.

Control cells- ○ , fluoride-treated cells- ● .


Glucose
μmoles/mg cells
1.5
1.0
0.5
0
G 6P
nmoles/mg cells
15
6
4
2
0
NaF added
0
4
6
MINUTES

As no loss of activity was observed at NaF concentrations as high as 24 mM, one must conclude that hexokinase is not the fluoride-sensitive component.

The role of ATP is more difficult to assess. Although the cellular ATP content decreases rapidly upon fluoride addition (Fig. 7), it was not possible from these results to determine whether the decrease was the cause or the effect of the decrease in the cellular glucose-6-P concentration. One might assume that ATP would be required for the phosphorylation of glucose, either within the cell by the hexokinase reaction or at the membrane during transport (116, 117). However, the fact that glucose transport in many bacterial species is mediated by the P-enolpyruvate-phosphotransferase system (see review, 118) indicates that ATP may not be involved in active glucose transport. Indeed, the fact that sugars are concentrated in membrane vesicles as their phosphorylated derivatives by this transferase system (105) raises the question as to the physiological role of hexokinase in these cells. The existence of the P-enolpyruvate-phosphotransferase system in S. salivarius and the suggestion that the mechanism of fluoride action may involve this transport system will be discussed in the next chapter.

2. Fluoride inhibition of polyglucose synthesis.

Regardless of the actual site of fluoride action which leads to the observed decline in the cellular glucose-6-P content, the consequences of this decrease will be considerable. Obviously, the flux of carbon through the glycolytic pathway will be severely curtailed, making less glucose-6-P available for synthesis and catabolism. The rapid decline

of both the cellular ATP concentration and glycogen synthesis attest to this fact. The finding that the glycogen synthesizing enzymes are not fluoride-sensitive, however, raises the question as to how glycogen formation can be prevented by fluoride at concentrations permitting appreciable exogenous glucose uptake (59, 79).

Obviously, an inadequate supply of either glucose-6-P and/or ATP would limit glycogen synthesis. Under conditions of fluoride inhibition, where entry of glucose into the cell is restricted, one would expect that glucose-6-P would be required preferentially for energy production. Supporting this suggestion are the theoretical considerations proposed by Atkinson (119) who has suggested that one mode of control of biosynthetic reactions is through the "energy charge" of the adenylate system, defined as ATP + ½ADP / AMP + ADP + ATP (120). A high energy charge in a cell will allow biosynthetic reactions to proceed, while low energy charge would inhibit such reactions. Thus, bacterial glycogen will not be synthesized when the demand for ATP utilization is equivalent to ATP production (121). In this condition, the intracellular steady state concentration of ATP, and hence the energy charge, would be low. Therefore, the ATP required as a substrate for the ADP-glucose pyrophosphorylase reaction in the glycogen synthetic pathway, as well as the required glucose-1-P (from glucose-6-P), would not be available. Furthermore, when the cellular ATP concentration is low, the concentration of the total of ADP, AMP and P_i must necessarily be high. These substances are generally feedback inhibitors of bacterial ADP-glucose pyrophosphorylase (71), although enzymes from different species are inhibited by different members, or combinations of members, of this group. Thus, even though some

glucose-6-P may accumulate in NaF-treated cells of S. salivarius the low concentration of ATP existing in the cell under these conditions would make it unavailable for glycogen synthesis.

CHAPTER V

FLUORIDE INHIBITION OF ENOLASE ACTIVITY *IN VIVO* AND ITS RELATIONSHIP TO THE INHIBITION OF GLUCOSE-6-P FORMATION

A. INTRODUCTION

Many purified metalloenzymes are known to be inhibited by fluoride (16, 24). The most widely studied of these is enolase, which for many years has been associated with the inhibition of carbohydrate metabolism. Indeed, early studies with crude muscle extracts clearly demonstrated that enolase was the most fluoride-sensitive enzyme in the glycolytic scheme (21). Warburg and Christian (26) subsequently confirmed these results with purified muscle enolase and demonstrated the importance of phosphate and magnesium ions in the inhibiting mechanism. But, as is the case with many *in vitro* enzyme studies with inhibitors, the fluoride sensitivity of enolase has generally been extrapolated to explain the *in vivo* fluoride inhibition of glycolysis. Hewitt and Nicholas (24) have pointed out, however, that the validity of this assumption is often questionable since very little information on the effects of fluoride *in vivo* has been reported.

Earlier studies with intact cells of *S. salivarius* has suggested that an early event in glycolysis prior to glucose-6-P formation was the prime site of fluoride inhibition of carbohydrate metabolism (59, 79).

The experiments reported in Chapter 4 confirmed this suggestion by demonstrating that the addition of fluoride to cells actively metabolizing glucose resulted in a drastic decrease in the intracellular content of glucose-6-P and ATP. Although the reason for this decrease was unknown, evidence that hexokinase was fluoride-insensitive indicated that fluoride was inhibiting some component involved in the glucose transport system or an associated process, such as the supply of energy for transport.

In view of the apparent differences in the results obtained with S. salivarius and the earlier findings with enolase, experiments were designed to include a comparison of the in vivo fluoride effect on enolase activity and on glucose-6-P synthesis. Evidence will be presented demonstrating the concomitant fluoride inhibition of both enolase activity and glucose-6-P synthesis under a variety of experimental conditions. Further data will indicate that these two separate effects are connected through the action of the P-enolpyruvate phosphotransferase transport system in this organism.

B. RESULTS

1. Inhibition as a function of pH.

The specific action of an inhibitor on an enzyme in a functioning pathway should result in the accumulation of the substrate and a decrease in the concentration of the product of the inhibited reaction. Therefore, the inhibition of enolase by fluoride should result in the accumulation of 2-P-glycerate and a decrease in the cellular content of P-enolpyruvate. Early studies with the propionibacteria, in fact, demonstrated that the

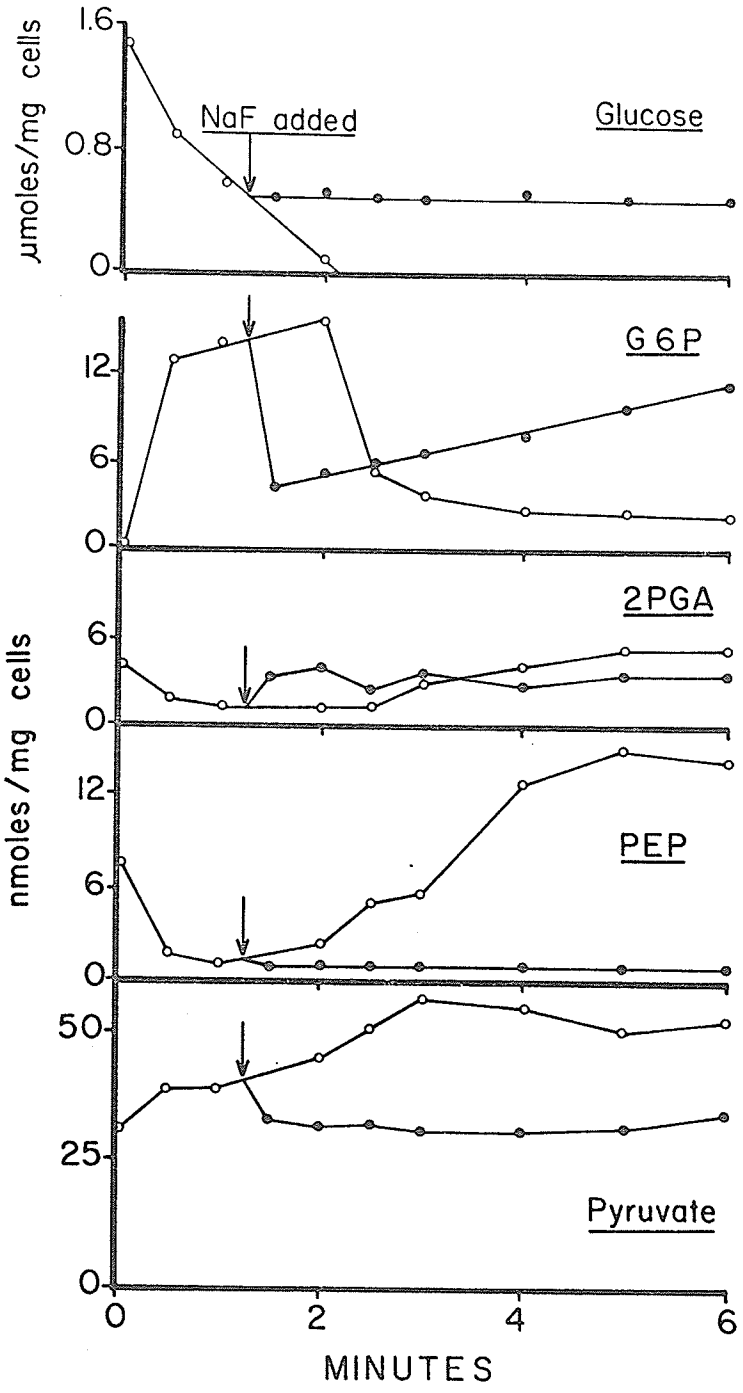
addition of fluoride to toluene-treated cells followed by overnight incubation, resulted in the accumulation of 2-P-glycerate in the medium (22, 23). With this in mind, experiments were undertaken to determine the effect of NaF on the activity of enolase by measuring the intracellular concentration of 2-P-glycerate and P-enolpyruvate (as well as pyruvate) in intact cells of S. salivarius metabolizing glucose in the presence and absence of the inhibitor. This effect, in turn, was compared to the effect of fluoride on exogenous glucose uptake and on glucose-6-P formation.

The addition of glucose to washed cells of S. salivarius, suspended anaerobically in phosphate buffer at a constant pH of 7.2, resulted in the immediate uptake of the substrate concomitant with an increase in the cellular content of glucose-6-P (Fig. 11). After the exogenous glucose had been depleted, the glucose-6-P level quickly decreased to a low level which, as will be seen later (Fig. 16), was maintained by the endogenous degradation of glycogen. As expected, the addition of 2.4 mM NaF to the cells 75 seconds after the onset of metabolism resulted in the rapid and complete inhibition of glucose uptake and an immediate decline in the cellular glucose-6-P content.

Further to the above effect was a small, but pronounced, increase in the cellular content of 2-P-glycerate and a decline in the level of P-enolpyruvate upon the addition of NaF indicating the in vivo inhibition of enolase. While 2-P-glycerate did not accumulate in large quantities in the presence of the inhibitor, P-enolpyruvate and pyruvate remained at significantly lower levels than those in the control cells. The P-enolpyruvate concentration in the control cells, on the other hand, although low in the presence of glucose, increased dramatically once the

Fig. 11. The effect of 2.4 mM NaF on exogenous glucose uptake and the intracellular content of glucose-6-P, 2-P-glycerate, P-enolpyruvate and pyruvate during anaerobic glucose metabolism at a constant pH of 7.2 by washed cells of S. salivarius.

Control cells- ○, fluoride-treated cells- ●.


μmoles/mg cells
1.6
0.8
0
NaF added
Glucose
G 6 P
12
6
0
2PGA
6
0
nmoles / mg cells
12
PEP
6
0
50
25
Pyruvate
0
0
2
4
6
MINUTES

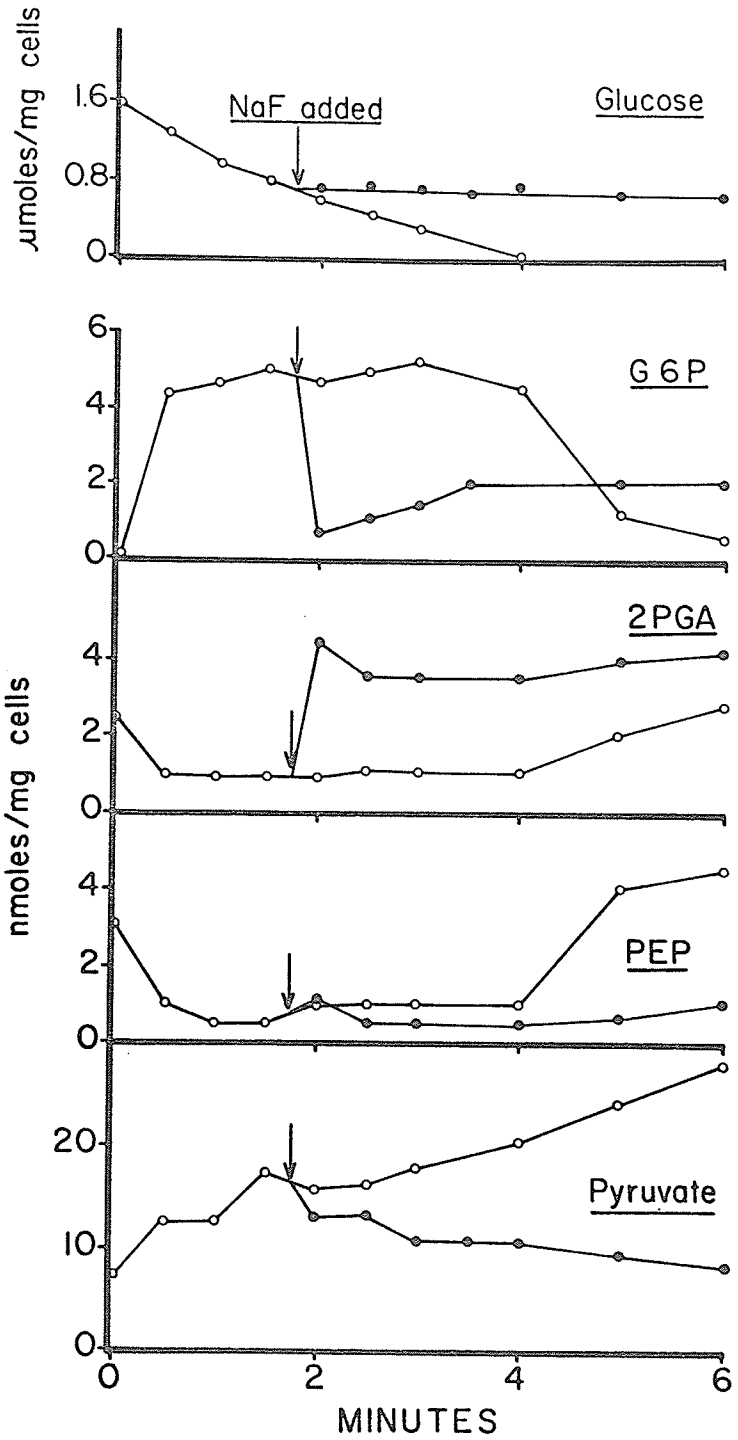
exogenous carbon source was depleted. These results, therefore, indicated two sites of fluoride inhibition: one at enolase and the other at a point prior to glucose-6-P formation.

As a consequence of these results, experiments were designed to determine whether the fluoride effects at these two sites were related to, or independent of each other. Unrelated sites of action would be indicated if, by changing the conditions of the experiment, one could selectively eliminate the inhibition at one site, while maintaining it at the other locus. To accomplish this, the pH of the medium and the concentration of the inhibitor were altered separately in subsequent experiments. pH was selected as a variable parameter since fluoride is known to have an increase inhibitory effect at low pH values (16).

The latter statement was substantiated when the experiment previously described was carried out at a constant pH of 5.8 instead of pH 7.2 (Fig. 12). At this suboptimal pH, the uptake of glucose and the glucose-6-P levels in both the control and fluoride-treated cells followed the previously established patterns, although the rate of glucose uptake by the control cells was depressed and, consequently, the cellular concentration of glucose-6-P at any one time was lower. Under these conditions, however, the 2-P-glycerate concentration increased dramatically upon the addition of fluoride to a much higher level than that observed at pH 7.2 and this level was maintained for the remainder of the experimental period. Both P-enolpyruvate and pyruvate followed somewhat the same pattern as seen at pH 7.2 except that the pyruvate concentration in the fluoride-treated cells decreased progressively with time.

Fig. 12. The effect of 2.4 mM NaF on exogenous glucose uptake and the intracellular content of glucose-6-P, 2-P-glycerate, P-enolpyruvate and pyruvate during anaerobic glucose metabolism at a constant pH of 5.8 by washed cells of S. salivarius.

Control cells- ○, fluoride-treated cells- ●.


μmoles/mg cells
1.6
0.8
0
NaF added
Glucose
6
4
2
0
G 6 P
2PGA
nmoles/mg cells
PEP
Pyruvate
20
10
0
0
2
4
6
MINUTES

A similar inhibitory pattern was observed for glucose-6-P and 2-P-glycerate following the addition of 2.4 mM NaF to cells metabolizing glucose at pH 8.0 (Fig. 13). Thus a variation in the pH of the external medium did not qualitatively alter the fluoride effect on glucose-6-P formation or on enolase activity. It was apparent, however, that the magnitude of the inhibition at enolase was greater at pH 5.8 than at the higher pH values.

2. Inhibition at low NaF concentrations.

In a further attempt to separate the fluoride effect at glucose-6-P formation from the observed inhibition at enolase, cells were incubated at varying NaF concentrations. The measured parameters remained the same, except that the cellular ATP content was also measured in order to determine the effect of low concentrations of NaF on the level of this energy source.

The addition of 0.36 mM NaF to cells anaerobically metabolizing glucose at pH 7.2 resulted in a strong but incomplete inhibition of glucose uptake (Fig. 14). Upon the addition of the inhibitor, the cellular glucose-6-P and ATP concentrations decreased while the level of 2-P-glycerate increased, again indicating enolase inhibition. The P-enolpyruvate concentration in the control cells was again high during endogenous metabolism and low in the presence of exogenous glucose, while the concentration in the treated cells remained low throughout the experiment. Fluoride had little effect on the pyruvate concentration.

Similar results were obtained with cells incubated at pH 7.2 with 0.12 mM NaF (Fig. 15), i.e., inhibition of glucose-6-P formation

Fig. 13. The effect of 2.4 mM NaF on exogenous glucose uptake and the intracellular content of glucose-6-P, 2-P-glycerate, P-enolpyruvate and pyruvate during anaerobic glucose metabolism at a constant pH of 8.0 by washed cells of S. salivarius.

Control cells- ○ , fluoride-treated cells- ● .

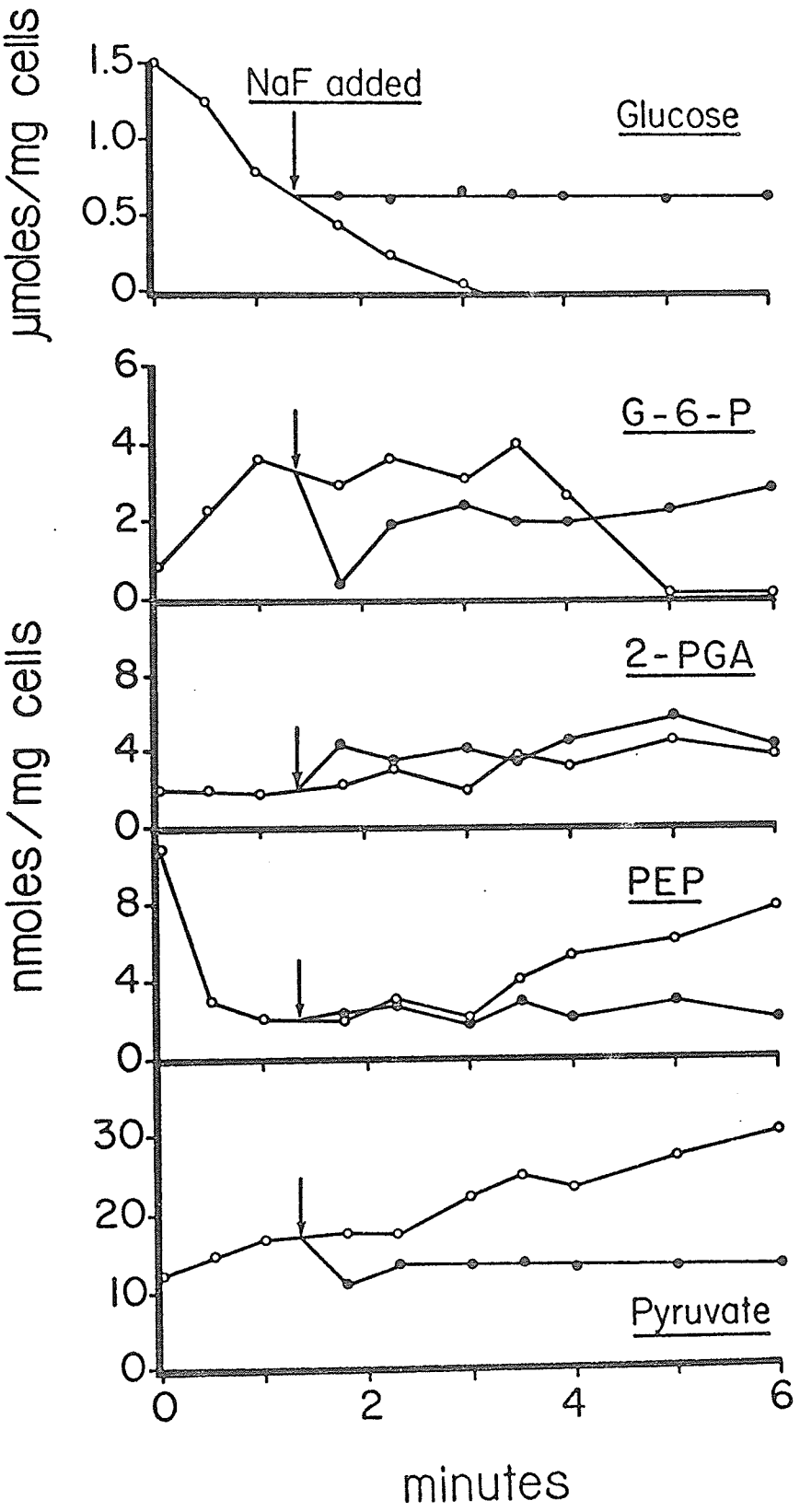

µmoles/mg cells
1.5
1.0
0.5
0
NaF added
Glucose
6
4
2
0
G-6-P
nmoles/mg cells
8
4
0
2-PGA
8
4
0
PEP
30
20
10
0
Pyruvate
0
2
4
6
minutes

Fig. 14. The effect of 0.36 mM NaF on exogenous glucose uptake and the intracellular content of glucose-6-P, 2-P-glycerate, P-enolpyruvate and pyruvate during anaerobic glucose metabolism at a constant pH of 7.2 by washed cells of S. salivarius.

Control cells- ○ , fluoride-treated cells- ● .

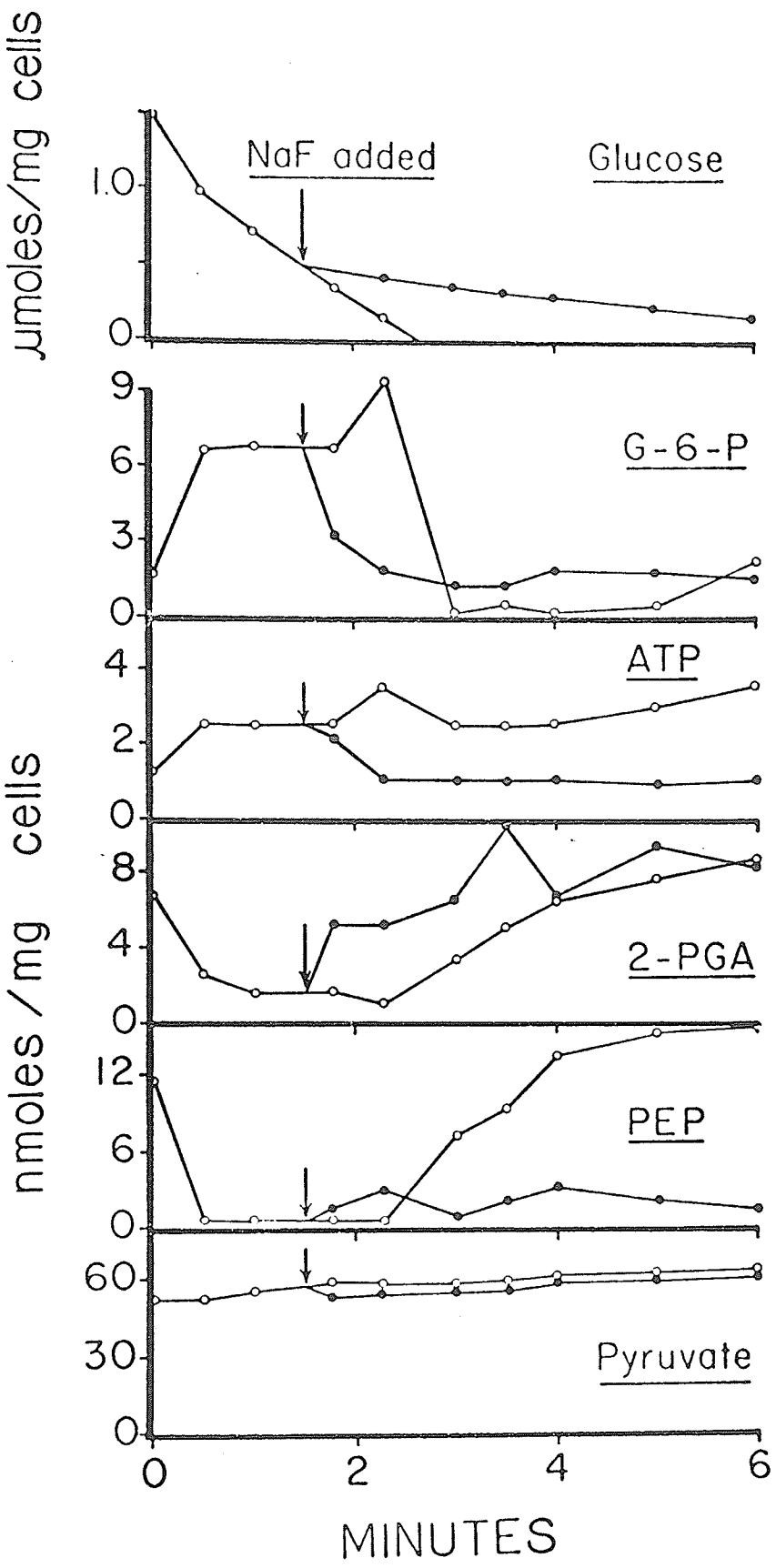
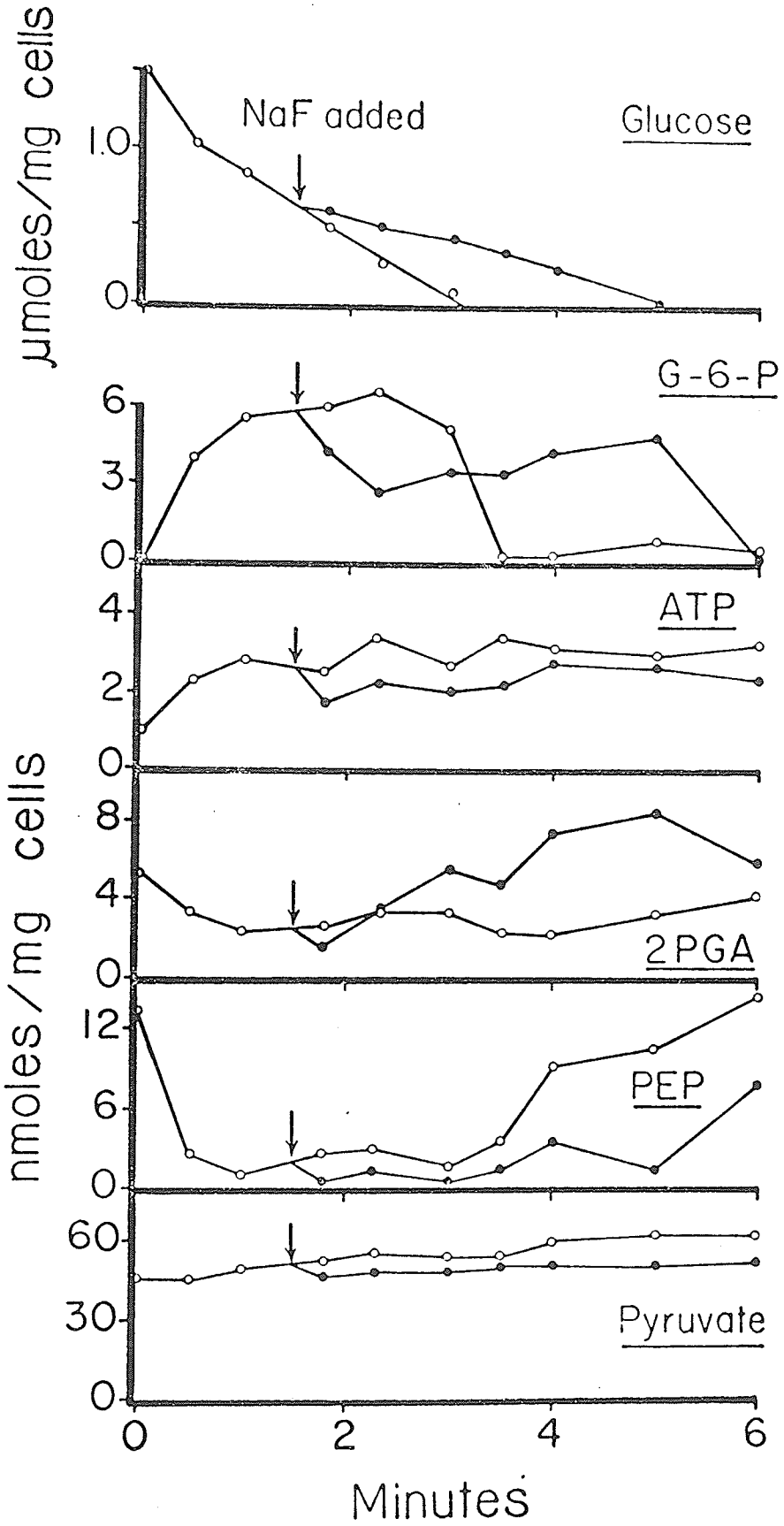

μmoles/mg cells
NaF added
Glucose
1.0
0
9
6
3
0
G-6-P
4
2
0
ATP
nmoles/mg cells
8
4
0
2-PGA
12
6
0
PEP
60
30
0
Pyruvate
0
2
4
6
MINUTES

Fig. 15. The effect of 0.12 mM NaF on exogenous glucose uptake and the intracellular content of glucose-6-P, ATP, 2-P-glycerate, P-enolpyruvate and pyruvate during anaerobic glucose metabolism at a constant pH 7.2 by washed cells of S. salivarius.

Control cells- ○ , fluoride-treated cells- ● .


µmoles/mg cells
NaF added
Glucose
1.0
0
G-6-P
6
3
0
ATP
4
2
0
nmoles/mg cells
8
4
0
2PGA
12
6
0
PEP
60
30
0
Pyruvate
0
2
4
6
Minutes

and enolase activity. As the inhibition of these two sites occurred under a variety of conditions, one must conclude that these two fluoride effects are related by a connecting mechanism.

3. P-enolpyruvate phosphotransferase activity.

The transport of glucose by some bacterial species is known to be mediated by the P-enolpyruvate phosphotransferase system (104, 106, 108). If P-enolpyruvate was involved in the glucose transport process in S. salivarius, such a system could be the logical mechanism connecting the inhibition of enolase with the reduction in glucose-6-P synthesis observed in the presence of NaF. Enolase inhibition would obviously reduce the P-enolpyruvate available for glucose phosphorylation during transport.

The presence of the phosphotransferase system in S. salivarius was tentatively suggested by the in vivo concentrations of P-enolpyruvate observed in Figures 11 to 15. The cellular level of this intermediate always decreased in the presence of glucose and always increased when this exogenous carbon source was depleted. This was confirmed by the direct assay of phosphotransferase activity in toluene-treated cells. In this experiment, 2-deoxyglucose-^{14}C was preferentially phosphorylated by P-enolpyruvate to 2-deoxyglucose-6-P-^{14}C (Table 8). While ATP would not substitute for P-enolpyruvate, 2-P-glycerate would phosphorylate the substrate almost to the same extent as P-enolpyruvate. This latter phosphorylation, however, was inhibited by 2.4 mM NaF, indicating that the 2-P-glycerate was initially converted to P-enolpyruvate by enolase before phosphorylation occurred. P-enolpyruvate phosphorylation of

TABLE 8

P-enolpyruvate phosphotransferase activity in toluene-treated cells of Streptococcus salivarius

Additions	Rate of phosphorylation
None	0.27[a]
ATP	0.25
P-enolpyruvate	4.10
2-P-glycerate	4.00
2-P-glycerate + 2.4 nM NaF	0.26

[a] nmoles 2-deoxyglucose-6-P-^{14}C formed from 2-deoxyglucose-U-^{14}C/mg dry wt cells/min.

glucose in the phosphotransferase system was itself not inhibited by NaF at concentrations as high as 4.8 mM (Table 9).

4. Inhibition of endogenous metabolism.

Glycogen degradation in S. salivarius is much less sensitive to fluoride than either glycogen synthesis or glucose uptake (48, 59). That the relative sensitivity of these latter processes is associated with inhibition of the transport process can be seen in the following experiment. Intact cells of S. salivarius were incubated at pH 7.2 with glucose-U-^{14}C to permit the synthesis of glycogen-^{14}C. When the exogenous glucose was exhausted, a zero time sample was withdrawn and 1.5 minutes thereafter a portion of the cells was added to 2.4 mM NaF. As shown in Fig. 16, the presence of the inhibitor resulted in a partial, but incomplete, inhibition of glycogen breakdown. Fluoride had little effect on the cellular concentrations of glucose-6-P and glucose-1-P despite the steady decrease in the ATP content of the cells. The lack of a pronounced fluoride effect on glucose-6-P synthesis during this endogenous metabolism indicates that the previously observed inhibition of glucose-6-P formation was associated solely with exogenous glucose metabolism.

Furthermore, the relative fluoride insensitivity of the endogenous pathway was not associated with a diminished NaF effect at enolase since the concentrations of 2-P-glycerate and P-enolpyruvate fluctuated upon the addition of fluoride in a manner which was characteristic of pronounced enolase inhibition. It is of interest that while the P-enolpyruvate concentration was at its characteristically low level in the

TABLE 9

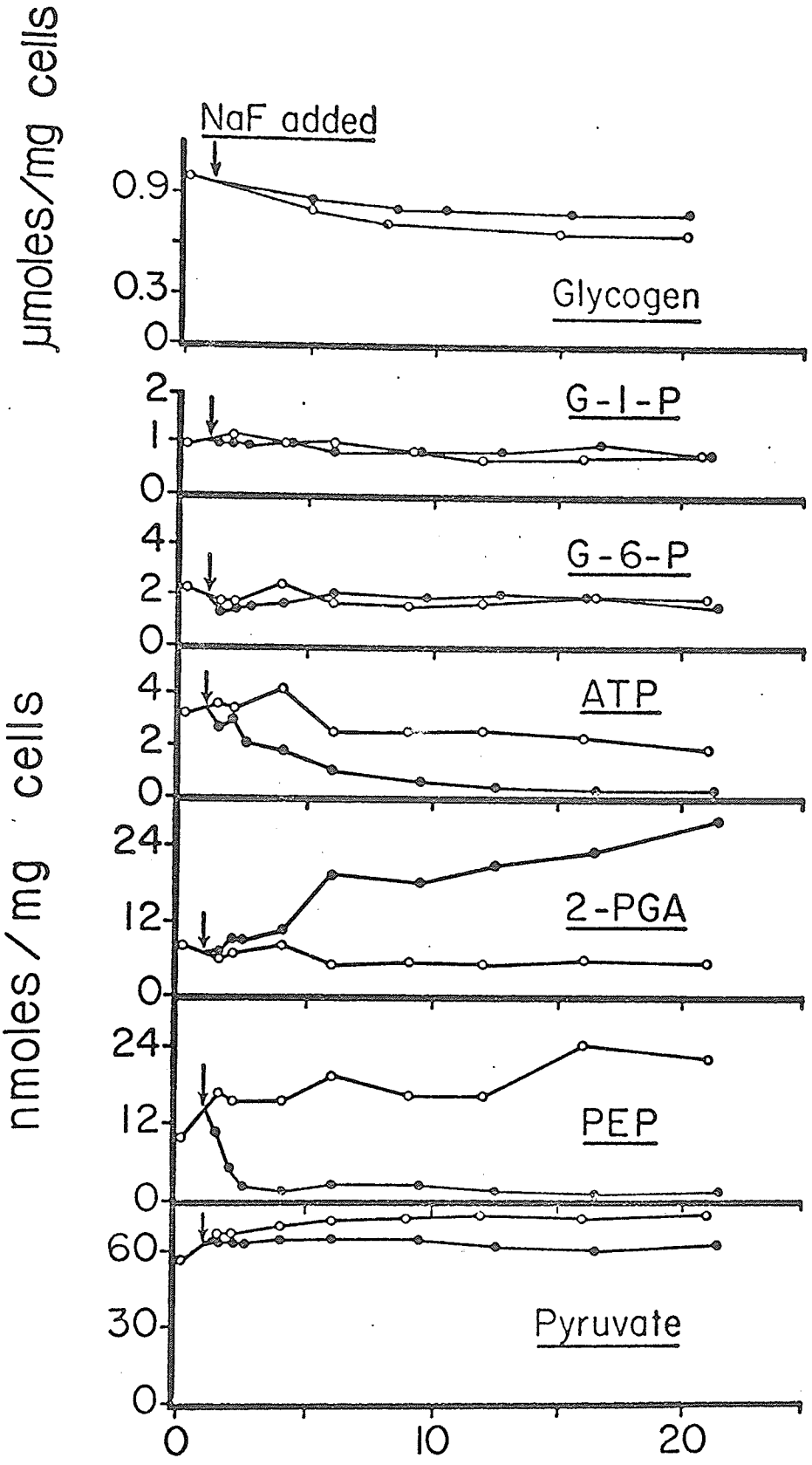
Effect of NaF on the P-enolpyruvate phosphotransferase activity in toluene-treated cells of S. salivarius

Additions	Rate of phosphorylation
None	6.38[a]
0.6 mM NaF	6.60
1.2 mM NaF	6.40
2.4 mM NaF	6.32
4.8 mM NaF	6.47

[a] nmoles 2-deoxyglucose-6-P-^{14}C formed from 2-deoxyglucose-U-^{14}C/mg dry wt cells/min. The assay system contained 2.0 mM P-enolpyruvate.

Fig. 16. The effect of 2.4 mM NaF on the intracellular content of glucose-6-P, glucose-1-P, ATP, 2-P-glycerate, P-enolpyruvate and pyruvate during anaerobic glycogen degradation by washed cells of S. salivarius incubated at a constant pH of 7.2.

Control cells- ○ , fluoride-treated cells- ◍ .


µmoles/mg cells
NaF added
0.9
0.3
0
Glycogen
2
1
0
G-1-P
4
2
0
G-6-P
4
2
0
ATP
nmoles/mg cells
24
12
0
2-PGA
24
12
0
PEP
60
30
0
Pyruvate
0
10
20

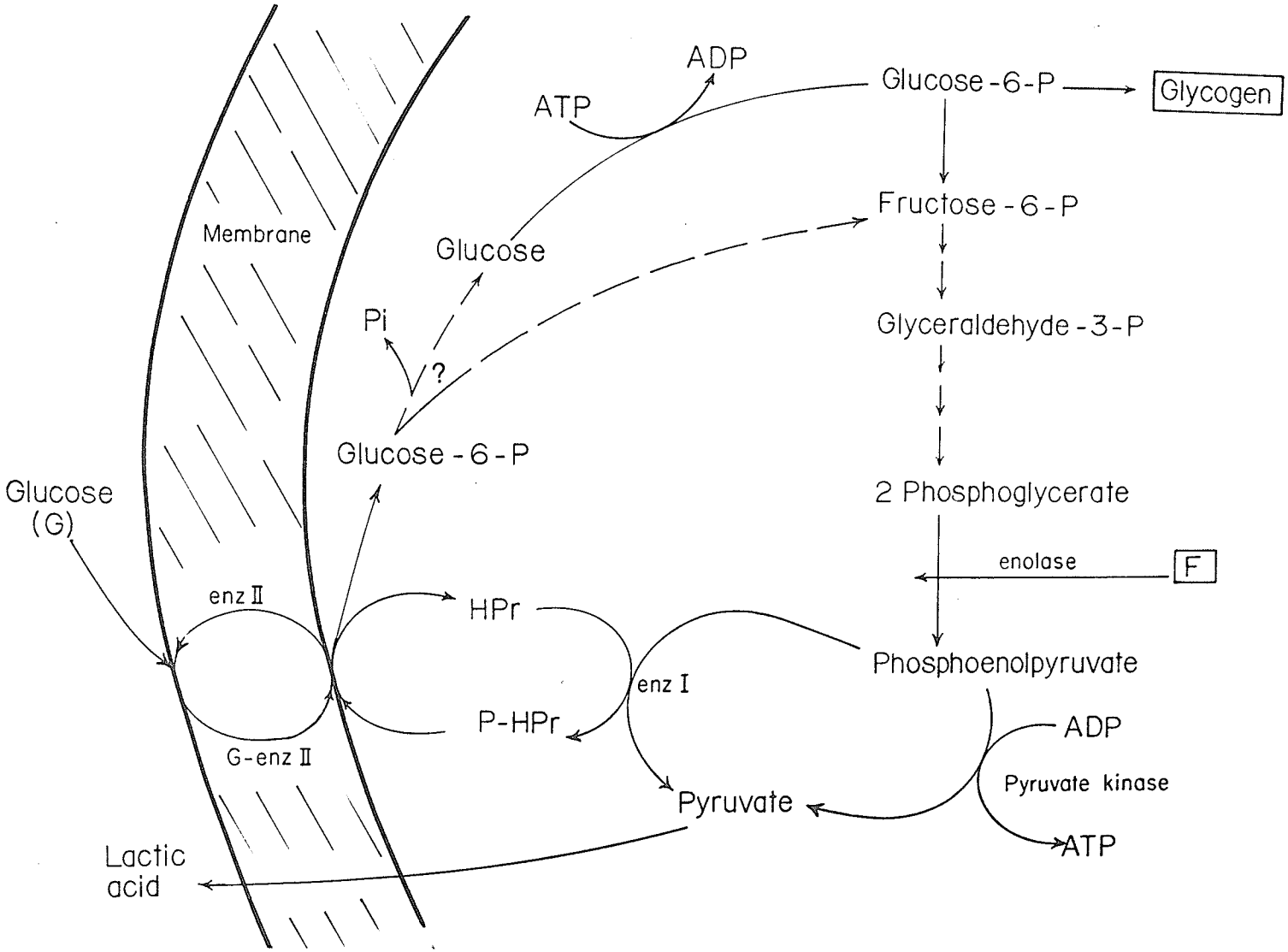
presence of the inhibitor, the cellular content of pyruvate was only slightly reduced by NaF.

C. DISCUSSION

Contrary to the long standing view that fluoride inhibits carbohydrate metabolism by its action on enolase, previous studies with intact cells of S. salivarius (48, 59) indicated that enolase inhibition was not the primary site of fluoride action. This suggestion arose from the fact that the addition of fluoride to cells actively metabolizing glucose resulted in the immediate inhibition of glucose uptake, while the percent conversion of glucose to lactic acid was not altered at fluoride concentrations permitting reduced glucose catabolism. Furthermore, exogenous glucose metabolism was much more sensitive to fluoride than was the endogenous degradation of glycogen, despite the fact that both types of metabolism employed the same pathway from glucose-6-P to lactic acid. This suggestion was fortified by the more recent demonstration that in vivo glucose-6-P synthesis was rapidly inhibited by NaF (Chapter 4).

The results of the present study, demonstrating the fluoride inhibition of in vivo enolase activity and its relationship to glucose-6-P formation via the P-enolpyruvate-phosphotransferase system, are consistent with the previous observations. Clearly, a reduction in enolase activity would result in less P-enolpyruvate being available for glucose transport, which in turn, would result in the decreased synthesis of glucose-6-P and subsequent intermediates, including 2-P-glycerate (Fig. 17). Thus, even

Fig. 17. Schematic representation of the pathways of glucose metabolism, and the site of fluoride action in intact cells of S. salivarius.


ADP
ATP
Glucose-6-P
Glycogen
Membrane
Fructose-6-P
Glucose
Pi
?
Glyceraldehyde-3-P
Glucose-6-P
2 Phosphoglycerate
Glucose
(G)
enolase
F
enz II
HPr
Phosphoenolpyruvate
enz I
P-HPr
ADP
G-enz II
Pyruvate kinase
Pyruvate
ATP
Lactic
acid

at fluoride concentrations low enough to permit reduced metabolism only a limited amount of 2-P-glycerate would accumulate in whole cells in the presence of exogenous glucose. In the case where glucose uptake was completely inhibited, as shown in Figures 11 - 13, 2-P-glycerate would accumulate as the result of endogenous metabolism (Fig. 16).

Since Romano and coworkers (108) have indicated that carbohydrate transport by the phosphotransferase system is a characteristic of facultative anaerobes and not of those organisms with oxidative physiology, one would expect that anaerobic metabolism by facultative anaerobes should be affected by fluoride in a manner similar to that observed with S. salivarius. This suggestion is supported by the report of McKay and coworkers (122), who demonstrated that 30 mM NaF completely inhibited lactose utilization and o-nitrophenyl-β-D-galactopyranoside hydrolysis by a "β-galactosidaseless" strain of S. lactis. Evidence was presented which indicated that the fluoride-sensitivity of this strain was associated with the availability of P-enolpyruvate for the phosphorylation of lactose during transport via the phosphotransferase system. On the other hand, another strain of the same species which contained β-galactosidase was not affected by fluoride since the phosphotransferase system was not involved in lactose transport. Similarly, the inhibition mechanism proposed for S. salivarius probably applies to the fluoride inhibition of glucose uptake observed with S. mitis (47) and with the microbes comprising salivary sediment (49), the majority of which are facultative anaerobes (123). This probably applies as well to earlier results with yeast (56), with E. coli (124) and with Propionibacterium pentosaceum (39), where anaerobic metabolism was shown to be much more

sensitive to fluoride than was respiration. In fact, the more recent results obtained by Carlson and Suttie (93, 125) with Hela cells might be interpretated in the same way, since fluoride decreased the rate of glucose utilization without altering the percent conversion of glucose to lactic acid.

The reduction in the amount of the P-enolpyruvate available for transport brought on by the fluoride inhibition of enolase activity in intact cells of S. salivarius may explain, in part, why exogenous catabolism is more sensitive to this inhibitor than is the endogenous degradation of glycogen. However, other factors, such as the difference in rates of enolase action during these two types of metabolism, or the interaction of fluoride at a second site on the cellular membrane, may also contribute to this sensitivity difference. With regard to the first possibility, it is clear that enolase in S. salivarius operates at a much slower rate during endogenous metabolism than during the exogenous fermentation of glucose (59). Earlier studies by Slater and Bonner (126), demonstrating the competitive inhibition of heart muscle succinic dehydrogenase by fluoride in the presence of phosphate, may be pertinent to the enolase situation in this organism. With succinic dehydrogenase, the degree of fluoride inhibition was shown to increase with increasing activity of the fluoride-free enzyme in the controls, thereby explaining why the enzyme, when assayed alone, was less sensitive to fluoride than was the same enzyme operating at a faster rate in the complete succinic oxidase system.

The possibility of fluoride interacting with the cell membrane has been suggested by earlier studies with a variety of systems, as well

as by recent evidence obtained with S. salivarius. With the latter organism, the fact that the fluoride effect on enolase and glucose-6-P formation was observed to occur very rapidly (within 15 sec) upon the addition of the inhibitor to the cells suggests that extensive penetration of the cell membrane fluoride may not be necessary to affect its action. This is particularly true for the experiments carried out at or near neutrality (i.e., pH 7.2 - 8.0), since fluoride apparently penetrates the cells as HF (51, 53) which in this pH range represents only about 0.01% of the total available fluoride (16). That little fluoride penetrates into cells of S. salivarius was substantiated by the fact that no measurable amount of fluoride was lost from the incubation medium, as measured by fluoride ion electrode analysis, even though glucose metabolism by these cells was inhibited (Hamilton, unpublished results). However, although no fluoride uptake into cells could be observed during the first 6 - 10 minutes of incubation with 0.44 mM NaF (at pH values between 7.5 and 5.0), considerable uptake was observed in 24 and 93 hours (21 and 39%, respectively).

While it is conceivable that fluoride interacts with the cellular membrane, it is also possible that enolase may be located on the membrane to facilitate the formation of P-enolpyruvate for the phosphotransferase system. The association of enolase activity with the membrane of E. coli was suggested by Kaback (105) following transport studies with isolated membrane preparations. α-methylglucoside uptake by these preparations was shown to be stimulated by 2-P-glycerate through its conversion of P-enolpyruvate since transport in the presence of 2-P-glycerate was selectively inhibited by 10 mM NaF.

The influence of the cellular membrane on the extent of fluoride inhibition in various microbial species has been known for some time. Early studies with yeast by Runnstrom and coworkers (50, 56, 127) demonstrated that cells with membranes altered by drying were more sensitive to NaF than were cells with intact membranes. Furthermore, Malm (128) demonstrated that increasing the permeability of yeast cells by starvation resulted in increased fluoride-sensitivity. Similarly, the early experiments of Wood, Stone and Werkman (23) with propionibacteria demonstrated that NaF was much more effective in causing the accumulation of 2-P-glycerate (i.e., enolase inhibition) if toluene was added to destroy the integrity of the cell membrane. Also of interest in this respect is evidence that fluoride-resistant bacteria are less permeable to fluoride (90, 91).

The more recent work by Kaback et al (see review, 118) with membrane preparations of E. coli illustrates most clearly the mechanism of fluoride action on microbial membranes. Evidence was presented for the transport of sugars into cells through a phosphorylation-mediated translocation of the cell membrane. This "vectorial phosphorylation", catalyzed by the P-enolpyruvate phosphotransferase system, was shown to be altered by NaF such that the formation of sugar-P occurred on the external rather than the internal surface of the membrane. Since the inhibitor had little or no effect on the actual phosphorylation of the sugar molecule, it was suggested that NaF altered the process of sugar translocation across the membrane.

Thus, it is conceivable that fluoride may have two sites of action on intact cells of S. salivarius, one at enolase to inhibit the

supply of P-enolpyruvate for phosphorylation and another at a second location in or on the membrane to alter the translocation of sugars into the cell. This would not only account for the increased sensitivity of exogenous glucose metabolism, but would also account for the rapid rate of fluoride action on cells incubated at neutral pH values.

CHAPTER 6

THE EFFECT OF FLUORIDE ON GLYCOLYSIS BY FLUORIDE-RESISTANT STRAINS OF STREPTOCOCCUS SALIVARIUS

A. INTRODUCTION

Although the action of fluoride in reducing the incidence of dental caries is well-known, the mechanism by which this is achieved has yet to be elucidated (36). One way by which fluoride appears to exert an effect is through the conversion of the enamel hydroxyapatite into the more acid-resistant fluorapatite, thereby producing a physical barrier to caries formation (see review, 129). However, since dental plaque contains a relatively high (mean, 3.5 mM) concentration of fluoride (68), most of which is apparently bound to bacterial cells (69), it is possible that the direct action of fluoride on acid production by oral bacteria also plays a role in caries reduction (42, 69, 70, 130).

In this connection, it is of interest to find that many micro-organisms, including oral lactobacilli(76, 77, 78, 79), have been shown to acquire resistance to fluoride after growth in the presence of this inhibitor. These strains, variously described as fluoride-adapted, fluoride-resistant or fluoride-trained are characterized by their ability to grow at fluoride concentrations which inhibit the growth and metabolism of the fluoride-sensitive parental strains. During studies on the

effect of fluoride on glycolysis by S. salivarius, Hamilton (79) isolated fluoride-resistant strains by step-wise adaptation to high fluoride levels and by selection of colonies from UV-irradiated spread-plates containing NaF (see Chapter 3). Both the step-wise adapted (A-100) and the UV-selected (UV-100) strains grew readily in the presence of 2.4 mM NaF, but they differed in their growth and metabolic characteristics. In the presence of 2.4 mM NaF, UV-100 cells always had shorter lag times, higher growth rates and higher cell yields than did A-100 cells. Furthermore, the percent conversion of glucose to lactate by the UV-100 cells (87%) was similar to that of wild-type cells (90%) and higher than that of A-100 cells (74%). In spite of these differences, however, the conversion to fluoride-resistance was genetic in nature, since both types of cells maintained their resistant characteristics even after passage through fluoride-free media for 300 generations.

Although many studies have described the isolation and growth characteristics of fluoride-resistant bacteria, the mechanism by which bacteria acquire this resistance is poorly understood. In view of this, the studies reported in Chapters 4 and 5 were expanded in order to determine the in vivo effect of fluoride on glucose metabolism by fluoride-resistant strains of S. salivarius.

As was done in the preceding chapters, the fluoride-sensitivity of glucose transport was monitored by measuring the rates of glucose uptake and glucose-6-P formation in whole cells metabolizing glucose in the presence and absence of NaF. The effect of fluoride on cellular enolase activity was determined by measuring the concentrations of 2-P-glycerate, P-enolpyruvate and pyruvate in the same cells. Energy

production during glycolysis was monitored by determining the cellular ATP content.

B. RESULTS

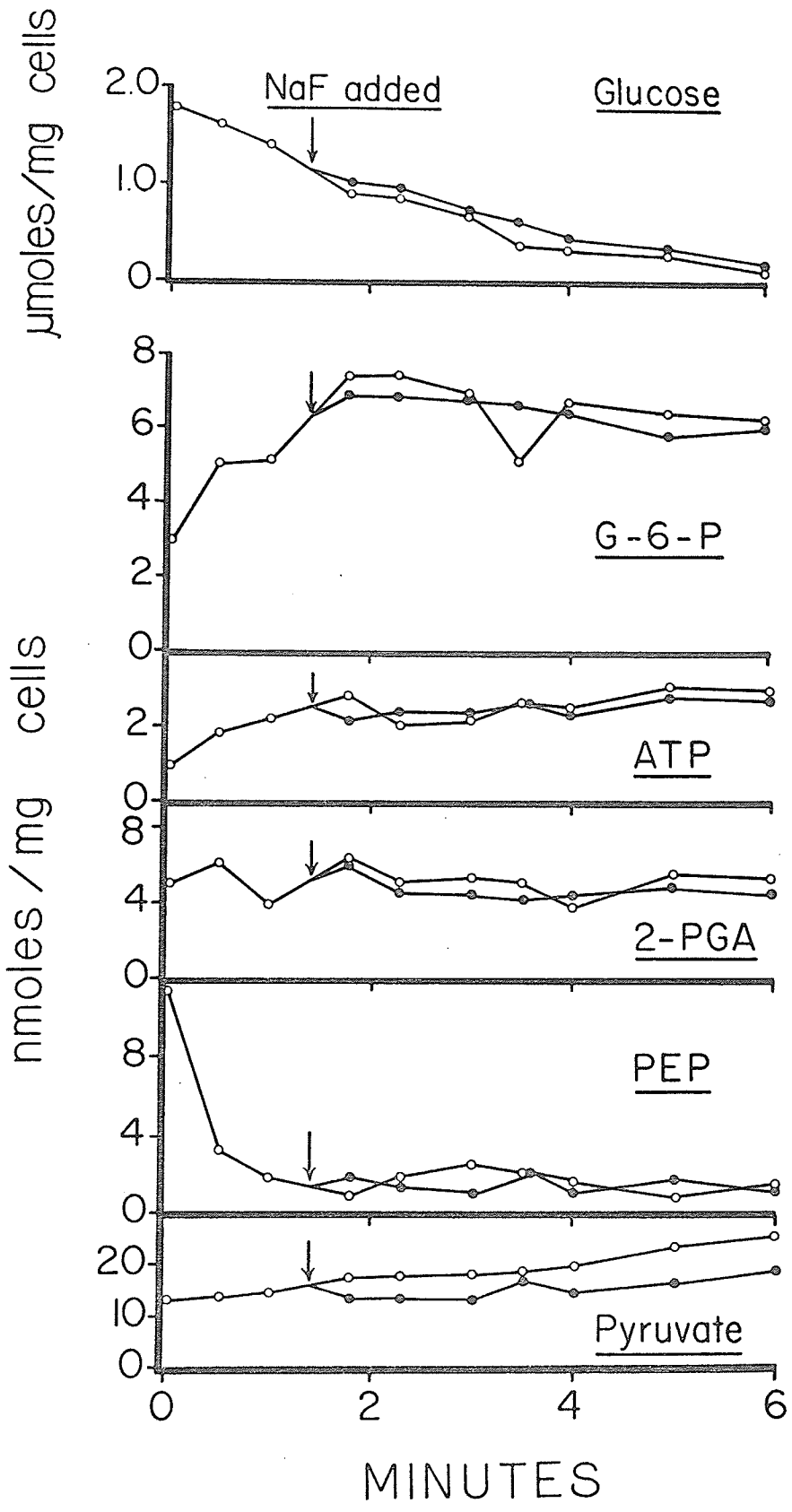
1. Effect of fluoride at neutral pH.

Both A-100 and UV-100 cells are able to metabolize glucose anaerobically at neutral pH in the presence of fluoride concentrations (2.4 mM NaF) which completely inhibit the uptake of this substrate by parental cells (48). Hence, it is likely that the glycolytic intermediate concentrations in resistant cells metabolizing glucose under these conditions would be unaffected by fluoride.

In order to test this possibility, A-100 cells were grown in media containing 4.8 mM NaF so that a high degree of tolerance to the inhibitor would be exhibited. The addition of glucose (1.8 μmoles/mg cells) to washed A-100 cells suspended anaerobically in phosphate buffer at a constant pH of 7.2 resulted in the immediate uptake of this substrate and an increase in the cellular concentration of glucose-6-P and ATP (Fig. 18). However, it can be seen from a comparison of Figures 18 and 11 that the rate of glucose uptake into A-100 cells in the absence of fluoride (0.28 μmoles/mg cells/min) was much lower than in parental cells incubated under the same conditions (0.50 μmoles/mg cells/min). The addition of fluoride to the cells resulted in a slight decrease (14%) in the rate of glucose utilization. Except for one point, the glucose-6-P content in fluoride-treated cells was slightly lower than in the controls, while there were no significant difference in ATP content.

Fig. 18. The effect of 2.4 mM NaF on exogenous glucose uptake and the intracellular content of glucose-6-P, ATP, 2-P-glycerate, P-enolpyruvate and pyruvate during anaerobic glucose metabolism at a constant pH of 7.2 by washed cells of S. salivarius A-100.

Control cells- ○ , fluoride-treated cells- ● .


μmoles/mg cells
2.0
1.0
0
NaF added
Glucose
8
6
4
2
0
G-6-P
nmoles / mg cells
2
0
ATP
8
4
0
2-PGA
8
4
0
PEP
20
10
0
Pyruvate
0
2
4
6
MINUTES

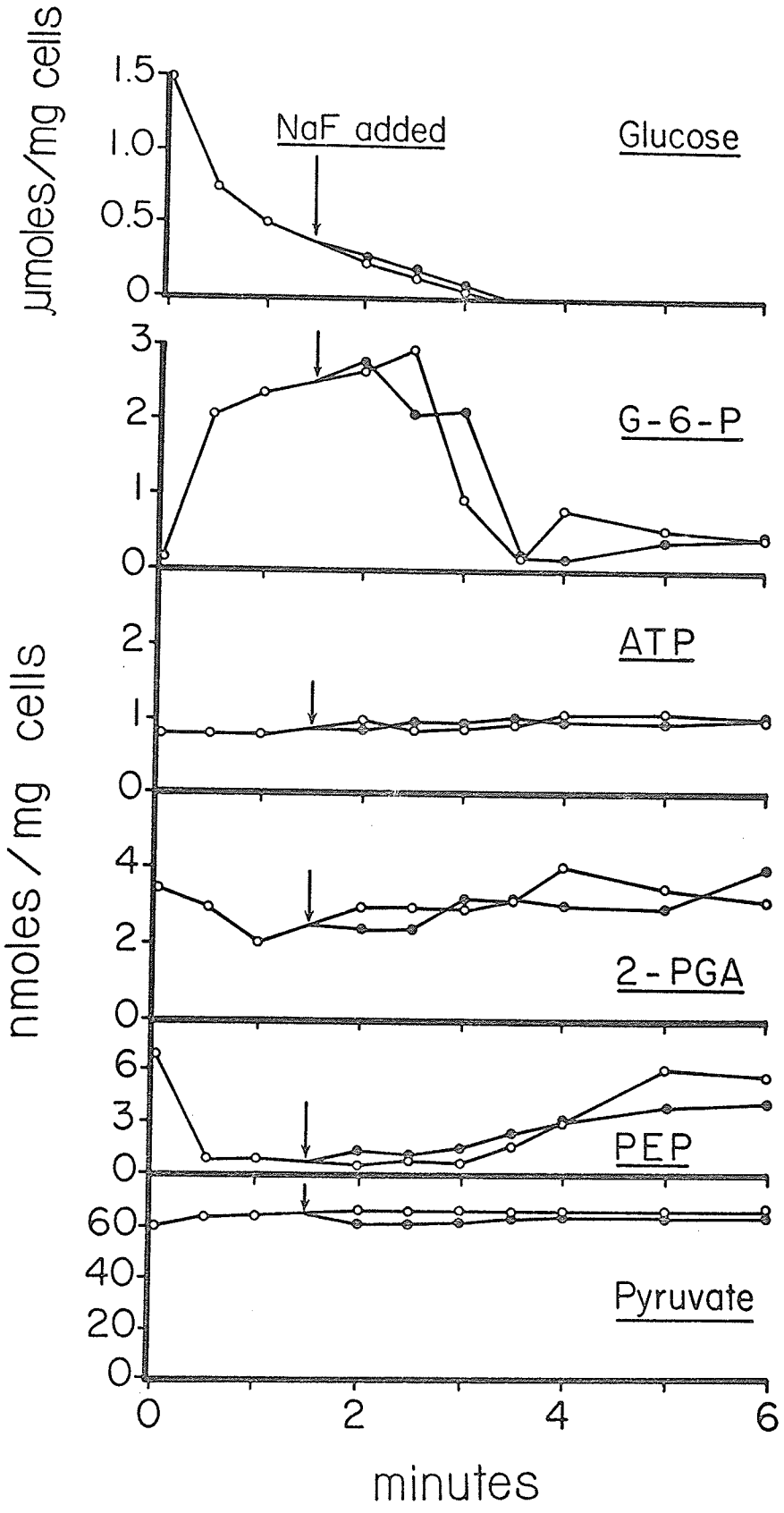
A comparison of the 2-P-glycerate and P-enolpyruvate concentrations in fluoride-treated and untreated cells revealed no significant differences. However, the cellular pyruvate concentration decreased slightly upon fluoride addition.

Similar results were obtained when glucose (1.5 μmoles/mg cells) was added to washed, fluoride-grown UV-100 cells suspended anaerobically in phosphate buffer at a constant pH 7.2 (Fig. 19). In contrast to the slow rate of glucose uptake by A-100 cells, UV-100 cells metabolized this substrate at a rate (0.45 μmoles/mg cells/min) almost equal to that of wild-type cells (Fig. 11). In contrast to the results obtained with wild-type and A-100 cells, however, the addition of the substrate did not result in an increase in the cellular ATP content although the glucose-6-P content increased in the usual fashion. The addition of 2.4 mM NaF after 1.5 minutes of metabolism resulted in a slight inhibition of glucose uptake. Fluoride had no immediate effect on the cellular glucose-6-P content, which decreased rapidly in both the control and fluoride-treated cells at the time when the exogenous glucose was depleted. As was the case with A-100 cells, the presence or absence of fluoride had no significant effect on the ATP content of UV-100 cells. The 2-P-glycerate concentration in the fluoride-treated cells was generally lower than in the control cells, while the P-enolpyruvate level under both conditions was highest when exogenous glucose was absent from the medium.

These results demonstrate that fluoride had, at most, only a slight inhibitory effect on glucose metabolism by the fluoride-resistant strains of S. salivarius at neutral pH. There was no sign of a significant inhibitory effect on cellular enolase activity.

Fig. 19. The effect of 2.4 mM NaF on exogenous glucose uptake and the intracellular content of glucose-6-P, ATP, 2-P-glycerate, P-enolpyruvate and pyruvate during anaerobic glucose metabolism at a constant pH of 7.2 by washed cells of S. salivarius UV-100.

Control cells- ○ , fluoride-treated cells- ● .


μmoles/mg cells
1.5
1.0
0.5
0
NaF added
Glucose
3
2
1
0
G-6-P
nmoles / mg cells
2
1
0
ATP
4
2
0
2-PGA
6
3
0
PEP
60
40
20
0
Pyruvate
0
2
4
6
minutes

2. Effect of fluoride at acid pH.

As mentioned previously, the inhibitory effect of fluoride on glucose metabolism by wild-type cells of S. salivarius was shown to increase as the pH decreased (48). Furthermore, the results presented in Chapter 5 demonstrated that fluoride caused an increased inhibitory effect on enolase in whole cells incubated at pH 5.8. Since the effect of pH on metabolism by fluoride-resistant bacteria has not been well-studied, the experiments reported in the last section were repeated at pH 5.8.

The addition of 2.4 mM NaF to A-100 cells metabolizing glucose anaerobically at a constant pH of 5.8 resulted in the complete inhibition of glucose uptake and a decrease in the cellular content of glucose-6-P, ATP and pyruvate (Fig. 20). The 2-P-glycerate content of the fluoride-treated cells rose to levels significantly higher than those in the untreated control cells demonstrating that enolase was inhibited. As usual, the P-enolpyruvate content in the control cells was higher during exogenous glucose metabolism while the P-enolpyruvate concentration in the fluoride-treated cells remained at a low level.

Substantially, the same fluoride effect was observed with UV-100 cells incubated at pH 5.8 under the previously described conditions (Fig. 21). The addition of the inhibitor resulted in the complete inhibition of glucose uptake and a decrease in the cellular content of glucose-6-P, ATP and pyruvate. The concentration of 2-P-glycerate in fluoride-treated cells immediately increased and was maintained at concentrations higher than those of the untreated cells again demonstrating the inhibition of enolase activity.

Fig. 20. The effect of 2.4 mM NaF on exogenous glucose uptake and the intracellular content of glucose-6-P, ATP, 2-P-glycerate, P-enolpyruvate and pyruvate during anaerobic glucose metabolism at a constant pH of 5.8 by washed cells of S. salivarius A-100.

Control cells- ○ , fluoride-treated cells- ● .

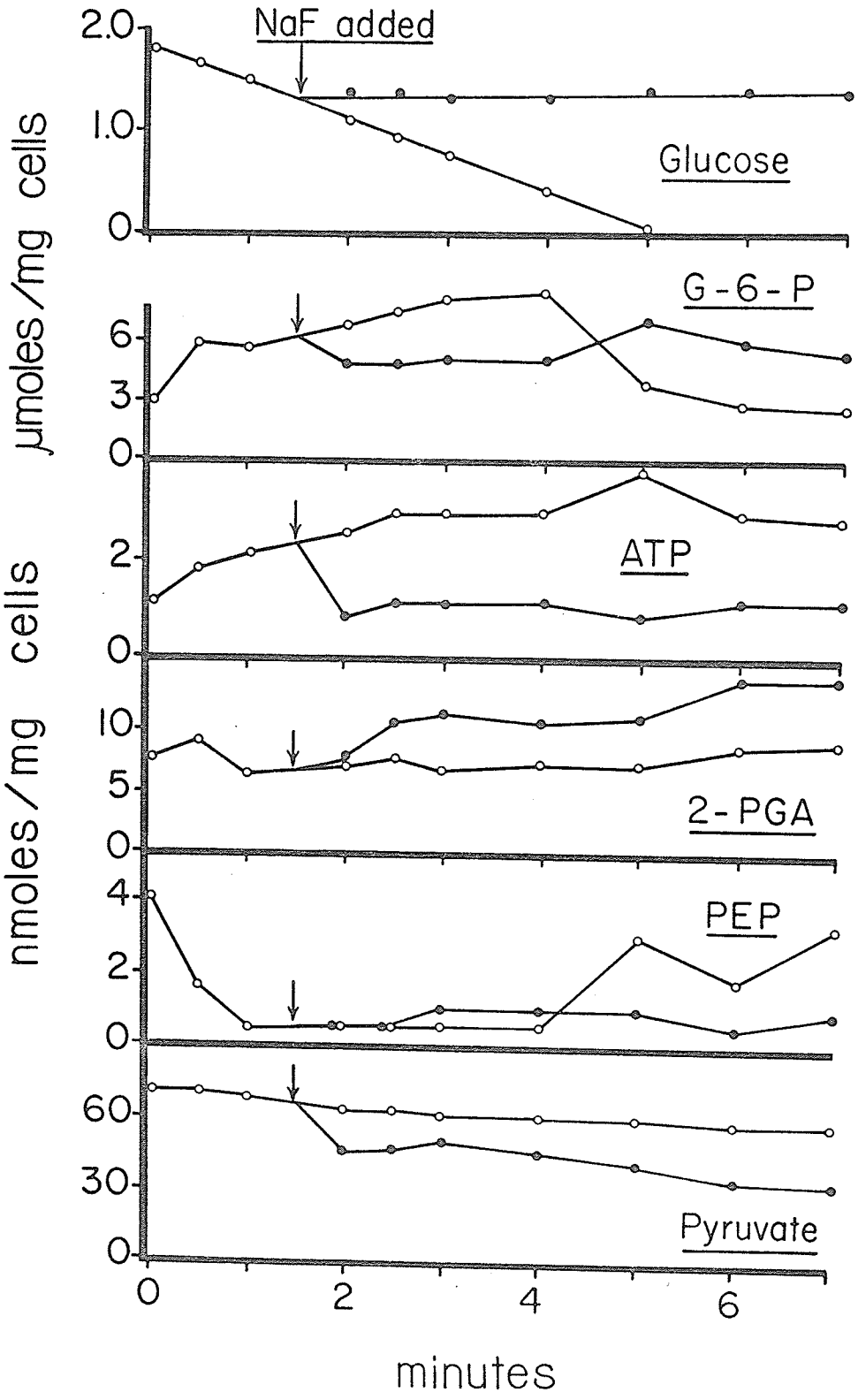
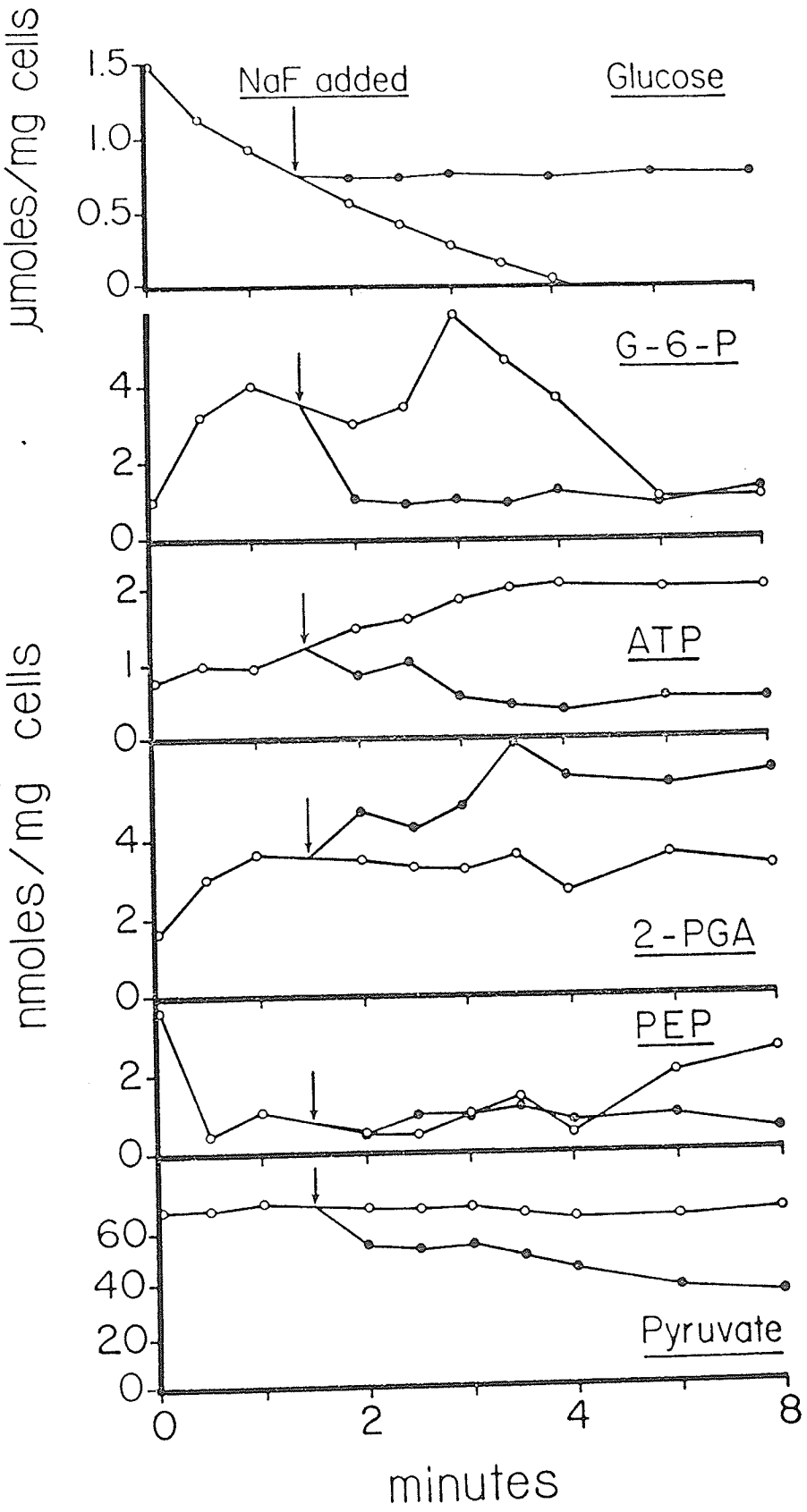

NaF added
Glucose
G-6-P
ATP
2-PGA
PEP
Pyruvate
μmoles/mg cells
nmoles/mg cells
minutes
2.0
1.0
0
6
3
2
10
5
4
60
30
0
2
4
6

Fig. 21. The effect of 2.4 mM NaF on exogenous glucose uptake and the intracellular content of glucose-6-P, ATP, 2-P-glycerate, P-enolpyruvate and pyruvate during anaerobic glucose metabolism at a constant pH of 5.8 by washed cells of S. Salivarius UV-100.

Control cells- ○ , fluoride-treated cells- ● .


μmoles/mg cells
nmoles/mg cells
NaF added
Glucose
G-6-P
ATP
2-PGA
PEP
Pyruvate
minutes
1.5
1.0
0.5
0
4
2
0
2
1
0
4
2
0
2
0
60
40
20
0
0
2
4
8

From these results, it is apparent that the property of fluoride-resistance exhibited by A-100 and UV-100 cells incubated at neutral pH is lost when these cells are incubated under slightly acidic conditions.

C. DISCUSSION

1. Mechanism of resistance.

Although fluoride may inhibit microbial metabolism at more than one site (see discussion, Chapter 5), the characterization of enolase as a major site of fluoride action in wild-type cells of S. salivarius indicates that in fluoride-resistant strains enolase must be either by-passed, fluoride-resistant or otherwise protected from the action of the inhibitor.

The enolase reaction could theoretically be by-passed through the development of a divergent glycolytic pathway, one which did not include this reaction. Although this type of mechanism was proposed to explain fluoride-resistance in Propionibacterium pentosaceum (89), later studies with the same organism demonstrated that the resistant strains were simply impermeable to fluoride and did not possess a divergent glycolytic pathway (90). The experiments reported in this chapter (Figs. 18 - 21) demonstrate that both the substrate and the product of the enolase reaction, 2-P-glycerate and P-enolpyruvate, respectively, were present in the cells in all cases. Hence, the enolase reaction is probably not by-passed through a divergent catabolic pathway.

A related mechanism would have the fluoride-resistant cells possessing an altered, fluoride-resistant enolase. The measurement of

glycolytic intermediates in control and "fluoride-trained" cells of the oral streptococcus HT25 by Williams (92) demonstrated significant differences between the two types of cells in the ratios of 3-P-glycerate and 2-P-glycerate to P-enolpyruvate. In all cases, the ratios P-enolpyruvate/2-P-glycerate and P-enolpyruvate/3-P-glycerate were higher in control cells than in the "fluoride-trained" cells, thereby indicating a reduced apparent equilibrium constant for the enolase reaction in the fluoride-trained cells. Williams suggested that this could be brought about by the formation of a low activity, fluoride-resistant enolase in the trained cells. From an examination of the pattern of glucose uptake and P-enolpyruvate production in resistant strains of S. salivarius (Figs. 20 - 21) it is evident that enolase is active when resistant cells were incubated without fluoride at pH 5.8. However, addition of fluoride to these same cells resulted in the pronounced inhibition of intracellular enolase activity. Thus, the enzyme molecule itself does not appear to be fluoride-resistant. However, the possibility that a form of enolase might exist which is fluoride-resistant at neutral pH but sensitive at acid pH cannot be discounted.

The results observed are, however, consistent with the hypothesis that intracellular enolase is somehow protected against the action of the inhibitor at neutral pH. One means by which this could be accomplished would be through the development of a permeability barrier to the inhibitor. Evidence obtained in studies with other fluoride-resistant bacteria supports such a mechanism. Destruction of the permeability barrier of fluoride-resistant P. pentosaceum cells by lyophilization resulted in the loss of resistance (90), while fluoride-trained cells

of streptococcus HT25 were less permeable to fluoride than were cells of the unadapted control culture (91).

Although the means by which fluoride-resistant cells could become impermeable to the inhibitor remain unknown, it is well-known that fluoride can react with many proteins (16). As mentioned previously, Kaback (118) has shown that fluoride can react with isolated membrane preparations of E. coli. If A-100 and UV-100 cell membranes were altered so that they could react with fluoride, the inhibitor would not reach enolase within the cell. The results of the present study indicate that this reaction would either be pH-dependent or would be reversed at acid pH. Alternatively, the membrane might react with hydrofluoric acid (HF), long thought to be the functional form of the inhibitor (51, 53) since the concentration of the HF form increases as the pH decreases (16). By this mechanism, the increased amount of HF present at low pH might simply overpower the resistance characteristic.

Removal of fluoride from inside the cell could also protect cellular enolase from the action of the inhibitor. Quissell and Suttie (95) have recently provided strong evidence for the existence of a fluoride-removing "pump" mechanism in fluoride-resistant mouse fibroblast L cells. As noted previously (Chapter 2), fluoride entered the cells at 0 C but at 37 C these same resistant cells were able to remove the accumulated intracellular fluoride against a concentration gradient. Experiments designed to test for the presence of a fluoride-removing pump in S. salivarius or in other bacteria have yet to be performed. The results reported in this chapter, however, reveal that if such a system were present it would function at pH 7.2 but not as pH 5.8.

2. Implications to oral microbiol ecology.

Edgar, Jenkins and Tatevossian (70) have demonstrated that dental plaque taken from subjects living in a "low-fluoride town" in England reached a lower pH (4.94) following incubation with sucrose than did similarly-treated plaque samples taken from subjects living in a town with a fluoridated water supply (mean final pH = 5.07). As a consequence of these results, these workers postulated that even if the plaque bacteria had acquired fluoride-resistance, the existence of the pH effect would negate the resistant characteristic and acid production by the plaque bacteria would still be inhibited by fluoride.

The results of the present study support this idea, since glycolysis by fluoride-resistant cells of S. salivarius was completely inhibited by fluoride at pH 5.8. However, during those times when the plaque pH is at or near neutrality, as is the case in fasting plaque (see review, 131), the property of the fluoride-resistance would allow bacterial glycolysis to proceed uninhibited.

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