Evidence on the constitutive nature of a *p*-nitrophenylphosphatase of *Streptococcus mutans*

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Cells of the cariogenic *Streptococcus mutans* (Ingbritt) were grown in media containing varying amounts of Trypticase and Phytone, carbohydrates, potassium and sodium ions and phosphates. None of the compounds studied induced or repressed the *p*-nitrophenylphosphatase of the cells, indicating that the enzyme was most likely constitutive.

Key-words: Phosphatases; streptococcus

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In previous studies a *p*-nitrophenylphosphatase of *Streptococcus mutans* was purified 3000-fold and the enzyme was partially characterized (*Knuuttila & Mäkinen*, 1972). This most likely membrane-associated enzyme was found specifically to hydrolyze *p*-nitrophenyl phosphate. The enzyme was also Mg-dependent, and phosphate ions slightly inhibited the rate of the hydrolysis of *p*-nitrophenyl phosphate, the inhibition constant, K_i, being 19.2 mM for K_2HPO_4 and 17.9 mM for Na_2HPO_4. The optimum pH of the enzyme was close to 8.0 and the isoelectric point was 5.05.

The regulatory effect of phosphate on phosphatases has been found in a variety of microorganisms including *Escherichia coli* (*Caren & Levinthal*, 1960; *Torriani*, 1960), *Bacillus subtilis* (*Takeda & Tsugita*, 1967; *Dobozy & Hammer*, 1968), *Pseudomonas fluorescens* (*Friedberg & Avigad*, 1967) *Aerobacter aerogenes* (*Wolfenden & Spence*, 1967), *Staphylococcus aureus* (*Shah & Blobel*, 1967), *Neurospora crassa* (*Kadner et al.* 1968) and *Bacillus licheniformis* (*Hulett-Cowling & Campbell*, 1971). However, alkaline phosphatases, nonrepressible by phosphate, were reported in *Salmonella typhimurium* by *Carillo-Castaneda & Ortega* (1967), and in *Escherichia coli* by *Dealy* (1966). A procedure for the isolation of the consititutive mutants of *Escherichia coli* for alkaline phosphatase has been described by *Torriani & Rothman* (1961).

The stimulating effect of potassium ions on the uptake of phosphorus by bacterial cells has also been emphasized by different authors (*Roberts & Roberts*, 1950; *Harold et al.* 1965; *Luoma*, 1968; *Luoma et al.*, 1971). Because it has been found that 2-phosphoenol pyruvate and glucose 1-phosphate are components in vectorial phosphorylation (*Kundig et al.*, 1964), it

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was assumed that the present *p*-nitrophenylphosphatase would be regulated by these phosphates presuming that the enzyme could be considered as a part of a vectorial phosphorylation system. *Carillo-Castaneda & Ortega* (1967) found that the activity of alkaline phosphatase of *Salmonella typhimurium* was increased in a medium containing lactate instead of glucose. Consequently, it was felt necessary to study whether, carbohydrates other than glucose could affect the synthesis of the *p*-nitrophenylphosphatase in the cells of *Streptococcus mutans*. This paper will thus provide information about the effect of potassium ions and different phosphates and carbohydrates on the synthesis of the above mentioned enzyme.

MATERIALS AND METHODS

1. Reagents and their sources

Trypticase™ (pancreatic digest of casein), Phytone™ (papaic digest of soybean meal) and Trypticase Soy Broth™ were obtained from BBL, Division of BioQuest (Cockeysville, Md., USA). Glucose 1-phosphate (disodium salt) and 2-phosphoenolpyruvic acid (trisodium salt) were products of Calbiochem (Los Angeles, Calif., USA). *p*-Nitrophenyl phosphate (disodium salt) and DL-β-glyceryl phosphate (disodium salt) were purchased from Sigma Chemical Company (St. Louis, Mo., USA), and all other reagents from Merck AG. (Darmstadt, Germany).

2. The organism and its cultivation

The microorganism (*Streptococcus mutans*, strain Ingbritt), which was the same as in previous studies (*Knuuttila & Mäkinen*, 1972), has been characterized by *Edwardsson* (1968). The maintaining of the cells as stab cultures, the preparation of the inoculum medium and cultivation procedures were as presented by *Knuuttila & Mäkinen* (1972). It may be mentioned, however, that the cells from the inoculum medium were transferred after harvesting to a 100 ml growth medium with the aid of the medium (the different media used are mentioned later). All centrifugations were performed with Sorwall Superspeed RC-2B centrifuge, using rotor SS-34 at 12 000 *g*, for 10 min and at +4°C. Usually the growth was measured as Klett-readings as earlier (*Knuuttila & Mäkinen*, 1972), but in some cases also dry weights of the cells were determined during the growth by washing the harvested cells twice with 0.9 % sodium chloride and by drying them at +70°C until the weights remained unchanged. If the pH of the growth media prior of the initiation of the growth was not between the desired 6.9—7.0, the adjustment was made using 0.2 M sodium hydroxide or 0.1 M hydrochloric acid.

The basic growth medium had the following composition: Trypticase (1.7 g per 100 ml), Phytone (0.3 g per 100 ml), sodium chloride (0.5 g per 100 ml), glucose (0.25 g per 100 ml) and dipotassium hydrogen phosphate (10 mM). In this basic medium the amount of different components was varied as follows:

a) Carbohydrates:

The amount of glucose was varied from 0.5 g per 100 ml to 0.05 g per 100 ml. The glucose was also replaced with 0.25 g fructose, sucrose, sorbitol or mannitol per 100 ml.

b) Phytone:

The above-mentioned medium containing 0.5 g glucose per 100 ml was modified by gradually reducing the quantity of Phytone. In one medium no Phytone was used.

c) Potassium ions:

When studying the effect of potassium ions, 5 g of Phytone was first dialyzed against water (35 l) at +4°C for 50 hours to reduce its high potassium content. After dialysis the Phytone preparation was freeze-dried and used at a concentration of 0.3 g per 100 ml. Besides dipotassium hydrogen phosphate, also disodium hydrogen phosphate (10 mM) was used, and thus the concentrations of the added sodium chloride and potassium chloride were as follows: 1) 0.050 M NaCl and 0.020 M KCl, 2) 0.068 M NaCl and 0.002 M KCl and 3) 0.070 M NaCl.

d) Phosphates:

In addition to dipotassium hydrogen phosphate, the following phosphates were used: disodium hydrogen phosphate, magnesium hydrogen phosphate, glucose 1-phosphate, DL-β-glyceryl phosphate and 2-phosphoenolpyruvic acid. The three last mentioned phosphates were added to the growth media simultaneously with the cells to avoid their apparent hydrolysis during autoclavization. All phosphates used were added at five concentrations: 1) 0.05 mM, 2) 0.1 mM, 3) 0.5 mM, 4) 1.0 mM and 5) 10.0 mM. When studying the effect of phosphates the ratio of Trypticase and Phytone was altered so that it was 0.2 g and 1.2 g per 100 ml, respectively, because of the high phosphate content of Trypticase (1.7 g per 100 ml contained 36 μg soluble phosphorus per ml). Phytone was not found to contain detectable amounts of soluble phosphorus. This alteration in the growth medium did not affect the growth of the cells in any noticeable way.

3. *Assay procedures*

Previous studies (*Knuuttila & Mäkinen*, 1972) on *Streptococcus mutans* have shown that after the disruption of the cells with ultrasound only one enzyme hydrolyzing *p*-nitrophenyl phosphate was demonstrated in the conditions used in the crude enzyme preparation. When using DEAE cellulose and molecular exclusion chromatography and isoelectric focusing in the purification, the idea of most likely only one *p*-nitrophenylphosphatase in the cells was ascertained. Consequently, the crude enzyme preparations resulting from sonications were used in this study. Cells obtained from 5 ml aliquots of the growth medium were harvested and the resulting pellets were suspended in 0.5 ml of cold 0.05 M borate buffer, pH 8.0. The suspensions were in principle sonicated as earlier (*Knuuttila & Mäkinen*, 1972). The sonicated mixtures were centrifuged and the supernatant fluids were used as the crude enzyme preparations. In these conditions one ultrasonic treatment was enough to release more than 90 % of the enzyme from cells in all cases.

The determination of the phosphatase activity was based on the method of *Bessey, Lowry & Brock* (1946), and it was carried out in 0.025 M borate buffer, pH 8.0, as described earlier (*Knuuttila & Mäkinen*, 1972). The phosphorus was measured according to the method of *Lowry & Lopez* (1946) and sodium and potassium were determined according to standard methods using the Perkin Elmer atomic absorption spectrophotometer, Model 303 (Perkin Elmer, Norwalk, Connecticut, USA).

RESULTS

The dry weight of the cells of the exponential growth phase was determined from the same cultures as shown in Figs. 2A, 3A and 4B. When the values of the dry weights from all three cultures were plotted against the respective Klett-readings a linear correlation was found in all cases (Fig. 1).

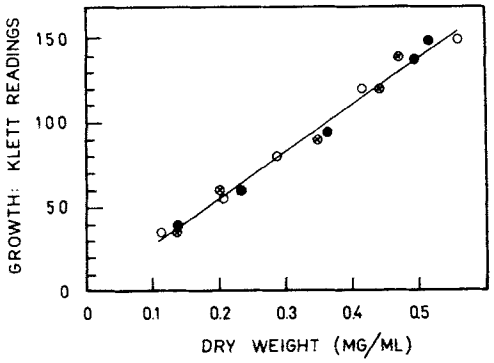

Fig. 1. Correlation between the dry weight of the cells and Klett readings during the exponential growth phase of *Streptococcus mutans*. Dry weights are expressed as milligrams per one milliliter of growth medium. The values were obtained from the following growth curves: Fig. 2A (o), Fig. 3A (•) and Fig. 4B (⊕).

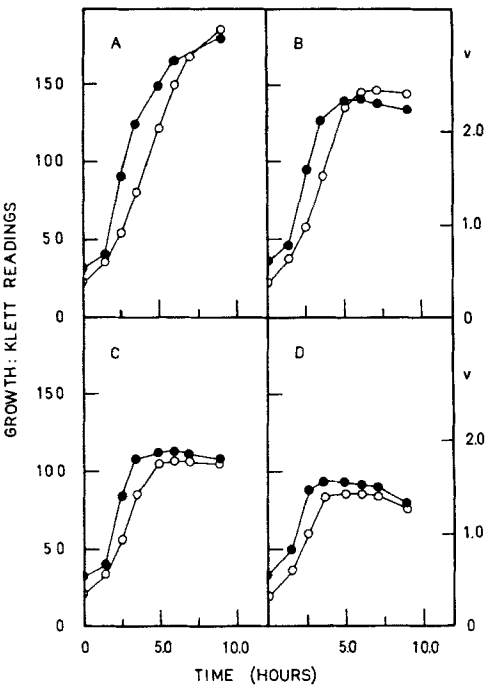

Fig. 2. The effect of different amounts of glucose on the growth of *Streptococcus mutans* and on the synthesis of *p*-nitrophenylphosphatase. Growth is expressed as Klett readings (o) and the enzyme activity as the rate, *v*, (in M x min^{-1} x 10^{-6}) of the hydrolysis of *p*-nitrophenyl phosphate (•). Cells of 5 ml aliquots of the growth medium were harvested and sonicated to make the enzyme preparations as mentioned in the Materials and Methods section. The initial glucose content was varied in the basal growth medium as follows: 0.50 g (A), 0.25 g (B), 0.10 g (C) and 0.05 g (D), per 100 ml of growth medium.

1) Effect of certain sugars

The effect of different amounts of glucose on the growth of the bacteria and the synthesis of *p*-nitrophenylphosphatase is shown in Fig. 2. When the amount of glucose was reduced gradually, the stationary growth phase was reached more rapidly. Therefore, during the first four hours the growth was in all cases almost the same. The synthesis of *p*-nitrophenylphosphatase followed the growth curve and only small differences were observed between the growth and enzyme curves in different glucose concentrations. The replacement of glucose with the respective amounts of sucrose or fructose (Figs. 3A and 3B) gave the same growth curves and did not change the relationship between the growth and the synthesis of the enzyme. However, the growth was somewhat retarded when polyols such as sorbitol or mannitol were used as carbon sources (Figs. 3C and 3D), but they had no repressive or inducive effect on the synthesis of *p*-nitrophenylphosphatase.

2) Effect of Phytone

Fig. 4 shows that Phytone (a papaic digest of soybean meal) was needed in this medium for the growth of *Streptococcus mutans*. The synthesis of the enzyme was again clearly dependent on the growth of the microorganism. Consequently, not even this component of the growth medium induced or repressed the enzyme.

3) Effect of potassium ions

The role of potassium ions in the growth of *Streptococcus mutans* and in the forma-

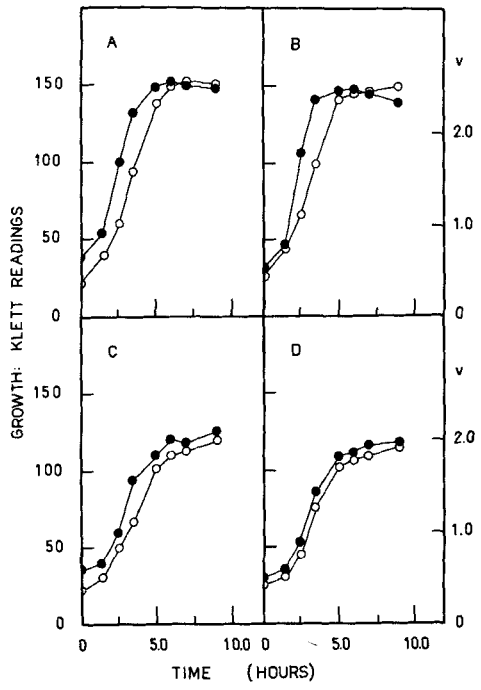

Fig. 3. The effect of sucrose (A), fructose (B), sorbitol (C) and mannitol (D) on the growth of *Streptococcus mutans* and on the synthesis of *p*-nitrophenylphosphatase. Glucose was replaced in the basal medium (100 ml) with 0.25 g the above mentioned sugars or polyols. Growth (o) and enzyme activity (•) are expressed as in Fig. 2.

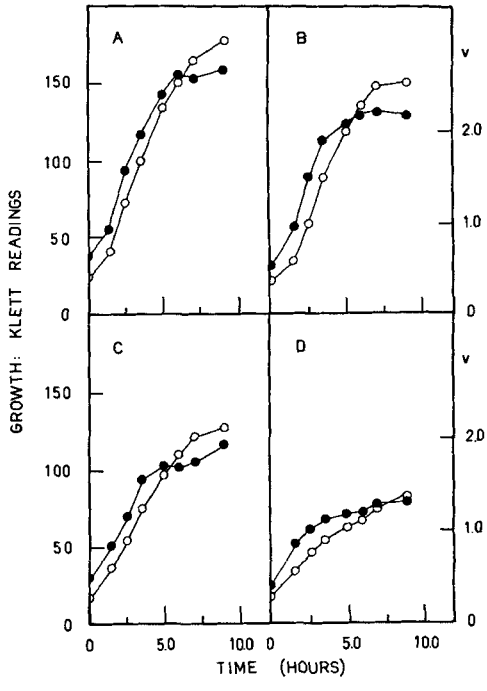

Fig. 4. The effect of different amounts of Phytone on the growth of *Streptococcus mutans* and on the synthesis of *p*-nitrophenylphosphatase. Growth (o) and enzyme activity (•) are expressed as in Fig. 2. Amounts of Phytone were: 0.3 g (A), 0.2 g (B) and 0.1 g (C), per 100 ml of growth medium. In one medium (D) no Phytone was used. Other components of the media are given in the Materials and Methods section.

tion of *p*-nitrophenylphosphatase is seen in Fig. 5. When the amount of potassium ions was reduced in the growth medium, the growth and also the synthesis of the enzyme were retarded. In the case where no potassium ions were added (Fig. 5D) the potassium content of the medium prior to inoculation was, however, 21 μg per ml. The results again indicated a clear dependence between the growth of the cells and the synthesis of the enzyme, when different concentrations of the potassium ions were used.

4) Effect of phosphates

As mentioned earlier in the Materials and Methods section the composition of the growth medium was changed so that the phosphate content was made low enough to study the effect of phosphate. The amount of soluble phosphorus in the medium used prior to inoculation was 4.2 μg per ml. Because phosphate was not found to have any regulatory effect on the synthesis of the enzyme only two of the phosphate concentrations used (the highest and lowest), are presented in Fig. 6 for disodium hydrogen phosphate and glucose 1-phosphate. Similar results were obtained with all other phosphates tested.

When the ingredients of the growth medium and related compounds were systematically tested for their possible

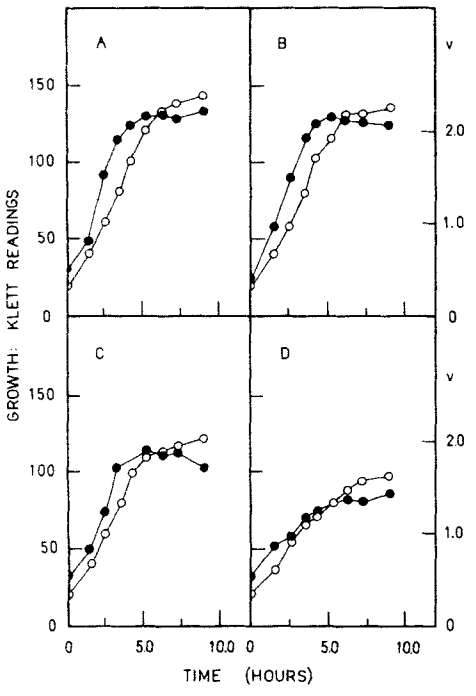

Fig. 5. The effect of potassium ions on the growth of *Streptococcus mutans* and on the synthesis of *p*-nitrophenylphosphatase. Growth (o) and enzyme activity (•) are expressed as in Fig. 2. The growth medium indicated in Fig. 5A was the same as for Fig. 2B, except that dialyzed Phytone used. Fig. 5A shows the effect of dialysis of Phytone. In other media indicated in Figs. 5B, 5C and 5D the dipotassium hydrogen phosphate of the basal medium was replaced with disodium hydrogen phosphate. Total concentrations of potassium ions were in the three last mentioned media (Figs. 5B, 5C and 5D) 801 μg per ml, 99 μg per ml and 21 μg per ml, respectively. No potassium ions were added to the medium indicated in Fig. 5D. The used sodium chloride concentrations were 0.050 M (B), 0.068 M (C) and 0.070 M (D), respectively.

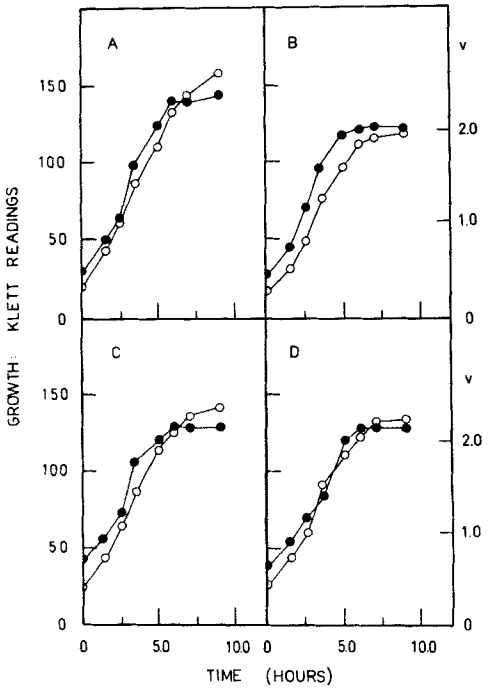

Fig. 6. The effect of high and low concentrations of disodium-hydrogen phosphate (A and B) and glucose 1-phosphate (C and D) on the growth of *Streptococcus mutans* and on the synthesis of *p*-nitrophenylphosphatase. Growth (o) and enzyme activity (•) are expressed as in Fig. 2. The growth media (100 ml) had the following composition: Trypticase 0.2 g, Phytone 1.2 g, glucose 0.25 g, sodium chloride 0.5 g, and the above mentioned two phosphates at concentrations of 10 mM and 0.05 mM, respectively. The concentration of water soluble phosphorus of the above media (without any added phosphate) was 4.2 μg per ml and the final concentrations were 35.2 μg per ml (A and C) and 4.4 μg per ml (B and D).

inducive or repressive effects, it was found that the present *p*-nitrophenylphosphatase was most likely a constitutive enzyme. This finding was considered important when evaluating the physiological significance of the *p*-nitrophenylphosphatase, which will be dealt with in subsequent papers.

DISCUSSION

The results showed that the *p*-nitrophenylphosphatase differed from most bacterial alkaline phosphatases in being nonrepressible at high phosphate concentrations. Because some strains of *Escherichia coli* with constitutive alkaline phosphatase have been described (*Torriani & Rothman*, 1961), it could be assumed that the repressive effect of phosphate upon the synthesis of alkaline phosphatase depends on the particular bacterial strain tested. *Echols et al.* (1961) have shown with *Escherichia coli* that the repression of alkaline phosphatase is under the control

of two regulator genes and the presence of a constitutive mutation in either of these genes prevents the repression of the enzyme. Consequently, because the synthesis of this type of *p*-nitrophenylphosphatase has not been studied on different streptococci, the possibility cannot be ruled out that there are strains of streptococcus with a similar enzyme of a repressive nature. However, in the conditions used the lowest phosphate concentration in the growth medium was considered to be low enough (cf. *Torriani*, 1960; *Shah & Blobel*, 1967) to cause the repressive effect of phosphate. Also the amount of phosphate in the enzyme preparations and reaction mixtures was too low to affect the enzyme reactions.

The regulation of the synthesis of *p*-nitrophenylphosphatase was in the present study investigated in a very complex growth medium. However, there would be no reason to assume that a possible repression would not arise in this medium (cf. *Hulett-Cowling & Campbell*, 1971). Also, the effect of components of this growth medium (other than phosphate) on the synthesis of this enzyme was studied. This was carried out in order to find out if there are other factors which could regulate the formation of the enzyme. The results indicated that potassium ions neither repressed nor induced the enzyme and that a possible suggestion for the significance of these ions as regulatory factors in the formation of the enzyme is not valid. The same result was found in the case of sodium ions.

The synthesis of the enzyme was not dependent on the sources of the carbohydrates. This may indicate that the function of the enzyme is not joined specifically to the metabolism of any of the sugars or polyols used, because, for example, glucose has generally been found to repress the formation of catabolic enzymes involved in the metabolism of other sugars (*Gibbons & Banghart*, 1967). It is difficult to evaluate the significance of this enzyme in sugar transport, although there are strong preliminary indications that glucose 1-phosphate in particular may be a central metabolite in the regulation of carbohydrate transport (*Kaback*, 1970). However, it may be concluded that this phosphate, as well as 2-phosphoenolpyruvic acid, had no effect on the synthesis of the present enzyme.

The small deviations in the curves representing the formation of the enzyme were probably due to the method used in making the enzyme preparations. The present *p*-nitrophenylphosphatase can thus be considered to be a basically constitutive enzyme in the conditions involved.

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