Significance of a *p*-nitrophenylphosphatase for *Streptococcus mutans*

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Knuuttila, M. L. E. Significance of a *p*-nitrophenylphosphatase for *Streptococcus mutans*. *Acta Odont. Scand.* 31, 21—30, 1973

The hydrolysis of *p*-nitrophenyl phosphate (added to the incubation medium), catalyzed by a Mg-dependent and highly specific *p*-nitrophenylphosphatase, stimulated the uptake of K^+ ions by the cells of *Streptococcus mutans*. The stimulation was detectable only in the presence of glucose and it was highest at 0.1—0.2 mM concentrations of *p*-nitrophenyl phosphate. The degree of stimulation was also dependent on the extracellular concentrations of K^+ ions. The rate of the hydrolysis of *p*-nitrophenyl phosphate, catalyzed by both the cells and the purified enzyme, was not affected by K^+ ions and dicyclohexylcarbodiimide. The other cleavage product of the hydrolysis of *p*-nitrophenyl phosphate (*p*-nitrophenol) inhibited the uptake of K^+ and phosphate ions, but it did not affect the activity of ATP:ase of the cells.

Key-words: Phosphatases; streptococcus; transport

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In recent years considerable information has been presented on an enzyme complex comprising an ATP:ase and a *p*-nitrophenylphosphatase. This enzyme complex has been suggested to regulate K^+ and Na^+ ions' translocations across the cell membrane. Although several authors (*Skou*, 1962; *Judah et al.*, 1962; *Fujita et al.*, 1966; *Emmelot & Bos*, 1966; *Albers & Koval*, 1966; *Israel & Titus*, 1967; *Rega et al.*, 1968; *Woodin & Wieneke*, 1968, 1970, 1971, *Tanaka & Sakamoto*, 1969; *Askari & Rao*, 1969, 1971; *Koyal et al.*, 1971, *Askari & Koyal*, 1971; *Rao et al.*, 1971) have studied the above mentioned enzymes, no exact mechanism for the function of the complex and no detailed explanation for the mutual relationship between its components have been described. However, the common feature of all models seems to be the postulate that ion movements are intimately related to the turnover of the phospho-enzyme intermediate within the membrane (*Mitchell*, 1967).

In the previous studies (*Knuuttila & Mäkinen*, 1972 a b; *Knuuttila*, 1972) a most likely membrane-associated phosphatase specifically hydrolyzing *p*-nitrophenyl phosphate has been purified and characterized from the cells of *Streptococcus mutans*. Evidence was also presented on the constitutive nature of this enzyme (*Knuuttila*, 1972). On the basis of these studies it became apparent that *p*-nitrophenyl phosphate could also be used

Received for publication, September 9, 1972.

as a substrate for the present *p*-nitrophenylphosphatase *in vivo*, when studying the uptake of K^+ ions by the cells. In this case *p*-nitrophenyl phosphate would either mimic the function of a natural substrate or it would act as a competitive inhibitor in the hydrolysis of the natural substrate. It is possible that the use of *p*-nitrophenyl phosphate in the present case as the substrate of the phosphatase would elucidate on the significance of the enzyme for the cells of *Streptococcus mutans*.

However, the effect of *p*-nitrophenol on the uptake of K^+ ions is also important, because the cleavage product of the substrate may affect the enzyme action. This may be important in this particular case, because it is known that the present enzyme has a very narrow substrate specificity and because it is evident that the *in vivo* substrate resembles in its structure that of *p*-nitrophenyl phosphate. It is likewise important to emphasize the fact that 2,4-dinitrophenol, (the structure of which resembles very much that of *p*-nitrophenol), inhibits the phosphate and K^+ ions uptake, and the protein and ribonucleic acid synthesis in different cells and cell organelles (*Hotchkiss*, 1943; *Nickerson & Mullins*, 1948; *Roberts et al.*, 1949; *Jarett & Hendler*, 1967; *Riemersma*, 1968; *Borst-Pauwels & Jager*, 1968).

Studies carried out by *Harold et al.* (1967, 1969), *Harold & Baarda*, (1968), *Schnebli & Abrams*, (1970) and *Abrams et al.*, (1960, 1972), have described an ATP:ase in *Streptococcus faecalis*. This enzyme, which probably plays a role in the transport of cations and other metabolites, was *in vivo* inhibited by dicyclohexylcarbodiimide. This compound is known to inhibit a number of energy-dependent transport processes, including the accumulation of K^+ ions and the uptake of phosphate and alanine.

This paper will provide further support for the earlier suggestion (*Knuuttila & Mäkinen*, 1972a) that the present phosphatase may be a component of an enzyme system which regulates the transport of K^+ ions. Consequently, this study describes the effect of *p*-nitrophenyl phosphate and *p*-nitrophenol on the ability of the cells of *Streptococcus mutans* to take up K^+ ions. The effect of dicyclohexylcarbodiimide on the present phosphatase was also studied *in vitro* and *in vivo*.

MATERIALS AND METHODS

Cells of *Streptococcus mutans*, which were cultivated as earlier described by *Knuuttila* and *Mäkinen* (1972a), were harvested at the end of the exponential growth phase using a refrigerated Sorvall Superspeed RC-2B centrifuge (12000 × g; for 5 min.). Prior to the subsequent experiments the harvested cells were stored for 15—20 hours in cold (+4°C) 0.9 % sodium chloride solution (about 1 g cells, dry weight, in 40 ml sodium chloride solution). Because the cells were not able to hydrolyze *p*-nitrophenyl phosphate, they were washed once with 4 M NaCl and water after storage in order to demonstrate the hydrolysis. The washings were performed at 22°C for 10 min with great excess (30—40 fold) of both NaCl and water.

The ability of the cells to take K^+ and phosphate ions or glucose was measured as changes of the concentration of these compounds in the incubation medium after removing the cells. The following incubation medium was used: 1.5 ml of 0.1 M tris-HCl buffer, pH 7.5, 1.5 ml of water, 1.5 ml of glucose, 1.0 ml of 15 mM $MgCl_2$, 150 µl of 10 mM Na_2HPO_4, 200 µl of KCl and 1.5 ml of the cell suspension. The suspension was prepared by suspending the washed cells (corresponding to

about 400 mg of dry cells) in 9.0 ml 0.1 M tris-HCl buffer, pH 7.5. When the effect of *p*-nitrophenyl phosphate or *p*-nitrophenol on the uptake of K^+ or phosphate ions was studied, the water (1.5 ml) was replaced with the same volume of freshly prepared aqueous solutions of the above mentioned reagents at various concentrations. The concentrations of potassium chloride and glucose were also varied, but they were usually added at concentrations of 0.10 M and 0.75 M, respectively. The incubations were carried out at 22°C, and samples of 0.5 ml, from which the cells were immediately removed by centrifugation, were taken from the incubation media usually four times during the experiment for the determination of potassium, *p*-nitrophenol, phosphate and glucose concentrations. The pH of all incubation media was simultaneously measured with glass and calomel electrodes at 22°C.

Potassium concentrations were determined with a Perkin Elmer Model 303 Atomic Absorption Spectrophotometer according to the manual instructions. Phosphorus determinations were carried out as described by *Lowry & Lopez*, (1946) and glucose was determined as presented by *Bergmeyer & Berndt*, (1965). The concentration of *p*-nitrophenol was measured spectrophotometrically at 410 nm in the presence of sodium hydroxide (pH above 10).

The effect of dicyclohexylcarbodiimide (Merck, Darmstadt, Germany) on the rate of the hydrolysis of *p*-nitrophenyl phosphate, catalyzed by the purified enzyme and the cell suspensions (prepared as mentioned above), was studied in the same way as that of the other enzyme modifiers (*Knuuttila & Mäkinen*, 1972a). When the cell suspensions were used as the enzyme preparations, the cells were removed by centrifugation before the spectrophotometric measurements. The effect of *p*-nitrophenol on the activity of ATP:ase was determined using the above mentioned cell suspensions and the partially purified preparations of the enzyme. The partial purification of ATP:ase was carried out by fractionation of DEAE cellulose as shown by *Knuuttila & Mäkinen* (1972a). The assay of ATP:ase and all reagents have been described in a previous paper (*Knuuttila & Mäkinen*, 1972a).

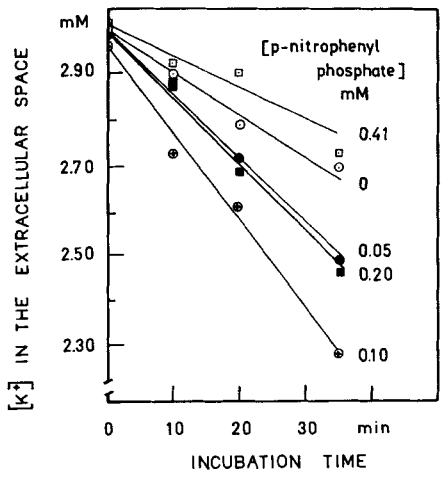

Fig. 1. The effect of the concentration of *p*-nitrophenyl phosphate on the change of the concentration of K^+ ions in the extracellular space, which could be considered as an uptake of these ions into the cells of *Streptococcus mutans*. It was not the mere presence of *p*-nitrophenyl phosphate in the incubation medium, but its enzymic hydrolysis, catalyzed by the cells, which led to the change of the concentration of K^+ ions.

RESULTS

1. Hydrolysis of p-nitrophenyl phosphate by whole cells

As earlier indicated in the Materials and Methods section it was found necessary, in order to demonstrate the ability of the cells to hydrolyze *p*-nitrophenyl phosphate, to treat the cells in a special way. There were

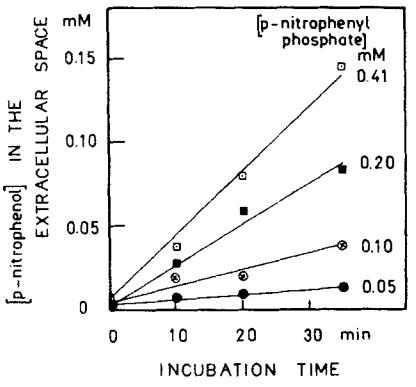

Fig. 2. The formation of *p*-nitrophenol in the extracellular space when incubating cells of *Streptococcus mutans* in the presence of *p*-nitrophenyl phosphate. The data were from the experiment shown in Fig. 1.

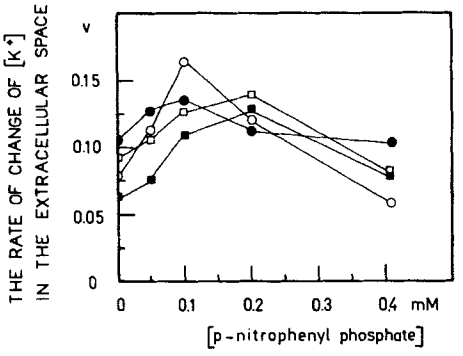

Fig. 3. The rate (v; M $\times$ min^{-1} $\times$ 10^{-4}) of the change of the concentration of K^+ ions in the extracellular space versus the initial concentration of *p*-nitrophenyl phosphate. The curves were obtained from four separate experiments, similar to that shown in Fig. 1.

three main reasons why sodium chloride was used for this purpose: 1) Sodium chloride is a chaotropic agent and it evidently has the ability to increase the water solubility of certain proteins and nonelectrolytes, and the tendency to make water more lipophilic (*Hatefi & Hanstein*, 1969). 2) Sodium chloride could reasonably be expected to reduce the potassium concentration in the cells. 3) Sodium chloride has an inhibitory effect on *p*-nitrophenylphosphatase and many other enzymes only at reasonably high concentrations. When the cells were washed with sodium chloride solutions of different concentrations and thereafter with water, 4 M sodium chloride was found to be suitable in causing the cell preparation to bring about a rapid hydrolysis of *p*-nitrophenyl phosphate (Fig. 2). After washings with 4 M sodium chloride and water no alterations in the cells could be seen microscopically. As a result of these washings the ability of the cells to metabolize glucose was reduced by about 10 % from that observed with cells washed only with water.

2. Effect of p-nitrophenyl phosphate on the uptake of K^+ ions

When different concentrations of *p*-nitrophenyl phosphate were used, it was found that concentrations ranging from 0.05 mM to 0.20 mM stimulated the decrease of the concentration of K^+ ions in the extracellular space during the 35 min incubation time used (Figs. 1 and 3). The decrease of the concentration of K^+ ions in the extracellular space was considered to represent an uptake of these ions by the cells. Fig. 3 shows this stimulation as alterations in the rate of the loss of K^+ ions (v, in M $\times$ min^{-1} $\times$ 10^{-4}) versus the concentrations of *p*-nitrophenyl phosphate. The results of Fig. 3 were obtained from four separate experiments. This stimulatory effect varied considerably when cell suspensions from different cultures were used, but in general the optimum stimulation was found at 0.10 mM or 0.20 mM concentrations of *p*-nitrophenyl phosphate. The highest concentration (0.41 mM) of *p*-nitrophenyl phosphate used did not affect or it only slightly inhibited the uptake of K^+ ions.

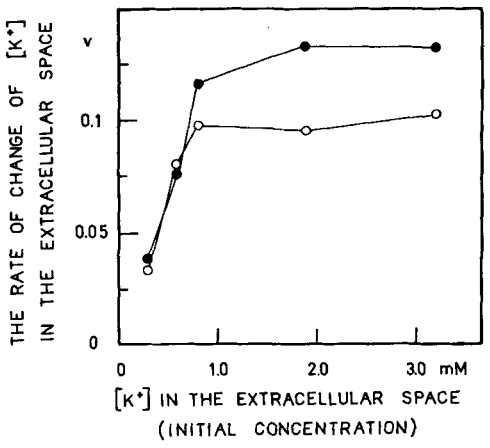

Fig. 4. The effect of the initial concentration of K^+ ions on the change of the concentration of these ions in the extracellular space. Incubation time: 15 min. in the presence of 0.1 mM *p*-nitrophenyl phosphate, (•); without added *p*-nitrophenyl phosphate, (o). The rate, v, is expressed in $M \times min^{-1} \times 10^{-4}$.

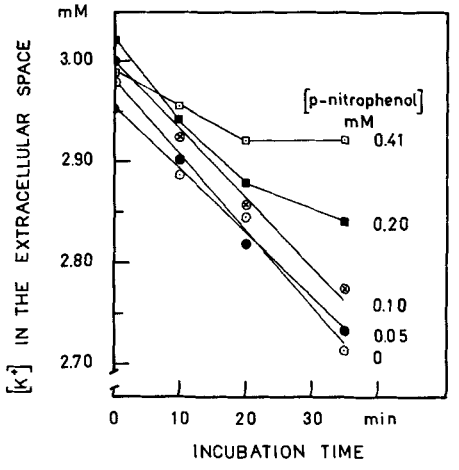

Fig. 5. The effect of *p*-nitrophenol on the concentration of extracellular K^+ ions when incubating the cells for 35 min.

The concomitant release of *p*-nitrophenol into the extracellular space during the incubation was, however, increased almost linearly at all the *p*-nitrophenyl phosphate concentrations used (Fig. 2). The values of Fig. 2 were obtained during the experiment shown in Fig. 1, but even in other experiments the amount of *p*-nitrophenol increased in the extracellular space during the incubation with increasing concentrations of *p*-nitrophenyl phosphate. However, the enzymic hydrolysis of *p*-nitrophenyl phosphate was necessary in order to observe the stimulation of the uptake of K^+ ions by the cells.

The *p*-nitrophenyl phosphate concentrations used were found to have no measurable effect on the consumption of glucose by the cells during the 35 min incubation, when compared to the situation where *p*-nitrophenyl phosphate was replaced with water. Glucose concentration in the medium decreased about 0.6 mg per ml during the 35 min period and the pH values simultaneously decreased from 7.30—7.40 to 7.15—7.25. When glucose was replaced with water no uptake of K^+ ions was found during the incubation in the absence or presence of *p*-nitrophenyl phosphate.

Fig. 4 shows the decrease of potassium ions in the extracellular space versus the initial amount of K^+ ions in the incubation medium. The upper curve was drawn with the aid of the values obtained in the presence of 0.10 mM *p*-nitrophenyl phosphate. The lower curve is from an experiment where *p*-nitrophenyl phosphate was replaced with water. The ordinate gives the rate of the uptake of K^+ ions during a 15 min incubation time. In order to explain the results of Fig. 4 it should be emphasized that the rate of the hydrolysis of *p*-nitrophenyl phosphate was not affected by the concentration of K^+ ions in the extracellular space. Additionally, K^+ ions, tested at concentrations ranging from 0 to 16.7 mM, were not found to affect the rate of the hydrolysis of *p*-nitrophenyl phosphate catalyzed by the purified enzyme. The curvature obtained resembled to a certain degree the Michaelis-Menten

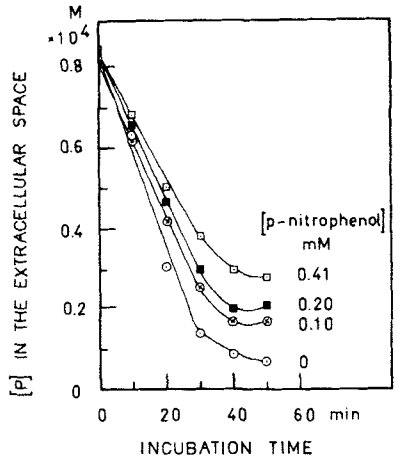

Fig. 6. The effect of *p*-nitrophenol on the concentration of extracellular phosphate ions when incubating the cells for 50 min.

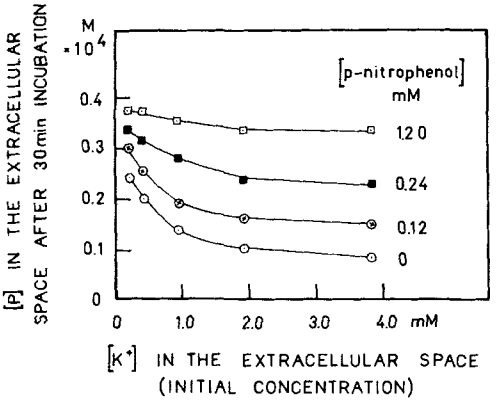

Fig. 7. The effect of *p*-nitrophenol on the concentration of extracellular phosphate ions when incubating the cells in the presence of different concentrations of K^+ ions. The values of phosphate concentrations were measured after 30 min. incubation. K^+ ion concentrations of the incubation media were measured before the incubation.

behaviour. This may indicate that the reaction(s) leading to the loss of K^+ ions in the extracellular space are of a possibly enzymatic nature. If so, *p*-nitrophenyl phosphate affected the maximum rate of the uptake of K^+ ions.

3. The effect of p-nitrophenol on the uptake of K^+ and phosphate ions

When the respective experiments as presented in Fig. 1 were carried out, replacing, however, *p*-nitrophenyl phosphate with *p*-nitrophenol of different concentrations, the results shown in Fig. 5 were obtained. This indicates that increasing concentrations of *p*-nitrophenol inhibited the decrease of K^+ ions in the extracellular space. Therefore, none of the concentrations of *p*-nitrophenol used had any stimulating effect on the uptake of K^+ ions. As already described, 2, 4-dinitrophenol is known to inhibit the uptake of phosphate ions by different cells. *p*-Nitrophenol had a similar effect in the case of *Streptococcus mutans* as shown in Fig. 6. This inhibition of the uptake of phosphate ions is evidently partly explained in terms of the effect of *p*-nitrophenol on the uptake of K^+ ions. This is supported by findings that increasing concentrations of *p*-nitrophenol reduced the stimulating effect of K^+ ions on the uptake of phosphate (Fig. 7). In addition, *p*-nitrophenol inhibited the uptake of phosphate ions although no K^+ ions were added to the incubation media. The concentrations of *p*-nitrophenol used had no effect on the consumption of glucose.

4. Other studies concerning the properties of p-nitrophenylphosphatase

Dicyclohexylcarbodiimide tested at concentrations ranging from 0.17×10^{-2} M to 0.17×10^{-8} M was not found to change the rate of the hydrolysis of *p*-nitrophenyl phosphate catalyzed by either the purified enzyme or the cell suspension. The same findings were made by *Schnebli & Abrams*, (1970) concerning the hydrolysis of *p*-nitrophenyl phosphate catalyzed

by the cells of *Streptococcus faecalis*. *p*-Nitrophenol tested at concentrations ranging from 0.013 mM to 1.30 mM also had no effect on the rate of the hydrolysis of ATP. This was determined both with the partially purified ATP:ase and with the cell suspension prepared as mentioned above.

DISCUSSION

The uptake of K^+ ions is an energy-providing process as reported by *Abrams*, (1960), *Zarlengo & Schultz*, (1960), *Harold & Baarda*, (1968), *Harold et al.*, (1967, 1969) in *Streptococcus faecalis*, and by *Roberts et al.*, (1949) in *E. coli*. The necessity of the presence of glucose for the uptake of K^+ ions by the cells of *Streptococcus mutans* most likely indicates the involvement of an active transport mechanism. The stimulation of the uptake of K^+ ions in the presence of certain concentrations of *p*-nitrophenyl phosphate found in this study was due to the action of the *p*-nitrophenylphosphatase. Without the hydrolysis of *p*-nitrophenyl phosphate no stimulation of the uptake was observed. Further, the stimulation was seen to be dependent on the concentration of *p*-nitrophenyl phosphate. In the present study the alterations in the concentrations of K^+ ions were measured only in the extracellular space. The decrease of the concentration of K^+ and phosphate ions in the extracellular space during the incubation was, however, most naturally explained as being an uptake of these ions by the metabolizing cells (rf. *Roberts et al.*, 1949).

Besides the concentration of *p*-nitrophenyl phosphate affecting the uptake of K^+ ions by the cells, the stimulation of the uptake, caused by *p*-nitrophenyl phosphate, was also dependent on the concentration of extracellular K^+ ions. This was the case even though the rate of the hydrolysis of the substrate was not changed. The result of Fig. 4 indicated that *p*-nitrophenyl phosphate most likely influenced the amount of a possible free carrier compound of K^+ ions rather than the reaction itself between K^+ ions and the proposed carrier.

Although the relationship between the phosphatase studied and the uptake of K^+ ions seems to be evident, the direct participation of this enzyme in the sodium-potassium pump was not shown. It is possible that the uptake of K^+ ions may also be electrically balanced by a concurrent uptake of anions. However, the findings of *Askari & Rao*, (1971), that the potassium-activated hydrolysis of *p*-nitrophenyl phosphate also stimulated the efflux of Na^+ ions into sodium free medium in red cell chosts, supports a possible exchange reaction between sodium and potassium ions involving the present *p*-nitrophenylphosphatase. The present bacterial enzyme differs from that of human red cells (*Judah et al.*, 1962; *Rega et al.*, 1968, *Askari & Rao*, 1971) and of many mammalian cells (*Fujita et al.*, 1966, *Askari & Koyal*, 1971, *Koyal et al.*, 1971) in not being activated by K^+ ions. A common feature of the above mammalian enzyme system, consisting of a *p*-nitrophenylphosphatase and an ATP:ase, and which is believed to participate in the transport of Na and K^+ ions, is the activation of the former enzyme by K^+ ions and of the latter by a mixture of K^+ and Na^+ ions. However, *Heller* and *Hanahan*, (1971) found that there were differences in the cation activation between the above mentioned enzymes in red cells of some animals. For example, adult cows and bovines lacked sodium and potassium activation of ATP:ase and potassium

activation of *p*-nitrophenylphosphatase activity in contrast to calves. However, it is not certain if adult cows and bovines also lacked a sodium-potassium pump. The participation of a membrane-bound ATP: ase of *Streptococcus faecalis* in the transport of Na^+ and K^+ ions has been described by *Harold et al.*, (1969) and *Abrams et al.*, (1972). This enzyme was not activated by the mixture of Na^+ and K^+ ions, as reported by *Schnebli & Abrams*, (1970). It may be important to study more exactly the relationship between the sodium-potassium pump and the potassium and/or sodium activation of *p*-nitrophenylphosphatase and ATP:ase both of which are linked to the transport of these cations.

Although the use of *p*-nitrophenyl phosphate gave interesting results in view of the possible significance of the enzyme, the effects of *p*-nitrophenol were found, however, to limit possibilities to explain the results. For example, it is difficult to state whether the partly opposite effects of *p*-nitrophenyl phosphate and *p*-nitrophenol on the uptake of K^+ ions are processes which are independent of each other, or whether the cleavage product of a natural substrate also has a direct regulatory effect on the function of the enzyme. The effect of liberated *p*-nitrophenol may explain the inhibitory effect of the high concentrations of *p*-nitrophenyl phosphate used in the uptake of K^+ ions.

The relationship between nitrophenols and the activity of ATP:ase has been studied by many authors (*Lardy & Wellman*, 1953; *Slater*, 1963; *Veldsema & Slater*, 1968; *Amons et al.*, 1968; *Cereijo-Santalo*, 1972). However, the effect of *p*-nitrophenol in the present study cannot be explained with the aid of the papers mentioned above, because *p*-nitrophenol had no effect on the activity of ATP:ase of *Streptococcus mutans*. The effect of dicyclohexylcarbodiimide on the rate of the hydrolysis of *p*-nitrophenyl phosphate also supports the results obtained with *Streptococcus faecalis* by *Harold et al.*, (1969).

Before any models for the function of the present *p*-nitrophenylphosphatase can be constructed, it is necessary to find the natural substrate of this enzyme. It is likewise important to study whether *Streptococcus mutans* has an ATP:ase which is linked to the transport of cations, as well as the relationship between the present *p*-nitrophenylphosphatase and the ATP-ase activity.

Acknowledgements. The author is grateful to Associate Professor Kauko Mäkinen for his stimulating criticism and to Miss Rauni Suominen for her skilled technical assistance. The author would like to thank the National Research Council for Medical Sciences of Finland and the Finnish Dental Society for financial support.

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