Journal of Bioenergetics and Biomembranes, Vol. 15, No. 1, 1983

Phosphate Transport System in *Paracoccus denitrificans*

Petr Zbořil,[1] Zdeněk Horák,[2] and Vladimír Dadák[1]

Received March 9, 1982; revised June 1, 1982

Abstract

P_i uptake in cells or spheroplasts of *Paracoccus denitrificans* is biphasic; only the first rapid phase represents net P_i transport. The second phase is limited by the rate of P_i utilization inside the cell, i.e., mainly by its esterification, and as such it was inhibited by DCCD. The P_i/dicarboxylate antiporter does not seem to be operative, and its inhibitor *n*-butylmalonate did not exert specific inhibition. P_i transport is inhibited by SH reagents; the most potent inhibitor is PCMB, and mersalyl is much less effective. However, neither inhibitor affects efflux of accumulated P_i. The gradient of potassium ions may be involved in the P_i uptake, which is lowered in the presence of valinomycin. FCCP alone does not release accumulated P_i from spheroplasts unless they are preincubated with SCN^-. The results indicate that P_i enters the cell by symport with protons.

Key Words: Bacterial P_i transport; P_i uptake into cells; P_i efflux; *Paracoccus denitrificans* cells; *Paracoccus denitrificans* spheroplasts; symport P_i/H^+; inhibitors of P_i uptake; SH reagents; *N,N'*-dicyclohexylcarbodiimide (DCCD) inhibitory effect.

Introduction

Inorganic phosphate transport across the inner mitochondrial membrane has been well investigated. It involves at least two carrier systems, the P_i/OH^- antiporter (or P_i/H^+ symporter) and the P_i/dicarboxylate antiporter. The former is inhibited selectively by NEM, the latter by *n*-butylmalonate; both of them are inhibited by mercury compounds such as PCMB[3] or mersalyl (Coty and Pedersen, 1974, 1975). Lately, energy-dependent P_i transport not corresponding to the P_i/OH^- antiport has been observed with inside-out vesicles of

[1]Department of Biochemistry, J. E. Purkyně University, 611 37 Brno, Czechoslovakia.
[2]Division of Nuclear Medicine, KUNZ, 657 15 Brno, Czechoslovakia.
[3]Abbreviations: PCMB, *p*-chlormercuribenzoate; NEM, *N*-ethylmaleinimide; DTNB, 5,5'-dithiobis-2-nitrobenzoate; DCCD, dicyclohexylcarbodiimide; FCCP, carbonylcyanide p-trifluoromethoxyphenylhydrazone.

0145-479X/83/0200-0001$03.00/0

the inner mitochondrial membrane. Its mechanism has not been understood; identically with the former it was inhibited by mercury componds and by NEM (Wehrle *et al.*, 1978).

The cytoplasmic membrane of *Paracoccus denitrificans* possesses a number of functional features common with the inner mitochondrial membrane (for a review see John and Whatley, 1977). There is but little information on the phosphate transport across this membrane. Burnell *et al.* (1975) described P_i transport in subcellular membrane vesicles of *P. denitrificans*; this corresponds to the mitochondrial transport since it is driven by a pH gradient (alkaline inside), implies a carrier sensitive to SH reagents, and is reversible. The order of its rate is, however, lower. Two major transport systems (Pit and Pst) for inorganic phosphate have been recognized in *Escherichia coli* (Willsky *et al.*, 1973). They differ in their sensitivity to uncoupling agents, only the Pit system being coupled to the protonmotive force (Rosenberg *et al.*, 1977). The uptake of P_i through both systems has an obligatory requirement for the presence of K^+ ions in the external medium (Russel and Rosenberg, 1980). The aim of the present paper is to study the P_i transport system operative in cells of *P. denitrificans*.

Materials and Methods

$^{32}P_i$ was obtained from Isocommerz (Berlin, G.D.R.); 2,5-diphenyloxazole, 1,4-bis-(5-phenyl-2-oxazolyl)benzene, mersalyl, and DTNB were from Sigma (München, G.F.R.); NEM was from Koch-Light (Colnbrook, U.K.); DCCD and PCMB were obtained from Lachema (Brno, Czechoslovakia); FCCP and valinomycin were gifts from Boehringer (Mannheim, G.F.R.); *n*-butylmalonate was synthesized in our laboratory; lysozyme was prepared from egg white according to Fevold and Alderton (1949).

Paracoccus denitrificans N.C.I.B. 8944 was grown in a medium of pH 7.4 containing 5 g of NaCl, 1 g of $NH_4H_2PO_4$, 1 g of K_2HPO_4, 0.2 g of $MgSO_4 \cdot 7H_2O$, 3 mg of ferric citrate, and 0.05 mol of sodium succinate in 1 liter. Inoculum was grown aerobically (agitation of 120 swings/min) for 16.5 hr at 37°C after it had been inoculated with 1 loop of the slanting agar culture per 300 ml of the medium. The cultivation was carried out under the same conditions with 30 ml of the inoculum per 1 liter of the medium for 14.5 hr. Bacteria were harvested by centrifugation at 2,600 *g* for 110 min, then washed in 0.9% NaCl with 10 mM Tris/HCl, pH 7.3, and again centrifuged (60 min); the obtained sediment was homogenized in 0.1 M Tris/HCl, pH 7.3 (40 mg d.w./ml). Spheroplasts were prepared by 30-min incubation of this suspension in 25 volumes of prewarmed 0.5 M sucrose with 10 mM Tris/HCl, pH 7.3, containing 25 mg of lysozyme/100 ml at 33°C with stirring. The mixture was

centrifuged at 20,000 g for 30 min, the sediment was homogenized in 0.5 M sucrose, and after further centrifugation it was homogenized in 0.25 M KCl. The spheroplast suspension was stored at 4°C up to one week. Protein was determined by the Folin reagent according to Lowry *et al.* (1951).

For P_i transport measurements, cells or spheroplasts were incubated under stirring to prevent oxygen exhaustion in 0.25 M KCl with 50 mM Tris/HCl, pH 7.3, and additional compounds as shown below. The reaction was started by addition of 0.5 mM (the final concentration) of potassium phosphate buffer, pH 7.3, labeled with ^{32}P (about 1 Ci/mol). Samples of 0.5 ml were withdrawn from the mixture and added into centrifugation cuvettes containing 5 ml of cold (0°C) dilution medium (0.25 M KCl) placed in an ice-bath to stop the reaction. Diluted samples were centrifuged with cooling at 20,000 g for 20 min, the supernatant was discarded, and the walls of cuvettes were well wiped. Then 5 ml of cold dilution medium was added, which flowed down the cuvette wall, and the process was repeated. The sediment was suspended in 2 ml of 0.1 *N* KOH, transferred into scintillation vials, and dried. After addition of 5 ml of the scintillation solution (1.25 g of 2,5-diphenyloxazole and 50 mg of 1,4-bis-(5-phenyl-2-oxazolyl)benzene in 1 liter of toluene), the samples were counted for radioactivity with a Tri-Carb spectrometer, model 3375 (Packard, U.S.A.). Alternatively, withdrawn samples were filtered through microporous filters of 0.45 μm pore size (Amicon, U.S.A.) which had been boiled in 50 mM phosphate buffer of pH 7.3 and washed with water before use to prevent P_i adsorption. Pellets collected on filters were washed twice with 5 ml of 0.25 M KCl, and the filter disks (13 mm in diameter) were counted for radioactivity. The specific activity of $^{32}P_i$ and a blank test for $^{32}P_i$ that adhered on the walls of cuvettes or filters were determined for each experiment.

For measuring ^{32}P bound in organic compounds, 0.5-ml samples were withdrawn from the same incubation medium and placed into 4 ml of 1.25 M $HClO_4$. Subsequently they were treated as described for ATP–P_i exchange measurement (Pullman, 1967).

Oxygen consumption was measured amperometrically with the Clark electrode. The reaction mixture was identical to that used for P_i transport measurement (with unlabeled P_i).

Results

Uptake of P_i

PCMB and mersalyl, which are most frequently used to stop P_i transport in the mitochondria, were found to be unsuitable for stopping P_i transport in *P. denitrificans*. This is due to the fact that mersalyl is a less potent inhibitor of P_i

uptake and PCMB is unable to prevent the efflux of accumulated P_i (see below). The uptake of P_i was followed in cells separated from the reaction medium by centrifugation or filtration. As can be seen from Fig. 1, both separation methods provided similar values. However, the centrifugation of cells appeared to be more reliable than the separation of cells by filtration. Blank values were much higher and the experimental variation larger with the latter method. The relatively longer time period needed for separation of cells by centrifugation was not the source of error of estimation because of the fact that the accumulation was considerably dependent on temperature. As follows from Fig. 2, the decrease of the incubation temperature from 20 to 0°C brought about a drop of the rate of P_i accumulation to only 4% of the original value. Two dilution media were compared: 0.25 M KCl and 0.25 M KCl with 50 mM Tris/HCl and 0.5 mM P_i. It was demonstrated that lower results were obtained when P_i was present in the dilution medium and, moreover, the first rapid phase of accumulation was absent. The difference between phosphate uptake without and with phosphate washing was constant whether 4 or 15

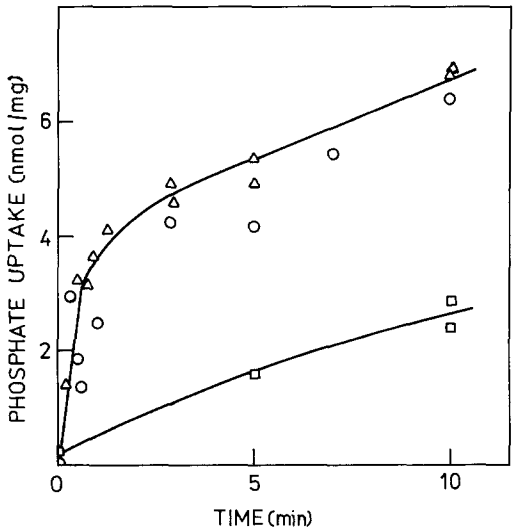

Fig. 1. Comparison of centrifugation and filtration methods for estimation of P_i accumulation. Cells (2.5 mg protein/ml) were stirred in 0.25 M KCl with 50 mM Tris/HCl, pH 7.3. At zero time, $^{32}P_i$ was added to make 0.5 mM concentration. Samples of 0.5 ml were withdrawn at intervals indicated and either added into the 5 ml of 0.25 M KCl at 0°C and centrifuged (△) or filtered through microporous filters (○). ^{32}P incorporated in organic compounds was determined in cells separated by centrifugation (□). For details see Materials and Methods.

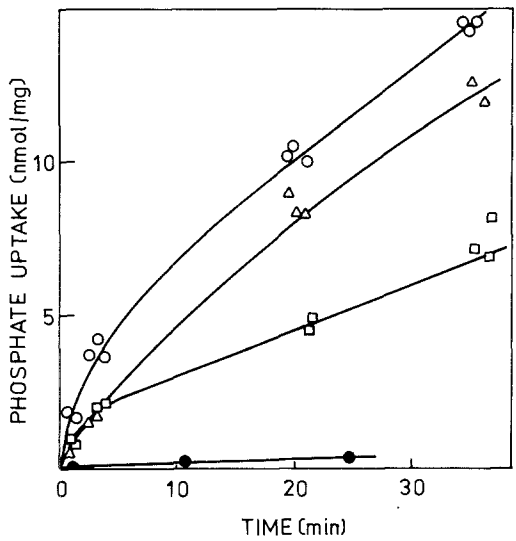

Fig. 2. Time course of P_i uptake and the effect of dilution medium and temperature. Conditions as in Fig. 1, centrifugation method, 2 mg of cell protein/ml. Incubation temperature: ●, 0°C; ○, △, □, 20°C. Substrate: □, endogenous; ●, ○, △, 20 mM L-lactate. Dilution medium: △, 0.25 M KCl with 50 mM Tris/HCl of pH 7.3 and 0.5 mM P_i; ●, ○, □, 0.25 M KCl.

nmol phosphate per mg protein have been taken up. We adjudged this phenomenon to the exchange of accumulated $^{32}P_i$ for outer unlabeled P_i which proceeded even at low temperatures. A constant difference in phosphate accumulated when using these two media for washing corresponds to the unesterified P_i pool within the cells which was exchanged when unlabeled P_i was present in the dilution medium. In the latter case the estimates of phosphate content in cells were represented mainly by esterified phosphate. The amount of ^{32}P-phosphate in cells in the dilution medium of 0.25 M KCl at 0°C was constant for at least 30 min. In the solution of pure KCl, P_i efflux did not occur, probably due to the sufficient pH gradient formed across the cell membrane which was maintained by the oxidation of endogenous substrates.

The uptake of P_i occurred in two phases. The first rapid phase was finished within several minutes (Figs. 1 and 2). The second, much slower phase showed a constant rate for tens of minutes, being almost equivalent to the rate of phosphate esterification. This could probably mean that only the rapid phase of P_i accumulation represented the actual P_i transport, the rate of which, under the described conditions, was apparently limited only by the activity of the carrier. The rate of P_i transport into cells or spheroplasts varied somewhat with individual cultivations, being about 2.7 nmol P_i/min · mg

protein by cells and 1.5 nmol P_i/min · mg protein by spheroplasts. It seems that after an internal P_i pool reaches its capacity, further P_i uptake is controlled by the rate of P_i utilization in the cytoplasmic space. The biphasic course of solute uptake was observed also by other authors. Medveczky and Rosenberg (1971) described this phenomenon for P_i uptake of *Escherichia coli*.

The energy demands of the transport were obviously covered by the endogenous substrate oxidation. The addition of L-lactate to the medium accelerated especially the slow phase of P_i accumulation. The effect on the rapid phase was not so pronounced (Fig. 2). The rate of oxygen consumption in the presence of 20 mM L-lactate was about twice as much as the endogenous respiration. The results with succinate were similar to those obtained with lactate. The P_i uptake was maximal in the range of pH 7.0–7.3.

Inhibition of P_i Uptake

Typical inhibitors of mitochondrial and bacterial P_i transport are compounds which react with SH groups. The effects of some inhibitors of this group on the P_i uptake by spheroplasts of *P. denitrificans* are compared in Fig. 3. At 1 mM concentration the most effective inhibitors were PCMB and

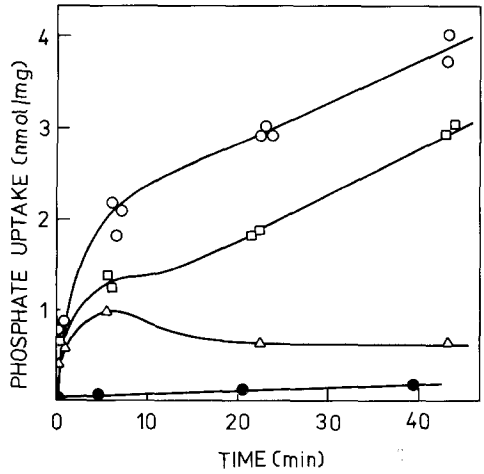

Fig. 3. Inhibition of P_i uptake by SH reagents. Conditions as in Fig. 1, 2 mg of spheroplast protein/ml, temperature 20°C, 20 mM L-lactate, centrifugation method. SH reagents (1 mM) were added 1 min before addition of $^{32}P_i$: ○, none; □, DTNB; △, mersalyl; ●, NEM or PCMB.

NEM, which inhibited almost completely both phases of accumulation. In contrast to its effect in mitochondria, mersalyl was much less effective and inhibited preferentially the slow phase of accumulation, while the first phase still proceeded at about 50% of the rate. Therefore it is not possible to use mersalyl as a specific inhibitor for abolition of P_i transfer across the cytoplasmic membrane of *P. denitrificans*. DTNB inhibited the first phase to approximately the same extent as mersalyl, but the slow phase was not inhibited. This could be because DTNB did not easily penetrate the membrane, as was reported by Guérin *et al.* (1970) with rat liver mitochondria.

The inhibitor of ATP synthesis, DCCD, inhibited both phases of P_i uptake (Fig. 4). The slow phase was more sensitive and was completely stopped by 2 μM DCCD, while the rapid phase was inhibited of about 50% at this concentration. At 10 μM DCCD concentration both phases of P_i accumulation were stopped.

The inhibitor of P_i/dicarboxylate exchange, *n*-butylmalonate, was almost without influence upon both phases of P_i accumulation at 1 mM concentration (now shown). At higher concentrations it inhibited markedly the slow phase; simultaneously the respiration was also inhibited. It followed from our results that under the described conditions, the P_i/dicarboxylate antiporter was not operative in the P_i uptake by cells of *P. denitrificans*. It seemed probable that most of P_i accumulated in the cell was passing through the P_i/H^+ symporter.

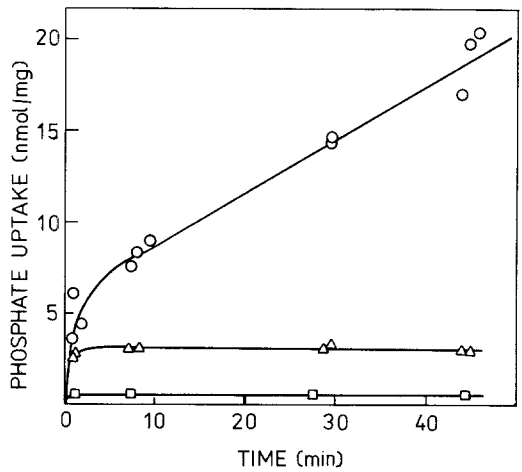

Fig. 4. Effect of DCCD on P_i uptake. Conditions as in Fig. 3, 2 mg of cell protein/ml: 20 mM succinate was used as substrate instead of lactate. DCCD was added 5 min before addition of $^{32}P_i$: O, none; Δ, 2 μM; □, 10 μM.

Efflux of Accumulated P_i

The addition of 1 mM PCMB stopped the P_i accumulation in spheroplasts but brought about an efflux of accumulated P_i, as can be seen from Fig. 5. The inability of PCMB to stop the efflux may be due to the lower accessibility of SH groups active in the transfer. In our experiments PCMB was added to the reaction mixture after the gradient of P_i on the cell membrane was generated ($c_{in} > c_{out}$). As a consequence of this gradient, the reorientation of the mobile P_i carrier to the cytoplasmic face of the membrane should occur (Guérin *et al.*, 1970). As proposed by Fonyó and Vignais (1980), the lower sensitivity of SH groups to the inhibitors of P_i transport observed after P_i accumulation was brought about by the change of the geometry of the polypeptide chain(s) of the P_i transporter. One μM FCCP neither induce nor accelerate the efflux of P_i caused by PCMB. FCCP was effective in releasing accumulated P_i only in spheroplasts preincubated with SCN^- ions (see Fig. 6). Burnell *et al.* (1975) have shown that the uptake of P_i by membrane vesicles of *P. denitrificans* was driven by the pH gradient. Our results could be interpreted to mean that the pH gradient was not effectively dissipated at 1 μM FCCP concentration or it could be continually regenerated under these conditions. In an earlier work, Scholes and Mitchell (1970) found that the preincubation of cells of *P. denitrificans* in the presence of SCN^- ions led to the marked enhancement of the FCCP-induced H^+ conductance of the

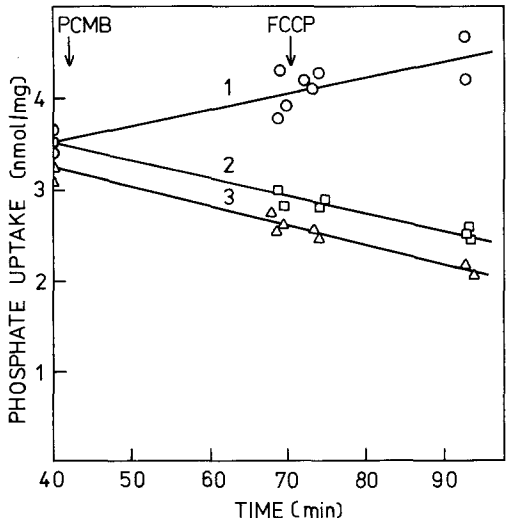

Fig. 5. PCMB-induced P_i efflux. Conditions as in Fig. 3, 1.8 mg of spheroplast protein/ml. Additions of 1 mM PCMB (experiments 2 and 3) or 1 μM FCCP (experiments 1 and 2) are indicated by arrows.

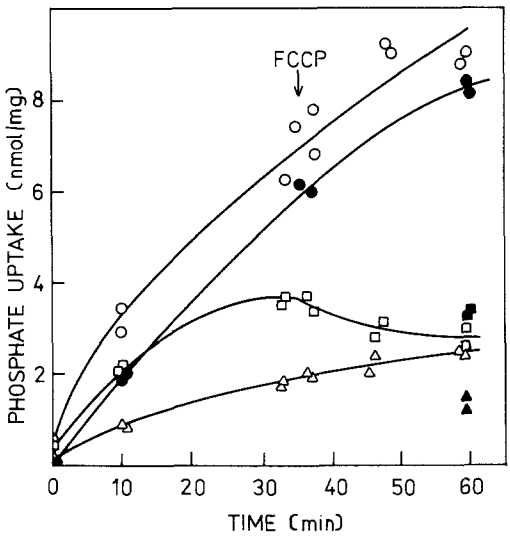

Fig. 6. Effect of FCCP on P_i transport in SCN^- or valinomycin-treated spheroplasts. Conditions as in Fig. 3. Spheroplasts were incubated 30 min with 25 mM KSCN (□, ■) or with 5 μg/ml valinomycin (△, ▲) before addition of $^{32}P_i$; ○, ●, controls. Open symbols represent total accumulated phosphate, and filled symbols represent organic phosphate.

membrane. The results of experiments carried out under these conditions indicated that the dissipation of pH gradient was accompanied by an efflux of accumulated P_i. FCCP released but a part of accumulated phosphate. Most of the accumulated phosphate is bound in organic compounds (see Fig. 5). The rate of organic phosphate formation was nearly identical with the slow phase of P_i uptake. After FCCP-induced phosphate efflux, all residual phosphate found seemed to be the organic phosphate. The P_i not esterified diffused out of the cytoplasma into the medium. Both SCN^- and especially valinomycin strongly decreased the rate and the amount of totally accumulated P_i. P_i transport was more sensitive to SCN^- in spheroplasts than in cells. SCN^- (5 mM) lowered the rate of uptake in spheroplasts; in cells, even 10 mM SCN^- produced mild stimulation. The decrease of P_i uptake after the spheroplasts were preincubated with valinomycin was apparently caused by the dissipation of the gradient of potassium ions. This indicates that potassium ion movement may play a role in the P_i transport in the *P. denitrificans* cells. However, the effect of potassium seems to be indirect, lying mainly in the interconversion between $\Delta\psi$ and ΔpH. Russell and Rosenberg (1979, 1980) have shown that the uptake of K^+ and P_i by the cells of *E. coli* are linked via the proton circulation.

Discussion

The P_i uptake by cells and spheroplasts of *P. denitrificans* proceeded in two phases, in contrast to the uptake of P_i by membrane vesicles derived from *P. denitrificans* (Burnell *et al.*, 1975). Our values of the rate of P_i transport calculated on the protein basis for the first minute of the reaction were considerably lower than 16 nmol P_i/min · mg protein found by Burnell *et al.* (1975). This could be due to the fact that in the cells and spheroplasts proteins related to transport processes represent only a minor part of total protein. The first rapid phase was not an artefact which could arise from P_i adsorption and binding to the outer surface of spheroplasts but represented the actual P_i transport. Evidence of it has come from the inhibitory effect of some specific inhibitors, as was found with NEM and PCMB which completely prevented the P_i uptake (Fig. 3). The rapid phase could not be attributed to the initial rapid exchange of external P_i for endogenous dicarboxylates. P_i/dicarboxylate exchange in mitochondria was not sensitive to NEM, but it was inhibited by *n*-butylmalonate (Coty and Pedersen, 1975). In spheroplasts the rapid phase of P_i accumulation was inhibited by NEM, but *n*-butylmalonate was almost without influence. The slow phase of P_i accumulation seemed to be limited by the rate of P_i esterification inside the cells.

The sensitivity of the P_i carrier of *P. denitrificans* to typical SH reagents resembled closely that of mitochondria with the exception of mersalyl. Mersalyl, which was recognized as a potent inhibitor of the P_i uptake and efflux in mitochondria (Guérin *et al.*, 1970), was only slightly inhibitory in spheroplasts. This can probably be explained by the lower accessibility of the carrier of *P. denitrificans* even in the case of spheroplasts, i.e., when the cell wall was removed. PCMB at 1 mM concentration completely prevented the P_i uptake, but its addition to spheroplasts preloaded with P_i brought about the P_i efflux. It is possible that this net efflux was directly induced by the presence of PCMB, e.g., it took place concomitantly with the P_i uptake and was made observable only after P_i uptake inhibition. The sensitivity of the P_i efflux to PCMB was much less in comparison to the sensitivity of the uptake. This was probably caused by the necessity of the penetration of the inhibitor through the cell membrane to reach the active site oriented to the cytoplasmic side. Burnell *et al.* (1975) used 10 mM concentration of methylmercuric chloride in order to stop the efflux of P_i accumulated in membrane vesicles of *P. denitrificans*, NEM at 10 mM concentration was found to be much less effective. However, at these concentrations both PCMB and NEM strongly affected the respiration of *P. denitrificans.*

The inhibition brought about by DCCD supports the view that the slow phase of P_i accumulation is limited by its utilization, thus being dependent on ATP formation. It is known that DCCD prevents the ATP formation at low

concentration, which can result in the observed inhibition of the slow phase of P_i uptake. The rapid phase was also inhibited, but to reach complete inhibition higher concentrations of DCCD were needed (Fig. 4). This could be related to the effect of DCCD on P_i transporter. Houštěk *et al.* (1981) described the binding of DCCD to the P_i/H^+ translocator which, however, was only slightly modified in its activity.

The mutual interdependence between the transport of P_i and K^+ and the K^+ cycling in *E. coli* were recently studied (Russell and Rosenberg, 1979, 1980; Kroll and Booth, 1981). Some of the findings could be valid even in the case of *P. denitrificans*, as was suggested in the work of McCarthy *et al.* (1981), who were able to demonstrate a decrease of the membrane potential by more than 20 mV and 2.3-fold stimulation of respiration in the media containing a high concentration of phosphate (100 mM) together with at least 1 mM K^+. Fonyó *et al.* (1981) suggested that K^+ accelerated the dissociation of $H_2PO_4^-$ in rat liver mitochondria, producing nondiffusible HPO_4^{2-}; this could increase the rate of $H_2PO_4^-$ entry. Erecińska *et al.* (1981) found that *P. denitrificans* accumulated K^+ at high velocity against a large concentration gradient and that this process included the ATP-driven transport systems. This could be particularly acute with regard to the apparent unidirectional properties of ATPase of *P. denitrificans* which cannot drive proton translocation (Ferguson *et al.*, 1976). Although the ability of the K^+ gradient to provide a significant energy storage capacity in *P. denitrificans* cells has not been unambiguously proved (McCarthy *et al.*, 1981), it cannot be excluded that the ATP-driven K^+ gradient may substitute for the gradient in pH and exert an effect on the transport of P_i.

References

Burnell, J. N., John, P., and Whatley, F. R. (1975). *FEBS Lett.* **58,** 215–218.

Coty, W. A., and Pedersen, P. L. (1974). *J. Biol. Chem.* **249,** 2593–2598.

Coty, W. A., and Pedersen, P. L. (1975). *Mol. Cell. Biochem.* **9,** 109–124.

Erecińska, M., Deutsch, C. J., and Davis, J. S. (1981). *J. Biol. Chem.* **256,** 278–284.

Ferguson, S. J., John, P., Lloyd, W. J., Radda, G. K., and Whatley, F. R. (1976). *FEBS Lett.* **62,** 272–275.

Fevold, H. L., and Alderton, G. (1949). In *Biochemical Preparations* (Carter, H. E., ed.), Vol. 1, Wiley-Interscience, New York, pp. 67–69.

Fonyó, A., and Vignais, P. V. (1980). *J. Bioenerg. Biomembr.* **12,** 137–149.

Fonyó, A., Lukács, G., and Ligeti, E. (1981). *Acta Biol. Med. Ger.* **40,** 907–914.

Guérin, B., Guérin, M., and Klingenberg, M. (1970). *FEBS Lett.* **10,** 265–268.

Houštěk, J., Pavelka, S., Kopecký, J., Drahota, Z., and Palmieri, F. (1981). *FEBS Lett.* **130,** 137–140.

John, P., and Whatley, F. R. (1977). *Adv. Bot. Res.* **4,** 51–115.

Kroll, R. G., and Booth, I. R. (1981). *Biochem. J.* **198,** 691–698.

Lowry, O., Rosenbrough, N., Farr, A., and Randall, R. (1951). *J. Biol. Chem.* **193,** 265–275.

McCarthy, J. E. G., Ferguson, S. J., and Kell, D. B. (1981). *Biochem J.* **196,** 311–321.

Medveczky, N., and Rosenberg, H. (1971). *Biochim. Biophys. Acta* **241,** 494–506.
Pullman, M. E. (1967). *Methods Enzymol.* **10,** 57–60.
Rosenberg, H., Gerdes, R. G., and Chegwidden, K. (1977). *J. Bacteriol.* **131,** 505–511.
Russell, L. M., and Rosenberg, H. (1979). *Biochem. J.* **184,** 13–21.
Russell, L. M., and Rosenberg, H. (1980). *Biochem. J.* **188,** 715–723.
Scholes, P., and Mitchell, P. (1970). *J. Bioenerg.* **1,** 61–72.
Wehrle, J. P., Cintrón, N. M., and Pedersen, P. L. (1978). *J. Biol. Chem.* **253,** 8598–8603.
Willsky, G. R., Bennett, R. L., and Malamy, M. H. (1973). *J. Bacteriol.* **113,** 529–539.