Properties of the Pantothenate Transport System in

Pseudomonas fluorescens P-2

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Features of the uptake system of pantothenate in *Pseudomonas fluorescens* P − 2 were studied. The entry of pantothenate into the resting cells occurred against a concentration gradient, following saturation kinetics.

A K_m value of 3×10^{-5}M, a temperature optimum of $40 − 45°$ and a pH optimum of $6.5 − 8.0$ were obtained.

The entry reaction was energy-dependent, being inhibited by 2,4-dinitrophenol and sodium azide. The reaction was also inhibited by some mono and dicarboxylic acids, and slightly by glucose, but not by glycerol or by some amino acids. The entry reaction was activated by β-alanine and formate.

Almost all the transported label was recovered from the cells by washing with cold water or diluted salt solutions. Na^+, K^+, Li^+, and NH_4^+ ions $(50 − 100$ mM) prevented the exit of pantothenate almost completely. The bivalent cations Mg^{2+} and Ca^{2+} were not so effective as the monovalent ones in preventing the exit.

The existence of inducible pantothenate binding material was found in shock fluid prepared from cells grown on pantothenate.

There is ample evidence that uptake of many substrates in a variety of micro-organisms is an active transport process, because the inhibitors of oxidative phosphorylation and electron transport, such as 2,4-dinitrophenol and azide, inhibit accumulation of the substrate against a concentration gradient.[1−3]

The mechanism of these transport processes is not yet fully understood. Many reports suggest that the phosphoenolpyruvate phosphoenoltransferase system is linked to carbohydrate transport and that sugars are accumulated as their phosphate ester.[4,5] According to another theory, permeation itself is not energy-dependent, but metabolic energy is required to raise the concentration levels of transported sugars within the cell.[6] Phibbs and Eagon[3] demonstrated that glucose, fructose, and mannitol are transported in the unaltered state in *Pseudomonas aeruginosa* and then trapped by phosphorylation *via* kinases. In noninduced cells carbohydrates may be transported by diffusion.

The mechanism of amino acid transport is likewise still obscure. Investigations with many micro-organisms suggested the existence of two pools, one of them being used for protein synthesis and the other allowing accumulation of amino acids.[7,8] Metabolic energy appears necessary to make transport irreversible.[9] Many of these amino acid transport processes are competitively inhibited by other amino acids and closely related compounds.[10-12] The entry and exit reactions are thought to be independent.

Koch [13] and Gryder and Adams [14] reported the effect of metabolic inhibitors on the influx and efflux to be different, and Horecker, Thomas and Monod [15] even presented the hypothesis that the exit of galactose in *Escherichia coli* ML is inducible and the uptake of galactose is constitutive.

Binding proteins for leucine,[16] arginine,[17] tryptophan,[18] phenylalanine,[19] galactose,[20] and arabinose [21] have been purified from shock fluid. Although the exact role of these binding proteins is not known, some of their features have been characterized. They have been shown to be surface proteins with molecular weights of *ca.* 25 000 − 35 000. They are fairly stable to heat and changes in pH and bind one substrate molecule.

In the present study evidence was obtained for the existence of an active uptake system for pantothenate in *Pseudomonas fluorescens* P-2. This transport was energy-dependent, was inhibited by some mono and dicarboxylic acids and activated by β-alanine and formate. The maximal uptake was found at $40 − 45°$ and at pH $6.5 − 8.0$. The K_m value of $3 \times 10^{-5}M$ at pH 7.4 was obtained.

The exit of label was prevented by K^+, Na^+, Li^+, and NH_4^+ and partly by the bivalent cations Mg^{2+} and Ca^{2+}. The inducible binding activity for pantothenate was found in the concentrated shock fluid.

EXPERIMENTAL

Materials. [14C]-D-Pantothenate was obtained from the New England Nuclear Corporation, Boston, Mass., USA, 2-oxoisovalerate from Sigma, St. Louis, Mo., USA, D-pantothenate, β-alanine, formic acid, glycine, citric acid, succinic acid, glutamic acid, iodoacetamide, propionic acid, and glyoxylic acid from Fluka AG, Buchs, Switzerland. Chloramphenicol was purchased from Parke, Davis & Co., Mich., USA.

Cultures. Pseudomonas fluorescens P-2 was grown with aeration on the pantothenate medium as described earlier [22,23] and growth was estimated from turbidity measurements made with a Klett-Summerson colorimeter, employing filter 62. The cells (0.170 mg of dry weight) used for uptake experiments were collected from the exponential phase in a refrigerated centrifuge and washed with cold 0.05 M phosphate buffer (pH 7.5). Activity was determined immediately.

Determination of transport activity. The reaction mixture contained 0.1 μmol of D-pantothenate, 40 000 cpm of [14C]-D-pantothenate (specific activity 4.75 mC/mmol), 0.17 mg of bacteria (dry weight) and 20 μg of chloramphenicol in 0.2 ml of salt solution.[22] Exit of the accumulated pantothenate was prevented by stopping the reaction after incubation for 1 min at 30° with cold 10 mM pantothenate medium containing 0.15 M sodium chloride (in some experiments 0.10 M sodium propionate was used). The cells were filtered on a 0.30 μm pore-size membrane filter (Millipore Corp. Bedford, Mass., USA) and washed for 2 min with the same cold sodium chloride solution. The filters were dried and radioactivity was measured in a Wallac liquid scintillation spectrometer, using toluene-based scintillation fluid.

In the exit studies the cells were incubated for 2 min at 30° and the accumulated radioactivity was washed out with different washing systems.

Preparation of shock fluid. The procedure of Neu and Heppel,[24] with some modifications, was used for the preparation of binding material. The freshly grown cells (820 mg

of dry weight) were suspended in 50 ml of 0.05 M Tris-HCl buffer (pH 7.5) containing 0.001 M EDTA and 20 % sucrose. The solution was incubated for 15 min at 30°. The cells were collected with centrifugation and osmotically shocked by resuspending them in 50 ml of cold 0.5 mM magnesium chloride. After the cells had been shaken at 0° for 10 min and centrifuged (15 000 g, 10 min), the supernatant was lyophilized and buffered by addition phosphate buffer (pH 7.5) to 0.05 M. The lyophilizate was used as binding material.

Binding assay. The shock fluid (20 μg of protein) was incubated for 1 min at 4° with 0.05 mM D-pantothenate and 40 000 cpm of [14C]-pantothenate in 0.4 ml of salt solution.[22] A 0.1 ml portion of the reaction mixture was passed through a Sephadex G-25 column (0.3 cm × 20 cm). The column was developed with the same radioactive pantothenate solution and the radioactivities of the fractions (0.09 ml) were measured in scintillation fluid containing 0.4 g PPO (2,5-diphenyloxazole), 40 mg dimethyl POPOP (1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene), 6 g naphthalene, 90 ml 1,4-dioxane, 10 ml methanol, and 2 ml ethylene glycol/100 ml.

Protein determination. Protein was determined by the method of Lowry *et al.*[25] using bovine serum albumin as standard.

RESULTS

[14C]-Pantothenate uptake began immediately after the addition of pantothenate to the cell suspension. This uptake continued at a linear rate for 2 min when the cells had been precultivated in the pantothenate medium (Fig. 1). When precultivated in the β-alanine or glucose media the uptake of radioactivity was linear for no longer than 2 min, although the initial rates
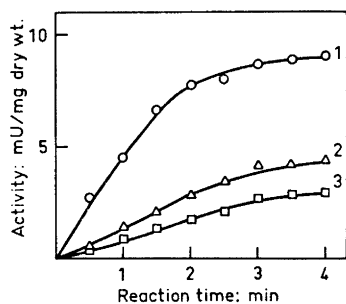

Fig. 1. The time course of D-pantothenate uptake. At zero time 0.17 mg of bacteria (dry weight) were incubated in 0.2 ml of salt solution [22] at 30° with 0.1 μmol of pantothenate and 40 000 cpm of [14C]-pantothenate (specific activity 4.75 mC/mmol). At intervals of 30 sec the reactions were stopped by adding 1 ml of ice-cold 0.15 M sodium chloride. The samples were immediately filtered and washed for 2 min with the same ice-cold salt solution. The filters were dried and counted in a liquid scintillation spectrometer. 1, precultivation was carried out in pantothenate; 2, precultivation was carried out in glucose; 3, precultivation was carried out in β-alanine.
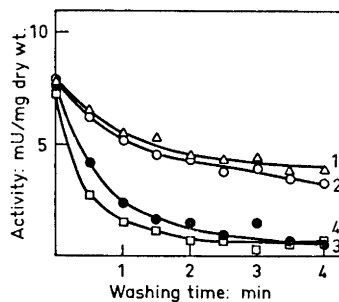
Fig. 2. Effect of different washing systems on the exit of pantothenate. The cells (0.17 mg dry weight in 0.2 ml) were incubated for 2 min at 30° in the presence of 0.1 μmol of pantothenate and 40 000 cpm of [14C]-pantothenate (specific activity 4.75 mC/mmol). After incubation, the cells were washed with different solutions; 1, with 10 mM K-pantothenate; 2, with 10 mM NaCl; 3, with 5 % TCA; 4, with water.

had strongly decreased. Not only entry rates, but also exit rates regulate the accumulation of substrates. When the cells were allowed to accumulate [^{14}C]-pantothenate for 2 min at 30° and the cells were then washed with cold water or 5 % TCA on a membrane filter, a rapid decrease in radioactivity was found (Fig. 2). When the cells were washed with cold 10 mM pantothenate or 10 mM sodium chloride the exit decreased sharply.

Fig. 3 shows the results of the experiments where the washings were carried out with 10, 25, 50, and 100 mM sodium chloride, potassium chloride, ammonium chloride, and lithium chloride. Since the concentration of these salt solutions was 50 mM, the initial exit rates were almost constant troughout the time of the experiments.

Magnesium chloride and calcium chloride were not so effective as the monovalent cations studied in preventing the exit of pantothenate (Fig. 3).

When chloride was displaced with sulphate, carbonate or nitrate, the results were almost identical with those of Fig. 3 (Fig. 4).
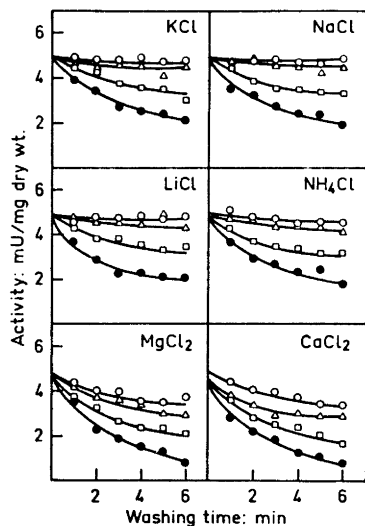

Fig. 3. Effect of several monovalent and two bivalent cations on the exit of pantothenate. Other conditions were the same as in Fig. 2. The cation concentrations were (●) 10 mM; (☐) 25 mM; (△) 50 mM; (○) 100 mM.
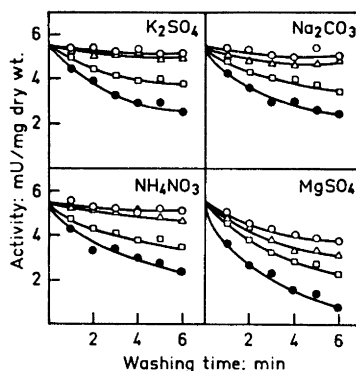
Fig. 4. Effect of some mono and bivalent anions on the exit of pantothenate. Other conditions were the same as in Fig. 2. The anion concentrations were (●) 10 mM; (☐) 25 mM; (△) 50 mM; (○) 100 mM.

The uptake of pantothenate followed saturation kinetics. Fig. 5 shows a reciprocal plot of initial velocity of uptake *versus* substrate concentration. The concentrations were varied from 10 to 500 μM and initial velocities were measured by stopping the reactions at intervals of 30 sec during the first 2 min. The apparent K_m for pantothenate uptake was 3×10^{-5} M. However, it seems evident that there also exists another uptake system for pantothenate, the K_m value of which is much higher (Fig. 5).
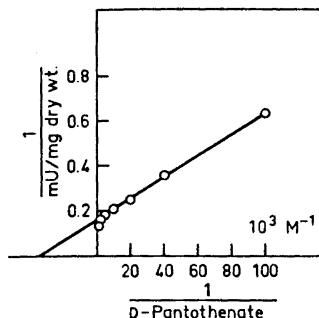
Fig. 5. A reciprocal plot of initial velocities of uptake *versus* substrate concentration. Experimental conditions were as described in the legend to Fig. 1.
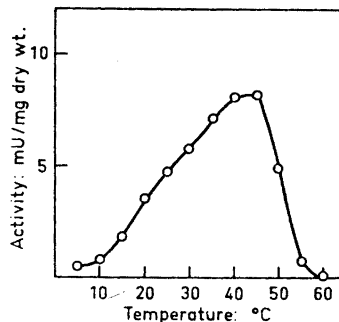
Fig. 6. Temperature dependence of pantothenate uptake in the cells precultured on pantothenate. Other conditions were as described in the legend to Fig. 1.

The uptake of pantothenate was also temperature-dependent. Below 10^c and above 50° the accumulation of label was negligible. The maximum activity was reached at $40-45°$ (Fig. 6). The components of the reaction mixture were preincubated for 2 min at the temperature to be studied. When the preincubation time was longer the uptake of radioactivity declined sharply at higher temperatures.

Hydrogen ion concentration had only a weak effect on the entry of pantothenate near neutrality. Between pH values of 6.5 and 8.0 the uptake was very similar. Below pH value 6.5 and above 8.0 a marked decline in activity was observed (Fig. 7).
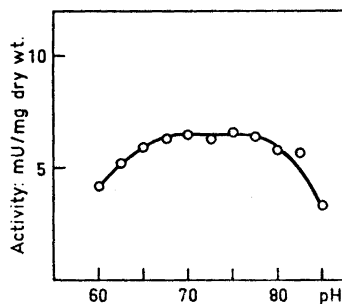
Fig. 7. pH dependence of pantothenate uptake. Other conditions were as described in Fig. 1.

The entry reaction was sensitive to energy poisons. If cells grown on pantothenate were preincubated for 10 min in the presence of 10^{-3} M 2,4-dinitrophenol or sodium azide, initial rates diminished by 55 % and 34 %, respectively. Iodoacetamide (10^{-3} M) and *p*-chloromercurybenzoate (10^{-4} M) were tested as inhibitors of entry and exit. Both compounds were found to inhibit the influx and enhance the release of label, although the inhibitions of entry were only 24 % and 17 % (Table 1).

Table 1. The effect of energy poisons and SH inhibitors on the uptake of pantothenate. The reaction mixture (0.2 ml) contained 0.5 μmol of pantothenate, 40 000 cpm of [^{14}C]-pantothenate (specific activity 4.75 mC/mmol), 0.17 mg of bacteria (dry weight) and an inhibitor. The radioactivity accumulated during 1 min was measured in a liquid scintillation spectrometer after the cells had been washed for 2 min with cold 0.1 M sodium chloride on a membrane filter (pore size 0.3 μm). In exit studies the washing solutions contained either 10^{-3} M iodoacetamide, 10^{-4} M p-chloromercurybenzoate or 10^{-1} M sodium chloride.

Inhibitor		Entry %	Exit %
—		100	—
2,4-Dinitrophenol	(10^{-3} M)	45	—
Sodium azide	(10^{-3} M)	66	—
Iodoacetamide	(10^{-3} M)	76	56
p-Chloromercury-benzoate	(10^{-4} M)	83	74
Sodium chloride	(10^{-1} M)	—	8
Cold water		—	90

A large number of other compounds inhibited the uptake of pantothenate. Fig. 8 is a summary of these data. Malate, succinate, propionate, and 3,3-dimethylmalate were the most effective inhibitors of pantothenate transport. β-Alanine and formate activated this transport process.
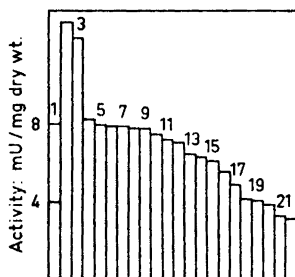

Fig. 8. Effect of metabolites on the uptake of pantothenate. The concentration of the metabolites was 2 mM. Other experimental conditions were those described in the legend to Fig. 1. 1, control; 2, β-alanine; 3, formate; 4, glycine; 5, L-alanine; 6, glycerol; 7, glucose; 8, citrate; 9, pantoate; 10, isobutyraldehyde; 11, glyoxylate; 12, glyoxylate + (NH$_4$)$_2$SO$_4$; 13, lactate; 14, 2-oxoisovalerate; 15, pyruvate; 16, acetate; 17, glucose (the cells were precultured for 2 h on glucose); 18, glutamic acid; 19, succinate; 20, 3,3-dimethylmalate; 21, propionate; 22, malate.
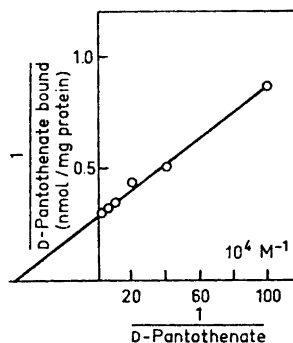

Fig. 9. Effect of pantothenate concentration on binding to concentrated shock fluid. The reaction mixture containing 5 μg of protein, 0.05 mM pantothenate and 10 000 cpm of [^{14}C]-pantothenate (specific activity 4.75 mC/mmol) in 0.1 ml was passed through a Sephadex G-25 column. The fractions collected were 0.09 ml.

When cells grown on pantothenate were osmotically shocked, they lost a notable proportion of their transport activity and the binding activity was easily demonstrated in concentrated shock fluid using equilibrium gel filtration and Millipore techniques. The binding reaction was very rapid and the binding rates were the same for 1-min and 10-min reactions. When the shock fluid had been prepared from cells grown on β-alanine, no detectable binding activity was found in the lyophilizate, which suggested that the binding material is closely linked with the pantothenate transport system in *Pseudomonas fluorescens* P-2 cells.

The true K_m value of the binding reaction was difficult to measure, because the background values were about 50 % above the values of the enzyme-substrate complex. Fig. 9 shows a reciprocal plot of binding *versus* pantothenate concentration. The apparent K_m value was 2×10^{-6} M.

DISCUSSION

The conclusion to be drawn from these studies is that an active pantothenate transport system exists in *Pseudomonas fluorescens* P-2 and is at least partly induced by pantothenate. The mechanism of this transport is still largely obscure, although some of its main features can be simulated with transport models.[2,9,13,26-28]

This uptake is a very rapid procedure, which depends on the concentration of pantothenate. The plateau region is achieved in 2 min. Analogously, the accumulation of tryptophan in *Escherichia coli*,[29] many amino acids in *Pseudomonas aeruginosa*[30] and folinate in *Pediococcus cerevisiae*[31] achieve the state of saturation in a few minutes. The limiting factor in pantothenate transport can be thought to be the slow intracellular metabolism. Although the specific activities of pantothenate hydrolase, β,β-dimethylmalate dehydrogenase, and the pantothenate transport system are about 3×10^{-1}, 4×10^{-2}, and 6×10^{-3} units/mg dry weight, it can be assumed, that the concentrations of intermediates cannot saturate all the degradative enzymes, and that this failure prevents the reaction from proceeding maximally.

Wong *et al.*[32] have reported that Na^+ prevents the release of α-aminoisobutyric acid from *Pseudomonas* species, and Stern and Sachan[33] that it inhibits the release of citrate from *Aerobacter aerogenes*. In Fig. 2 we can see that the accumulation rate continued linearly for over 10 min when the cells were washed with cold water. This suggests that the exit velocity is very rapid at a low ion concentration.

The effect of mono and bivalent cations on the transport of pantothenate indicates the independence of the entry and exit mechanisms. Although K^+, Na^+, Li^+, NH_4^+, and to some extent Mg^{2+} and Ca^{2+} prevented the release of pantothenate from the cells, NH_4^+ or Ca^{2+} could not replace K^+, Na^+ or Li^+ in the entry reaction. Furthermore, the concentration needed for the optimal entry reaction was different from the concentration needed to prevent the release of accumulated pantothenate. Mg^+ enhanced the entry of pantothenate catalyzed by K^+ (results not shown).

Pseudomonas aeruginosa was found to accumulate [^{14}C]-valine maximally at 45°, although no growth occurred at this temperature.[30] No growth of

Pseudomonas fluorescens P-2 was found at temperatures above 35°, although the transport activity was maximal at 40 − 45°. Nurmikko *et al.*[34] reported that pantothenate hydrolase, the first enzyme on the pathway to pantothenate degradation, was very sensitive to heat, and that denaturation of the enzyme was almost complete in 5 min *in vitro*. The situation may be the same *in vivo*. Although pantothenate transport is rapid at high temperatures. it cannot protect pantothenate hydrolase against inactivation.

The effect of energy poisons and some other inhibitors on pantothenate uptake was similar to other transport systems, suggesting an active uptake mechanism of pantothenate. There are also several reports in the literature concerning inhibition of transport systems by glucose, amino acids and many metabolites.[6,10,11,12,35] Some of these inhibitions are competitive, the inhibitors being transported by the same entry system. If β-alanine is an activator of pantothenate uptake, it can be argued that malate, succinate, propionate, 3,3-dimethylmalate, *etc.*, cause inhibition by occupying the activator site. However, because the uptake of pantothenate started immediately and continued linearly for 2 min, the concentration of the β-alanine formed ought to be sufficient for the activation from the very beginning of the reaction. and the site of hydrolysis ought to be near the transport system.

The effect of formate is also difficult to understand. Boezi and deMoss [35] explained that formate is unable to serve as precursor of inhibitor but only to provide energy for the transport system. However, it is improbable that all carbon compounds give rise to entry inhibitors in a few minutes. For example, glucose may be totally inactive or it may even activate the transport system.[14,27] When *Pseudomonas fluorescens* P-2 was precultivated on pantothenate or pantoate and then for only 2 h in a glucose medium, the inhibitory effect was strongly enhanced (Fig. 8). I can therefore suggest that although the addition of glucose to the uninduced cells has only a weak inhibitory effect on pantothenate uptake "*in vitro*", the induction of the glucose pathway produces metabolites which inhibit the entry of pantothenate *in vivo*.

The transport systems as well as the binding proteins have a high affinity for their substrates, the K_m values usually being $10^{-4} - 10^{-6}$ M. Pantothenatebinding material had an affinity for pantothenate about 15 times as great as that of whole cells. Analogously, the K_m for the binding of phenylalanine has been shown to be less than one-hundredth of that of whole cells [19] and for the binding of arabinose it has a K_m value one-twentieth of that of whole cells.[21] These results suggest that binding material is only part of the whole permeation system. Leive *et al.*[36] have reported the release of lipopolysaccharides from *Escherichia coli* and some *Salmonella* species, and Tucker and White [37] found the release of phospholipids from *Haemophilus parainfluenzae* during incubation in EDTA and EDTA − Tris buffer. These results and others suggest that also lipids may play a role in this transport process.

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