Correlations between Pantothenate Uptake, Phospholipid Synthesis and Pantothenate-binding Protein Formation in *Pseudomonas fluorescens* P-2

P. MÄNTSÄLÄ

Department of Biochemistry, University of Turku, SF-20500 Turku 50, Finland

Correlations were investigated between increased pantothenate uptake and the formation of pantothenate-binding protein and phospholipids in *Pseudomonas fluorescens* P-2. Ethanolamine, choline, sarcosine, glyoxylate, and serine increased pantothenate uptake in whole cells, even in the presence of chloramphenicol. On the other hand, pantothenate, glycerol, and glucose were the most effective of the compounds investigated in synthesizing phospholipids both in whole cells and in membrane vesicles prepared from *Pseudomonas fluorescens* P-2. Although growth on ethanolamine, choline, and sarcosine was negligible over several hours, incorporation of ^{32}P continued linearly from the very beginning. The concentration of total phospholipids and phosphatidyl ethanolamine followed cell growth quite closely.

Pantothenate-binding protein was formed only in the presence of pantothenate and pantoate.

Correlation was observed between pantothenate uptake and formation of pantothenate-binding protein when the cells were cultured on pantothenate and pantoate, whereas phospholipid synthesis was rapid on any of several good energy sources.

Active transport systems of several B-group vitamins and related compounds and their regulation in micro-organisms have been reported.¹⁻⁷ It is sometimes difficult to determine the type of regulation operating in the accumulation of compounds, because regulation of intracellular concentrations of the vitamins as well as other metabolites involves control not only of their biosynthesis but also of entry and exit of the compounds. It has long been recognized that this process is rarely one of

passive diffusion: it involves specific carrier- or binding-proteins having affinities for their substrates.⁸ Furthermore, suggestions have been made that a lactose transport system might require the synthesis of membrane lipids.^{9,10}

In previous studies we have found pantothenate transport activities in *Pseudomonas fluorescens* P-2 to increase during growth on glyoxylate, glycolate, and glycine, even when growth was inhibited with chloramphenicol.⁷ The present work investigates whether there are correlations between pantothenate transport activities in the whole cells, phospholipid synthesis and the formation of pantothenate-binding protein during the growth of *Pseudomonas fluorescens* P-2.

METHODS

Organism and growth conditions. *Pseudomonas fluorescens* P-2 was cultured aerobically at 30°C in media containing, per litre, 1.35 g of KH_2PO_4 , 0.26 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 3 mg of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 2.1 mg Na_2MoO_4 , 1.5 mg of MnSO_4 and 10 mmol of a carbon source.¹¹ 0.66 g of $(\text{NH}_4)_2\text{SO}_4$ was added as a source of nitrogen when carbon compounds containing no nitrogen groups were used as energy sources. The media were adjusted to pH 7.0 and applied immediately.

The strain was maintained on slopes of a pantothenate agar medium. Turbidity measurements were made with a Klett-Summerson colorimeter employing filter 62.

Preparation of protoplasts and membrane vesicles. Protoplasts and membrane vesicles were prepared by the lysozyme-EDTA method of Kaback.¹² Cells, taken from different growth

phases (20 mg of dry weight), were centrifuged at 10 000 *g* for 30 min and washed twice with 10 mM Tris-HCl (pH 8.0). The washed cells were suspended in 30 mM Tris-HCl buffer containing sucrose (20 %), dipotassium magnesium EDTA (15 mM), and lysozyme (0.5 mg/ml, Sigma Chemical Company, St. Louis, Mo., U.S.A.) and incubated for 20 min at 25°C. Protoplasts were collected by centrifugation for 15 min at 16 000 *g*. Lysis of protoplasts was produced by osmotic treatment in the presence of DNase II (Sigma Chemical Company, St. Louis, Mo., U.S.A.). Incubations were carried out at 30°C and centrifugations at 27 000 *g*. The purity of membrane vesicles was established by electron and phase contrast microscopy methods.

Measurement of pantothenate uptake into whole cells. An assay method for [^{14}C]-labelled pantothenate uptake into whole cells was described earlier by Mäntsälä.¹³ The initial velocities of pantothenate uptake were determined by incubation of the cells (250 μg of dry weight), [^{14}C]-pantothenate (75 000 cpm, specific activity 4.75 mCi/mmol, The New England Nuclear Corporation, Boston, Mass., U.S.A.), potassium pantothenate (0.05 μmol) and $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.5 μmol) in 0.2 ml of the reaction mixture at 30°C for various reaction times.

Measurement of pantothenate uptake into membrane vesicles. In total volume of 0.2 ml, the reaction mixtures contained 4 μmol of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 10 μmol of potassium phosphate buffer (pH 6.6), 75 000 cpm of [^{14}C]-pantothenate, 0.01 μmol of potassium pantothenate, of 4 μmol ascorbate, 0.02 μmol of phenazine methosulfate, and 0.16 mg of membrane protein. Reactions were terminated by addition of 2 ml of 0.2 M NaCl, and membrane vesicles were washed immediately with 8 ml of 0.2 M NaCl on Millipore membrane filters (0.3 μm pore-size). The filters were dried and activities were measured in a scintillation spectrometer.

Phospholipid extraction and estimations. Samples (1 mg of dry weight) were taken at intervals during growth and centrifuged at 5000 *g*. The cell pellets were washed with phosphate-free medium and re-centrifuged. The samples of whole cells and membrane vesicles (as prepared above) were inoculated into 20 ml of media containing about 2 μCi of [^{32}P]-labelled phosphate/ml, (The Radiochemical Centre, Amersham, U. K.), 10 μmol of potassium phosphate and other compounds, as described in the "Organism and growth conditions section". After incubation at 30°C, the samples were precipitated with 2 ml of cold 50 % TCA, filtered and washed twice with 3 ml of 5 % TCA and 3 ml of distilled water on a Millipore filter (0.3 μm pore-size). The filters were then dried and placed in extraction vessels from which phospholipids were eluted with 5 ml of a methanol-chloroform solution (1:2, by vol).

The extraction was repeated twice at room temperature overnight. The solutions were poured into scintillation vials, the solvent removed *in vacuo* and activities counted in toluene-based scintillation fluid.

Chromatography was carried out on glass plates with a layer of silicic acid. Two-dimensional separations were made. The first solvent used was chloroform-methanol-water (70:30:5 by vol) and the second chloroform-methanol-7 M NH_4OH (60:35:5 by vol). After development the plates were sprayed with 0.2 % ninhydrin and then with molybdenum reagent.¹⁴ The standard phospholipids were purchased from Koch-Light Laboratories Ltd., Colnbrook, Buckinghamshire, U.K. For quantitative determination of phosphatidyl ethanolamine and phosphatidyl glycerol, the spots were scraped from the plates and the phospholipids were eluted with 5 ml of scintillation fluid.

Protein was determined by the method of Lowry *et al.*¹⁵

Preparation of crude extract and shock fluid. Crude cell extracts were prepared by freezing and thawing as described by Mäntsälä and Nurmikko,¹⁶ and shock fluid by the procedure of Neu and Heppel.¹⁷ Samples (80 mg of dry weight) were taken from the cultures at different phases of growth. The shock fluid was concentrated by lyophilization.

Assay of pantothenate-binding activity. Binding activity towards crude extracts and shock fluids were assayed as described earlier¹³ and by equilibrium dialysis. The dialysis sacs containing binding material (2 mg of protein) were shaken for 24 h at 4°C in tubes containing 600 000 cpm of [^{14}C]-pantothenate in 0.1 ml. After dialysis the samples were pipetted onto Millipore filters, dried and counted in a scintillation spectrometer.

RESULTS

Pantothenate transport into whole cells and membrane vesicles of *Pseudomonas fluorescens* P-2. In a previous study the uptake of pantothenate into whole cells of *Pseudomonas fluorescens* P-2 was found to increase in the presence of glyoxylate, glycolate, and glycine, even when growth was inhibited with chloramphenicol, but not in the absence of nitrogen.⁷ Ethanolamine, choline, sarcosine, and serine without added pantothenate have now also been found to increase pantothenate transport activities (Fig. 1). As can be seen, while the most effective compounds in the early phases of growth are glyoxylate and ethanolamine, after several hours pantothenate and pantoate become the most effective. Activities begin to decrease on glyoxylate, ethanolamine, choline,

Fig. 1. Effect of pantothenate, pantoate, glyoxylate, ethanolamine, choline, serine, and sarcosine on pantothenate transport activity in *Pseudomonas fluorescens* P-2. Pre-cultivations were carried out on pantothenate. The reaction mixture contained [$1\text{-}^{14}\text{C}$]-pantothenate (75 000 cpm, specific activity 4.75 mCi/mmol), potassium pantothenate (0.05 μmol), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.5 μmol) and about 250 μg dry wt cell suspension in 0.2 ml. The reaction mixture was incubated at 30° and reactions were terminated at intervals of one minute by washing the cells on Millipore filters with 10 ml of 0.2 M NaCl solution.

(a) Turbidity of the culture (Klett-Summerson colorimeter, filter 62). The concentrations of the compounds were 10 mM. ●, pantothenate; ■, pantoate; ○, glyoxylate; □, ethanolamine; △, choline; ●, serine; ▲, sarcosine.

(b) Specific activity of pantothenate transport. The symbols are the same as above.

(c) Specific activity of pantothenate transport in the presence of chloramphenicol (200 $\mu\text{g}/\text{ml}$ of the culture). The symbols are the same as above.

(d) Specific activity of pantothenate transport. ○, ethanolamine + pantothenate; ●, ethanolamine + pantothenate + chloramphenicol.

sarcosine, and serine during the exponential phase of growth. In these cases moreover, the rise in transport activities was not prevented by added carbon compounds.

Membrane vesicles prepared from cells taken from the lag, acceleration, exponential, and stationary phases of growth on ethanolamine, choline, sarcosine, and serine accumulated [$1\text{-}^{14}\text{C}$]-pantothenate almost equally and without dependence on the growth phase (Fig. 2). Accumulation of pantothenate, negligible without an added electron donor system, is in-

creased about 5–10 times when an artificial electron donor system, ascorbate-phenazine methosulfate, is used.

Incorporation of ^{32}P into phospholipid fractions of whole cells, protoplasts and membrane vesicles. Although ethanolamine, choline, sarcosine, and serine are the most effective compounds on pantothenate uptake into whole cells (Fig. 1), incorporation of ^{32}P is most rapid into cells grown on pantothenate, glycerol, and glucose and, in general, on compounds that allow rapid growth (Fig. 3). However, although growth

Fig. 2. Accumulation of $[1-^{14}\text{C}]$ -pantothenate in membrane vesicles from *Pseudomonas fluorescens* P-2 grown on ethanolamine, choline, sarcosine, and serine. The reaction mixtures contained in a total volume of 0.2 ml, 4 μmol of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 10 μmol of potassium phosphate buffer (pH 6.6), 75 000 cpm of $[1-^{14}\text{C}]$ -pantothenate, 0.01 μmol of potassium pantothenate and about 0.16 mg of membrane protein. Ascorbate-phenazine methosulfate was used as an artificial electron donor. Other experimental conditions were the same as in the legend to Fig. 1.

O, pantothenate (the reaction mixture contained ascorbate + phenazine methosulfate); ●, pantothenate; △, ethanolamine; □, choline; ▲, sarcosine; ■, serine.

is almost negligible for several hours on ethanolamine, choline, sarcosine, and serine, incorporation of ^{32}P into phospholipids increases from the very beginning. When the growth reaches the acceleration phase, ^{32}P incorporation into phospholipid fractions also increases.

The results given in Table 1 are in good agreement with those presented in Fig. 3. Protoplasts and membrane vesicles prepared from the cells grown on pantothenate, glycerol, and glucose incorporate $[^{32}\text{P}]$ -labelled phosphate more rapidly than the protoplasts and membrane vesicles prepared from the cells grown on ethanolamine, choline, sarcosine, and serine.

Because no correlation between total phospholipid synthesis and pantothenate uptake was observed when bacteria were cultured on glyoxylate, ethanolamine, choline, sarcosine, and serine, the synthesis of individual phos-

Fig. 3. Incorporation of ^{32}P into phospholipids of *Pseudomonas fluorescens* P-2. The reaction mixture contained 2 μCi $[^{32}\text{P}]$ -labelled phosphate/ml, 10 μmol of potassium phosphate, 1 mg (dry weight) of the cell pellet and other compounds as described in the "Organism and growth conditions" section. The reaction mixtures were incubated at 30° and the samples were precipitated at intervals of 10 min with 2 ml of cold 20 % TCA, filtered and washed twice with 3 ml of 5 % TCA on Millipore filters (0.3 μm pore-size). The filters were then dried and phospholipids were extracted with methanol-chloroform solution (1:2, by vol).

(a) Turbidity of the culture. The concentrations of the compounds were 10 mM (Casamino acids 0.5 %). □, Casamino acids; ●, pantothenate; ○, glycerol; ■, glucose; ×, ethanolamine, choline, serine, sarcosine or glyoxylate.

(b) ^{32}P incorporation activity. The symbols are the same as above.

(c) ^{32}P incorporation activity. ■, ethanolamine; △, choline; □, serine; ▲, sarcosine.

(d) ^{32}P incorporation activity. ●, glyoxylate + pantothenate; ○, glyoxylate + 5 mM $(\text{NH}_4)_2\text{SO}_4$; ⊙, glyoxylate without ammonium sulfate.

pholipids during growth was investigated. It can be seen that concentrations of phosphatidyl ethanolamine are high throughout all growth phases in whole cells and parallel total incorporation of ^{32}P very closely (Fig. 4). In contrast, the synthesis of phosphatidyl glycerol, another common phospholipid in *Pseudomonas fluorescens* P-2, ceases very rapidly when the cells are transferred from pantothenate or

Table 1. Incorporation of [32 P]-labelled phosphate into phospholipids of protoplasts and membrane vesicles from *Pseudomonas fluorescens* P-2. Protoplasts and membrane vesicles were prepared by the method of Kabaek (1971)¹² as described in the "Preparation of protoplasts and membrane vesicles" section. Activities were determined as described in the legend to Fig. 3.

Growth Klett ₆₂	[32 P] Incorporation (log cpm/mg protein)													
	Pantothenate		Glucose		Glycerol		Ethanol-amine		Choline		Serine		Sarcosine	
	P ^a	M ^a	P	M	P	M	P	M	P	M	P	M	P	M
30	3.10	2.40	2.90	2.30	2.94	2.30	2.40	2.18	2.36	2.25	2.40	2.14	2.26	2.10
40	3.28	—	3.10	—	3.30	—	2.68	2.44	2.52	—	2.63	—	2.26	2.26
50	3.44	3.10	3.26	2.90	3.62	2.34	2.90	2.68	2.88	2.60	2.40	2.10	2.56	2.38
70	3.62	—	3.48	—	3.84	—	—	—	3.10	—	2.90	2.65	—	—
120	3.90	3.60	3.70	3.33	4.12	3.76	—	—	3.44	2.90	—	—	—	—
180	3.67	—	—	—	—	—	—	—	—	—	—	—	—	—

^a Abbreviations: P = protoplasts; M = membrane vesicles.

Table 2. Incorporation of [32 P] labelled phosphate into phosphatidyl ethanolamine and phosphatidyl glycerol of protoplasts and membrane vesicles from *Pseudomonas fluorescens* P-2. The experimental conditions were the same as in the texts of Table 1 and Fig. 4.

Growth Klett ₆₂	[32 P] Incorporation (log cpm/mg protein)											
	into phosphatidyl ethanolamine						into phosphatidyl glycerol					
	Pantothenate		Ethanolamine		Serine		Pantothenate		Ethanolamine		Serine	
	P ^a	M ^a	P	M	P	M	P	M	P	M	P	M
30	1.91	2.41	1.72	1.74	2.04	2.10	1.21	1.41	1.27	1.13	1.44	1.41
35	2.20	—	2.10	2.48	2.37	2.40	1.23	1.30	1.18	1.20	1.47	1.44
50	2.63	2.83	2.73	3.06	2.79	2.56	1.42	—	1.41	1.56	1.63	1.76
100	3.10	—	—	—	—	—	1.77	1.64	1.65	—	—	—
160	2.94	3.14	—	—	—	—	1.83	—	1.70	—	—	—

^a Abbreviations: P = protoplasts; M = membrane vesicles.

glycerol media into ethanolamine, choline, sarcosine, or serine media. Phosphatidyl glycerol synthesis accelerates again when growth continues. At the end of the exponential phase of growth, phosphatidyl glycerol synthesis ceases and some degradation of the phospholipid is observed.

Table 2 shows the results of 32 P incorporation into phosphatidyl ethanolamine and phosphatidyl glycerol in protoplasts and membrane vesicles. The results resemble those obtained for whole cells (Fig. 4).

Formation of pantothenate-binding activity during growth on pantothenate, pantoate, ethanol-

amine and choline. Pantothenate-binding activity follows growth only in pantothenate and pantoate media (Fig. 5). In the course of two generations, the cells grown on pantothenate and pantoate lose their binding activity on ethanolamine and choline both in the shock fluids and the crude extracts. These results suggest the inducible nature of the pantothenate-binding protein and that there is a correlation between pantothenate transport activities in whole cells and pantothenate-binding activity on pantothenate and pantoate but not on ethanolamine and choline.

Fig. 4. Relative amounts of phosphatidyl ethanolamine and phosphatidyl glycerol in *Pseudomonas fluorescens* P-2 cells. Phosphatidyl ethanolamine and phosphatidyl glycerol were separated and quantitatively determined by thin-layer chromatography, radioactivity of the spots being measured in a scintillation spectrometer.

(a) ^{32}P incorporation in phosphatidyl ethanolamine versus ^{32}P incorporation in total phospholipids. O, ethanolamine; □, choline; ●, serine; Δ, sarcosine. (b) ^{32}P incorporation in phosphatidyl glycerol versus ^{32}P incorporation in total phospholipids. The symbols are the same as above.

DISCUSSION

Although the data presented in this paper demonstrate that there are no correlations between pantothenate transport activities in whole cells and phospholipid synthesis, they do reveal some general properties of the transport process and phospholipid synthesis in *Pseudomonas fluorescens* P-2. It has been found that pantothenate transport is an inducible process requiring cell multiplication.⁷ The results concerning phospholipid synthesis in *Ps. fluorescens* P-2 are in good agreement with those of Ballesta and Schaechter¹⁸ for *E. coli*.

Acta Chem. Scand. B 28 (1974) No. 1

Fig. 5. Formation of pantothenate-binding protein during the growth of *Pseudomonas fluorescens* P-2. Pantothenate-binding activity was measured by gel filtration and equilibrium dialysis. The dialysis sacs contained 2 mg of protein and dialysis tubes 600 000 cpm of $[1-^{14}\text{C}]$ -pantothenate (specific activity 4.75 mCi/mmol).

(a) Binding activity in shock fluid. O, pantoate. ●, pantothenate; Δ, ethanolamine; □, choline. (b) Binding activity in crude extracts. The symbols are the same as above.

They found that the total phospholipid synthesis as well as the synthesis of phosphatidyl ethanolamine, the main phospholipid component, is related to growth and cell division. This may be due to the fact that phosphatidyl ethanolamine is normally rather stable compared, for instance, with phosphatidyl glycerol.¹⁸⁻²⁰ The linear incorporation of ^{32}P into phospholipids at the beginning of growth on the compounds mentioned in Fig. 3 probably reflects a turnover of phospholipids rather than a net synthesis because the transfer of the cells from a richer to a poorer medium causes strong inhibition of phospholipid metabolism. Concentrations of phosphatidyl glycerol decrease about 40 % during 4–6 h growth on ethanolamine, choline, sarcosine, and serine. These results and those of Ballesta and Schaechter¹⁸ suggest that the metabolism of phosphatidyl glycerol is related not to growth and cell

division but to other cell processes. Cronan²¹ reported that the phospholipid composition of the cells was altered when cultures of *E. coli* progressed from exponential growth to the stationary phase. Bertsch *et al.*²² reported similar findings for *Bacillus magaterium* cells. When cells of *Pseudomonas fluorescens* are transferred from a rich medium to a poor one some phospholipids may be altered, thus preventing phospholipid degradation in the new growth conditions.

Several reports suggest a close relationship between the transport system and binding-protein.^{8,23-26} Correlations were observed between pantothenate transport and formation of binding-protein in this work as well.

The finding that pantothenate uptake in *Pseudomonas fluorescens* P-2 is an inducible process⁷ implies the possibility that growth on ethanolamine, choline, sarcosine, and serine stimulates the synthesis of pantothenate-binding protein. Nevertheless, binding activities remain low, at least on ethanolamine and choline. Unpublished results in our laboratory suggest that pantothenate uptake into membrane vesicles from *Pseudomonas fluorescens* P-2 is stimulated (except in the presence of the artificial electron donor system, ascorbate-phenazine methosulfate) in the presence of ethanolamine, choline, and sarcosine too. On the other hand, it is not clear whether the above-mentioned compounds can serve as direct electron donors in whole cells as well. Further results concerning pantothenate transport into membrane vesicles from *Pseudomonas fluorescens* P-2 will be published later.

The author thanks Professor V. Nurmikko for his help during this work and Mr. T. Hut-tunen for technical assistance.

REFERENCES

1. Kawasaki, T., Miyata, I., Esaki, K. and Nose, Y. *Arch. Biochem. Biophys.* 131 (1969) 223.
2. Cooper, B. A. *Biochim. Biophys. Acta* 208 (1970) 99.
3. Mandelbaum-Shavit, F. and Grossowicz, N. *J. Bacteriol.* 104 (1970) 1.
4. Roth, J. A., McCormic, D. B. and Wright, L. D. *J. Biol. Chem.* 245 (1970) 6264.
5. Di Girolamo, P. M. and Bradbeer, C. J. *Bacteriol.* 106 (1971) 745.
6. Taylor, R. T., Norell, S. A. and Hanna, M. L. *Arch. Biochem. Biophys.* 148 (1972) 366.
7. Mäntsälä, P., Kokkonen, H. and Nurmikko, V. *Acta Chem. Scand.* 26 (1972) 136.
8. Pardee, A. B. *Science* 162 (1968) 632.
9. Fox, C. F. *Proc. Nat. Acad. Sci. U. S. A.* 63 (1969) 850.
10. Hsu, C. C. and Fox, C. F. *J. Bacteriol.* 103 (1970) 410.
11. Goodhue, C. T. and Snell, E. E. *Biochemistry* 5 (1966) 393.
12. Kaback, H. R. In Jacoby, W. B., Ed., *Methods in Enzymology*, Academic, London 1971, Vol. 22, p. 99.
13. Mäntsälä, P. *Acta Chem. Scand.* 26 (1972) 127.
14. Dittmer, J. C. and Lester, R. L. *J. Lipid Res.* 5 (1964) 126.
15. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. *J. Biol. Chem.* 193 (1951) 265.
16. Mäntsälä, P. and Nurmikko, V. *Suomen Kemistilehti B* 43 (1970) 414.
17. Neu, H. C. and Heppel, L. A. *J. Biol. Chem.* 240 (1965) 3685.
18. Ballesta, J. P. G. and Schaechter, M. *J. Bacteriol.* 107 (1971) 251.
19. Kanfer, J. and Kennedy, E. P. *J. Biol. Chem.* 238 (1963) 2919.
20. Golden, N. G. and Powell, G. L. *J. Biol. Chem.* 247 (1972) 6651.
21. Cronan, J. E., Jr. *J. Bacteriol.* 95 (1968) 2054.
22. Bertsch, L. L., Bonsen, P. P. M. and Kornberg, A. *J. Bacteriol.* 98 (1969) 75.
23. Wiley, W. R. *J. Bacteriol.* 103 (1970) 656.
24. Barash, H. and Halpern, Y. S. *Biochem. Biophys. Res. Commun.* 45 (1971) 681.
25. Lengeler, J., Hermann, K. O. and Unsöld, H. J. *Eur. J. Biochem.* 19 (1971) 457.
26. Tsay, S.-S., Brown, K. K. and Gaydy, E. T. *J. Bacteriol.* 108 (1971) 82.

Received July 25, 1973.