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Some Characteristics and Control of Pantothenate Transport in *Escherichia coli* U-5/41

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Properties and formation of the pantothenate transport system in *Escherichia coli* U-5/41 were investigated. The results in this paper suggest the existence of an active transport process, which is formed constitutively.

Transport activity was inhibited by 2,4-dinitrophenol and sodium azide. The K_m value for pantothenate uptake was approximately 2.5×10^{-5} M. Pantothenate transport was temperature- and pH-dependent, the respective optima being 45 – 50° and 4.0 – 4.5.

Exit was prevented by some monovalent ions and also by glucose ($10^{-2} - 5 \times 10^{-1}$ M) when the cells were precultured for at least 2 h in the glucose minimal medium.

Pantothenate was transported into the cells of *E. coli* without phosphorylation and transport activity was reduced by osmotic shock. Pantothenate-binding activity was demonstrated in the concentrated shock fluid, in the ammonium sulphate precipitate and in the Sephadex G-100 eluate.

Biological transport systems have received much attention during the last few years. Attention has also been paid to the systems by which most vitamins of the B group are taken up by microorganisms.[1–5] It has long been recognized that the transport process is usually not one of passive diffusion but involves the functioning of an active permeation process, which may exhibit general regulatory patterns. Although much has been learned about the specific components and their possible relationships to the transport of carbohydrates,[6,7] little is known of the manner in which charged compounds, including several vitamins, are transported. Kawasaki *et al.*[1] reported that membrane thiamine kinase participated in the uptake of thiamine, a process by which this vitamin is accumulated as thiamine pyrophosphate against a concentration gradient. DiGirolamo and Bradbeer [4] found that *E. coli* has an initial vitamin B_{12}-binding system with a high affinity for vitamin B_{12} coupled with a large B_{12} storage capacity.

In a preliminary report of this work some properties of the pantothenate transport system in *E. coli* were outlined.[8] In the present study this system is further characterized. Evidence is presented that the uptake of pantothenate

is dependent on temperature, pH and energy supply. Furthermore, formation of the uptake system has been shown to be constitutive, and acumulation of pantothenate occurs without phosphorylation. Pantothenate-binding activity was demonstrated in the shock fluid.

EXPERIMENTAL

Materials. [^{14}C]-D-Pantothenate was obtained from the New England Nuclear Corporation, Boston, Mass. All chemicals were from commercial suppliers.

Organism and growth media. The test organism, *Escherichia coli* U-5/41, was cultured in a minimal salt medium [9] containing 0.2 % or 10 mmol of a carbon compound.

Cultivation. Bacteria were grown with aeration at 37° until the exponential phase of growth. In the experiments on the formation of the pantothenate transport system during growth, samples were withdrawn at intervals of 1 h. Growth was estimated from the turbidity measurements made with a Klett-Summerson colorimeter fitted with a No. 62 filter. The cells (about 0.1 mg of dry weight) were collected by centrifugation at 4° and washed with cold minimal salt solution.

Measurement of pantothenate uptake. The samples were preincubated for 3 min at 45° in the presence of chloramphenicol (100 μg/ml) and the uptake process was then initiated by adding 0.1 ml of the preincubated minimal salt solution containing 60 000 cpm of [^{14}C]-D-pantothenate and 0.05 mM D-pantothenate. Initial velocities were measured by stopping incubation at intervals of 30 sec with cold 0.1 M sodium chloride containing 10 mM pantothenate. The cells were filtered on a Millipore membrane filter (0.45 μ pore size) and washed for 3 min with the same cold sodium chloride solution. The filters were removed from the suction apparatus immediately, dried, and placed in counting vials containing 5 ml of toluene-based scintillation fluid. Uptake rates are expressed as nanomoles of labelled compound per milligram of bacteria (dry weight) per minute.

Identification of accumulated compounds. The cells (0.4 mg) were incubated for 2, 4, 10, 20, and 60 min, then deposited on a Millipore filter and washed for 10 min as described above. [^{14}C]-Pantothenate was extracted from the cells by incubating them for 15 min in 50 % ethanol at 25° or for 15 min in 0.01 M sodium acetate buffer, pH 4.5, at 85°. The suspensions were centrifuged at 5 000 *g* and the supernatant was lyophilized and dissolved in 0.1 ml of water. The samples were chromatographed on paper with a 1-butanol : acetic acid : water (25:4:10) developing system.

Exit measurements. The cells (0.1 mg dry weight) from the exponential phase of growth were centrifuged and washed with mineral salt solution. The samples were then incubated at 45° for 30 min in the presence of 0.1 ml of [^{14}C]-pantothenate. After incubation the cells were washed with various salt solutions or with glucose.

Measurement of binding activity. For the preparation of binding material the washed cells were treated by the method of Neu and Heppel [14] with 0.05 M Tris-HCl buffer (pH 7.5), which contained 0.001 M EDTA and 20 % sucrose. The solution was shaken for 10 min at 30°, and the cells, when they had been centrifuged, were osmotically shocked with 5×10^{-4} M $MgCl_2$. The supernatant (15 000 *g*) was lyophilized and the binding activity determined by equilibrium dialysis. The dialysis bags contained 0.2 ml of concentrated shock fluid and this was dialyzed for 24 h at 4° against 0.05 M Tris-HCl buffer (pH 7.5) containing 5000 cpm/ml of [^{14}C]-pantothenate (specific activity 4.75 mCi/mmol). The radioactivity of the samples (0.1 ml) was measured as described above.

Partial purification of the binding material. Unless specified otherwise, purification procedure was carried out at 4 – 6°. The lyophilizate (140 mg) was dissolved in cold 0.05 M potassium phosphate buffer (pH 7.0). Centrifugation of the resulting solution (50 000 *g*, 1 h) was followed by concentration with ammonium sulphate up to 40 % saturation. The insoluble material was discarded. Additional ammonium sulphate (up to 80 % saturation) was added, the pH adjusted to 7.0, and the precipitate collected by centrifugation. The precipitated material was dissolved in 10 ml of potassium phosphate buffer (pH 7.0) and applied to a column of Sephadex G-100, which had been pre-equilibrated with the same buffer. The eluted fractions were tested for binding activity and the active fractions were combined and concentrated by addition of ammonium sulphate. Disc electrophoresis was carried out as described originally by Ornstein and Davies and modified by Chang *et al.*[11]

RESULTS

The time course of the uptake of pantothenate by the cells of strain U-5/41 at various temperatures is shown in Fig. 1. The transport reaction proceeded linearly for at least 10 min under the conditions used. However, cells incubated

Table 1. Effect of 2,4-dinitrophenol and sodium azide on pantothenate uptake.[a]

Compound		Activity (%) Preincubated	Added to the reaction mixture
None		100	
2,4-Dinitrophenol	0.1 mM	94	98
»	0.5 mM	65	85
»	1.0 mM	28	60
Sodium azide	1.0 mM	90	95
»	5.0 mM	70	90
»	10.0 mM	33	82

[a] Experimental conditions are the same as those given in the legend to Fig. 4.

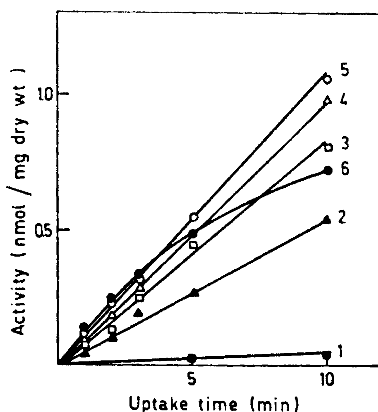

Fig. 1. Effect of temperature on the time course of pantothenate uptake. Cells of *E. coli* U-5/41 were harvested from exponentially growing cultures in glucose minimal medium and resuspended in 0.1 ml of mineral salts solution. The cell suspension (0.1 mg dry weight) was then incubated in the presence of 60 000 cpm of [^{14}C]-D-pantothenate and 0.05 mM D-pantothenate. Initial velocities were measured as described in the text. 1, at 0°; 2, at 30°; 3, at 40°; 4, at 45°; 5, at 50°; 6 at 55°.

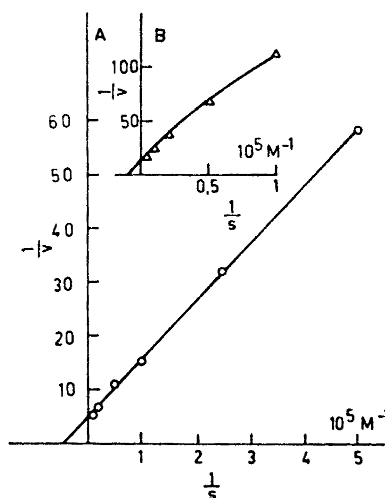

Fig. 2. Concentration dependence of pantothenate transport. Time course curves were obtained for each substrate concentration. Other experimental conditions are described in the text. A, radioactivity taken up at 45°; B, radioactivity taken up at 25°. Activity is expressed as nmol per min per mg dry weight.

for 20 min in the presence of 1 mM 2,4-dinitrophenol or 10 mM sodium azide had only about 30 % of their initial transport activity (Table 1). Thus, the accumulation process depends on metabolic energy. Furthermore, concentration gradients of pantothenate can be produced through transport into the cells of *E. coli* U-5/41. These results suggest the active transport of pantothenate under the conditions used.

In this system, the uptake of pantothenate into the cells of strain U-5/41 followed saturation kinetics. Fig. 2 shows a reciprocal plot of initial rate of uptake *versus* substrate concentration. Initial rates were computed from points taken at intervals of 30 sec during the first 4 min. The concentrations ranged from 2 to 100 μM. A value of 2.5×10^{-5} M for K_m and of 0.3 nmol per min per mg dry weight of cells for V_{max} was obtained. At temperatures below 30° the K_m value for pantothenate transport was approximately 1×10^{-4} M and the uptake system was not inhibited by 1 mM 2,4-dinitrophenol, which suggests that pantothenate was transported into the cells by diffusion.

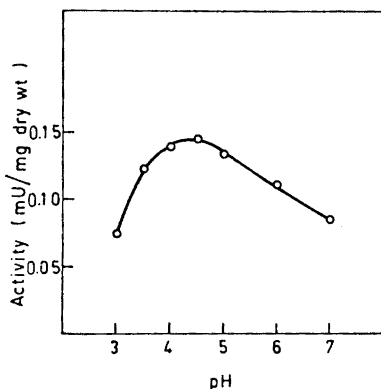

Fig. 3. Uptake of pantothenate as a function of pH. Uptake reactions were carried out in citrate buffers at 45°. Other experimental conditions are the same as in the legend to Fig. 1.

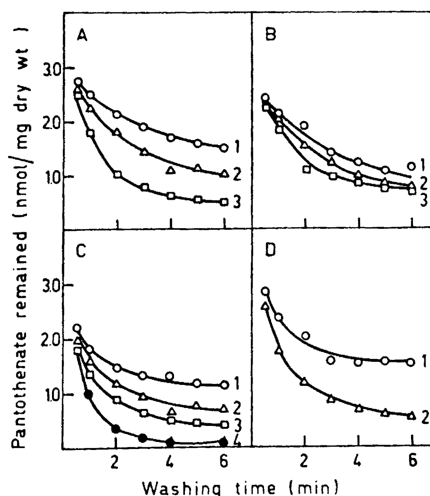

Fig. 4. Effect of temperature and glucose concentration on exit reaction. A. Exit against mineral salts solution 1, at 0°; 2, at 30°; 3, at 45°. B. Exit against glucose solution (20 mM) 1, at 0°; 2, at 30°; 3, at 45°. C. Exit against glucose solution at 30° 1, 50 mM; 2, 20 mM; 3, 10 mM; 4, H_2O. D. Exit against mineral salts solution containing 1 mM iodoacetamide at 0°, 1; at 45°, 2.

The effect of pH on pantothenate uptake was studied in citrate buffers. As shown in Fig. 3, the rate of pantothenate accumulation was maximal at 4.0 – 4.5.

The effect of temperature and various compounds on exit was investigated by allowing the cells to accumulate [^{14}C]-pantothenate at 45° for 10 min and then chilling, harvesting by centrifugation at 4°, and resuspending the bacteria in solutions of different salts with pantothenate added. Fig. 4 shows the results of the experiment. At 45° exit was much more rapid than at 30° and at 30° more rapid than at 4°. When the cells were resuspended in medium containing only 10^{-4} M pantothenate the exit rates were marked even at 4°. Glucose, when added to the pantothenate solution, prevented the exit of [^{14}C]-pantothenate at 45°, 30°, and 4° in cells precultured for at least 2 h in the glucose minimal medium.

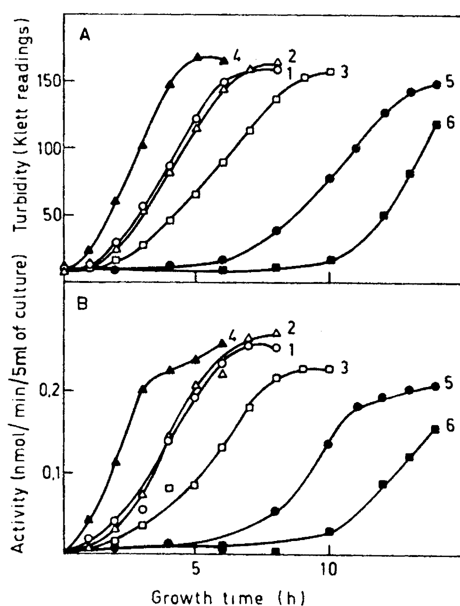

Fig. 5. Formation of the pantothenate transport system during growth of *E. coli* U-5/41 in various media. Experimental conditions are the same as in the legend to Fig. 1. A. Growth 1, on glucose; 2, on glucose + pantothenate; 3, on glycerol; 4, on casamino acids; 5, on succinate; 6, on glutamate. The concentration of carbon compound was 10 mM (casamino acids 0.1 %). B. Activity of the transport system. The symbols are the same as above.

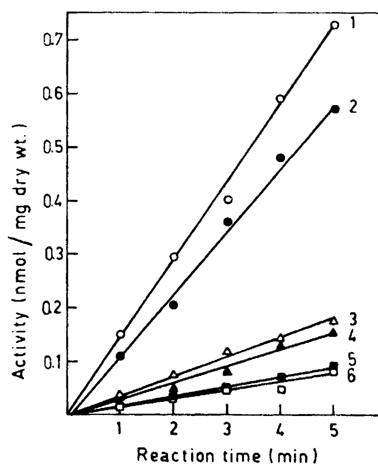

Fig. 6. Effect of shock treatment and freezing and thawing on the time course of pantothenate uptake. Experimental conditions are described in the text. 1, the cells were taken from the early logarithmic phase of growth; 2, the cells were taken from the end of the logarithmic phase of growth; 3, the osmotically shocked cells taken from the early logarithmic phase; 4, the osmotically shocked cells taken from the end of the logarithmic phase of growth; 5, the cells were taken from the early logarithmic phase of growth and disrupted by freezing and thawing; 6, the cells were taken from the end of the logarithmic phase of growth and disrupted by freezing and thawing.

The pantothenate accumulated by the cells was not phosphorylated, because in cells pulsed for a short time at 45° only one radioactive spot identical with [^{14}C]-pantothenate was found on a chromatogram. The presence of pantothenate kinase and absence of pantothenate hydrolase in *E. coli* U-5/41 were demonstrated and when the cells were pulsed for a longer period at 37° various spots were obtained by chromatography.

A pantothenate transport system was formed constitutively in the cells of strain U-5/41. Formation of the transport system on glucose, glucose + pantothenate, glycerol, glutamate, casamino acids, or succinate almost completely paralleled the growth curves, the activities reaching maxima at the end of the exponential phase of growth (Fig. 5).

Transport activity was reduced by osmotic shock or by freezing and thawing. Fig. 6 shows that after osmotic shock approximately equal proportions of the ability to transport pantothenate were lost in cells taken from the early exponential phase and those from the end of the exponential phase of growth. When the shock fluid was concentrated by lyophilization, it was possible to demonstrate binding of pantothenate to the lyophilized material.

Table 2. Partial purification of pantothenate-binding material from *E. coli* U-5/41.

	Protein (mg)	Binding activity (nmol)	Specific activity (nmol/mg protein)
Shock fluid	70	18	0.25
Lyophilizate	71	14	0.20
$(NH_4)_2SO_4$ precipitate (80 % saturation)	41	18	0.44
Sephadex G-100	22	17	0.78

Table 2 shows the results of purification procedures on the binding material. The Sephadex G-100 fractions appeared to be purified only about 3-fold over the shock fluid. Four or five proteins with molecular weights in order of tens of thousands were observed in the active Sephadex G-100 fractions by using disc electrophoresis.

DISCUSSION

From the results described above it is obvious that transport of pantothenate into the cells of *E. coli* U-5/41 is an active process. The results reported here resemble those described in several previous papers,[12–17] except for the low pH and high temperature optima in pantothenate transport. High transport activity at low pH values may indicate weak dependence on ionic attraction for binding of pantothenate and the relatively greater importance of van der Waals forces. The rate of uptake increased with increasing temperature up to 50°, then decreased rapidly at higher temperatures, the later behavior probably being due to partial cell destruction or death. Mandelbaum-Shavit and Grossowicz [18] have found two uptake systems for folinate in

Pediococcus cerevisiae, one of them dependent on temperature, pH and glucose, the other independent of temperature and glucose. No different phases, such as were found in the transport of vitamin B_{12} in *E. coli*,[4] were observed in pantothenate transport. On the other hand, the initial phase of B_{12} uptake was extremely rapid and it may be difficult to demonstrate its counterpart in other systems. Furthermore, the total counts during the initial phase were only about 15 % of the counts during the second phase.

Although the existence of pantothenate kinase in these cells was demonstrated by the method of Ward *et al.*,[19] pantothenate was accumulated without phosphorylation. On the other hand, formation of 4'-phosphopantothenate requires ATP, which is generated by oxidative phosphorylation. Because, although pantothenate was transported without phosphorylation, uptake was inhibited by 2,4-dinitrophenol, energy must be required for some other process involved in the accumulation of pantothenate. It is suggested that energy may be required for passage of pantothenate across the membrane as in the case of β-galactosides in *E. coli*.[20] Thiamine is accumulated as thiamine pyrophosphate in *E. coli* [1] and in this system energy is required for accumulation of thiamine, but not for the passage of thiamine across the membrane. Glucose was found to increase the internal steady-state concentration of pantothenate only if the cells had been grown on glucose for at least 2 h. It is therefore suggested that the effect of glucose in preventing exit is involved in the supply of energy after induction of the pathway of degradation of glucose.

The uptake of pantothenate in *Pseudomonas fluorescens* P-2 is an inducible process.[5] On the other hand, the uptake system in *E. coli* U-5/41 is a constitutive one. Although pantothenate uptake in *P. fluorescens* was controlled by induction and repression [5] and thiamine uptake in *E. coli* by repression and derepression,[21] pantothenate, when added to the growth medium, had no influence upon the growth of *E. coli* and only a slight influence upon pantothenate transport. Rogers and Lichstein [22] reported that the activity of the biotin transport system in *Saccharomyces cerevisiae* was not inhibited by intracellular free biotin pools, although the synthesis of the biotin transport system may be repressed during growth in a medium containing a high concentration of biotin. Although pantothenate, when added to the growth medium at a high concentration, decreased transport activity, this can hardly be regarded as repression of the transport system, because over 10 mM pantothenate was required to achieve a 20 % decrease in transport activity. More likely the intracellular pantothenate pool tends to decrease pantothenate transport. Furthermore, although Iwasima *et al.*[23] found that formation of thiamine-binding protein was repressed by thiamine added to the growth media, no repression of pantothenate-binding material was observed when pantothenate was added to the growth media.

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