LIPOIC ACID UPTAKE IN *ESCHERICHIA COLI*

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Escherichia coli **Crookes strain transports lipoic acid via a temperature- and energy-dependent system which is saturable. Because of lipoic acid's lipophilic nature special precautions must be taken in stopping uptake to prevent artifactual results. There is no sulfhydryl group requirement of the transport system. Both chloramphenicol and ammonium sulfide inhibited the incorporation of lipoic acid into the protein-bound form. Lipoic acid transport was sensitive to osmotic shock. Although a slight increase in the specific lipoic acid binding to certain fractions of the osmotic shock fluid was obtained, the existence of a lipoic acid-binding protein was not established. Membrane vesicles transported lipoic acid.**

INTRODUCTION

Lipoic acid functions as a cofactor in the oxidative decarboxylation of α-keto acids by the pyruvate and α-ketoglutarate dehydrogenase complexes (1). Activating enzymes catalyze the conversion of free lipoic acid to the protein-bound active form in both *Escherichia coli* and *Streptococcus faecalis* (2,3). This protein-bound form is linked through the ξ-NH_2 group of lysine (4) and does not turn over in *E. coli* (5). Exogenously supplied lipoic acid is used preferentially by *E. coli* even though the organism synthesizes lipoic acid (6).

A constitutive system capable of concentrating lipoic acid 100-fold in a temperature- and energy-dependent reaction has been described in *S. faecalis* (7,8). Furthermore, the lipoic acid activating system in both *S. faecalis* 10C1 and *E. coli* Crookes was found entirely in the soluble fraction (9), suggesting that this system was not directly involved in the transport of lipoic acid into the cell.

This paper reports a study of lipoic acid transport in *E. coli* with special emphasis on the specificity and properties of the system, the effect of osmotic shock and the possible involvement of binding proteins, and interaction of lipoic acid with the cell membrane. Preliminary results of some of these studies have appeared (10,11).

MATERIALS AND METHODS

Materials

Unlabeled *DL* - α - lipoic acid, 5- (1,2-dithiolan-3-yl) pentanoic acid, and 8-methylthioctic acid, 5-(1,2-dithiolan-3-yl) hexanoic acid, were generously supplied by Dr. D. S. Acker of du Pont. Dr. L. J. Reed of the University of Texas provided unlabeled lipoic acid and both the C_7, 4-(1,2-dithiolan-3-yl) butyric acid, and C_9, 6- (1,2-dithiolan-3-yl) hexanoic acid, analogs. Radioactive lipoic acid labeled with ^{35}S was prepared as described by Leach (12). The specific activity of the lipoic acid was 46 μCi/mg.

Growth of *Escherichia coli*

E. coli Crookes ATCC 8739 cells were grown in M-9 minimal medium (13) supplemented with 0.2% glucose on a New Brunswick R-10 reciprocating shaker at 37 C. After overnight growth the cells were diluted 20-fold into fresh medium and incubated with shaking for 3 hr. The culture was then diluted with an equal volume of fresh medium and grown for 45 min.

Measurement of lipoic acid uptake

Cell preparations containing 1.1 to 1.3 × 10^9 cells/m*l* were incubated for 10 min in M-9 containing 0.2% glucose plus chloramphenicol (0.1 mg/m*l*) at 37 C. The radioactive substrate was added to a final concentration of 1 μM and the mixture was incubated with shaking for 10 min at 37 C. The uptake process was stopped by pipetting 0.5-m*l* samples onto 1 m*l* of crushed, frozen M-9. After centrifugation at 4 C and one wash with ice-cold M-9, the cell pellet was suspended in one m*l* of water, transferred to a scintillation vial and counted in 10 m*l* of Bray's scintillation cocktail (14). Samples were counted to a 1% standard counting error.

If incorporation (into protein-bound form) was being determined, the chloramphenicol was omitted and the reaction was

stopped with crushed, frozen M-9. The cell pellet was treated with 1 m*l* of 70% ethanol. Unless otherwise noted, data are presented from typical experiments which were repeated 2-3 times but not averaged.

Osmotic shock procedure (15)

Ten m*l* of cells grown on M-9 medium to the early stationary phase were added to 200 m*l* of fresh M-9 and incubated for 4-5 hr to obtain midlog phase cells. The cells were harvested and washed twice with 0.03 *M* Tris-HCl (pH 7.1). The washed cells (250 mg) were suspended in 20 ml of 20% sucrose in 0.03 *M* Tris with 10^{-4} *M* EDTA and the suspension slowly rotated at room temperature for 10 min. The cell suspension was then centrifuged at $10^4 \times g$ for 10 min at 4 C. The supernatant fluid was removed. The cell pellet was immediately subjected to shocking by adding 20 ml of ice-cold 5×10^{-4} *M* $MgCl_2$ solution. After 3 min, an equal volume of 0.05 *M* Tris-HCl (pH 7.1) containing 1×10^{-3} *M* $MgCl_2$ was added to the cell suspension and the mixture centrifuged at $10^4 \times g$ for 10 min. To count the viable cells, aliquots from the untreated and shocked cells were plated on agar plates containing 1% tryptone broth plus 0.5% NaCl.

Membrane Preparation

Membranes were prepared according to the lysozyme/EDTA method of Kaback (16), except that since the Crookes strain is so susceptible to the lysozyme/EDTA treatment that few viable cells remain (less than 1 in 10^3 by plate count), the ultracentrifugation step was omitted. The final preparation was suspended in 0.5 *M* potassium phosphate buffer, pH 6.6, and stored in small aliquots at - 15 C. Membrane protein was assayed by the method of Lowry, et al. (17), using bovine serum albumin as the standard protein.

Equilibrium dialysis

All equilibrium dialysis experiments were done using Chemical Rubber Company five-chamber equilibrium dialysis cells with a volume of 1 m*l* for each chamber. A single thickness of dialysis tubing (Arthur Thomas Co., 48 Å pore), cut to appropriate length and treated with 1 m*M* EDTA, was used as the dialysis membrane. Equilibrium dialysis suspensions contained 10 m*M* glu-

TABLE 1. *Measurement of lipoic acid uptake*

A. Effect of Method of Stopping the Reaction

Treatment	Net counts taken up
Quick freezing with liq. N_2	1280
Slow freezing	1002
Mushy medium	1092
Millipore filtration	40

B. Distribution of free and protein-bound lipoic acid

Treatment	pmols		
	Pool	Protein-bound	Total
Boiling	16.6	54.4	71.0
Ethanol	15.2	55.8	71.0
None	—	—	72.8

Part A. A 2 mg/m*l* suspension of cells was centrifuged and suspended twice in M-9 medium and equilibrated at 20 C for 15 min. Glucose was added to a final concentration of 1 mg/m*l* and the incubation continued for 15 min. longer. Then 20 μg/m*l* of radioactive lipoic acid was added and 0.5-m*l* samples were taken after 5 min incubation for treatment as indicated. A background count was obtained in the absence of cells was subtracted from each value.

Part B. A 2 mg/m*l* suspension of cells was washed twice with M-9 medium and was incubated 10 min at 37 C with 0.2% glucose. Then 0.4 μg/m*l* of radioactive lipoic acid was added and the incubation was continued for another 10 min. The reaction was stopped by pipetting 0.5-m*l* samples onto 0.5 m*l* of slushy M-9. The cells were washed once with 4 C M-9 and then boiled for 5 min or suspended in 70% ethanol. The precipitate and supernatant solution were separated by centrifugation.

cose and 1 m*M* $MgSO_4$, unless the effect of either glucose or Mg^{2+} was being studied, and penicillin/streptomycin to a concentration of 114 and 0.1 μg/m*l*, respectively. Stirring was accomplished by means of a plastic bead inside each cell and a Chemical Rubber Company rocker motor (8 rocks/ min), and the cell was held at the desired temperature (usually 22 C) for 15 - 24 hr. Aliquots were taken from each side of the cell, one side containing membranes or protein and ligand and the other side containing ligand only. These were counted in 10 m*l* of Bray's solution in a Packard Tri-Carb and results were corrected by comparison to appropriately quenched standards.

RESULTS

Measurement of Lipoic Acid Uptake

Table I, Part A shows a comparison of various methods for stopping the uptake of lipoic acid in whole *E. coli* cells. Quick freezing by ejecting the cell suspension into a centrifuge tube cooled in liquid nitrogen, slow freezing by ejecting the cell suspension into a centrifuge tube in an ice bath followed by freezing in a - 15 C room, and ejecting the cell suspension onto 2 volumes of ice medium frozen and chopped into fine crystals all gave equivalent results. No correction was made for any nonspecific adsorption, but the kinetic curves suggest that any such effect is minimal.

Because of a high background adsorption of lipoic acid by membrane filters, the usual method for terminating uptake was not suitable. About 10% of the lipoic acid is retained by the filters in the absence of bacterial cells and this background retention increases linearly with increasing lipoic acid concentrations. The background was not reduced by treatment of the filters with nonradioactive lipoic acid or by exhaustively washing the membrane filters with EDTA-or glycerol-containing solutions.

Part B of Table I shows the equivalence of results obtained when separating free pool lipoic acid from that which is protein bound, by either boiling or ethanol precipitation. Previous results showed that trichloroacetic acid treatment of cells to measure protein-bound lipoic acid leads to artifacts in the distribution of lipoic acid (7). Reaction termination with ice-slushy medium and precipitation by 70% ethanol treatment were the procedures adopted for the analyses reported in this paper.

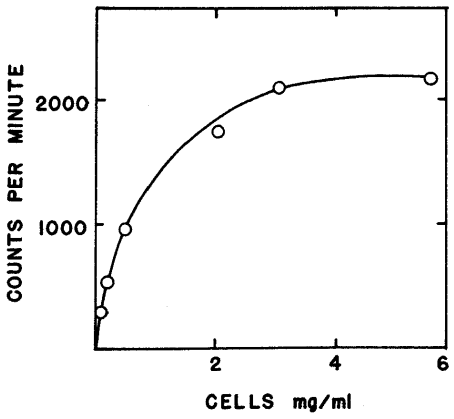

FIGURE 1. Effect of *Escherichia coli* concentration on lipoic acid uptake.
Washed cells in M-9 at the indicated concentrations were incubated 15 min at 20 C and glucose (1 mg/m*l*) was added. After 15 min incubation at 20 C, 10 μg/m*l* of radioactive lipoic acid was added. Samples (0.5 m*l*) of the cell suspension were ejected onto slushy M-9. The cells were washed twice with ice-cold M-9. The radioactivity was determined as indicated in the Materials and Methods Section.

Effect of Cell Concentration

Figure 1 shows the effect of various concentrations of *E. coli* cells on the uptake of lipoic acid during a 5-min incubation at 20 C. The amount of radioactive lipoic acid taken up is proportional to cell concentration down to 1 mg of cells/m*l*.

Effect of Lipoic Acid Concentration

The saturation curves for both free pool lipoic acid and protein-bound lipoic acid are shown in Fig. 2. The protein-incorporating system is one-half saturated at a lipoic acid concentration of about 30 m*M* while lipoic acid transport into the pool is one-half saturated at 50 μM.

Kinetics of Lipoic Acid Uptake

The time course was determined at 20 C over a short period as seen in Fig. 3A. Maximum uptake occurred rapidly within one min and after three min the uptake decreased gradually to a plateau reached at five min. A similar time course of lipoic acid uptake was observed with *S. faecalis* 10C1, with the maximum uptake being obtained at 30 sec. Figure 3B shows uptake over an extended incubation period at 37 C accompanied by cell growth. The incorporation of labeled lipoic acid into the pro-

tein-bound form parallels growth for the first 2 hr of incubation. The uptake into the free pool shows the typical overshoot kinetics.

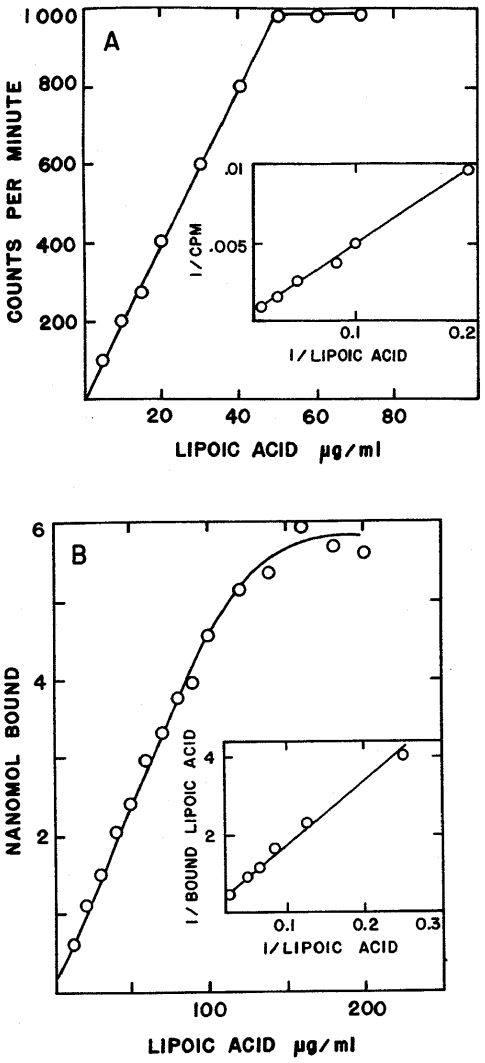

FIGURE 2. Effect of lipoic acid concentration on lipoic acid uptake and incorporation in the protein-bound form.

A. Lipoic acid uptake. The washed cell suspensions (2 mg/m*l*) in M-9 medium were incubated at 20 C for 15 min prior to the addition of 1 mg/m*l* glucose, which was followed by another 15-min incubation. Radioactive lipoic acid was added at the indicated concentrations. After 5 min incubation at 20 C, the uptake was stopped on slushy M-9. The cells were washed twice with ice-cold M-9 and the radioactivity in the cell pellet was determined as described in the Materials and Methods Section. The inset shows a Lineweaver-Burk plot of the data.

B. Bound lipoic acid. Aliquots of a mid-phase log culture were incubated with various concentrations of [^{35}S]-lipoic acid for 10 min at 37 C. The uptake reaction was stopped and the protein-bound lipoic acid was measured as described in the Materials and Methods Section. Duplicate samples were taken. The inset shows a Lineweaver-Burk plot of the data.

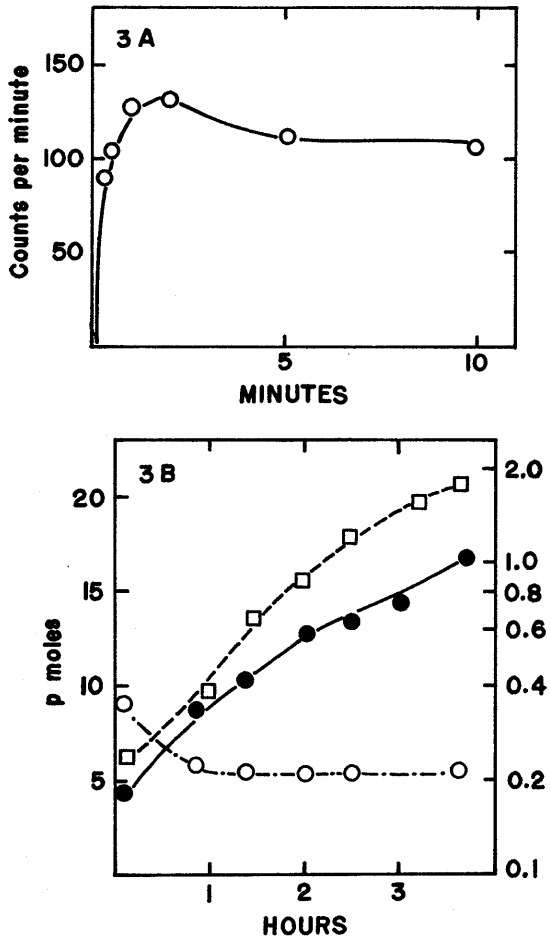

FIGURE 3. Time course of lipoic acid uptake.

A. Short time course. The washed cell suspension (1 mg/m*l*) was equilibrated at 20 C for 15 min and then incubated with glucose (1 mg/m*l*) for 15 additional min. After radioactive lipoic acid (10 µg/m*l*) was added, samples were taken at the indicated times and ejected onto slushy M-9. The cells were washed twice with ice-cold M-9 and the radioactivity in the cells was determined as described in the Materials and Methods Section.

B. Long-term uptake during growth. Mid-log phase *E. coli* cells were suspended to an A_{600} of 0.2 in fresh M-9 medium containing 10 µ*M* [^{35}S]-lipoic acid. At the indicated times 4-m*l* samples (done in duplicate) were placed into chilled centrifuge tubes and centrifuged at $10^4 \times g$ for 10 min at 4 C. The absorbance at 600 nm was measured, ●. The protein-bound lipoic acid, □, and the free pool lipoic acid, O, were determined as described in the Materials and Methods Section.

Effect of Temperature on Lipoic Acid Uptake

When the lipoic acid uptake was measured at 10 and 20 C, the amount of uptake obtained at 20 C was twice that obtained when the cells were incubated at 10 C.

Effect of Sulfhydryl Reagents and Compounds on Lipoic Acid Uptake

Many transport systems require a sulfhydryl group or groups for activity and thus are inhibited by sulfhydryl-reactive reagents. Part A of Table 2 shows that 7-amino-1,3-naphthalenedisulfonate, *p*-chloromercuribenzoate, and *N*-ethylmaleimide do not inhibit lipoic acid uptake.

Table 2, Part B shows the effect of various sulfhydryl compounds on the free pool of lipoic acid. This experiment was done in the presence of chloramphenicol, which inhibits incorporation of lipoic acid into the protein-bound form (see the following section). Both cysteine and cystine were potent inhibitors and 2-mercaptoethanol was slightly stimulatory. The inhibition requires a concentration of at least 10 times that of lipoic acid, suggesting a low specificity of inhibition. A sulfhydryl component is probably not essential for the transport of lipoic acid.

Effect of Inhibitors and Analogs on Lipoic Acid Uptake

Experiments to determine the influence of various inhibitors on the accumulation of lipoic acid into the pool and incorporation of lipoic acid into the protein-bound form were performed by incubating midlog phase cells with the inhibitor 10 min before adding radioactive lipoic acid and measurement of accumulation and incorporation after 10 min incubation. Table 3 shows these results: sodium azide, chloramphenicol, and ammonium sulfide have no effect on the accumulation. 2,4-Dinitrophenol and potassium cyanide, which inhibit energy production, reduce the accumulation of lipoic acid. As seen before, cystine reduces accumulation and also incorporation into

TABLE 2. *Effect of sulfhydryl reagents and compounds on lipoic acid uptake*

Part A

Treatment	Counts taken up
None	3255
$2.5 \times 10^{-3}M$ 7-Amino-1,3-naphthalenedisulfonate	3223
$3 \times 10^{-4}M$ *p*-Chloromercuribenzoate	3100
$1 \times 10^{-3}M$ *N*-Ethylmaleimide	3090

Part B

Additive	Conc., m*M*	uptake, pmol.	% control
None		128	100
2-Mercaptoethanol	1.0	154	120
2-Mercaptoethanol	10.0	152	119
Cysteine	1.0	15	12
Cystine	0.01	137	107
Cystine	0.1	25	19
Cystine	1.0	11	9
CoA (red)	0.01	123	96
CoA (red)	1.0	101	79
Glutathione (red)	0.01	124	97
Glutathione (red)	1.0	127	99
Glutathione (ox)	0.01	114	89
Glutathione (ox)	1.0	108	84
Dithiothreitol	0.01	104	81
Dithiothreitol	0.1	121	95
Dithiothreitol	1.0	137	107

Part A. The cells (2 mg/m*l*) were incubated for 30 min at 20 C with the sulfhydryl reagent. The cells were washed with M-9 and then incubated 15 min before glucose was added. After 15 min additional incubation radioactive lipoic acid was added and the uptake was determined after one min.

Part B. The sulfhydryl componds were added to midlog phase cells 10 min before the addition of radioactive lipoic acid. After 10 min of uptake the reaction was stopped with slushy M-9. The results shown are the average of triplicate samples.

the protein-bound form. The most effecttive inhibitors of incorporation of lipoic acid into the protein-bound form are chloramphenicol and ammonium sulfide. This inhibition coupled with the lack of effect on accumulation makes these compounds selective reagents for differentiation of accumulation and incorporation of lipoic acid into the protein-bound form. Since the effect of 2,4-dinitrophenol and potassium cyanide was greater on accumulation than on incorporation, the level of lipoic acid accumulated in the pool is greater than that required to saturate the lipoic acid-activating system for incorporation of lipoic acid into the protein-bound form.

Table 3, Part B shows the effect of incubation of cells for 3 min with 1 m*M* lipoic acid analogs before addition of 10 μM lipoic acid and measurement of incorporation and accumulation. The C_7 and C_9 analogs of lipoic acid slightly inhibit both accumulation and incorporation. Further, oleic acid inhibits incorporation but not accumulation. These results suggest that the transport system for lipoic acid is fairly specific.

Effect of Osmotic Shock on Lipoic Acid Uptake

Treatment of *E. coli* cells with osmotic shock has been used to implicate binding proteins in the transport of several specific compounds. Figure 4 compares the effect of shocking of *E. coli* on the transport of lipoic acid and of valine. The osmotic shock procedure reduces the uptake of both substrates; about 30% of the original uptake activity remains for lipoic acid while the reduction of valine uptake was 80%. Attempts to restore lipoic acid transport by the addition of the shock fluid to the treated cells have been unsuccessful.

Measurement of the binding of lipoic acid to the concentrated protein of the osmotic shock fluid by the technique of equilibrium dialysis revealed 7 pmol of lipoic acid and 9 pmol of leucine were bound per mg of protein.

Protein fractionation experiments on the osmotic shock fluid involving ammonium sulfate precipitation, DEAE-cellulose chromatography, and gel filtration all failed to separate a protein peak with increased

TABLE 3. *Effect of inhibitors and analogs of lipoic acid uptake*

Part A. Inhibitors

Additive	Conc., m*M*	% control	
		Free pool	Protein-bound
Cystine	1.0	32	8
2,4-Dinitrophenol	0.1	65	90
KCN	1.0	60	67
NaN_3	1.0	99	103
$(NH_4)_2S$	1.0	97	7
Chloramphenicol	400 μg/m*l*	99	7

Part B. Analogs

Additive	% control	
	Free pool	Protein-bound
None	100	100
Oleic acid	102	58
Octanoic acid	106	101
Hexanoic acid	105	96
Acetic acid	116	100
C_7 Lipoic acid	71	90
C_9 Lipoic acid	78	75
8-Methylthioctic acid	105	91

Part A. The indicated concentrations of inhibitors were added 10 min before the addition of 10 μ*M* radioactive lipoic acid. The uptake was stopped after 10 min with slushy M-9. All values are the average of two experiments each with triplicate samples except for the ammonium sulfide, which was done only once.

Part B. Each compound was dissolved in 95% ethanol and added at a concentration of 1 m*M* three min before the addition of 10 μ*M*[^{35}S]-lipoic acid. The data shown were collected after 10 min incubation. Similar results were obtained when the samples were incubated 3 min.

lipoic acid binding activity. The highest activity achieved with lipoic acid was 10.1 pmol/mg while with thiamine-binding protein an ammonium sulfate fractionation yielded a binding activity of 25.8 pmol/mg.

The uptake of lipoic acid by membrane vesicle preparations showed a biphasic concentration dependence (Fig. 5) which is reminescent of the pattern shown for thiamine uptake (18). When the proteins and lipids were separated from these vesicles and lipoic acid binding was measured on each component separately, only nonsaturable binding was observed.

DISCUSSION

Vitamins are transported into bacterial cells by systems which are just as diverse as those systems which function in the transport of other compounds (19). The vitamin transport systems are as one would expect—efficient and specific. There are advantages in studying transport systems for vitamins: 1) the substrates have a rather limited function, 2) the low concentration of vitamins present in the natural environment is reflected by high-affinity systems, 3) the metabolic functions of most of the vitamins are known, and 4) the whole spectrum of transport mechanisms can be studied with one biologically functional class of compounds.

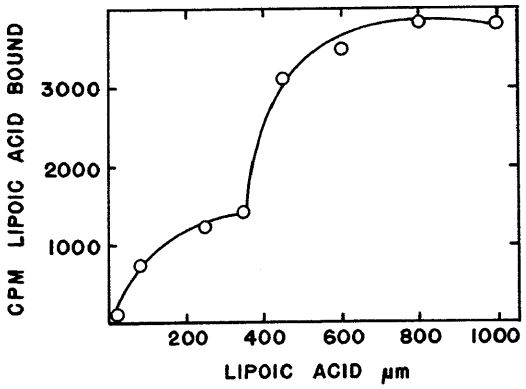

FIGURE 5. Uptake of lipoic acid by membrane vesicles.
Membrane vesicles were prepared as described in the Materials and Methods Section. They were incubated with various concentrations of lipoic acid as indicated. The uptake of lipoic acid was determined as described in the Materials and Methods Section.

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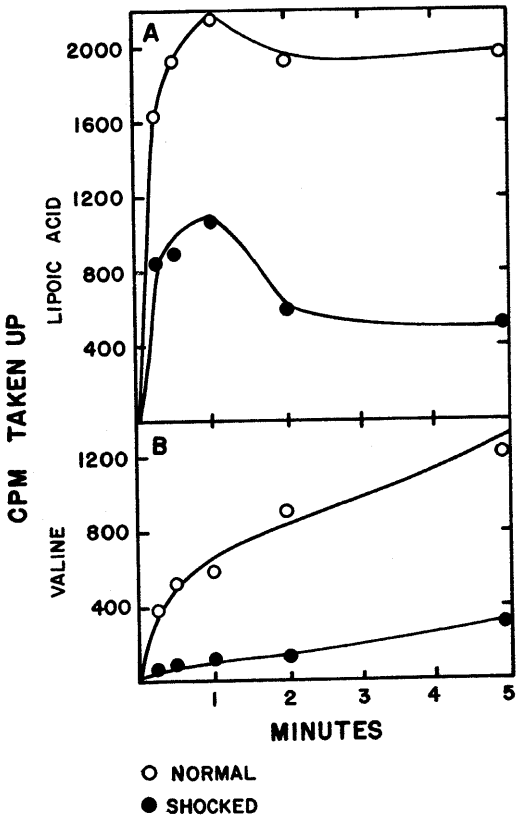

FIGURE 4. Effect of osmotic shock of *E. coli* on the uptake of lipoic acid and valine.
E. coli cells were grown as described in the Materials and Methods Section and divided into two parts. One part served as the control (untreated). The other part was treated by the osmotic shock procedure given in the Materials and Methods Section.
A. Lipoic Acid Uptake. The uptake of lipoic acid by the control, O, and the treated, ●, cells was determined as described in Figure 3A.
B. Valine Uptake. The uptake of valine (0.025 μmol/ml) was determined for control cells, O, and treated cells, ●. The procedure was analogous to that used with lipoic acid.

Because we were familiar with the enzymatic reactions for the conversion of lipoic acid to its functional form and had developed an interest in peptide transport, we undertook a study of the transport of lipoic acid. Several aspects of our research concerning lipoic acid transport in *S. faecalis* have been published (7-12). In this paper we have characterized the transport of lipoic acid in *E. coli.*

The uptake of lipoic acid by *E. coli* is proportional to the cell concentration (Fig. 1), is saturable with lipoic acid (Fig. 2), and is temperature-dependent. The uptake of lipoic acid is rapid (Fig. 3) and the same type of overshoot phenomenon is noted with lipoic acid in *S.*

other compounds. Becker and Lichstein (20) ascribe this commonly observed overshoot as a transinhibition of uptake (active accumulation inhibited by the intracellular pool) and the continued efflux of intracellular substrate.

Osmotic shock (Fig. 4) and 2,4-dinitrophenol (4 m*M*) reduced lipoic acid transport. Attempts to purify a lipoic acid-specific binding protein from the osmotic shock fluid were unsuccessful. Because of the lipophilic nature of lipoic acid, there are considerable nonspecific hydrophobic interactions. Those lipid solubility characteristics of lipoic acid required special precautions in the procedure used for stopping uptake and preventing of artifactual interactions.

Lipoic acid is accumulated by membrane vesicles (Fig. 5) of *E. coli*. Because of the lipophilic nature of lipoic acid and of the membrane preparations and/or vesicles, we turned our attention to thiamine transport in *E. coli.*

We have demonstrated the existence of a lipoic acid transport system in *E. coli* and defined some of its properties. Because of the nonspecific hydrophobic interactions that occur with lipoic acid, the study of vitamin transport systems is easier when other substrates are used.

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