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Active Transport of Biotin in *Escherichia coli* K-12

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The transport of [^{14}C]biotin into cells of a biotin prototroph, *Escherichia coli* K-12 strain Y10-1, was investigated. The vitamin taken up by the cells in this strain existed primarily in the free form. Addition of glucose enhanced the rate of uptake six- to eightfold and the steady level was reached in 2 to 3 min resulting in accumulation of biotin against a concentration gradient. The uptake showed marked dependence on temperature (Q_{10}, 2.3; optimum, 37 C) and pH (optimum 6.6) and was inhibited by iodoacetate. Energy of activation for glucose-dependent uptake was calculated to be 16,200 cal per mol. The rate of biotin uptake with increasing biotin concentrations showed saturation kinetics with an apparent K_m and V_{max} values of 1.4×10^{-7} M and 6.6 pmol per mg of dry cells per min respectively. The cells also accumulated biotin against a concentration gradient in the absence of added glucose, although at a much lower rate. This accumulation was much more susceptible to inhibition by azide and uncouplers of oxidative phosphorylation suggesting that the energy source was supplied through the electron-transport chain. Inhibition studies with a number of biotin analogues indicated the requirement for an intact ureido ring. The biotin uptake was inhibited in cells grown in biotin-containing medium and was shown to be the result of repression of the transport system, suggesting the control of the biotin transport.

Biotin transport has been extensively investigated in the two natural biotin auxotrophs, *Lactobacillus plantarum* and *Saccharomyces cerevisiae* (2, 14, 15, 18, 19). Lichstein and Ferguson (9) were the first to report an energy requirement for the transport of biotin into the cells of *L. plantarum* and that transport was inhibited by the addition of the biotin analogue, homobiotin. Subsequent studies revealed that biotin transport was not only dependent on an energy source but also on temperature, pH, and other factors which are indicative of an active transport process (18). More direct evidence for a protein-mediated transport system was obtained with *S. cerevisiae* when Rogers and Lichstein (15) discovered that biotin uptake was controlled by the biotin levels in the growth medium. High levels of biotin decreased the rate of biotin uptake and protein synthesis was required to restore this capacity. In support of a membrane-bound transport system is the finding of Becker, Wilchek, and Katchalski (1) that the *p*-nitrophenyl ester of biotin inactivates biotin uptake in yeast irreversibly, presumably by forming a covalent bond with some component of the membrane. More recently, Griffith and Leach (6) observed that osmotic shock treatment of *Escherichia coli* cells did not affect the biotin transport activity of the cells. They concluded that a soluble biotin-binding protein was not a component of the biotin transport system and therefore the latter must be firmly associated with the inner cell membrane.

Biotin may also enter cells by processes which are not indicative of active transport. In *L. plantarum* a portion of the biotin uptake has been shown to be independent of an energy source, temperature, and pH. It is not inhibited by iodoacetic acid, but still exhibits analogue inhibition, suggesting a facilitated diffusion mechanism (18). In yeast cells in which the active transport is inhibited by biotin repression, a small biotin uptake is observed which shows the kinetics of a passive diffusion process (15).

Biotin transport systems in biotin prototrophs have not yet been examined in any great detail. Cicmanec and Lichstein (Abstr. Annu. Meet. Amer. Soc. Microbiol. 1973, P195, p. 173) claimed that in two strains of *E. coli*, K-12 and Crookes, biotin was taken up by facilitated diffusion and the uptake occurred in dividing cells but not in resting cells. Pai (11, 12), on the other hand, was able to demon-

strate biotin uptake in resting cells of *E. coli* K-12. His study was not directed towards the mechanism of biotin transport but rather towards determining whether a derepression of the biotin biosynthetic pathway was due to an alteration in the regulatory gene of the *bio*A operon or an altered permeability. An interesting offshoot of this investigation was the indication that the transport system and the biotin biosynthetic pathway were independently regulated by the biotin levels in the growth medium, suggesting separate repressors.

The present study is part of a larger investigation of a series of α-dehydrobiotin-resistant mutants. Certain of these mutants have been shown to be regulatory mutants of the biotin biosynthetic pathway, repressor mutants (R^-) and operator constitutive mutants (O^c) (4), and others appear to have an altered transport system for biotin. The factors affecting the uptake and accumulation of biotin were explored in detail and evidence is presented to support an active process in *E. coli* K-12, Y10-1.

MATERIALS AND METHODS

Chemicals. Crystalline *d*-biotin was purchased from Sigma Chemical Co. Carbonyl-labeled [^{14}C]biotin (20 mCi/mmol) was purchased from Amersham-Searle Corp. *d*-Dethiobiotin, 7,8-diaminopelargonic acid and *d*-diaminobiotin were prepared as previously described (8). α-Dehydrobiotin was a gift from L. J. Hanka, Upjohn Co., and was used after chromatography on a Dowex-1-formate column. Homobiotin was a gift of Hoffmann-LaRoche. Vitamin-free casein hydrolysate was purchased from Nutritional Biochemical Corp. Carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) was kindly provided by R. Hanson.

Bacterial strains. *E. coli* K-12 strain Y10-1 was employed in all experiments. Mutants of this strain have been used extensively to study biotin biosynthesis and its regulation (16). A wild strain of *E. coli*, W3110 (*str*^{r}), a biotin-deletion mutant, T50-1, and a biotin auxotroph, *bio*B-105, were used to show the diversity in the levels of free and bound biotin.

Growth media and cultural conditions. The medium described by Vogel and Bonner (17) was used after supplementing with 0.5% glucose, 0.01% L-leucine, 0.0005% thiamine, and 2% (vol/vol) casein hydrolysate. The final medium was sterilized by Millipore filtration. Biotin was added aseptically when desired; a concentration of 0.2 ng per ml was used for the growth of the biotin auxotrophs.

Uptake studies. Cells grown either to early stationary phase or to mid-log phase were used. The flask containing a 10% inoculum of an overnight grown culture was incubated for 4 to 5 h to reach the early stationary phase of growth (Klett$_{66}$: 250-280). A 1% inoculum was used for mid-log grown cells which required an incubation period of 3.5 h (Klett$_{66}$: 120). The cells were washed once with distilled water warmed to 37 C and resuspended in 0.05 M phosphate buffer, pH 6.6. Unless indicated otherwise, the uptake medium contained per ml: 50 μmol of potassium phosphate, pH 6.6, 20 μmol of glucose, 1 μmol of magnesium sulfate, 100 μg of chloramphenicol, and 1.0 to 1.2 mg of cells (dry weight). The reaction mixture was equilibrated for 10 min at 37 C and 75 ng of [^{14}C]biotin was added to start the reaction. Portions of 2 ml were withdrawn at various time intervals and rapidly added to 4 ml of cold 0.9% saline previously placed on the Millipore filter (0.65 μm pore size; 47 mm diameter). The cold saline stopped the transport process (see effect of temperature). Filtration was completed within 10 to 12 s and the filters were washed once with 4 ml of cold saline. Larger wash volumes did not further reduce the [^{14}C]biotin levels retained on the filter. The filters were placed in counting vials and dried at 80 C for 1 h, and 15 ml of toluene, containing per liter 6 g of 2,5-diphenyloxazole (PPO) and 0.3 g of 1,4-bis[2-(5-phenyloxazolyl)] benzene (POPOP), was added. The radioactivity on the filters was counted at 4 C in an Ansitron liquid scintillation counter. Corrections for quenching were applied with the aid of the external standard and disintegrations per min were converted to nanograms of biotin per milligram of cells (dry weight). A control was run at 0 C under similar conditions to determine the nonspecific binding of [^{14}C]biotin and this correction was applied to all the experimental values. The results are averages of at least two determinations. When free and bound biotin were to be differentiated, additional samples were taken into Nalgene centrifuge tubes containing 4 g of ice and centrifuged for 5 min at 12,000 rpm. The cells were resuspended in 5 ml of hot water and heated for 10 min in a boiling-water bath. The heated cells were collected on Millipore filters and washed with 20 ml of hot water. The filters were dried and counted in the manner previously described. These samples represented bound biotin.

RESULTS

Preliminary experiments conducted on the intracellular distribution of biotin in cells of *E. coli* K-12 strain Y10-1 indicated that essentially all the biotin was in the free form whether the cells were grown to mid-log or stationary phase. A check on three other strains of *E. coli* showed marked differences in the levels of free and bound [^{14}C]biotin compared to strain Y10-1 (Table 1). After 10 min, nearly all the biotin taken up by the deletion mutant (T50-1) was in the bound form. The biotin auxotroph (*bio*B-105) had the same amount of [^{14}C]biotin in the bound form, but also substantial amounts in the free form. The large amount of bound [^{14}C]biotin in the auxotrophs is due to the accumulation of biotin apoenzymes in cells grown either in biotin-sufficient or -deficient medium (10). When such cells are resuspended in solutions of higher biotin concentrations there ensues a rapid conversion of the apoenzyme form to the holoenzyme form. In the

TABLE 1. *Distribution of intracellular [^{14}C]biotin in various strains of E. coli K-12*[a]

Strain	[^{14}C]biotin uptake (ng/mg dry cells)	
	Free	Bound
Y10-1	1.12	<0.05
W3110	0.66	0.34
Y10-1 (*bioB*-105)	1.70	3.60
T50-1 $(bio)_{del}$	0.05	3.60

[a] The uptake studies were performed as described in Materials and Methods. Cells collected from cultures grown to stationary phase were used in all cases and free and bound biotin were determined at the steady state level (10 min, except for the deletion mutant where it was 30 min).

prototroph, W3110, 34% of the [^{14}C]biotin taken up is in the bound form. It would thus appear that in these prototrophs the biotin levels within the cell are insufficient to completely saturate the apoenzymes formed during growth. It is also evident from the data that in biotin auxotrophs a major part of [^{14}C]biotin taken up by the cells is covalently bound.

Effect of energy source. *E. coli* cells showed very rapid uptake of [^{14}C]biotin in the presence of 0.02 M glucose, reaching approximately 65% of the steady state level in about 0.3 min (Fig. 1). Higher glucose concentrations did not further enhance the uptake rate or the steady state level of biotin. In the absence of glucose, the biotin was taken up at a lower initial rate and did not reach a plateau even after 10 min. In the presence of glucose the initial rate of biotin uptake was enhanced six- to eightfold, although total accumulation after 10 min was only 1.3-fold greater than in its absence. It is also evident from Fig. 1 that there is a small uptake of [^{14}C]biotin at 0 C which is not influenced by prolonged incubation in the presence or absence of glucose. This uptake represents nonspecific binding of [^{14}C]biotin to the filter, cells, or both.

Effect of temperature and pH. The uptake of biotin in *E. coli* cells was markedly temperature dependent (Fig. 2A). A sharp maximum was observed at 37 C with 50% reduction in the uptake rate at 30 and 50 C, respectively. No uptake was observed at 7 C or below or at 58 C and above. The Q_{10} value between 25 and 35 C was approximately 2.3 and the energy of activation between 30 and 37 C as determined from the Arrhenius plot (Fig. 2B) was calculated to be approximately 16,200 cal per mol of biotin accumulated.

The biotin uptake in the cells was also markedly influenced by the pH of the uptake medium as shown in Fig. 3, with an optimal value for accumulation at pH 6.6.

Effect of biotin concentration. The rate of biotin uptake increased with increasing external biotin concentration, approaching saturation at approximately 0.5 μM biotin (Fig. 4). The apparent K_m and V_{max} values calculated from the Lineweaver-Burk plot (insert, Fig. 4) were 1.4×10^{-7} M and 6.6 pmol of biotin per mg of cells per min, respectively.

Establishment of concentration gradient. An important consideration in identifying an active transport system is to determine if the transport molecules are taken up against a concentration gradient. Thus, estimations of the intracellular [^{14}C]biotin concentrations were made at the steady state using different extracellular biotin concentrations. Figure 5 indicates that the biotin gradient (ratio of intracellular to extracellular biotin concentration) is a function of the extracellular biotin concentration. It was empirically found that a replot of the reciprocal of the gradient versus the extracellular concentration was linear as shown in Fig. 5 (insert) and an extrapolation to the ordinate gave a ratio of 36 at vanishingly small extracellular biotin concentrations. Although a similar set of experiments was not performed in the absence of glucose, it was found that at an extracellular biotin concentration of 15 ng/ml the ratio was 9.8 as compared to 11 in the presence of glucose. The positive ratio obtained when glucose is omitted from the uptake medium suggests that this is also an active process with the energy derived from endogenous reserves. Additional support for an active trans-

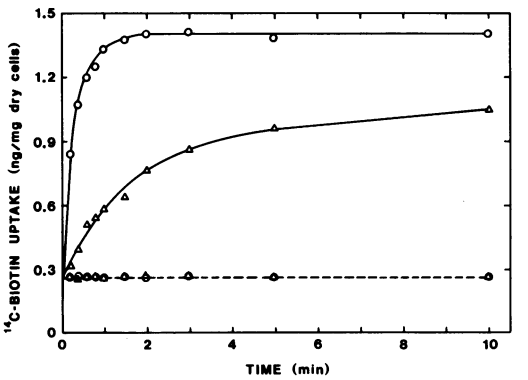

FIG. 1. *Time course of [^{14}C]biotin uptake by E. coli cells in the presence (○) and absence (△) of 0.02 M glucose at either 0 C (dotted line) or at 37 C (solid lines) in 0.05 M phosphate buffer, pH 6.6, with 75 ng of [^{14}C]biotin per ml of uptake medium. Other conditions were described in Materials and Methods.*

port process was also obtained with the study of the effect of metabolic inhibitors on the biotin transport system.

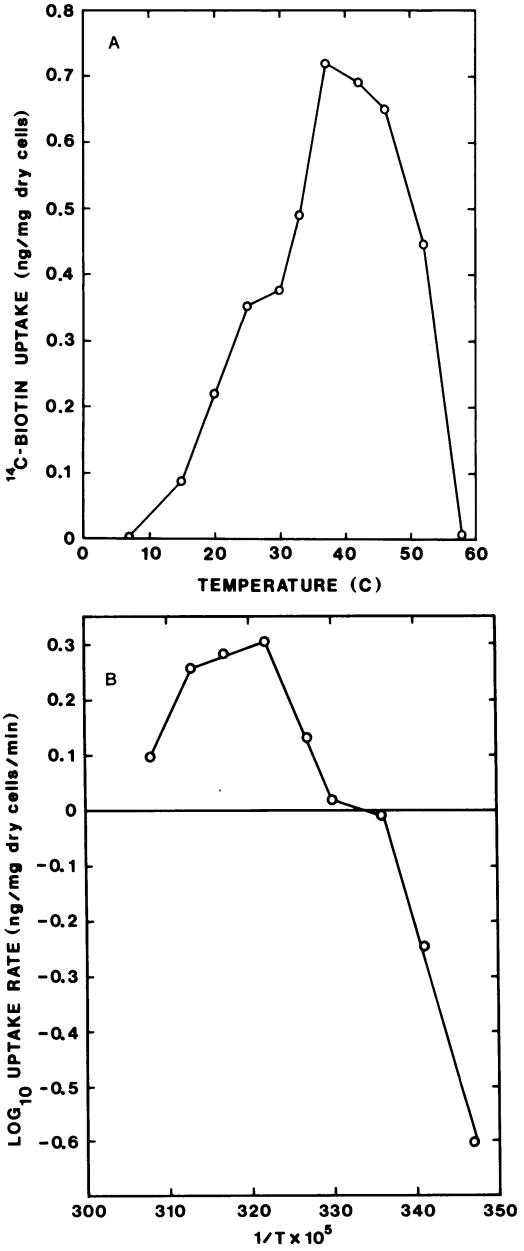

FIG. 2. *(A) Effect of temperature on the uptake of [^{14}C]biotin in the presence of glucose. Experimental conditions were as for Fig. 1, except that the cells were equilibrated for 10 min in the uptake medium at the indicated temperatures prior to addition of [^{14}C]biotin. Incubation time was 0.3 min. (B) Arrhenius plot of temperature data.*

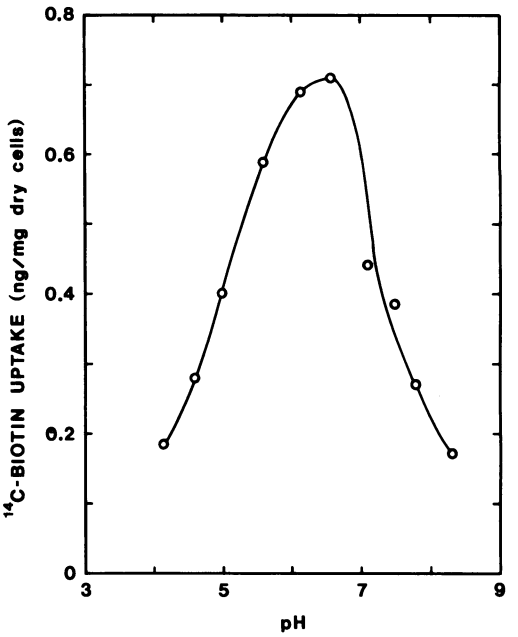

FIG. 3. *Effect of pH on the uptake of [^{14}C]biotin. Experimental conditions were as for Fig. 1, except that the cells were equilibrated for 10 min in the uptake medium containing glucose at the indicated pH values prior to addition of [^{14}C]biotin. Incubation time was 0.3 min.*

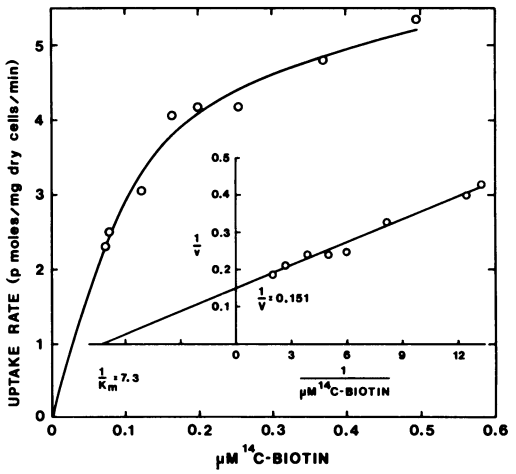

FIG. 4. *Uptake of [^{14}C]biotin as a function of concentration. Experimental conditions were the same as those for Fig. 1 with added glucose. Since biotin uptake is extremely rapid, an automatic 2-ml delivery syringe was used for sampling at 0.1-min intervals and an incubation temperature of 30 C was maintained. Each point on the curve represents the rate of biotin uptake calculated by the least square plot of data obtained from triplicate experiments.*

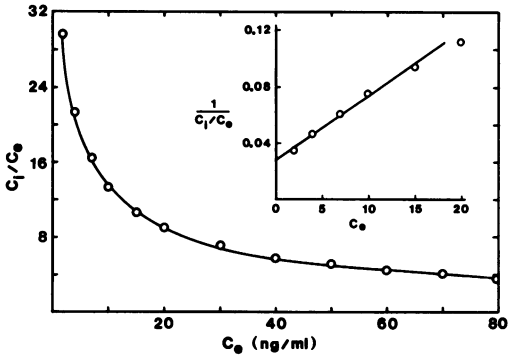

FIG. 5. *Effect of extracellular biotin concentration (C_e) on intracellular to extracellular biotin gradient (C_i/C_e). Experimental conditions were the same as those for Fig. 1 with added glucose. Biotin uptake was measured after 10 min of incubation in each case. C_i was calculated as ng of biotin per ml assuming an intracellular free space of 0.25 μliters per 2.5 × 10^8 cells (13).*

Effect of inhibitors. Table 2 summarizes the effects of various metabolic inhibitors on [^{14}C]biotin uptake in the presence and absence of glucose. Iodoacetate, an inhibitor of glycolysis, showed marked inhibition in the presence of glucose than in the absence in contrast to azide, an inhibitor of electron transport. The combined effect of both the inhibitors appeared to be additive. The uncouplers of oxidative phosphorylation, 2,4-dinitrophenol and CCCP, were more effective inhibitors in the absence of glucose. Complete elimination of biotin uptake in the presence and absence of glucose was obtained by the combination of iodoacetate and 2,4-dinitrophenol. Cyanide, which is also an inhibitor of electron transport, is not as effective as azide.

Effect of biotin analogues. To determine the specificity of the biotin transport system in *E. coli*, the effect of several structurally related biotin analogues on [^{14}C]biotin uptake was investigated (Table 3). The analogues, containing an intact ureido group but altered at the position of the sulfur atom by deletion, substitution, or oxidation, inhibited biotin uptake more than 50%. Transport appeared to be more sensitive to biotin sulfone as only one-tenth the concentration was required to produce the same degree of inhibition. Those analogues altered in the side chain by addition of a methylene group or a double bond were also effective inhibitors of biotin transport. The biotin analogues, diaminobiotin and 7,8-diaminopelargonic acid, which lack the ureido group, exerted only a minimal effect on the uptake system even at concentrations higher than that used with the other analogues.

Control of biotin transport. Growth of cells in a medium containing increasing concentration of biotin decreased the [^{14}C]biotin uptake. Addition of 5 ng of biotin per ml to the growth medium, which normally represses the biotin biosynthetic enzymes more than 95%, produced only an 18 to 20% decrease in the transport of biotin, whereas addition of 10 ng of biotin resulted in a 40% decrease. This inhibitory effect of biotin on the transport system in *E. coli* was previously indicated by Cicmanec and Lichstein (Abstr. Annu. Meet. Amer. Soc. Microbiol. 1973, P195, p. 173) and Pai (12) but no

TABLE 2. *Effect of metabolic inhibitors on [^{14}C]biotin uptake*[a]

Inhibitor	Percent inhibition[b]	
	With glucose	Without glucose
Iodoacetate (10 mM)	70	20
Azide (10 mM)	18	78
Iodoacetate + azide	86	90
Cyanide (10 mM)	46	18
Cyanide (20 mM)	47	31
2,4-Dinitrophenol (2 mM)	73	97
Iodoacetate + 2,4-dinitrophenol	100	100
CCCP (5 μM)	11	60

[a] The uptake studies were performed as described in Fig. 1. The inhibitor(s) was present during the 10-min equilibration period prior to addition of [^{14}C]biotin.

[b] Percent inhibition as compared to uptake in the absence of inhibitor(s) after 0.3 min of incubation.

TABLE 3. *Inhibition of [^{14}C]biotin uptake by biotin analogues*[a]

Analogue	Concn (ng/ml)	Percent inhibition[b]
Biotin sulfone	300	59
Oxybiotin	3,000	54
Dethiobiotin	3,000	65
Homobiotin	3,000	52
α-Dehydrobiotin	3,000	56
Diaminobiotin	4,500	16
7,8-Diaminopelargonic acid	4,500	9

[a] The uptake studies were performed with 45 ng of [^{14}C]biotin per ml of uptake medium containing glucose. The analogues, in concentrations indicated, were added simultaneously with [^{14}C]biotin after the equilibration period.

[b] Percent inhibition as compared to uptake in the absence of analogue after 0.3 min of incubation.

information was provided as to the mechanism involved and, therefore, this aspect was examined in greater detail. The procedure was essentially similar to what was used previously to determine if control of the biotin biosynthetic enzymes was due to feedback inhibition or repression (5). The results are presented in Fig. 6. It can be seen that the transfer of cells grown in a high biotin-containing medium to a biotin-depleted medium for 2 h resulted in a twofold increase in their capacity to take up [^{14}C]biotin. This increase was not observed when chloramphenicol, an inhibitor of protein synthesis, was incorporated in the medium. Similar results were also obtained when rifampin, a specific inhibitor of DNA-dependent RNA polymerase was used instead of chloramphenicol. These results suggest that the mechanism of control of the biotin transport system is through enzyme repression. A similar mechanism for the regulation of biotin transport by biotin in *S. cerevisiae* has been reported by Rogers and Lichstein (15).

DISCUSSION

The rate of biotin uptake in *E. coli* K-12 strain Y10-1 is linear for about 0.3 min and reaches a steady state concentration in about 2 min. Biotin uptake is observed in cells grown to mid-log or stationary phase, contrary to the observations of Cicmanec and Lichstein (Abstr. Annu. Meet. Amer. Soc. Microbiol. 1973, P195, p. 173). The overshoot phenomenon observed in yeast at high biotin concentration is not evident in *E. coli*. The transport process shows a sharp pH optimum at 6.6 and is markedly temperature dependent with a Q_{10} of 2.3. The calculated energy of activation, 16,200 cal per mol, is of the same order of magnitude reported for the active transport system in *L. plantarum* (18). Rapid kinetic measurements permitted the estimation of an apparent K_m of 1.4×10^{-7} M which is comparable to the values obtained for both yeast and *L. plantarum* (2).

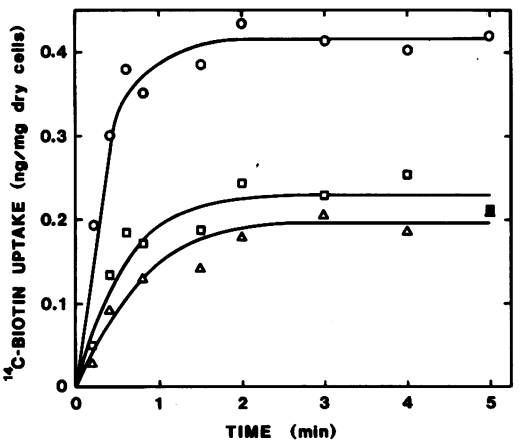

FIG. 6. *Repression of biotin transport system by biotin. E. coli cells were grown to mid-log phase in minimal medium containing 15 ng of biotin per ml. Cells were washed in cold saline and distributed into minimal medium with zero biotin* (○), *15 ng of biotin per ml* (△) *or 100 μg of chloramphenicol* (□). *The suspensions were shaken for 2 h at 37 C, cells were harvested, and the* [^{14}C]*biotin uptake was measured.*

The strain of *E. coli* used in these experiments accumulates biotin primarily in the free form, but there is considerable variation in the free and bound biotin accumulation with three other strains used in our laboratory. The time interval in which the steady state was reached in strain Y10-1 is about one-tenth that observed by Pai (11) with another *E. coli* K-12 strain at the same external biotin concentration. It is difficult to reconcile the marked differences in the rates in these two studies, except to indicate that extended time intervals to reach the steady state level were observed in the three strains, which show varying amounts of bound biotin. There was no indication as to the amount of bound biotin present in the strain used for the biotin transport studies reported by Pai.

Free biotin accumulation by *E. coli* cells proceeds against an apparent concentration gradient, which is indicative of an active transport process. However, the degree of accumulation is low compared to that observed with yeast and *L. plantarum* (14, 18). An active transport process, rather than facilitated diffusion, is also supported by the energy requirement for biotin uptake. Addition of metabolic inhibitors shows differential effects on the glucose-dependent and -independent biotin transport processes. Whether the biotin transport system is driven directly by phosphate-bond energy formed by either oxidative phosphorylation or glycolysis (3) or through a portion of the electron-transport chain as in vesicles (7) is not evident from the inhibitor studies and must await further investigation with vesicle preparations.

In addition to the saturation kinetics observed with biotin the presence of a carrier-mediated transport process is further supported by two additional pieces of evidence. The inhibitory effects of the biotin analogues in this organism, as in yeast (14), show the specificity for an intact ureido ring for inhibition. Alterations in the side chain or in the tetrahydrothiophene ring have little effect. This specificity is

reminiscent of the affinity of avidin for biotin analogues. Secondly, the transport process in *E. coli* is under negative control by biotin as previously indicated by Cicmanec and Lichstein (Abstr. Annu. Meet. Amer. Soc. Microbiol. 1973, P195, p. 173) and Pai (12) and now supported by our own studies on cells grown in high biotin media. The decreased biotin uptake was shown to be due to the repression of the transport system since the biotin transport could be restored by placing the cells in a biotin-free media for 2 h. During this time interval protein synthesis is required, as was evident from the inhibition by chloramphenicol or rifampin. Pai (12) recently presented evidence that the repression of biotin transport and the biotin biosynthesis of enzymes by biotin are independent processes. In our system, much higher concentrations of biotin are required for repressing biotin transport than biotin biosynthesis, which could support the suggestion for different repressors in the two systems.

ACKNOWLEDGMENTS

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