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Uptake of Extracellular Biotin by *Escherichia coli* Biotin Prototrophs

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Uptake of exogenous biotin by two *Escherichia coli* biotin prototroph strains, K-12 and Crookes, appeared to involve incorporation at a fixed number of binding sites located at the cell membrane. Incorporation was characterized as a binding process specific for biotin, not requiring energy, and stimulated by acidic pH. Constant saturation quantities of exogenous biotin were incorporated by these cells, and the amounts, which were titrated, depended on whether the cells were resting or dividing. Resting cells incorporated exogenous biotin amounting to 2% of their total intracellular biotin content. Fifty percent of the exogenous biotin was incorporated into their free biotin fraction, and 50% was incorporated into their bound biotin fraction. On the other hand, dividing cells incorporated exogenous biotin into all of their intracellular sites, 88% going into the intracellular-bound biotin fraction, and 12% going into the free biotin fraction. Calculations suggested that each cell contained approximately 3,000 binding sites for biotin. It was postulated that biotin incorporation sites might have been components of acetyl coenzyme A carboxylase located at or near the membrane.

Biotin is a vitamin essential to the metabolism of *Escherichia coli,* an organism capable of its synthesis but preferentially utilizing an exogenous source. Indeed, an exogenous concentration of 5 ng/ml of biotin (0.2×10^{-7} M) resulted in nearly total repression of the enzymes for biotin biosynthesis in two strains of *E. coli,* Crookes and K-12 (13, 15). Employing these two strains, which had been used extensively in our laboratory for studies of biotin biosynthesis, we carried out an investigation to determine the process by which exogenous biotin entered these cells.

Previous studies with two biotin auxotrophs, *Lactobacillus plantarum* and *Saccharomyces cerevisiae,* have demonstrated the presence of energy-dependent transport systems which concentrated biotin in the free biotin fractions within these cells (21, 17). Following transport by *Lactobacillus,* the free biotin fraction contained more biotin than the initial bound biotin fraction by twofold, and in *Saccharomyces,* by 360-fold. Since the bound biotin fractions of both organisms remained constant once saturated, they appeared to represent binding of the prosthetic group by apoenzymes, whereas the free biotin fractions, which fluctuated with the external concentrations of biotin, might have been an intracellular pool maintained in reserve. In contrast, initial studies with the two *E. coli* strains have revealed that biotin was taken mainly in the bound biotin fraction. Moreover, in our two *E. coli* strains, intracellular free biotin fractions did not appear to exist to any great extent. In cells of the Crookes strain under conditions where the biotin requirement was satisfied by biosynthesis, about 90% of the total intracellular biotin was in the bound biotin fraction (13).

Although the nature of the bound biotin fractions has not been determined in our strains, other investigators have shown that *E. coli* cells contain only one major biotin-containing polypeptide corresponding to the biotin carboxyl carrier protein (BCCP) component of acetyl coenzyme A (CoA) carboxylase (6). This observation was consistent with the absence of pyruvate and propionyl CoA carboxylases in this organism (1). The BCCP, which was covalently bound to a biotin molecule, was one component of the acetyl CoA carboxylase of *E. coli* and acted as the CO_2 carrier between the biotin carboxylase and transcarboxylase subunits (7).

The present report examines the mechanism of incorporation of biotin by the two *E. coli* prototrophic strains used in our laboratory, both of which maintain intracellular biotin predominantly in its bound form.

MATERIALS AND METHODS

Bacterial strains. Two biotin prototroph strains, *E. coli* Crookes (ATCC 8739) and *E. coli* K-12, were employed in all experiments. Both of these strains had been used in previous studies on biotin biosynthesis (12, 14), and the K-12 strain was the parent

strain used in subsequent studies on biotin uptake by Pai and Lichstein (12). All experiments were conducted at least once with each strain, with nearly duplicate results obtained in all cases.

Growth medium and conditions. The basal medium employed was that of Davis and Mingioli (5), which consisted of 7 g of K_2HPO_4, 3 g of KH_2PO_4, 1 g of $(NH_4)_2SO_4$, 0.5 g of sodium citrate · 3 H_2O, 0.1 g of $MgSO_4 \cdot 7\ H_2O$, and 2 g of glucose per liter, with a final pH of 6.8. Growth was carried out at 37°C on a rotary shaker.

Uptake studies. Cells were grown to late mid-log phase, washed two times with distilled water by centrifugation, and suspended in phosphate buffer (20 mM, pH 6.8) for use in incorporation mixtures. The incorporation mixtures (total volume, 80 ml) consisted of washed cells (approximately 0.5 mg of dry weight per ml), potassium phosphate buffer (20 mM, pH 6.8), $MgSO_4$ (1.0 μM), glucose (44 mM), $(NH_4)_2SO_4$ (7.56 mM), and [^{14}C]biotin at various concentrations. These constituents allowed cell division to take place. For resting cells, $(NH_4)_2SO_4$ was omitted from the incorporation mixtures. The washed cells suspended in buffer were added to the incorporation mixtures at zero time, and the mixtures were incubated at 37°C with reciprocal shaking. At various intervals, 5.0-ml portions were removed and filtered through 0.45-μm type HA membrane filters (47 mm; Millipore Corp.). At these times, cell concentrations were measured by turbidity with a Klett-Summerson photoelectric colorimeter using the red filter, 640 to 700 nm. Klett readings of 250 units for the Crookes strain and 269 units for the K-12 strain were determined to be equivalent to 1 mg of dry weight per ml or 4×10^9 cells per ml. The membrane filters were washed two times with 50-ml portions of distilled water, dried, and counted for radioactivity using Bray (2) counting solution in a Packard Tri-Carb scintillation spectrometer.

Avidin treatment. Cells were prepared as for uptake studies and suspended in avidin treatment mixtures, which had the same constituents as incorporation mixtures except that [^{14}C]biotin was omitted and avidin (0.02 U/ml) was added. After 2 h of incubation at 37°C with reciprocal shaking, the cells were washed three times with 20-ml portions of distilled water and used in uptake studies.

Determination of free and bound biotin. Membrane filters containing washed 1-ml portions of cells from uptake reaction mixtures were added to tubes containing 7 ml of water. The cells were boiled in a boiling water bath for 20 min and cooled, and the cell debris was separated by centrifugation (5,000 × *g* for 5 min) from the supernatant, which contained the free biotin released during boiling. The cell debris was washed once with 7 ml of water and centrifuged. The decanted wash was combined with the original supernatant, and one 1-ml sample of this was counted for radioactivity. The remaining cell debris was suspended in distilled water to its original volume, and a 1-ml portion was counted for bound biotin. In a few instances, the bound biotin was also determined after acid treatment, i.e., autoclaved for 1 h in 6 N H_2SO_4 according to the published procedure (13, 21), and the quantities of bound biotin were found to be the same by either method. The 1-ml samples of free or bound biotin were counted in 15 ml of Bray solution.

Microbiological assay. Measurement of biotin was determined microbiologically, employing *L. plantarum* 17-5 (ATCC 8014). The procedures and the methods for preparing samples for the microbiological assay have been described previously (17).

Chemicals. D-[*carbonyl*-^{14}C]biotin with a specific activity of 58 mCi/mmol was purchased from Amersham/Searle, Arlington Heights, Ill. Avidin, which we assayed for biotin-binding activity (1 U/mg) by microbiological assay with *L. plantarum*, was purchased from Worthington Biochemicals Corp., Freehold, N.J.

RESULTS

Initial uptake rate and steady-state level with resting cells. Our initial experiments were to identify the K_m for any biotin transport system in our organisms so that the appropriate concentration of exogenous biotin would be known for subsequent experiments. One hundred and eighty-seven experiments were carried out over a 500-fold concentration range of exogenous biotin. One half of the experiments employed cells of *E. coli* K-12; the other half used *E. coli* Crookes. With either strain, the results were similar. Uptake was measured with resting cells suspended in media that lacked a nitrogen source.

No significant difference in the uptake rates measured over a 500-fold concentration range of extracellular biotin was observed (Table 1). The mean initial uptake rate was 0.020 ± 0.016 pmol of biotin per mg of dry cell weight per min. Initial uptake rates were determined for initial time intervals extending from 5 to 40 min when uptake rates were constant.

Intracellular steady-state concentrations of biotin were achieved between 10 and 80 min after biotin was introduced into the uptake media. The steady-state concentration was not influenced by the extracellular biotin concentration over the 500-fold range that was examined (Table 1). For 187 experiments, the mean intracellular concentration of biotin at steady state was 0.42 ± 0.33 pmol/mg of dry cell weight. Since the mean intracellular content of biotin for these cells has been reported to be 20 pmol/mg of dry weight (13), that amount of biotin incorporated by resting cells was only 2% of the total intracellular content. A similar rate and steady-state biotin uptake occurred in these cells at 4°C (data not shown) and at 37°C.

Time course of incorporation by resting and dividing cells. A time-course study with resting and dividing cells revealed a different initial rate and steady-state level for each condition (Fig. 1). Resting cells had an initial rate of biotin incorporation of 0.016 pmol/mg of dry weight per min and reached a steady-state level of biotin incorporation after about 60 min of

TABLE 1. *Initial rates and steady-state levels of biotin incorporation at various exogenous biotin concentrations by resting cells of E. coli Crookes and K-12*[a]

No. of expts	Extracellular [^{14}C]-biotin concn ($\times 10^{-7}$ M)	Initial rate of incorporation[b] (pmol/mg of dry wt per min)		Steady-state concn of intracellular biotin[c] (pmol/mg of dry wt)	
		$\bar{x}$	s	$\bar{x}$	s
42	0.02	0.016	0.011	0.59	0.27
11	0.08	0.019	0.022	0.65	0.10
25	0.08	0.018	0.011	0.44	0.27
4	0.3	0.024	0.020	0.50	0.43
10	0.6	0.023	0.017	0.32	0.11
11	1.0	0.026	0.006	0.33	0.14
19	1.0	0.023	0.022	0.50	0.22
21	1.0	0.016	0.013	0.25	0.09
7	1.2	0.021	0.016	0.32	0.13
14	2	0.028	0.023	0.61	0.59
12	5	0.016	0.016	0.30	0.18
11	10	0.020	0.016	0.36	0.26

[a] Cell concentration was approximately 0.5 mg of dry weight per ml in resting incorporation mixtures described in Materials and Methods.

[b] Measured by time-course samples over the initial linear incorporation period, which varied from 5 to 40 min. $\bar{x}$, Mean value; s, standard deviation.

[c] Measured by time-course samples; steady state usually reached in 10 to 80 min.

0.60 pmol/mg of dry weight. During the 140-min time course, these cells showed no growth, remaining at a turbidity of Klett 100 for the duration. The apparent intracellular concentration of biotin at the steady-state level, assuming for the moment that all the incorporated biotin was dissolved in intracellular free water and taking 1 mg of dry weight to be equivalent to 2.8 μl of cell water (22, 23), was 2.1×10^{-7} M, which was 105-fold greater than the concentration in the medium.

In contrast, dividing cells showed an increase in mass, going from Klett 82 to 206 over 160 min. During this growth period, an initial rate of biotin incorporation remained constant at 0.050 pmol/mg of dry weight per min for 60 min until Klett 125, at which point the incorporation reached a peak (3.70 pmol/mg of dry weight) and abruptly dropped. The drop in incorporation was the result of the cells exhausting all of the available extracellular biotin, thus diluting out the incorporated biotin with growth. Upon increasing the extracellular concentration of biotin fourfold to 0.08×10^{-7} M, incorporation continued to a fourfold-higher steady-state level (11.2 pmol/mg of dry weight) (Table 2). The maximum apparent intracellular concentration of biotin of 3.2 pmol/mg of dry weight (11.4×10^{-7} M) was 571-fold greater than the extracellular concentration of 0.02×10^{-7} M (Fig. 1).

Resting and dividing cells of the K-12 strain yielded time-course curves similar to those of the Crookes strain (data not shown).

Steady-state levels of incorporation by dividing cells. Since the steady-state concentrations were found to be dependent upon the extracellular biotin concentrations with dividing cells, these levels were measured over a wide concentration range of exogenous biotin. Intracellular incorporation reached a maximum steady-state level varying between 18.8 and 23.1 pmol/mg of dry weight (Table 2). Increasing the extracellular biotin concentration above 0.2×10^{-7} M did not alter this steady-state level of incorporation. At or above 0.2×10^{-7} M extracellular biotin, all of the intracellular biotin as determined by microbiological assay was [^{14}C]-biotin derived from outside the cells. At the two concentrations less than 0.2×10^{-7} M tested, the biotin that was available in the media was exhausted before the cells could incorporate it to their maximum steady-state level. At these

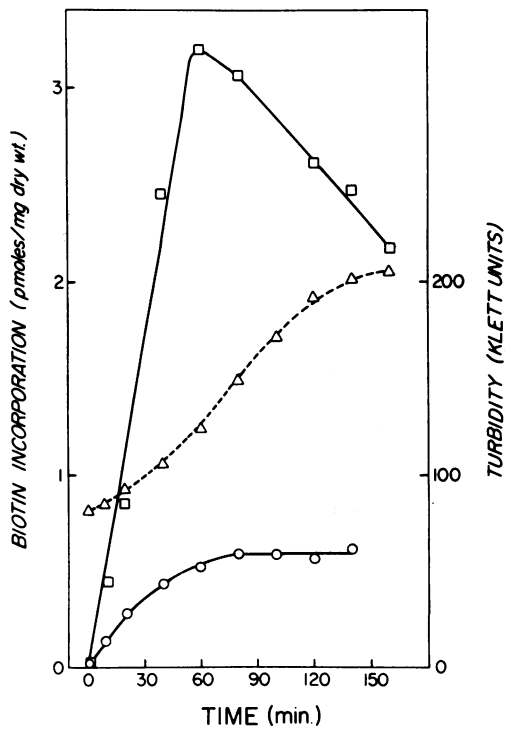

FIG. 1. *Biotin incorporation by resting and dividing cells of E. coli Crookes. Cells were suspended in incorporation mixtures containing 0.02×10^{-7} M [^{14}C]biotin. Dividing cells (□) had a nitrogen source available; resting cells (○) did not. Cell division was followed by turbidity and is represented by the broken line (△). Resting cells showed no increase in turbidity.*

TABLE 2. *Intracellular steady-state levels of biotin incorporation at various extracellular biotin concentrations by dividing cells of E. coli Crookes and K-12*

No. of expts	Extracellular [^{14}C]biotin concn in uninoculated growth medium[a] ($\times 10^{-7}$ M)	Intracellular [^{14}C]biotin concn at steady-state levels[b] (pmol/mg of dry wt)		Total intracellular biotin concn at steady-state levels[c] (pmol/mg of dry wt)
		$\bar{x}$	s	
3	0.02	2.4	0.4	20
13	0.08	11.2	3.5	18
3	0.2	21.2	3.6	21
5	0.4	23.1	1.2	23
8	2	22.2	4.4	22
2	20	18.8	4.5	19

[a] Growth medium (100 ml) was inoculated with 1 mg of dry weight cells.

[b] Measured after growth to stationary phase. $\bar{x}$, Mean value; s, standard deviation.

[c] Measured by microbiological assay for one experiment at each extracellular [^{14}C]biotin concentration.

two concentrations, the total intracellular biotin determined by microbiological assay was approximately 20 pmol/mg of dry weight, part of which was [^{14}C]biotin and the rest of which was [^{12}C]biotin synthesized de novo.

Effect of energy and protein inhibitors on incorporation. Since incorporation of extracellular biotin by either resting or dividing cells led to intracellular concentrations that were considerably greater than the outside concentrations, the possibility that an energy requirement was needed to establish such a concentration gradient was investigated. However, as shown in Table 3, an energy requirement was not found to be the case. Initial rates and steady-state levels of incorporation by resting cells were similar in media lacking ammonium sulfate but containing glucose, lacking glucose, or containing the inhibitors sodium azide, iodoacetate, sodium fluoride, sodium cyanide, sodium arsenate, and 2,4-dinitrophenol. In these experiments, biotin incorporation was followed over a 2-h time course, during which time the resting cells did not increase significantly in mass and exhibited incorporation time curves (not shown) similar to that of resting cells in Fig. 1. The inhibitor chloramphenicol did not alter the incorporation into resting cells, suggesting that this process did not require protein synthesis in these cells. A distinctly greater initial rate and steady-state level of biotin incorporation were observed only with dividing cells.

Effect of pH on incorporation by resting cells. Initial rate of biotin incorporation and steady-state concentrations were influenced by the pH of the uptake reaction mixture (Table 4). Incorporation was stimulated at acid pH and inhibited at alkaline. A similar degree of stimulation at pH 4.0 also occurred when the cells were maintained at 4°C (not shown).

Effect of biotin analogs on incorporation of biotin by resting and dividing cells. Although the incorporation process did not require an energy source, its sensitivity to pH conditions suggested that some cellular components were involved. To further investigate the nature of this process, several analogs of biotin were used to determine the degree of specificity of incorporation. Homobiotin, desthiobiotin, and oxybiotin had no significant effect on biotin incorporation when present at 100-fold excess concen-

TABLE 3. *Effect of energy inhibition on initial rates and steady-state levels of biotin incorporation by resting cells of E. coli Crookes*

Incorporation media[a]	Condition of cells	Initial rate of incorporation[b] (pmol/mg of dry wt per min)	Steady-state concn of intracellular biotin[c] (pmol/mg of dry wt)
Complete	Dividing	0.128	4.29
(+) Chloramphenicol (50 μg/ml)	Resting	0.024	0.59
(−) $(NH_4)_2SO_4$	Resting	0.016	0.49
(−) Glucose	Resting	0.014	0.66
(−) $(NH_4)_2SO_4$, (−) glucose	Resting	0.023	0.71
(+) Azide (10^{-2} M)	Resting	0.023	0.63
(+) Arsenate (10^{-2} M)	Resting	0.015	0.49
(+) Iodoacetate (10^{-2} M)	Resting	0.023	0.54
(+) Fluoride (10^{-2} M)	Resting	0.017	0.42
(+) Cyanide (10^{-2} M)	Resting	0.035	0.60
(+) 2,4-Dinitrophenol (10^{-4} M)	Resting	0.020	0.45

[a] [^{14}C]biotin concentration was 0.02×10^{-7} M; (+) indicates additions, (−) indicates deletions.

[b] Measured as for Table 1.

[c] Measured as for Table 1 after 120-min time course.

TABLE 4. *Effect of pH on initial rates and steady-state levels of biotin incorporation by resting cells of E. coli Crookes*

pH of resting incorporation media[a]	Initial rate of incorporation[b] (pmol/mg of dry wt per min)	Steady-state concn of intracellular biotin[c] (pmol/mg of dry wt)
4.0	0.045	1.06
6.6	0.026	0.48
7.4	0.030	0.32
8.8	0.003	0.06

[a] [^{14}C]biotin concentration was 0.2×10^{-7} M.

[b] Measured as for Table 1.

[c] Measured as for Table 1 after 120-min time course.

tration. At higher concentrations, the three analogs affected the initial rate and steady-state level to varying extents (Table 5). Desthiobiotin, when present 3,000-fold in excess of biotin, lowered the initial rate of incorporation by resting and dividing cells by about 45% and decreased the steady-state level achieved by dividing cells by 30%. A more profound effect occurred with homobiotin at concentrations 10,000-fold or greater than that of biotin, at which incorporation of biotin was completely inhibited. Since the concentrations of analog needed for inhibition of biotin incorporation were extremely high in comparison with concentrations for analog inhibition of other metabolic processes, it was concluded that incorporation was a rather specific process for biotin.

The nature of intracellular biotin. The intracellular nature of the incorporated biotin was investigated to discriminate between an intracellular free pool and intracellular binding sites. Although it has been shown that biotin was apparently concentrated intracellularly as much as two logs greater than the extracellular concentration (Table 1), this was accomplished without the expenditure of energy. Thus, active transport against a concentration gradient into an intracellular pool did not seem to be the likely explanation.

Indeed, a time course of incorporation of biotin into the bound and free fractions of dividing cells revealed that most of the biotin was tightly bound to these cells (Fig. 2). In this experiment, bound biotin was defined as that biotin that remained incorporated in the cells after boiling for 20 min. Free biotin released by boiling was determined as the difference between the total biotin before boiling and the bound biotin remaining subsequently. For cells in the late exponential growth phase, the released free biotin amounted to 1.5 pmol/mg of dry weight or 10% of the saturated total 14.8 pmol/mg of dry weight. The percentage of free biotin was negligible during the early and mid-exponential growth phase, at which time most biotin was bound, but was established in the late exponential phase and maintained into the stationary phase. Possibly, an accelerated rate of formation of binding sites during early and mid-exponential growth accounted for most of the biotin being bound. Other than during the early and mid-exponential phase, the bound:free ratio of intracellular biotin was constant over a wide range of intracellular biotin concentrations. In several different experiments, cells which were allowed to accumulate total intracellular biotin concentrations ranging from 6 to 30 pmol/mg of dry weight maintained free biotin fractions that were 10 to 14% of the total and bound fractions that were 87 to 89% of total (Table 6). In these experiments, the bound biotin could be released from the cells only after autoclaving for 1 h in 6 N H_2SO_4. Such drastic treatment suggested that this biotin fraction was covalently

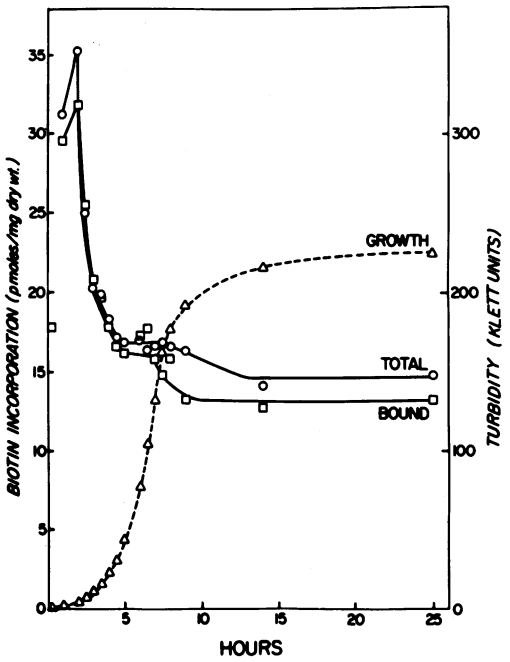

FIG. 2. *Biotin incorporation into free and bound fractions during growth. Cells (1 mg dry weight per100 ml of medium) were inoculated into growth medium with 1 × 10^{-7} M [^{14}C]biotin. Cell division was followed by turbidity* (△), *and total* (○) *and bound* (□) *biotin incorporation was determined in samples by procedures given in Materials and Methods.*

TABLE 5. *Effect of biotin analogs on initial rates and steady-state levels of biotin incorporation by resting and dividing cells of E. coli Crookes*[a]

Condition of cells	Analog in incorporation mixtures	Analog concn[a]/biotin concn	Initial rate[b] (pmol/mg of dry wt per min)	Steady-state level (pmol/mg of dry wt)
Resting	None	0	0.026	0.65
Resting	Homobiotin	1,000	0.013	0.49
Resting	Homobiotin	10,000	0.000	0.00
Resting	Desthiobiotin	250	0.038	0.68
Resting	Desthiobiotin	3,000	0.015	0.64
Dividing	None	0	0.101	13.9
Dividing	Homobiotin	2,500	0.093	13.7
Dividing	Desthiobiotin	250	0.112	13.4
Dividing	Desthiobiotin	3,000	0.055	0.5
Dividing	Oxybiotin	550	0.098	13.8

[a] [^{14}C]biotin concentration was 0.08 × 10^{-7} M.

[b] Measured as for Table 1; each value is the average of two experiments.

bound to an intracellular apoenzyme, most likely acetyl CoA carboxylase, the only known biotin apoenzyme in these cells (6).

Incorporation of biotin by resting cells similarly to dividing cells resulted in a constant, although different, ratio of bound to free biotin. Under resting conditions (Table 7), approximately one half of the biotin was incorporated into the free fraction and one half into the bound fraction. By definition, free biotin was that which was released from the cells by boiling (21). However, whether this free biotin escaped from intracellular free pools or from weak intracellular bonds has not been determined by past studies.

Intracellular:extracellular biotin exchange by resting and dividing cells. To determine if the free biotin fraction was derived from an intracellular pool of biotin, an exchange study was undertaken. In the data from the previous section, the concentration of free biotin in dividing cells, 1.5 pmol/mg of dry weight (Fig. 2), and in resting cells, 0.32 pmol/mg of dry weight (Table 7), presented intracellular concentrations of 4×10^{-7} and 0.8×10^{-7} M, respectively. For these two cases, the extracellular concentration of biotin was 1×10^{-7} M. Thus, a possible incorporation of biotin into the free fraction of dividing cells may have occurred against a concentration gradient. However, the lack of an energy requirement, already discussed, argued against an intracellular free pool. To verify the absence of a free pool in the free fraction, cells were grown with [^{14}C]biotin at a concentration to saturate their incorporation requirement and subsequently tested for exchange with [^{12}C]biotin. Since the [^{12}C]biotin entered the cells without an energy requirement, apparently by diffusion if a pool existed, it should have exchanged with any such pool of [^{14}C]biotin. However, this did not occur (Fig. 3). In resting cells no exchange occurred, and in dividing cells the rate of [^{14}C]biotin loss was the same from the free and bound fraction and corresponded to a diluting out by cell division. By exchanging isotopes in a duplicate control experiment (not shown), no [^{14}C]biotin was observed to be incorporated into the dividing and resting cells. Thus, the [^{12}C]biotin entered the cells without a resulting loss of [^{14}C]biotin already incorporated, other than a diluting out with growth. These data suggested that the free fraction of intracellular biotin was not a pool.

TABLE 6. *Levels of biotin incorporated into bound and free intracellular fractions by dividing cells of E. coli Crookes and K-12*

Total[a] (pmol/mg of dry wt)	Bound (pmol/mg of dry wt)	Free (pmol/mg of dry wt)	Bound/total (%)	Free/total (%)
25.22	21.96	3.58	87	14
30.17	26.80	3.37	89	11
6.25	5.45	0.80	87	13
9.14	8.01	1.12	88	12
14.80	13.20	1.50	89	10

[a] Measured in dividing cells at stationary phase after growth on various concentrations of biotin, in some instances limiting concentrations.

TABLE 7. *Levels of biotin incorporated into bound and free intracellular fractions with time by resting cells of E. coli K-12*[a]

Time (min)	Total (pmol/mg of dry wt)	Bound (pmol/mg of dry wt)	Free (pmol/mg of dry wt)	Free/total (%)[b]
1	0.17	0.10	0.07	41
3	0.20	0.11	0.09	45
10	0.33	0.14	0.19	58
15	0.33	0.14	0.19	58
20	0.36	0.15	0.21	58
30	0.43	0.21	0.22	51
40	0.48	0.20	0.28	58
60	0.43	0.24	0.19	44
90	0.43	0.26	0.17	40
105	0.49	0.25	0.24	49
116	0.57	0.25	0.32	56

[a] Incorporation mixture contained 1×10^{-7} M [^{14}C]biotin, and no $(NH_4)_2SO_4$.

[b] Mean value = 51; standard deviation = 7.3.

Avidin treatment. Since an intracellular free pool of biotin did not seem to be present, experiments were designed to explore the possibility of the existence of a limited number of intracellular binding sites for biotin. Cells were treated with the biotin-binding protein avidin, which cannot penetrate the cell membrane (11). Harvested, washed cells were suspended in either resting or dividing medium containing avidin and incubated for 2 h, washed thoroughly to remove excess avidin, and tested for biotin incorporation.

At an extracellular concentration of 0.02×10^{-7} M biotin, almost all of the biotin became incorporated into the avidin-treated cells within the first min (Table 8). Of the 1.8 pmol of biotin per ml available in the medium, 1.7 pmol/ml or 3.32 pmol/mg of dry weight was incorporated. On the other hand, cells treated in a duplicate manner, but in the absence of avidin in the treatment mixture, incorporated 0.57 pmol of biotin per mg of dry weight over a 40-min span.

Since all of the available biotin had been incorporated immediately by the avidin-treated cells, a higher concentration (0.072×10^{-7} M) of biotin was offered to these cells in an attempt to reach a saturation point. However, once again, essentially all of the initial extracellular biotin (7.2 pmol/mg of incorporation mixture) became associated with the cells (7.1 pmol/ml of incor-

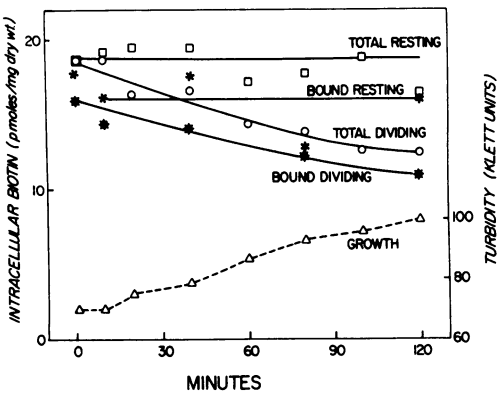

FIG. 3. *Exchange of extracellular [^{12}C]biotin with preloaded intracellular [^{14}C]biotin in both free and bound fractions. Cells which had been grown in medium containing 0.2 × 10^{-7} M [^{14}C]biotin were harvested and suspended in resting or dividing incorporation mixtures in the presence of 0.2 × 10^{-7} M [^{12}C]biotin. The disappearance of the preloaded [^{14}C]biotin was measured over 120 min. Symbols: resting cells, total intracellular biotin (□); resting cells, bound biotin fraction (*); dividing cells, total intracellular biotin (○); bound biotin fraction (●); growth by dividing cells (△).*

TABLE 8. *Effect of avidin pretreatment on biotin uptake by resting and dividing cells of E. coli K-12*

Expt no.	Metabolic state of cells	[^{14}C]biotin concn (×10^{-7} M)	Type of pretreatment[a]	Intracellular biotin steady-state concn (pmol/mg of dry wt)	Time to reach steady state (min)
1	Resting	0.02	Avidin	3.32	1
	Resting	0.02	None	0.57	40
2	Resting	0.072	Avidin	8.6	1
	Resting	0.072	None	0.44	25
3	Resting	1.8	Avidin	15	1
	Resting	1.8	None	0.61	20
4	Dividing	0.06	Avidin	20.5	10
	Dividing	0.06	None	13.7	100

[a] 2-h pretreatment with or without 0.02 U of avidin per ml at 37°C.

poration mixture) within 1 min. Thus, the avidin-treated cells incorporated 8.6 pmol/mg of dry weight (Table 8), an increase proportional to the increase in extracellular biotin concentration. Incorporation into control cells was similar to that at the lower biotin concentration, as was to be expected, and agreed with the data in Table 1. According to these data, resting cells incorporated a similar level of intracellular biotin over a wide extracellular concentration range.

By increasing the extracellular concentration to 1.8 × 10^{-7} M (180 pmol/ml), biotin incorporation by avidin-treated cells reached saturation before exhausting all of the extracellular biotin. The intracellular level attained in 1 min was 15 pmol/mg of dry weight or 9.9 pmol/ml of incorporation mixture (Table 8). Although not shown, a similar initial intracellular level was obtained at 4°C. The increase from 7.2 to 9.9 pmol/ml of intracellular incorporation in these cells was not in proportion to the increase in extracellular biotin concentration from 7.2 to 180 pmol/ml. The saturation level (15 pmol/mg of dry weight) of incorporation by avidin-treated resting cells was similar to the steady-state level of incorporation by dividing non-avidin-treated cells (Tables 2 and 8).

It was of interest to see if the incorporation and loss of biotin by avidin-treated cells would be altered under dividing cell conditions. To accomplish this, dividing cells were treated with avidin (0.02 U/ml) for 2 h, washed thoroughly, and suspended in dividing incorporation mixture containing 0.06 × 10^{-7} M biotin (6 pmol/ml). Intracellular incorporation reached a saturation concentration of 20.5 pmol/mg of dry weight (4.52 pmol incorporated per ml of incorporation mixture) after 10 min (Table 8). This was not as rapid an incorporation as by avidin-treated resting cells, presumably because the avidin content of these cells had been diluted out by cell division during the 2-h treatment.

Dividing non-avidin-treated cells, which served as the control, incorporated biotin with an initial rate of 0.279 pmol/mg of dry weight per min for 100 min before exhausting the extracellular biotin and beginning to dilute out the incorporated biotin. At 100 min, 0.43 mg of dry cell weight per ml had incorporated 13.7 pmol of biotin per mg of dry weight, which was 5.9 pmol of biotin per ml of incorporation mixture of the original 6 pmol/ml.

The results with avidin-treated and non-treated dividing cells as well as the data for avidin-treated resting cells suggest a constant number of sites in the cell to which biotin was incorporated. Provided that the concentration of the biotin in the incorporation mixture was not limiting, approximately 15 to 25 pmol of biotin per mg of dry cell weight was incorporated, either to avidin molecules, which presumably covered these sites, or to the sites themselves. Computations made from these data indicated that approximately 3,000 biotin molecules are incorporated per cell (20 pmol of biotin per 4 × 10^{9} cells).

DISCUSSION

Biotin incorporation by resting or dividing cells of *E. coli* did not appear to involve a

membrane transport process. First, the initial rates and steady-state levels of incorporation of biotin were constant over a wide range of extracellular biotin concentrations. Since this range included low extracellular concentrations of biotin that were insufficient to satisfy the metabolic requirements of the cells, the constant rate of uptake could not have resulted from saturation by substrate of all possible transport enzymes. Secondly, intracellular biotin concentrations were established which were greater than the extracellular concentrations and would have required active transport, yet this possibility was ruled out by the lack of energy requirement. Thirdly, the intracellular biotin did not exchange freely with extracellular biotin that was being incorporated, although this would have been expected if it were in an intracellular free pool. Lastly, that resting cells were capable of taking up biotin at 4°C at the same rate and to the same extent as at 37°C was further evidence for a physical binding process rather than a mediated transport process.

These data suggest that the free intracellular biotin existed as a weakly bound moiety that could be released by boiling. It is postulated that this might have been free biotin undergoing carboxylation that attached noncovalently to the active sites of the biotin carboxylase or transcarboxylase subunits of the *E. coli* acetyl CoA carboxylase (9). Along these lines, we observed an increase in biotin incorporation into resting cells under acidic conditions, presumably because a [^{14}C]biotin molecule was able to replace a [^{12}C]biotin molecule at these sites. These data are consistent with an earlier report that the carboxylated free biotin was relatively unstable at acid pH (9).

It would seem that the constant level of incorporation by dividing cells was a consequence of a fixed number of binding sites. Indeed, support for this comes from an earlier study on biosynthesis of biotin with the same strain as used in this study, *E. coli* Crookes (13). In the earlier study, the quantity of intracellular biotin and its distribution in bound and free intracellular fractions which were established during intracellular biosynthesis of biotin were found to be between 15 and 25 pmol/mg of dry weight distributed 88% in bound form and 12% in the free fraction. The same results were obtained in the present study. Therefore, the quantity of intracellular biotin and its intracellular distribution in dividing cells were constant values whether the biotin was derived from an extracellular source or made de novo. Moreover, it had been reported in the earlier study that the biosynthetic pathway was repressed by an extracellular concentration of biotin of 5 ng/ml (0.2×10^{-7} M) (13). In the present study, this was observed to be the minimum amount of extracellular biotin sufficient to satisfy all of the binding sites during incorporation. Thus, no biosynthesis was required by the cells because extracellular biotin at this concentration was available to bind all of the intracellular sites.

The number of intracellular sites is further confirmed by the data from avidin-treated cells. The same amount of biotin was taken up by avidin-treated cells in 1 min as is found in these cells at steady state. It is postulated that this occurred because the avidin had first bound the [^{12}C]biotin that was present at each of the binding sites. Avidin, being a tetrameric complex, would then have been capable of binding another molecule of [^{14}C]biotin at each site. The rapidity of the [^{14}C]biotin incorporation was not surprising considering that avidin binds free or enzyme-bound biotin with a remarkable affinity (K_d of biotin = 10^{-15} M) (9).

Moreover, the data suggested that biotin binding occurred at the cell membrane. Since avidin bound to the sites quantitatively, this protein, which was impermeable to the cells (9), must have had access to the [^{12}C]biotin at those sites. A second piece of evidence for a membrane location for the binding sites was the apparent lack of any transport system for biotin. Yet extracellular biotin seemed to have access to the binding sites, even at 4°C.

The identity of the binding sites must be left for future investigation. Since the only biotin-requiring enzyme in *E. coli* appears to be acetyl CoA carboxylase, which binds biotin covalently by a peptide linkage to a lysine residue of its BCCP (6, 9), it is tempting to speculate that BCCP might be the binding site. Our data showed a specificity of incorporation similar to BCCP for biotin alone and not its analogs. It can be speculated that since this is the only biotin-requiring enzyme in these cells, it would be in the interest of cellular economy if this enzyme were located in the membrane with access to biotin molecules on either side. No information has been published as to the exact location of the acetyl CoA complex in *E. coli*. However, it has been shown that acyl carrier protein of the fatty acid synthetase is located on or near the inside surface of *E. coli* (19). Perhaps a similar organization will be found to exist for acetyl CoA carboxylase.

Incorporation of extracellular biotin by binding was suggested as an alternative to transport by Campbell et al. (3) to explain the effects of an *E. coli* mutant with reduced net uptake. However, these investigators were dissatisfied that the bound biotin would possibly have to serve as a regulatory molecule. Subsequently,

Pai (14) resolved this difficulty by showing that low biotin uptake might have been the consequence rather than cause of the mutant's overproduction of biotin. A fixed number of binding sites suggested by our data would be consistent with the data from both these studies.

A binding mechanism of uptake has been suggested for citrate incorporation by *Aerobacter aerogenes*. In this organism, an inducible biotin-dependent oxaloacetate decarboxylase is tightly bound to the cytoplasmic membrane. At this location it functions as a carrier protein in the transport of citrate as well as participating in its catabolism (18). Similarly to our observation on biotin incorporation, citrate is not found to accumulate free inside the cells, but remains bound to this enzyme as it undergoes further catabolism on the inner side of the membrane. Another biotin-dependent enzyme, methylmalonyl CoA decarboxylase of *Micrococcus lactilyticus*, is believed to be ribosome bound but associated with the cell membrane. As it catalyzes a terminal step in lactate fermentation, its membranous location allows for a more rapid diffusion of the products into the medium (1).

A recent report of active transport of biotin by wild-type *E. coli* K-12 strains showed that biotin may enter some strains by active transport (16). However, unlike the strains used in this study, which contained predominantly bound biotin, strains possessing a transport system for biotin contained almost all of their intracellular biotin in the free form. Future research should address itself to uncovering possible different structural components for biotin uptake in different strains of *E. coli*. In addition, knowledge is needed to explain the purpose of different biotin-uptake mechanisms.

LITERATURE CITED

1. **Alberts, A. W., and P. R. Vagelos.** 1972. Acetyl-CoA carboxylases, p. 37-82. *In* P. D. Boyer (ed.), The enzymes, vol. 6, 3rd ed. Academic Press Inc., New York.
2. **Bray, G. A.** 1960. A simple efficient liquid scintillator for counting aqueous solutions in a liquid scintillation counter. Anal. Biochem. **1**:279-285.
3. **Campbell, A., A. del Campillo-Campbell, and R. Chang.** 1972. A mutant of *Escherichia coli* that requires high concentrations of biotin. Proc. Natl. Acad. Sci. U.S.A. **69**:676-680.
4. **Cronan, J. E., and P. R. Vagelos.** 1972. Biochim. Biophys. Acta **265**:25-60.
5. **Davis, B. D., and E. S. Mingioli.** 1950. Mutants of *Escherichia coli* requiring methionine or vitamin B_{12}. J. Bacteriol. **60**:17-28.
6. **Fall, R. R., A. W. Alberts, and P. R. Vagelos.** 1975. Analysis of bacterial biotin-proteins. Biochim. Biophys. Acta **379**:496-503.
7. **Fall, R. R., and P. R. Vagelos.** 1975. Biotin carboxyl carrier protein from *Escherichia coli*. Methods Enzymol. **35**:17-25.
8. **Green, N. M.** 1975. Avidin. Ad. Protein Chem. **29**:85-133.
9. **Lane, M. D., J. Moss, and S. E. Polakis.** 1974. Acetyl coenzyme A carboxylase. Curr. Top. Cell. Regul. **8**:139-195.
10. **Moss, J., and M. D. Lane.** 1971. The biotin-dependent enzymes. Ad. Enzymol. Relat. Areas Mol. Biol. **35**:321-442.
11. **Oxender, D. L.** 1972. Membrane transport. Annu. Rev. Biochem. **41**:777-814.
12. **Pai, C. H.** 1973. Biotin uptake in biotin regulatory mutant of *Escherichia coli*. J. Bacteriol. **116**:494-496.
13. **Pai, C. H., and H. C. Lichstein.** 1965. The biosynthesis of biotin in microorganisms. I. The physiology of biotin synthesis in *Escherichia coli*. Biochim. Biophys. Acta **100**:28-35.
14. **Pai, C. H., and H. C. Lichstein.** 1966. Biosynthesis of biotin in microorganisms. IV. Repression and derepression of (+)-biotin synthesis from (±)-desthiobiotin. Arch. Biochem. Biophys. **114**:138-144.
15. **Pai, C. H., and H. C. Lichstein.** 1967. Biosynthesis of biotin in microorganisms. VI. Further evidence of desthiobiotin as a precursor in *Escherichia coli*. J. Bacteriol. **93**:1930-1933.
16. **Prakash, O., and M. A. Eisenberg.** 1974. Active transport of biotin in *Escherichia coli* K-12. J. Bacteriol. **120**:785-791.
17. **Rogers, T. O., and H. C. Lichstein.** 1969. Characterization of the biotin transport system in *Saccharomyces cerevisiae*. J. Bacteriol. **100**:557-564.
18. **Sachan, D. S., and J. R. Stern.** 1971. Studies of citrate transport in *Aerobacter aerogenes:* binding of citrate by a membrane bound oxalacetate decarboxylase. Biochem. Biophys. Res. Commun. **45**:402-408.
19. **Vagelos, P. R.** 1971. Regulation of fatty acid biosynthesis. Curr. Top. Cell. Regul. **4**:119-166.
20. **Volpe, J. J., and P. R. Vagelos.** 1973. Annu. Rev. Biochem. **42**:21-60.
21. **Waller, J. R., and H. C. Lichstein.** 1965. Biotin transport and accumulation by cells of *Lactobacillus plantarum*. I. General properties of the system. J. Bacteriol. **90**:843-852.
22. **West, I. C.** 1970. Lactose transport coupled to proton movements in *Escherichia coli*. Biochem. Biophys. Res. Commun. **41**:655-661.
23. **Winkler, H. H., and T. H. Wilson.** 1966. The role of energy coupling in the transport of β-galactosides by *Escherichia coli*. J. Biol. Chem. **241**:2200-2211.