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BIOTIN TRANSPORT BY A BIOTIN-DEFICIENT STRAIN OF *ESCHERICHIA COLI*

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Biotin uptake has been investigated using an *Escherichia coli* biotin requiring auxotroph grown under biotin-deficient conditions. This strain accumulated biotin in the free and bound form. In agreement with a previous report by O. Prakash and M.A. Eisenberg (J. Bacteriol. 120 (1974) 785–791), the biotin entry proved to be an active process which depended on an energy source and was inhibited in the presence of uncouplers. The kinetic parameters have been determined ($K_M = 0.05\ \mu M$, $V_{max} = 7$ pmol/min per mg dry weight). The pool of free biotin could be readily exchanged with external biotin and decreased to a very low level in the absence of an energy source. The use of several biotin analogues revealed that this transport system was quite specific for biotin: slight modifications, for instance in the valeric chain, lowered drastically the affinity for the carrier.

Introduction

When biotin is present in a medium, microorganisms use the exogenous vitamin for their growth. Biotin controls its own biosynthesis [1–5] and Pai and Lichstein have shown that an external concentration of 5 ng of biotin per ml is sufficient to repress the biosynthesis of the vitamin by *Escherichia coli* [3]. Microorganisms have developed very efficient ways to extract biotin from the growth medium and biotin uptake has been studied with several microorganisms. First studies with *Lactobacillus arabinosus* [6,7] and *Saccharomyces cerevisiae* [8–10] revealed that this transport is, to a large extent, an active process: biotin is accumulated within the cell against a concentration gradient and an energy source is necessary. In addition, this uptake is specific for biotin and is inhibited by biotin analogues [7,9]. Becker et al. [11] demonstrated that this process is mediated by a specialized protein component while studying the inhibition of biotin uptake by the paranitrophenyl ester of biotin. This reagent forms a covalent bond with the system [11] and Viswanatha et al. [12] showed that this inhibition can be reversed by thiol compounds. Using *S. cerevisiae* strains, Rogers and Lichstein showed that biotin uptake is regulated by the level of external biotin [10].

Biotin uptake by prototrophic strains of *E. coli* K-12 was examined some years later by Prakash and Eisenberg [13] who concluded that biotin is also transported uphill by this microorganism. More recently, Cicmanec and Lichstein [14] investigated the biotin uptake with *E. coli* and observed that the transport was not dependent on the presence of an energy source. They concluded that biotin uptake was not an active process.

According to these authors, the discrepancies with former reports are due to the different strains used and more precisely they emphasize the differences of bound and free biotin pools of the *E. coli* strains used in these studies.

In contrast with some important biological

molecules, the breakdown of biotin by microorganisms is slow compared to uptake and does not interfere with uptake measurements (Piffeteau, A., unpublished results). However, the existence of two pools, a pool of free biotin and a pool of bound biotin (i.e., biotin bound covalently to carboxylases) complicates the study of biotin uptake.

When prototrophic strains are used, the pool of bound biotin will be almost saturated, biotin being either synthesized by the bacteria or of exogenous origin. On the other hand, the pool of free biotin will depend strongly on the growth conditions and on the concentration of exogenous biotin.

Such a problem is not met with strains that depend on biotin for their growth, since, in the absence of interfering biosynthesized biotin, it is possible to control accurately the content of the two pools of biotin.

In this study, we have investigated the uptake of biotin by *E. coli* using a biotin-dependent strain, with special emphasis on the energy dependence, the nature and properties of the free biotin pool and the specificity of the permease system.

Experimental

Materials

[*carbonyl*-^{14}C]Biotin (50 Ci/mol) was purchased from Amersham International (U.K.). (+)-Biotin (+)-norbiotin, (+)-homobiotin, 2'-thiobiotin and α-dehydrobiotin were gifts from Hoffman La Roche Co. (Basel). α-Methylbiotin was obtained through the courtesy of Dr. Hanka (Upjohn Company, Kalamazoo) and (±)-oxybiotin through that of Dr. Detitta (Buffalo). Dr. Wormser (Detroit) is acknowledged for providing us with carbobiotin and azabiotin. Most biotin analogues have been synthesized in our laboratory according to known procedures: selenobiotin [15], (+)-biotin sulfoxide, (−)biotin sulfoxide [16], biotin sulfone [17], dethiobiotin [18], *N,N'*-dimethylbiotin [19], biotinol [20]. (+)-Biotin chlorosulfoxide was prepared by chlorination of (+)-biotin sulfoxide (Moreau, B., unpublished data) and α-ethyl biotin by alkylation of biotin (Bory, S., unpublished data). Carbonyl cyanide *m*-chlorophenylhydrazone (C-CCP) was purchased from Calbiochem.

E. coli C162 (bio B$^-$, his$^-$) a mutant derived from *E. coli* K-12 strain was a generous gift from Dr. P. Cleary.

E. coli C162 was grown on synthetic medium 63 (13.6 g KH_2PO_4, 2 g $(NH_4)_2SO_4$, 0.2 g $MgSO_4 \cdot 7\,H_2O$, 0.5 mg $FeSO_4 \cdot 7\,H_2O$ per litre; adjusted to pH 7.0 with 4 M potassium hydroxide [21] and supplemented with 2 g glucose and 35 mg L-histidine. Traces of biotin were removed by treatment with Norit before autoclaving. Biotin was then added aseptically.

To obtain biotin deficient bacteria, synthetic medium containing biotin (0.1 ng/ml) was inoculated to a final concentration of $1.1 \cdot 10^8$ bacteria per ml ($A_{570nm} = 0.2$) with an overnight culture (0.1 ng/ml biotin). Bacterial growth was carried out at 37°C on a reciprocal shaker and monitored at 570 nm for two generations.

Methods

Uptake studies. During the preparation of bacterial suspensions used for uptake experiments, we have attempted to minimize the temperature or osmotic shocks that could have modified the properties of the uptake system. Centrifugations were carried out at room temperature and washing was achieved with medium 63, avoiding distilled water [29] or cold saline [22].

Cells were harvested in the mid exponential phase ($(5.5–6.0) \cdot 10^8$ cells/ml) at 13000 × *g*. After a wash with medium 63, cells were suspended in uptake medium (13.6 g KH_2PO_4, 2.6 g K_2SO_4, 0.2 g $MgSO_4 \cdot 7\,H_2O$, 0.5 mg $FeSO_4 \cdot 7\,H_2O$, 3.6 g glucose, 0.1 g chloramphenicol per l adjusted to pH 6.6 with 4 M potassium hydroxide) at a concentration of $3.7 \cdot 10^9$ cells per ml.

After equilibration at 37°C for 10 min, uptake experiments were initiated by addition of [^{14}C]biotin. 1 ml samples were pipetted, diluted with 3 ml medium 63 on a Millipore filter (47 mm diameter, 0.45 μm pores) immediately filtered under vacuum and washed three times with 3 ml portions of medium 63 (total time: 1 min). Filters were then cut, placed in counting vials and dried at 80°C for 1 h, and the radioactivity was estimated by adding 10 ml of toluene containing 6 g 2,5-diphenyloxazole (PPO) and 0.3 g 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]-benzene (dimethylPOPOP) per l and counting in an SL 30 Intertechnique Scintillation counter. Results were corrected for quenching

by the external standard method.

When biotin uptake was followed as a function of time, the concentration of [^{14}C]biotin was 70 ng/ml, unless otherwise stated, and the uptake was followed during 60 min.

For K_m, V_{max} and K_i determinations, samples were pipetted after 2 min incubations for five different concentrations of biotin and three different concentrations of inhibitors.

All the data are the averages of at least two independent determinations.

Bound and free biotin. Cells were washed twice with distilled water. Free biotin was extracted by autoclaving the bacteria for 20 min at 120°C in water. After centrifugation cells were washed with distilled water and free biotin was estimated by counting aliquots in Bray's scintillator [23]. Bound biotin was then extracted by autoclaving bacteria for 2 h at 120°C in 2 M H_2SO_4. After neutralization with concentrated KOH, Bound biotin was estimated by counting aliquots [24]. In both cases correction for quenching is achieved by the external standard method.

Results and Discussion

Biotin content of E. coli C162 cells

In order to study the energy dependence and the kinetic parameters of biotin uptake, it is necessary to measure rates of entry in a pool of free biotin which is almost empty. This is why the use of prototrophs can raise some problems linked to the difficulty in controlling the level of free biotin within the cell.

In the studies by Prakash and Eisenberg [13] the *E. coli* strain Y 10-1 used probably synthesized enough biotin to saturate its pool of bound biotin, as shown by the very low uptake of [^{14}C]biotin in that pool. Assuming that parent strain *E. coli* Y 10-1 and mutant strain *E. coli* Y 10-1 bioB105 have almost identical potential pools of biotin, it is possible to figure out that *E. coli* Y 10-1 pool of free biotin is filled to about 30% of its maximum content [13]. Prakash and Eisenberg [13] have thus examined the entry of vitamin in a pool of free biotin that was initially far from saturation. On the other hand, Cicmanec and Lichstein have studied resting cells of bacteria that have accumulated, before the uptake experiment, about 98% of the biotin that can be incorporated [14]. It is thus probable that they have examined the entry of biotin in a pool of free biotin that was almost saturated, and fewer clear conclusions can be derived from their results with resting cells.

The biotin requiring *E. coli* C162 strain that we used accumulated biotin in the free and bound form and the biotin content depended on growth or uptake conditions (Table 1). When grown in the presence of biotin in excess (5 ng/ml) the pools of bound and free biotin were very similar. The value that we observed (3.7 ng biotin in the bound form per mg dry weight) is very similar with this obtained by Prakash and Eisenberg with auxotroph *E. coli* Y10-1 bioB105 * [13]. When the amount of biotin in the growth medium was minimal (0.1 ng/ml), the content of both pools fell to less than 10% of the maximum value. With such biotin-depleted bacteria, we have thus been able to follow the entry of more than 90% of the maximal biotin content of the cells.

Biotin uptake by E. coli C162 cells

General features Our results are in good agreement with an active transport as proposed by Prakash and Eisenberg for *E. coli* Y10 [13].

The uptake by resting cells was linear for about 3 min and reached a plateau in 30 min (Fig. 1. For an external biotin concentration of 70 ng/ml (0.3 μM) the initial rate was 0.85 ng (3.5 pmol) biotin/min per mg dry weight and the intracellular concentration at the plateau was 8 ng/mg dry weight. Two-thirds of the biotin was bound (4.5 ng/mg dry weight) and one-third was free (2.3 ng/mg dry weight). No overshoot phenomenon was detected, as described with *S. cerevisiae* [8] but the uptake at 37°C appeared to be dependent on the growth phase during which bacteria were harvested (Fig. 2). Uptake was maximum for bacteria collected in the middle of the exponential phase and decreased when bacteria were collected later in the exponential phase or in the stationary phase. All the experiments have thus been run

* *E. coli* Y10-1 bioB105 biotin depleted cells (i.e., grown in the presence of 0.2 ng biotin per ml) incorporated 3.6 ng biotin per mg dry weight in the bound form when incubated with high biotin concentrations [13].

TABLE I

BIOTIN CONTENT OF *E. coli* C162 CELLS GROWN ON BIOTIN SUFFICIENT (5 ng/ml) AND BIOTIN-DEFICENT MEDIUM (0.1 ng/ml)

Synthetic growth medium supplemented with biotin were inoculated with a suspension of biotin-deficient cells to a concentration of $1.1 \cdot 10^8$ cells/ml. After growth, cells were harvested and washed. The biotin contents of both pools were determined after extraction with boiling water (free biotin) and 2 M sulfuric acid (bound biotin) as described under Experimental.

Growth conditions		Biotin content (ng/mg dry cells)	
Phase (cells/ml)	[^{14}C]Biotin (ng/ml)	Free	Bound
Mid log ($5.5 \cdot 10^8$)	5	3.5	3.7
Stationary ($1.2 \cdot 10^9$)	5	4.8	3.6
Mid log ($5.5 \cdot 10^8$)	0.1	0.12	0.28

with bacteria collected in the middle of the exponential phase.

Kinetic parameters and inhibition by biotin analogues. The plateau value (measurements at 30 min) was almost independent of biotin concentration when the external level of vitamin was raised above 10 ng/ml and the system showed Michaelis-Menten kinetics: measurements under initial rate conditions (sampling at 2 min) plotted according to Lineweaver and Burk lead to kinetic parameters $K_m = 50 \pm 20$ nM and $V_{max} = 1.7$ ng (7 pmol)/min per mg dry weight. Those figures are close to those

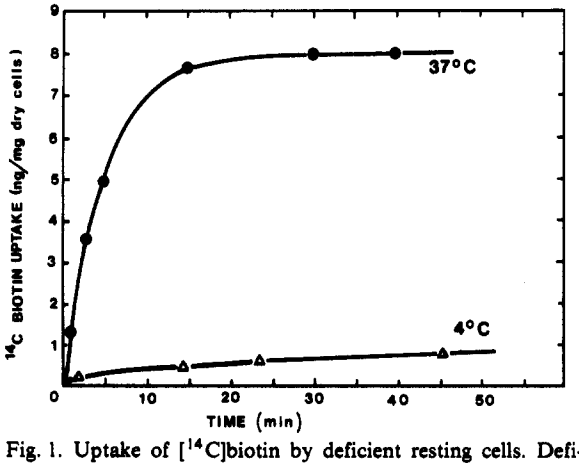

Fig. 1. Uptake of [^{14}C]biotin by deficient resting cells. Deficient *E. coli* C162 cells grown in the presence of 0.1 ng/ml biotin were resuspended in the uptake medium ($3.7 \cdot 10^9$ cells/ml). Incubations were carried out at 37°C (●) or at 4°C (△) and uptake was initiated by addition of 70 ng/ml [^{14}C]biotin. Aliquots were filtered and counted as described under Experimental.

TABLE II

INHIBITION OF [^{14}C]BIOTIN TRANSPORT BY DEFICIENT RESTING CELLS OF *E. COLI* C162 BY BIOTIN ANALOGUES

Incubations of the cells ($3.7 \cdot 10^9$ cells/ml) were carried out at 37°C for 2 min. Aliquots were filtered and washed as indicated in the experimental part and counted. Results were plotted according to Lineweaver and Burk and K_i values determined from three different inhibitors concentrations.

Biotin analogue	K_i analogue [a] / K_m biotin
Replacement of the sulfur atom	
Dethiobiotin	NI
(+)-Selenobiotin	1
(±)-Carbobiotin	30
(±)-Azabiotin	NI
(±)-Oxybiotin	NI
Modification of the thiophane ring	
(+)-Biotin sulfoxide	3
(+)-Biotin chlorosulfoxide	7
(−)-Biotin sulfoxide	11
Biotin sulfone	3.5
(±)-Methyl *cis*-biotin	3
(±)-Methyl *trans*-biotin	30
Modification of the ureido ring	
2′-Thiobiotin	2.5
(+)-N,N′-Dimethylbiotin	100
Modification of the side chain	
(+)-Biotinol	26
(+)-Homobiotin	40
(+)-Norbiotin	NI
(+)-α-Dehydrobiotin	5
α-Methylbiotin	7
α-Ethylbiotin	50

[a] Analogues were competitive inhibitors, or non inhibitors (NI) over the concentration range tested (up to 1.7 μM).

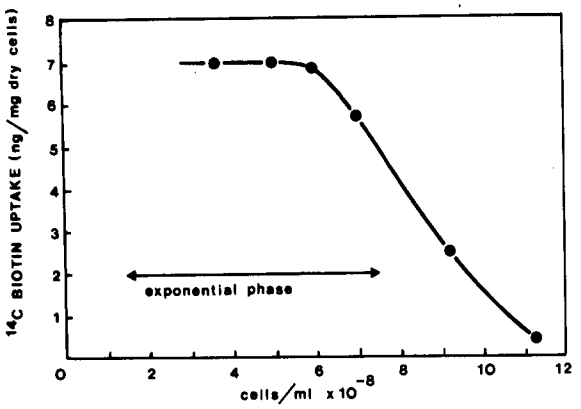

Fig. 2. Influence of the growth phase on the uptake of [^{14}C]biotin by deficient resting cells. Deficient *E. coli* C162 cells, grown in the presence of 0.1 ng/ml biotin were harvested at different phases as indicated by the number of cells/ml. The uptake was then measured for 10 min at a cell concentration of $3.7 \cdot 10^9$ cells/ml, in the presence of 70 ng/ml [^{14}C]biotin.

determined for *E. coli* strain Y 10 ($K_m = 140$ nM, $V_{max} = 6$ pmol/min per mg) [13].

The inhibition of biotin transport by biotin analogues supported the existence of a carrier mediated process. The method was first tested with unlabelled biotin, which proved to be a competitive inhibitor of [^{14}C]biotin uptake, with a K_i/K_m ratio equal to 1. Results quoted in Table II show that most biotin analogues were competitive inhibitors, the K_i of which depended strongly on the structure of the analogue.

The absence of the five-membered ring as in dethiobiotin suppressed all the inhibition properties and the replacement of the sulfur atom by another element had a large influence on the behavior of the analogue: selenobiotin was an excellent competitive inhibitor and carbobiotin a poor one, whereas azabiotin and oxybiotin were not inhibitory over the concentration range tested. Results with oxybiotin were in agreement with Cicmánec and Lichstein, who noticed no inhibition [14], and with Prakash and Eisenberg, who observed only 54% inhibition at an oxybiotin-to-biotin ratio of 67 [13]. Oxidation of the sulfur atom lowered somewhat the affinity and revealed that (−)-biotin sulfoxide had a lower affinity than the (+)-isomer or the sulfone.

In contrast to Prakash and Eisenberg's conclusion [13] we observed that the side chain of biotin was a very sensitive area where modifications can induce dramatic variations of the affinity. The carboxyl group was essential, as shown by the low affinity of biotinol, and its position was also crucial: an increase of one methylene group (homobiotin) diminished the affinity drastically, whereas a decrease of one methylene group (norbiotin) suppressed any affinity for the binding site. The introduction of a double bond α to the carboxyl group which does not modify the length nor the bulkness of the side chain, nevertheless multiplied that dissociation constant by 5. The presence of alkyl group α to the carboxyl led to lower affinities as the size of the alkyl group increased.

To enter a cell by an active process, products go through two steps, one of binding at the active site of the system and one of translocation through the membrane.

When an analogue of a substrate is a competitive inhibitor, it binds to the active site, but this does not produce information concerning the translocation step. We have been able to demonstrate that biotin analogues were going through the translocation step in two cases, that of α-dehydrobiotin [24] and of selenobiotin [25].

Oxybiotin has a good growth promoting activity for microorganisms and animals [26,27] and this implies that it enters the cells and is used as prosthetic group by biotin-dependent carboxylases. However it was not an inhibitor of biotin transport, which suggested that oxybiotin does not use the biotin transport system to go through the cell membrane.

Exchange of intracellular biotin. .Biotin-depleted bacteria were allowed to saturate both biotin pools by incubation with [^{14}C]biotin. After 30 min, external [^{14}C]biotin was diluted by addition of a 20-fold excess of unlabelled biotin in the uptake medium and the radioactivity of the cells was monitored (Fig. 3a). After a sharp decrease, the radioactivity reached a plateau value corresponding to about 2/3 of the initial value. In a complementary experiment, bacteria were loaded with unlabelled biotin (70 ng/ml). After 40 min incubation, [^{14}C]biotin (70 ng/ml) was added to the medium and the radioactivity entering the cells was monitored. Fig. 3b shows that this radioactivity reached a plateau. This pool of biotin ex-

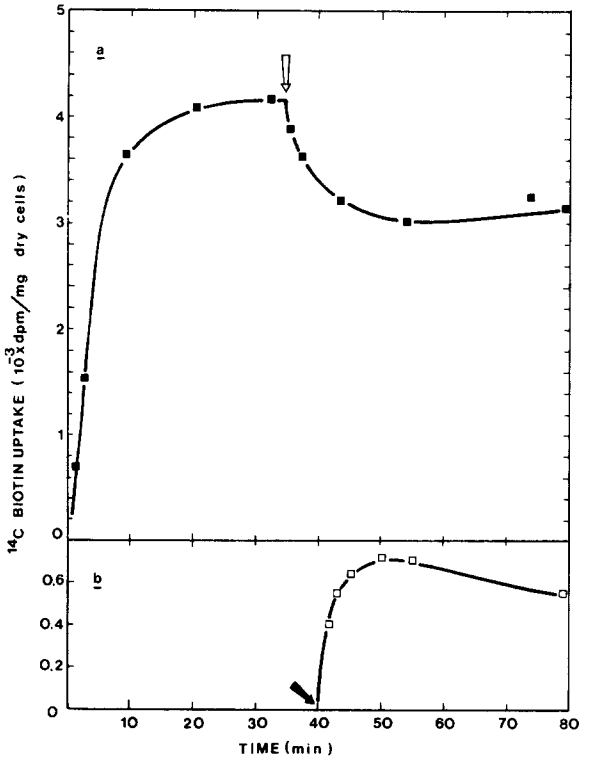

Fig. 3. Exchange of intracellular biotin by resting cells. The same suspension of deficient *E. coli* C162 cells was used in both experiments. (a) ■, Deficient cells ($3.7 \cdot 10^9$ cells/ml) incubated at 37°C in the uptake medium in the presence of 70 ng/ml [^{14}C]biotin. After 34 min 1.4 μg/ml unlabelled biotin was added to the medium (⇓). (b) □, Deficient cells ($3.7 \cdot 10^9$ cells/ml) incubated at 37°C in the uptake medium in the presence of 70 ng/ml unlabelled biotin. After 40 min, 70 ng/ml [^{14}C]biotin was added to the medium (⇓).

changeable by countertransport was identical with the pool of biotin which had been released in previous experiment (Fig. 3a) and corresponded roughly to one-third of the total biotin content of the cells. As judged from its size and properties, it was logical to admit that the pool of biotin that could be exchanged corresponded to that of free biotin.

Energy dependence. Biotin transport was strongly dependent on energy. It was temperature-dependent, as shown by the low value of the uptake at 4°C. (Fig. 1). This uptake at low temperature was inhibited by biotin analogues and decreased by lack of energy and thus cannot be totally accounted for by nonspecific binding as stated previously [13] but reflects probably, at

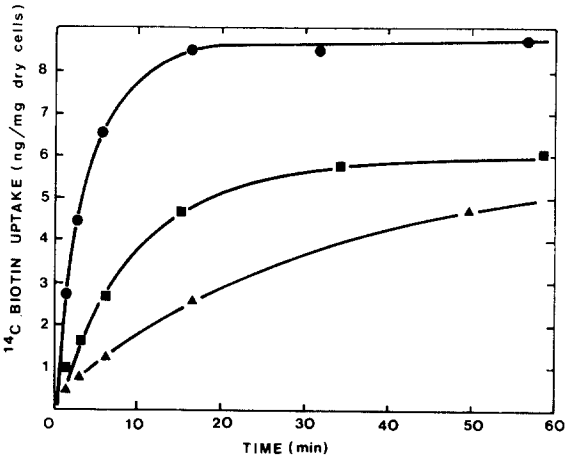

Fig. 4. Influence of CCCP and glucose on the [^{14}C]biotin uptake by deficient resting cells. Experimental conditions as in Fig. 1. [^{14}C]Biotin at 70 ng/ml. (●) Standard conditions; (■) 10 μM CCCP added 10 min before [^{14}C]biotin; (▲) no glucose in the uptake medium and 10 μM CCCP added 10 min before [^{14}C]biotin.

least in part, some specific binding as already suggested in the case of thiamin [28].

On addition of the uncoupler carbonyl-cyanide *m*-chlorophenylhydrazone (CCCP) to the uptake medium, the initial rate of uptake decreased by a factor of 3 and the plateau value was reduced to about 2/3 of the value for the standard assay without CCCP (Fig. 4). When glucose was omitted and CCCP added, the initial rate was divided by 6 and the plateau value was reduced again to about 2/3 of the normal value (Fig. 4). When glucose was omitted, the initial rate was halved but the plateau was not changed (curve not shown). The effect of glucose alone on initial rate was unexpected: the biotin uptake concerns such a small number of molecules that we would have expected enough energy to be available initially in the cell to achieve the transport without using immediately exogenous glucose.

We examined then the effect of an uncoupler on the rate and extent of exchange of biotin. When 10 μM CCCP was added to a suspension of bacteria preloaded with [^{14}C]biotin, a rapid outflow of [^{14}C]biotin was observed (Fig. 5) and a plateau value was reached corresponding to about 2/3 of the initial radioactivity, that is to the plateau value observed when the initial loading of bacteria is allowed to proceed in the presence of CCCP

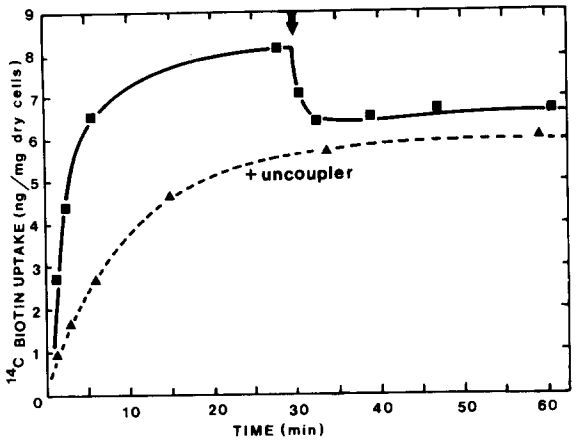

Fig. 5. Influence of CCCP on the intracellular [^{14}C]biotin content of resting cells. Experimental conditions as in Fig. 1. (■) CCCP 10 μM was added after 29 min incubation with 70 ng/ml [^{14}C]biotin (↓); (▲) CCCP 10 μM added 10 min before starting incubation with 70 ng/ml [^{14}C]biotin.

(dotted line). Thus when energy was no longer available, an outflow of biotin was induced to an extent corresponding to the pool of free biotin.

All these facts can logically be accounted for if the role of the energy source is to increase the rate of entry of biotin into the cell. When biotin enters the biotin-deficient cell, it becomes linked to the carboxylases till saturation of the bound pool. A dynamic equilibrium is then established between external and internal biotin (free pool) with a concentration gradient depending on the ratio of the constant k_{cat}/K_m for entry and outflow of biotin. This concentration gradient is maintained, as long as energy is available to increase the rate of entry.

We have taken advantage of the properties of biotin-deficient *E. coli* strains to study the transport of biotin. The deficient bacteria proved to be very useful for controlling the experimental conditions (i.e., the contents of the two pools of biotin) and have been of great help to examine very thoroughly the properties of the pool of free biotin, and the dependence of the phenomena on energy.

We intend now to take advantage of the experience that we have already accumulated with auxotroph strains to move on studying prototroph strains and to determine if both kinds of strain have the same behavior.

Acknowledgements

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