Mechanism of the Antibiotic Action of α-Dehydrobiotin†

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ABSTRACT: α-Dehydrobiotin, a naturally occurring biotin analogue, exhibits antibiotic properties [Hanka, L. J., Reineke, L. M., & Martin, D. G. (1969) *J. Bacteriol. 100*, 42–46]. It is shown in this paper that in addition to its activity as corepressor of the transcription of the biotin locus [Guha, A., Saturen, Y., & Szybalski, W. (1971) *J. Mol. Biol. 56*, 53–62] α-dehydrobiotin acts at the enzyme level. The synthesis of specifically tritiated α-dehydrobiotin has been achieved. By use of this labeled compound and of a biotin-dependent strain of *Escherichia coli*, it has been demonstrated that α-dehydrobiotin can be linked covalently to proteins without further transformation. The fixation of biotin to apocarboxylases is inhibited irreversibly after preincubation with α-dehydrobiotin. This strongly supports the hypothesis that α-dehydrobiotin can be specifically linked to apocarboxylases in place of biotin and leads to carboxylases that are inactive. Thus, the antibiotic properties of α-dehydrobiotin would be partly due to the fact that it competes with biotin for the fixation on the apocarboxylases, producing irreversibly inactive enzymes.

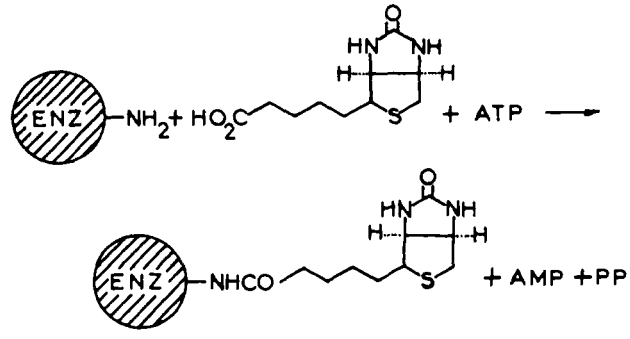
α-Dehydrobiotin (α-DHB)[1] is the first product met along the biochemical degradation pathway of biotin (Mc Cormick, 1975; Mc Cormick & Wright, 1971; Eisenberg, 1973) (see Figure 1). This catabolite has been isolated from cultures of *Streptomyces lydicus* during a systematic screening of antibiotic derivatives (Hanka et al., 1966). α-DHB exhibits antibiotic properties in synthetic medium against microorganisms such as *Escherichia coli*, *Bacillus subtilis*, and several strains of mycobacteria (Hanka et al., 1969, 1972; Hanka, 1967). However, these antibiotic properties are lost in the presence of biotin, and this suggests that α-DHB acts as an antimetabolite of biotin.

α-DHB has been shown to act, as does biotin (Pai & Lichstein, 1965), as a corepressor of the transcription of the biotin locus. This property, originally demonstrated by the technique of hybridization of DNA strands of biotransducing phages with pulse-labeled [^{3}H]RNA of *E. coli* (Guha et al., 1971; Vrancic & Guha, 1973), has been confirmed since by a study based on the measurement of the activities of two biotin biosynthesizing enzymes, the 7,8-diaminopelargonic acid aminotransferase and the dethiobiotin synthetase (Eisenberg, 1975).

No study has yet considered hypotheses where α-DHB acts as an antagonist of biotin at the enzymatic level, for instance, by interfering with biotin during the last step of the biosynthesis of biotin-dependent carboxylases. During this step, biotin is linked to the apocarboxylases according to Scheme I (Murthy & Mistry, 1972).

The specificity of the synthetases toward biotin analogues has been tested in vitro with rat and bacterial synthetases (Kosow et al., 1962; Lane et al., 1964; Blaschkowski, 1969). Some biotin analogues act as antagonists (Lane et al., 1964), but, in most cases, their fixation on the apoenzyme has not been investigated. The only example where the fixation has been rigorously demonstrated is that of 2′-thiobiotin

Scheme I: Covalent Attachment of Biotin on Apocarboxylases



(Blaschkowski, 1969) on apo β-methylcrotonyl-CoA carboxylase. However, the holoenzyme thus obtained is inactive. With oxybiotin (Lane et al., 1964), there is evidence indicating that it is probably bound to the apotranscarboxylase of *Propionibacterium shermanii*, but no carboxylation activity has been detected. Thus, if fixation has really occurred, the holoenzyme is again inactive.

However, in vivo studies show that oxybiotin exhibits intrinsic growth factor properties for several organisms without transformation into biotin, which means that it becomes linked to the apoenzymes and gives rise to active holocarboxylases. This is the only analogue, among those which have been tested, which is able to substitute for biotin (Mc Coy et al., 1948).

Recently, selenobiotin (Bory & Marquet, 1976) proved to be as efficient as biotin in supporting growth of biotin-requiring mutants (Piffeteau et al., 1976). In this case, acetyl-CoA carboxylase of *E. coli* with selenobiotin as the prosthetic group is in vitro 60% as active as the normal enzyme (Piffeteau et al., 1976).

Enzymatic carboxylations by biotin-dependent carboxylases involve two active sites and biotin, which is covalently bound to the apoenzyme through a long arm, acts as a carboxyl carrier between the carboxylation and the transcarboxylation sites. Nothing is known concerning the spatial relationship of these sites except in the case of transcarboxylase of *P. shermanii* where some indications were obtained by nuclear

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[1] Abbreviations used: α-DHB, α-dehydrobiotin; Me_2SO (DMSO in Schemes I and II), dimethyl sulfoxide; DCCI, dicyclohexylcarbodiimide; HABA, 2-(4′-hydroxyazobenzene)benzoic acid; PACA, *p*-(dimethylamino)cinnamaldehyde.

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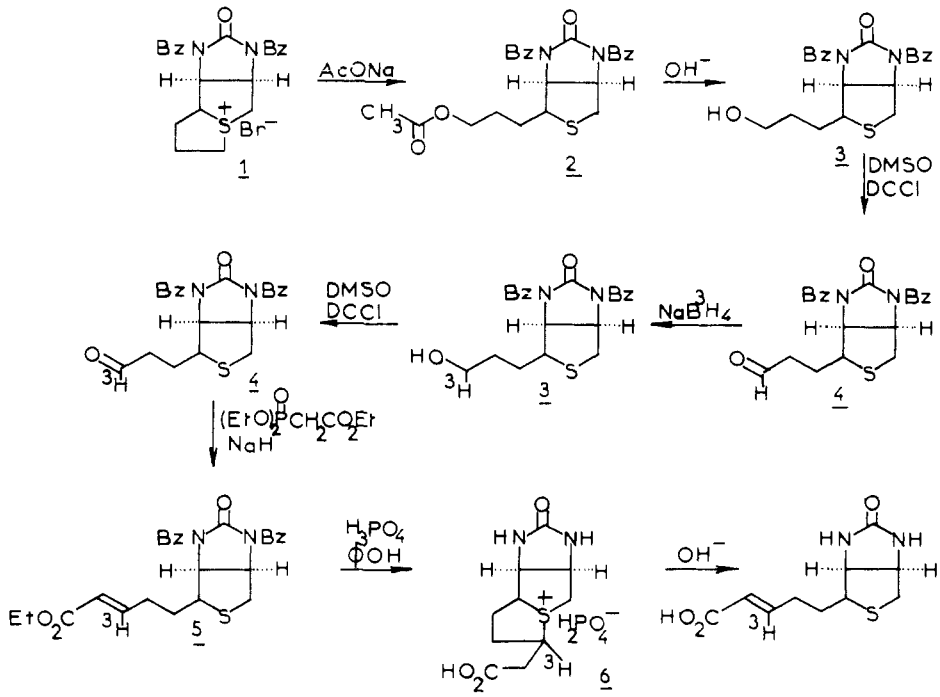
Scheme II: Synthesis of [3-^{3}H]-*d*-α-DHB



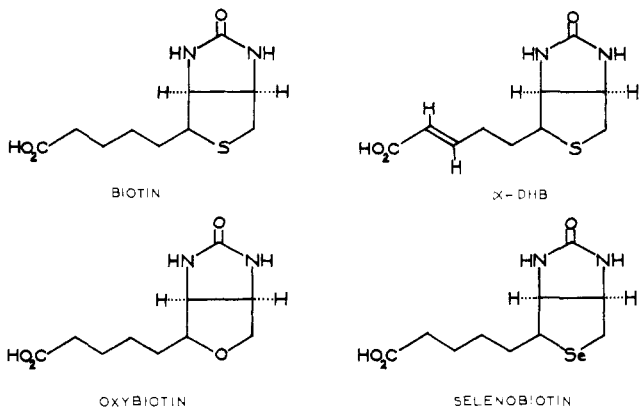


FIGURE 1: Structures of biotin, α-DHB, oxybiotin, and selenobiotin.

magnetic resonance (Fung et al., 1976). It is obvious that the oscillation rate and positioning of biotin at the active site and consequently the specific activities of carboxylases depend on the length and conformational mobility of this arm.

We propose the hypothesis that α-DHB might be also a substrate for the synthetase and might be linked covalently to the carboxylases whose zero activity would be partially responsible for the antibiotic properties of α-DHB.

Experimental Section

Pyridine, dicyclohexylcarbodiimide (DCCI), phenol, orthophosphoric acid, L-histidine, and Norite were purchased from Prolabo (France); triethyl phosphonoacetate was from Aldrich Chemical Co. (Europe division); 2-(4′-hydroxyazobenzene)benzoic acid (HABA), avidin (12 units/mg), and chloramphenicol were from Sigma Chemical Co.; glucose, bactotryptone, and yeast extract were from Difco; dimethyl sulfoxide (Me_2SO) was from S.D.S. (France); trifluoroacetic acid was from Merck Co. (RFA); *p*-(dimethylamino)cinnamaldehyde (PACA) was from Fluka (Switzerland). *d*-Biotin and *l*-3,4-(1′,3′-dibenzyl-2′-ketoimidazolido)-1,2-trimethylenethiophanium bromide are gifts from Hoffmann-La Roche Inc. (Basel). A sample of *d*-α-dehydrobiotin (α-DHB) was obtained through the courtesy of Dr. Hanka, Upjohn Co. (Kalamazoo). Sephadex G-10 was purchased from Pharmacia Fine Chemicals, and AG1X2 was from Bio-Rad. [2′-^{14}C]-*d*-Biotin (42 mCi/mmol) and sodium [^{3}H]borohydride (660 mCi/mmol) were purchased from Amersham/Searle (England). All other chemicals and reagents were of the highest purity available.

Synthesis of [^3H]-*d*-α-*DHB*. The synthesis of [^{3}H]-*d*-α-DHB was achieved according to the general synthesis (Scheme II) designed for *d*-α-DHB (Field et al., 1972, 1976).

l-1,3-Dibenzyl-2-oxohexahydrothieno[3,4-d]imidazole-4-propanal (**4**) was prepared in an overall yield of 40% starting from *l*-3,4-(1′,3′-dibenzyl-2′-ketoimidazolido)-1,2-trimethylenethiophanium bromide (**1**) by treatment with sodium acetate in absolute ethanol, saponification, and oxidation by Me_2SO, DCCI, pyridine, and trifluoroacetic acid (1.1 mL, 3 mmol, 0.08 mL, and 0.06 mL per mmol of alcohol **3**).

[*1-^3H*]-*l*-1,3-*Dibenzyl-2-oxohexahydrothieno[3,4-d]-imidazole-4-propanol* (**3**). **4** (90 mg, 0.237 mmol) is reduced with [^{3}H]$NaBH_4$ (25 mCi, 661 mCi/mmol, 0.038 mmol) in anhydrous tetrahydrofuran (1 mL) under argon at room temperature for 72 h. After addition of water, the mixture of [1-^{3}H]-**3** and -**4** is extracted with methylene chloride (86 mg, 12 mCi).

[*1-^3H*]-*d*-**4**. The above mixture of [1-^{3}H]-**3** and -**4** (86 mg, 12 mCi) is oxidized, as described previously, with Me_2SO (0.6 mL), DCCI (180 mg), pyridine (30 μL), and trifluoroacetic acid (21 μL) under argon for 20 h. The DCCI in excess is destroyed by addition of 150 mg of oxalic acid dissolved in 0.6 mL of methanol. After filtration and extraction with ether, the crude extract is purified by thin-layer chromatography on silica gel (eluant: ethyl acetate–hexane, 1:1) and yields 34 mg (40%) of [1-^{3}H]-**4**.

[*3-^3H*]-*l-1,3-Dibenzyl-2-oxohexahydrothieno[3,4-d]-imidazole-4-pent-2-enoic Acid Ethyl Ester* (**5**). One equivalent of the reagent prepared from triethyl phosphonoacetate (1 equiv) and sodium hydride (50% oil suspension; 1 equiv washed twice with anhydrous benzene) is added to 44 mg of [1-^{3}H]-**4** dissolved in 2 mL of anhydrous tetrahydrofuran at 4 °C under argon. After 90 min at room temperature, 4 mL of water is added, and the mixture is extracted with dichloromethane. The

crude extract is purified by chromatography on alumina and yields 30 mg of [3-^{3}H]-**5** (57%).

[3-^{3}H]-d-2-Oxohexahydrothieno[3,4-d]imidazole-4-pent-2-enoic Acid (α-DHB). The deprotection of the ureido ring is the critical step of the synthesis of this molecule. Before attempting this crucial debenzylation on the radioactive product, we have run the experiment on **5** and checked that debenzylation followed by extensive desalting over a Sephadex G-10 column (vide infra) led to α-DHB as the unique product, identified by ^{1}H NMR and comparison with an authentic sample by thin-layer chromatography on silica gel (eluant: 1-butanol–benzene–water–methanol, 2:1:1:1; spot test with PACA) and anion-exchange chromatography (AG1X2 resin, formate form, elution with 0.05 N formic acid).

[3-^{3}H]-**5** (30 mg) is heated at 150 °C for 3 h in 400 mg of orthophosphoric acid containing 10 mg of phenol. After addition of water (4 mL), the mixture is extracted with ether. The aqueous layer, neutralized with potassium carbonate, is made strongly alkaline with solid potassium hydroxide and heated for 1 h on a steam bath (95 °C). After neutralization with phosphoric acid (pH 7–7.5), the product is purified on the Sephadex G-10 column (height = 1 m).

Since the contaminating salts (potassium phosphates) could not interfere in our experiments, the desalting has not been as thorough as described above and the radioactive α-DHB has been titrated colorimetrically with PACA (Mc Cormick & Roth, 1970) by comparison with a standard curve established with nonradioactive α-DHB. This step yields 3.5 mg of [3-^{3}H]-*d*-α-DHB (21%; 64.6 mCi/mmol).

Bacterial Strain. *E. coli* C162 (bioB$^-$, His$^-$) was a generous gift from Dr. P. Cleary.

Growth Conditions. The microorganism is grown either in a complex medium (bactotryptone, 10 g/L; yeast extract, 5 g/L; NaCl, 5 g/L; pH 7.3) or in a synthetic medium [KH_2PO_4, 13.6 g/L; $(NH_4)_2SO_4$, 2 g/L; $MgSO_4$, 0.2 g/L; $FeSO_4$, 0.5 mg/L; pH 7.0 (KOH)] supplemented with glucose (2 g/L) and L-histidine (35 mg/L). Traces of biotin in synthetic medium are removed by treatment with Norite before autoclaving. The selected amount of biotin or *d*-α-DHB is then added aseptically.

Biotin-Deficient Bacteria. A synthetic medium provided with 0.1 ng/mL biotin is inoculated with a small volume (1:200 v/v) of a dilute suspension (10^7 cells/mL) of bacteria, grown on a synthetic medium containing biotin (1 ng/mL) and extensively washed with 0.85% saline. The growth is then monitored at 570 nm.

Influence of α-DHB on the Biotin Uptake by Resting Cells. Biotin-deficient bacteria grown in the mid-log phase are collected by centrifugation, washed once with distilled water (37 °C), and placed in the incubation buffer for 30 min in the presence of α-DHB. After centrifugation and washing with distilled water, the bacteria are incubated (37 °C) in the same buffer in the presence of [^{14}C]biotin. The incorporation of biotin is monitored by filtering and counting aliquots. The blank is determined by incubating bacteria under the same conditions without adding α-DHB.

[3-^{3}H]-d-α-DHB Uptake by Resting Cells. Biotin-deficient bacteria grown and washed as described above are incubated in the incubation buffer in the presence of radioactive *d*-α-DHB (1.25 μg/mL) for 2 h at 37 °C. After incubation the bacteria are harvested and washed twice with distilled water, and the α-DHB is extracted.

Measurement of Free and Bound α-DHB. After collection and washing of the cells with distilled water, the free α-DHB is released by heating in water (110 °C; 20 min; 33 mL/10^{12} cells). After centrifugation, the bound α-DHB is extracted by acid hydrolysis (H_2SO_4, 4 N; 110 °C; 2 h; 10 mL/10^{12} cells) (Pai & Lichstein, 1965; Ogata, 1970). This treatment converts α-DHB into sulfonium salt **6**. After neutralization (KOH, 4 N), the solution is made strongly alkaline with solid potassium hydroxide and heated on a steam bath for 1 h. Aliquots are then counted, leading to a value of 2.4 ng of α-DHB bound per mg of dry cells. α-DHB is identified by ion-exchange chromatography on an AG1X2 column and by recrystallization.

AG1X2 Chromatography of Extracted Bound d-α-DHB. Bound α-DHB is chromatographed on a 43.5 × 1.0 cm AG1X2 (formate form) column. After washing of the column with distilled water, elution is performed with 0.05 N formic acid, and the radioactivity is determined by counting 1-mL aliquot fractions in 10 mL of Bray's liquor.

Recrystallization of Bound d-α-DHB. Authentic *d*-α-DHB is added to the extract of bound *d*-α-DHB. After dissolution with normal potassium hydroxide, the concentration of α-DHB is determined by titration with avidin (Green, 1970), and the radioactivity is determined and corrected for quenching. α-DHB is then crystallized by careful addition of normal hydrochloric acid. After crystallization, the tube is centrifuged and the supernatant is discarded. The crystals are dissolved with normal potassium hydroxide, and titration with avidin and counting are performed on an aliquot. The next crystallization step is then induced by acidification.

Results and Discussion

A single mutation in the B cistron of the biotin locus prevents *E. coli* C162 from transforming dethiobiotin into biotin and makes it dependent on exogenous biotin for growth. We have shown with such a strain that α-DHB cannot replace biotin as a growth factor in synthetic medium after careful elimination of any contaminating biotin. We have observed that α-DHB cannot support the growth of bacteria at concentrations as high as 500 ng/mL, when the culture medium has been treated with active charcoal and inoculated with a small volume of a dilute suspension of extensively washed bacteria.

The reported antibiotic properties of α-DHB prove that it is able to penetrate into the cell. The lack of growth observed here, with a mutant where no repression of biotin biosynthesis can occur, shows that α-DHB cannot replace biotin. The question is to know whether α-DHB can be fixed to the carboxylases, giving inactive enzymes.

In this hypothesis, α-DHB occupies sites which are normally devoted to biotin. A preincubation of biotin-deficient bacteria with α-DHB must lower the number of sites available for biotin.

To test this, we have studied the incorporation of [^{14}C]biotin in biotin-deficient bacteria. Figure 2 shows that preincubation with α-DHB (7.5 μg/mL) significantly lowers the amount of biotin that enters resting cells of *E. coli* C162.

However, in all living cells, biotin is distributed among two pools, the free biotin and the bound biotin which is covalently linked to carboxylases (Waller & Lichstein, 1965). We have measured the influence of preincubation with α-DHB on the contents of these two pools.

Results of Table I show that the amounts of free and bound biotin are very low in bacteria that have been grown in the presence of suboptimal concentrations of biotin. When resting biotin-deficient bacteria are incubated in the presence of biotin, the vitamin enters the cells, presumably by an active transport system (Prakash & Eisenberg, 1974; Cicmanec & Lichstein, 1978), and very rapidly binds covalently to the carboxylases.

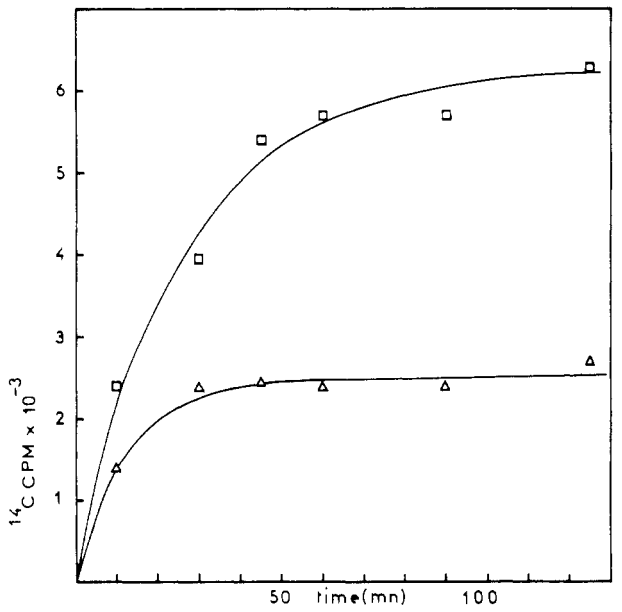


FIGURE 2: Influence of α-DHB on the [^{14}C]biotin uptake by resting cells of *E. coli* C162. *E. coli* C162 cells grown on synthetic medium supplemented with glucose (2 g/L), histidine (35 mg/L), and biotin (0.1 ng/mL) are harvested in the mid-log phase (5×10^8 cells/mL), washed with bidistilled water, and suspended in the incubation buffer (3.85×10^9 cells/mL). Incubation is carried out at 37 °C during 30 min in the presence (7.5 μg/mL) or in the absence of α-DHB. After centrifugation and washing with bidistilled water, the cells are resuspended in the incubation buffer (3.85×10^9 cells/mL) and [^{14}C]biotin is added (100 ng/mL). 2-mL aliquots are filtered over 0.6-μm pore filters which are dried at 80 °C for 1 h and counted. (△) Incubation with α-DHB; (□) incubation without α-DHB.

Table I: Influence of Growth Conditions and Preincubation with α-DHB on the Content of the Free and Bound Pools of Biotin of *E. coli* C162

bacteria	free biotin[f] (ng/mg of dry cells)	bound biotin[g] (ng/mg of dry cells)
normal cells[a]	3.5	3.6
deficient cells[b]	0.12	0.28
deficient cells incubated with biotin[c]	2.3	4.5
deficient cells preincubated with α-DHB[d]	0.35	2.7
deficient cells preincubated without α-DHB[e]	0.51[h]	5.8

[a] Cells grown on synthetic medium supplemented with glucose (2 g/L), histidine (35 mg/L), and [^{14}C]biotin (5 ng/mL) were harvested in the mid-log phase. [b] Cells grown on synthetic medium supplemented with glucose (2 g/L), histidine (35 mg/L), and [^{14}C]biotin (0.1 ng/mL) were harvested in the mid-log phase. [c] Biotin-deficient cells grown in the presence of biotin (0.1 ng/mL) were incubated for 30 min in the uptake buffer containing [^{14}C]-biotin (140 ng/mL). [d] Biotin-deficient cells grown in the presence of biotin (0.1 ng/mL) were preincubated in the uptake buffer containing α-DHB (7.5 μg/mL) for 30 min, harvested, and washed. These cells were then incubated for 30 min in the uptake buffer containing [^{14}C]biotin (100 ng/mL). [e] Biotin-deficicient cells that were treated as described above with the exception that preincubation was carried out in the absence of α-DHB. [f] Extracted by boiling 20 min in water. [g] Extracted by sulfuric hydrolysis (H_2SO_4, 4 N; 2 h; 120 °C). [h] It appears that the pool of free biotin is significantly modified by preincubation. At the present time we have no explanation for this decrease.

Results of Table I indicate that preincubation with α-DHB lowers significantly the level of the bound-biotin pool of biotin-deficient bacteria that have been incubated with biotin and that most of the modifications observed in Figure 2 are due to a change in the pool of bound biotin.

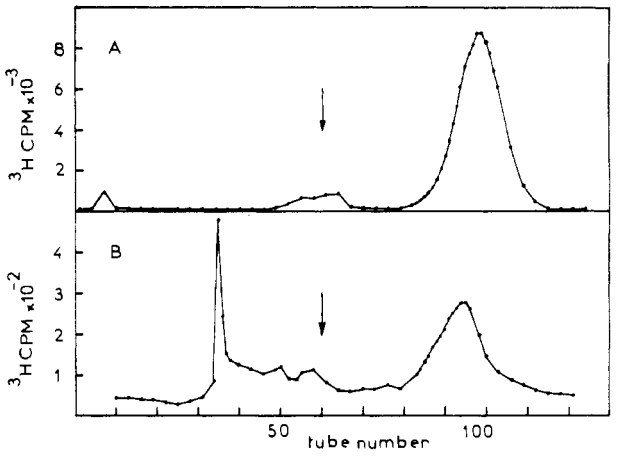


FIGURE 3: Identification of α-DHB. The AG1X2 column (1 × 43.5 cm) is eluted with distilled water for 25 tubes and then with 0.05 N formic acid. (A) Sample of authentic [3-^{3}H]-*d*-α-DHB; (B) fraction of bound α-DHB. The arrow corresponds to the elution of biotin.

Table II: Identification of Bound α-DHB by Recrystallization to Constant Specific Radioactivity

crystallization no.[a]	α-DHB crystals[b] (mg)	sp act.[c] (dpm/mg)
1	3.62	26 733
2	3.01	26 485
3	2.61	25 617
4	2.32	25 880

[a] Crystallization has been carried out in a conical centrifuge tube. After addition of 4.7 mg of α-DHB to the bound fraction, crystallization is induced by careful acidification (HCl, 1 N) of a solution of the potassium salt of α-DHB in water. Crystals are collected by centrifugation and subjected to the next crystallization step. [b] Titration of α-DHB with avidin. [c] Correction for quenching by the double-channel and external standard method. Samples are counted in 10 mL of Bray's liquor.

These experiments provide evidence for the binding of α-DHB, but to get unambiguous proof, we have synthesized the specifically tritiated compound according to Scheme II (Field et al., 1972, 1976; see Experimental Section) which could also allow us to check that the incorporation occurs without transformation of the α-DHB.[2]

After incubation in the presence of [^{3}H]-*d*-α-DHB, biotin-deficient cells were treated as follows. One part of the bacteria was disrupted by sonication and the proteins were precipitated with trichloroacetic acid. Most of the radioactivity incorporated by resting cells precipitated and was thus bound to proteins.

Free and bound α-DHB's were extracted (Ogata, 1970; Pai & Lichstein, 1965) from the rest of the bacteria. The bound radioactive material was identified as α-DHB by using ion-exchange chromatography (Figure 3) and recrystallization with an authentic sample of *d*-α-DHB to constant radioactivity (Table II).

As shown above (Table I), the incorporation of biotin, which occurs specifically on carboxylases, is inhibited in an irreversible manner by preincubation with α-DHB. Furthermore, the decrease of the pool of bound biotin which arises from the

[2] The possible transformations of α-DHB, before or after incorporation, include conversion to biotin. This was a priori eliminated on the basis of growth experiments. The possible catabolites bisnor- and tetranorbiotin would have lost the label. The other degradation products with modified ureido and thiophane rings have very different chromatographic properties.

preincubation with α-DHB (3.1 ng of biotin per mg of dry cells) corresponds roughly to the amount of α-DHB which becomes linked to proteins during incubation with [^{3}H]-α-DHB (2.4 ng of α-DHB per mg of dry cells).

This strongly suggests that a large part of the α-DHB binds specifically at sites, which are normally devoted to biotin, on apocarboxylases, leading to carboxylases which are inactive.

d-α-DHB thus has this interesting ability to act specifically at two levels. The first action, the repression of the biotin biosynthesis, is very likely completed at the enzyme level by a competition with biotin for the sites available on the apocarboxylases.

As judged from molecular models of biotin and α-DHB, the length of the arm that links the prosthetic group to the apocarboxylases is almost unaffected by the presence of the double bond. This suggests that in carboxylases that have incorporated α-DHB, the prosthetic group can potentially occupy the correct positions in the active sites of carboxylation and transcarboxylation. The lack of activity of these enzymes may thus not be a consequence of a mispositioning of the cofactor but rather may derive from the loss of conformational freedom that lowers the rate or even prevents the oscillation between the two active sites.

The study of other biotin analogues modified on the side chain and the determination of the effect of different modifications on the enzymatic activity will bring useful information concerning the spacial relationship of the two active sites.

Acknowledgments

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