The Biosynthesis of Biotin in an Auxotrophic Strain of *Humicola*

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INTRODUCTION

The biosynthesis of biotin has been widely studied, but even now the biochemical pathway is not entirely clear (McCormic & Wright, 1971). Two different schemes of biotin synthesis have been proposed. That proposed by Pai and co-workers in *Escherichia coli* leads from pimelic acid to biotin via 7-keto-8-aminopelargonic acid, 7,8-diaminopelargonic acid and desthiobiotin (Pai & Lichstein, 1965, *a, b, c*, 1966, 1967; Birnbaum, Pai & Lichstein, 1967; Pai, 1968; Pai, 1970). However, the mechanism for the incorporation of the sulphur atom in the molecule is still unknown (Pai, 1972). It is thought that cysteine is the sulphur donor in the conversion of desthiobiotin into biotin (Umbarger & Davis, 1962). In *Achromobacter* a different pathway, which does not include the formation of desthiobiotin, has been proposed (Lezius, Ringelman & Lynen, 1963). In this the sulphur atom enters the molecule as a cysteamine residue and biotin is formed from cysteine, pimelyl-CoA and carbamyl phosphate.

In the present work the biosynthesis of biotin was studied in a biotin-requiring strain of *Humicola*, strain 16-1,† isolated from soil (de Bertoldi & Verona, 1970; de Bertoldi, Lepidi & Nuti, 1972). In this mould the biotin requirement can be satisfied by thiamine, although on minimal medium containing thiamine the growth rate is lower. Thiamine is not a known precursor of biotin, and it was therefore postulated that since the $R-CH_2-S-CH_2-R$ radical is common to both thiamine and biotin the block in the synthesis of biotin was at this step, and that the mould could utilize this radical in the conversion of thiamine to biotin. This paper describes experiments designed to examine this hypothesis and clarify the pathway of biotin biosynthesis in this mould.

METHODS

Organisms and media. The origin, morphology and cytology of strain 16-1 of the genus *Humicola* was described by de Bertoldi (1972) and de Bertoldi *et al.* (1972). It was maintained in the laboratory at 26 °C on complete medium agar (CMA) prepared with minimal medium agar (MM agar) plus complete supplements (CS) according to Pontecorvo (1953). Microbiological assays were done with *Lactobacillus plantarum* (ATCC8014) on Bacto biotin assay medium (Difco).

Assay of compounds which can replace biotin for growth. This was done by measuring the diameter of single colonies of the mould on MMA plus one of the following compounds at the final concentration of 1 or 10 µg/ml: biotin, desthiobiotin, thiamine, methionine, thiophene, tetrahydrothiophene, thiodiacetic acid, thiodiethanol, trithiane, dimethyl sulphide, penicillin G, penicillin V, methicillin, ampicillin, 6-amino-penicillanic acid, actithiazic acid,

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† This strain is now deposited in the American Type Culture Collection.

Table 1. *Growth of strain 16-1 on MMA supplemented with various compounds*

Each number represents the average diameter of nine colonies grown for 7 days at 26 °C.

Compound	Growth (mm)	
	1 µg/ml	10 µg/ml
Biotin	29.1	29.3
Desthiobiotin	25.6	25.4
Thiamine*	18.2	24.1
Methionine	15.6	19.3
Thiophene	0	0
Tetrahydrothiophene	6.5	0
Thiodiacetic acid	9.7	14.9
Thiodiethanol	7.2	9.2
Trithiane	0	0
Dimethylsulphide	13.5	0
Penicillin G	11.0	0
Penicillin V	20.2	22.1
Methicillin	18.9	18.6
Ampicillin	0	0
6-Amino penicillanic acid	15.6	14.1
Actithiazic acid	0	0
Cephalosporin C	9.3	9.3
Cephalosporin N	8.6	8.9
Bacitracin	17.2	18.7

* At concentrations of 2 or 20 µg/ml.

cephalosporin C, cephalosporin N, bacitracin. All compounds, except the antibiotics, were tested with *L. plantarum* to establish that they were biotin-free.

Isolation of biotin from mould. The preparation of acid hydrolysate from the mould and purification of biotin, involving complexing with avidin (Sigma) and separation of the complex on a column of Sephadex G-25, were done as described by Lezius *et al.* (1963), except that the acid hydrolysate was concentrated by evaporation; charcoal adsorption and the ion-exchange chromatography steps were omitted to improve yield.

Experiments with labelled thiamine. The mould was grown in Roux bottles in 300 ml MM + thiamine [^{35}S]-hydrochloride (1.7×10^{-3} mCi/ml) or thiamine (thiazole-2- ^{14}C) hydrochloride (1.7×10^{-4} mCi/ml), the specific activities of isotopes (Radiochemical Centre, Amersham, Buckinghamshire) being 146 mCi/mmol (^{35}S) and 18.9 mCi/mmol (^{14}C) respectively. Before addition to the medium the labelled compounds were diluted five times with unlabelled thiamine to achieve a final concentration of 20 µg/ml. After 10 days' incubation at 26 °C, acid hydrolysates of mycelia were prepared and biotin isolated as described. The radioactivity of chromatographic fractions was counted with a liquid scintillation spectrometer (Packard Tricarb scintillation spectrometer, model 352), using Insta-gel (Packard) as scintillation solution.

Microbiological assay. Biotin in the acid hydrolysate used for chromatography was assayed with *L. plantarum* by the method of Wright & Skeggs (1944). Chromatographic fractions were also tested for biotin after autoclaving at 125 °C for 1 h. To render the avidin biotin-free, an aqueous solution was autoclaved at 125 °C for 1 h before assay. To verify if desthiobiotin, thiamine, methionine and penicillin V (the four compounds which best satisfied the biotin requirement) were utilized in biotin synthesis by the mould, an initial test was carried out in which the mould was grown in flasks of liquid MM containing separately each one of these compounds. The cultures were shaken for 10 days at 26 °C and mycelia

Fig. 1. G-25 Sephadex-column chromatography of the biotin-avidin complex contained in the acid hydrolysates from the culture grown in the presence of $[^{14}\text{C}]$ thiamine (a) or $[^{35}\text{S}]$ thiamine (b). A 2 ml sample was applied to the column and eluted with 1 % (w/v) NaCl, pH 10, at a flow rate of 15 ml/h; 2.5 ml fractions were collected. The biotin content of fractions determined by microbiological assay with *L. plantarum* is reported. ●, Absorbance at 280 nm; ○, radioactivity; △, biotin.

were harvested and acid hydrolysates prepared as described, and assayed for biotin with *L. plantarum*. As a control the same test was carried out adding water, biotin, desthiobiotin, thiamine, methionine or penicillin V to the assay medium.

RESULTS

Sulphur compounds which can replace biotin

The ability of the fungus to grow on compounds containing the $\text{R}-\text{CH}_2-\text{S}-\text{CH}_2-\text{R}$ group common to biotin and thiamine was tested. Before use all compounds, with the exception of the antibiotics, were shown to be biotin-free in microbiological assay. Most of the compounds examined supported growth although to differing extents (Table 1). The best growth responses were to desthiobiotin, thiamine, methionine, penicillin V or bacitracin in MM agar; these compounds almost completely substituted for the biotin requirement. At the higher concentration (10 $\mu\text{g}/\text{ml}$) some substances (thiamine, methionine and thiodiacetic acid) supported increased growth, while others (tetrahydrothiophene, dimethyl sulphide and penicillin G), were inhibitory. There was no growth in the presence of ampicillin, acthithiazic acid, thiophene or trithiane at either concentration, perhaps because they could not be metabolized by the mould or because they were toxic.

Microbiological assay

Acid hydrolysates from the mould grown on MM in the presence of desthiobiotin, thiamine, methionine or penicillin V, showed differing concentrations of biotin. After growth on desthiobiotin, the extract had no significant effect on the growth of *L. plantarum*. It could have been that desthiobiotin was not transformed into biotin by the mould, but was directly utilized as a vitamin. However, after growth on thiamine, methionine or penicillin V, the extracts stimulated growth of *L. plantarum*; when these substances were added directly to the biotin assay medium they did not stimulate growth of the test bacterium, but penicillin V completely inhibited growth of *L. plantarum*, presumably through its action as an antibiotic.

Biosynthesis of biotin from thiamine

Thiamine replaced biotin as a growth factor and supported 80% of the growth response to biotin when it was converted by the mould into a substance which stimulated growth of *L. plantarum*. We therefore attempted to examine the possibility that the sulphur atom and one of the adjacent carbon atoms of thiamine were incorporated into biotin. This was done by using thiamine labelled with ^{35}S or ^{14}C in the 2-position of the thiazole ring. When the radioactivity of biotin isolated from the mould was assayed, it was found that both ^{14}C and ^{35}S were incorporated into biotin. Fig. 1 (*a*), (*b*) shows that a peak of radioactivity was associated with avidin in the gel-filtration chromatography in the experiments with both ^{14}C - and ^{35}S -labelled thiamine. When the chromatographic fractions were assayed for biotin, it was observed that biotin eluted with the main protein peaks and with the radioactivity. As a control, amounts of avidin equal to that present in the chromatographic fractions were tested in the same way. After an appropriate correction, necessary because of the probable presence of biotin in the avidin, the amount of biotin in each fraction, for both experiments involving ^{35}S - or ^{14}C -incorporation, is shown in Fig. 1 (*a*), (*b*). The biotin peaks in both experiments are associated with the radioactivity and avidin peaks.

DISCUSSION

The biotin requirement of strain 16-1 can be satisfied by compounds containing in their molecule the thioester group, $\text{R-CH}_2\text{-S-CH}_2\text{-R}$, this being the minimal chemical structure required by the mould for growth (de Bertoldi, unpublished data). Thiamine, methionine and penicillin V are converted by the mould into biotin; desthiobiotin completely replaced the biotin requirement and it seems to be utilized by the mould without conversion into biotin. Both the sulphur and the adjacent carbon atoms of thiamine are incorporated into biotin, confirming that a part of the thiamine molecule is utilized in biotin biosynthesis. Since the sulphur atom enters the molecule of biotin as an organic compound (i.e. bound to carbon), it appears that desthiobiotin is not a precursor of biotin in this mould. It is suggested that, in 16-1 strain, the block in biotin biosynthesis is in the formation of the sulphur-carbon bond and that the sulphur atom is introduced early in the biosynthetic pathway. This hypothesis seems to accord with the pathway of biotin biosynthesis proposed by Lezius *et al.* (1963) rather than with that reported by Pai and co-workers which involves, as the penultimate step, the formation of desthiobiotin.

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