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The Biosynthesis of Biotin in Growing Yeast Cells

THE FORMATION OF BIOTIN FROM AN EARLY INTERMEDIATE

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1. Yeast cells grown in the presence of an unknown radioactive biotin vitamer produced by *Penicillium chrysogenum* incorporated the vitamer into the newly synthesized biotin. 2. The biotin was isolated as the avidin–biotin complex and after hydrolysis the biological activity and radioactivity were shown to be coincidental. 3. The specific activity of the biotin was identical with that of the pimelic acid used in a previous investigation to label the unknown vitamer. 4. The role of the unknown biotin vitamer as an intermediate in biotin biosynthesis is discussed.

In an investigation with *Penicillium chrysogenum* it was shown that [1,7-$^{14}C_2$]pimelic acid is incorporated into an unknown biotin vitamer (i.e. a substance capable of overcoming biotin deficiency) that accumulates in the culture medium (Eisenberg & Maseda, 1966). The biosynthesis of this vitamer, as well as desthiobiotin, was controlled by the biotin concentration of the medium. The present study provides evidence for the incorporation of the labelled unknown vitamer into the newly synthesized biotin of growing yeast cells. These findings firmly support an earlier suggestion of an intermediary role for this vitamer in the biogenesis of biotin (Eisenberg, 1962).

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MATERIALS AND METHODS

The yeast turbidity assay and the disk assay procedures were carried out in the manner described by Eisenberg (1963). Ascending paper chromatography was done on Whatman no. 1 paper at 0° with butan-1-ol–acetic acid–water (12:3:5, by vol.). A Packard Tri-Carb liquid-scintillation spectrometer was used for radioactivity measurements and corrections were made for quenching. Measurements of E_{280} were made with the Beckman DU spectrophotometer. A purified sample of avidin was a gift from Dr N. M. Green and had an activity of 10 units/mg. The ^{14}C-labelled unknown vitamer was the purified material obtained by Eisenberg & Maseda (1966) and had a specific activity of $5{\cdot}12 \times 10^4$ disintegrations/min./μg. of biotin equivalents.

Growth of yeast cells. The yeast basal medium used for bioassay was distributed in duplicate flasks in 10, 50 and 500ml. quantities. After sterilization, 0·001ml. of the radioactive material was added to the flask containing 10ml. of medium, which was then inoculated with a drop of a dilute suspension of yeast cells. After 24hr. of incubation at 30° the entire contents of the flask were transferred to the flask containing 50ml. of medium to which had been added 0·005ml. of a solution of the labelled vitamer. The contents of this flask were used in turn to inoculate the largest volume of medium to which had been added 0·01ml. of a solution of the labelled vitamer. The cells were harvested by centrifugation and washed twice with water, yielding 3g. wet wt. of yeast. The supernatant fluid and the water washes were combined and concentrated to 100ml. *in vacuo*. This material was assayed for residual biological activity and radioactivity.

Isolation of biotin. The cells were suspended in 50ml. of 4N-H_2SO_4 and the suspension was autoclaved for 1hr. at 120°. After cooling, the pH was brought to 6·8 with saturated $Ba(OH)_2$, the precipitate was washed three times with water and the washings were added to the supernatant fluid. The combined solutions were evaporated to dryness, the residue was dissolved in a small volume of water and the small amount of precipitate was removed by centrifugation. Paper chromatography revealed the presence of biotin, biotin (+)-sulphoxide and biocytin. The solution was adjusted to pH2·5 and placed on a Dowex 50 (Na^+ form) column (1·5cm.$\times$15cm.). The column was washed with water, which removed the biotin and left the biocytin and other amino acids on the column. The initial effluent and the water washings were combined and evaporated to a small volume. The pH of the solution was adjusted to 6·5 and the material placed on a Dowex 1 (acetate form) column (1cm.$\times$20cm.). The column was washed with water and 0·5N-acetic acid and the biotin was eluted with 1·5N-acetic acid. The acetic acid was removed by evaporation and avidin solution (1·0ml. containing 0·5mg. in 0·9% NaCl, pH9·5) was added to the flask. The mixture was left at room temperature for 10min. before chromatography on a Sephadex G-25 column (0·9cm.$\times$25cm.) that

had been previously equilibrated with the same salt solution. More avidin solution (1·0 ml.) was used to rinse the flask and this was also added to the column, which was developed with 0·9% NaCl solution. Samples (1 ml.) were collected and E_{280} of each was measured. Portions were removed from each tube, treated with an equal volume of 4N-acetic acid and autoclaved for 1 hr. at 120°. The autoclaved and original solutions were assayed for biological activity and radioactivity.

RESULTS

The analysis of the samples obtained from the column chromatography of the avidin–biotin complex is shown in Fig. 1. The avidin used was in tenfold excess of that required to bind all the biotin present. The biotin–avidin complex appears as a shoulder on the main absorption band and has its peak E_{280} in tube 8. For comparison the elution pattern of an identical concentration of avidin chromatographed on the same column under similar conditions is also shown. The radioactivity and biological activity coincide with each other and with the avidin–biotin complex. Paper chromatography of the hydrolysed material obtained from tubes 7, 8 and 9 revealed two components, namely biotin and biotin (+)-sulphoxide.

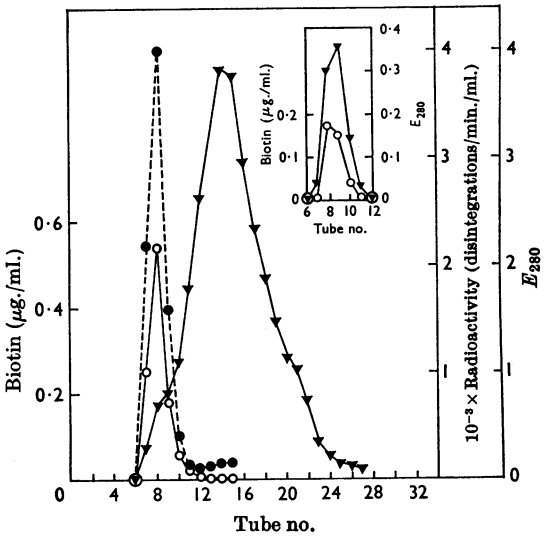

Fig. 1. Column chromatography of avidin–biotin complex (main Figure) and purified avidin (insert). The preparations (see the text) were added to a Sephadex G-25 column (0·9 cm. × 25 cm.) and eluted with 0·9% NaCl, pH 9·5. ○, Biological activity after hydrolysis; ●, radioactivity; ▼, E_{280}.

If the unknown vitamer were a true intermediate, then one would expect the labelled biotin derived from it to have the same specific activity as the pimelic acid used initially to label the unknown vitamer. Specific-activity measurements made on the hydrolysed samples gave values that were too low. However, on calculation of the percentage of incorporation of the unknown vitamer on the basis of both biological activity and radioactivity, it was found that the former gave very much higher values, suggesting a source of biotin contamination. On hydrolysis of the chromatographed avidin fractions, the biological activity curve shown in the insert in Fig. 1 was obtained, indicating that the avidin contained a substantial amount of bound biotin. To correct for dilution with unlabelled biotin, 0·2 ml. samples from tubes 7–11 of the avidin–biotin chromatography were combined and an equal volume of 4N-acetic acid was added. The same procedure was carried out for the contents of tubes 7–11 of the avidin chromatography. Both sets were hydrolysed as described above and each was assayed by the yeast turbidity method. The avidin–biotin preparation was also assayed for radioactivity. After correction for bound biotin it was found that the biotin isolated from the yeast cells has the same specific activity as the original labelled pimelic acid (Table 1).

DISCUSSION

It was originally proposed that the first step in the biosynthesis of biotin is the condensation of

Table 1. *Comparison of the specific radioactivity of biotin with that of pimelic acid used as precursor*

[^{14}C]Pimelic acid was used to obtain labelled biotin vitamer (Eisenberg & Maseda, 1966). Yeast cells were grown in the presence of the vitamer (see the text). Biotin was isolated as a complex with avidin and its specific activity was determined. The biotin content of the complex is corrected for the presence of biotin in the purified avidin preparation.

	Biotin–avidin (mμmoles/ml.)	Avidin (mμmole/ml.)	Difference (mμmole/ml.)	Radioactivity (disintegrations/ min./ml.)	10^{-6} × Sp. activity (disintegrations/ min./μmole)
Biotin	1·2	0·5	0·7	2950	4·5
Pimelic acid	—	—	—	—	5·1

pimelic acid with a C_3 amino acid in a reaction similar to that demonstrated for the formation of δ-aminolaevulic acid (Eisenberg, 1963; Shemin, Russell & Abramsky, 1955). This would of necessity require that all intermediates on the pathway to biotin become labelled when the organism is grown in the presence of labelled pimelic acid. The unknown vitamer, desthiobiotin and a number of other vitamers have been shown to become labelled under these conditions (Eisenberg & Maseda, 1966). The fact that the labelled vitamer gives rise to labelled biotin with the same specific activity as the original pimelic acid strongly supports the role of the unknown vitamer as an intermediate in the biogenesis of biotin.

The original hypothesis of Lezius, Ringelman & Lynen (1963), that cysteine is a precursor in the synthesis of biotin, is no longer tenable in light of the more recent evidence. (It is, of course, possible that there is another pathway operating in *Achromobacter*, but the fact that the unknown vitamer and desthiobiotin have now been found in the filtrates of such a large variety of microorganisms is much more suggestive of a common pathway.) If the C_3 unit condensing with pimelic acid were indeed cysteine, then all intermediates would contain sulphur. However, studies with [^{35}S]sulphate indicated the absence of sulphur from the unknown vitamer (Eisenberg, 1966). It has also been shown by Pai & Lichstein (1965*a*,*b*) that biotin has a repressive action on the desthiobiotin synthesis in resting cultures of *Escherichia coli*, and a similar action of biotin has also been observed on both the unknown vitamer and desthiobiotin in growing cultures of *Penicillium chrysogenum* (Eisenberg & Maseda, 1966). This argues for desthiobiotin and the unknown vitamer as intermediates in the biosynthesis of biotin and relegates the incorporation of sulphur to a later stage, as suggested by Tatum (1945).

In a study with resting cells of *Bacillus sphaericus*, Iwahara, Tochikura & Ogata (1965) observed that the addition of alanine, aspartic acid or glutamic acid together with pimelic acid enhanced the total biotin yield over that of pimelic acid alone. Alanine was the most effective of the three amino acids. When pyruvic acid was added with either of the dicarboxylic amino acids the yield increased to a value approaching that of alanine, suggesting the formation of the latter by transamination. This suggests that alanine is the C_3 precursor condensing with pimelic acid and the product of this reaction is 8-amino-7-oxopelargonic acid. However, in a study of the distribution of vitamers in the culture medium of a variety of moulds and bacteria, Ogata *et al.* (1965*a*,*b*) found an unknown vitamer with an electrophoretic mobility pattern identical with that of the unknown in *Penicillium chrysogenum*. A similar pattern was also obtained for a synthetic sample of 7-amino-8-oxopelargonic acid, which they believe to be the unknown vitamer. It is difficult at present to see how this compound could be the postulated condensation product of alanine with pimelic acid. However, since only the electrophoretic pattern was given with no additional information, the final proof of structure must await analysis of the purified material.

Note added in proof. In a recent communication (Iwahara, Kikuchi, Tochikura & Ogata, 1966) the unknown vitamer was shown to have the same R_F in three solvent systems as 8-amino-7-oxopelargonic acid. Both compounds were converted into desthiobiotin in a resting cell suspension of *Bacillus sphaericus*. It was also shown by paper chromatography that the radioactive unknown vitamer gives rise to radioactive desthiobiotin, confirming the results obtained in the present study.

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