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An Early Intermediate in the Biosynthesis of Biotin

INCORPORATION STUDIES WITH [1,7-$^{14}C_2$]PIMELIC ACID

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1. An unknown biotin vitamer was obtained in high yields in culture filtrates of *Penicillium chrysogenum*. 2. Production of this vitamer and desthiobiotin is controlled by the biotin concentration in the medium. 3. The unknown vitamer becomes labelled when the organism is grown in the presence of radioactive pimelic acid. 4. Chromatographic procedures were developed for the purification of the radioactive vitamer. 5. The vitamer is extremely stable in concentrated acid but gives rise to new vitamers under certain conditions. 6. The intermediate role of this vitamer in the synthesis of biotin is discussed.

In a previous study with *Phycomyces blakesleeanus*, an unknown biotin vitamer was found to be excreted into the culture medium together with biotin and desthiobiotin (Eisenberg, 1963). The concentration of this vitamer was too low for isolation and chemical characterization and therefore indirect methods were utilized to obtain information on its chemical structure. Thus with the aid of paper electrophoresis it was possible to demonstrate the presence of both an amino and a carboxyl group with the charged groups separated. In addition, this vitamer did not combine with the protein avidin, indicating the absence of a ureido structure. More recently, the absence of sulphur from this compound was demonstrated by the lack of uptake of [^{35}S]sulphate (Eisenberg, 1966). There were also indications that the unknown vitamer became labelled with ^{14}C when the organism was grown in the presence of [^{14}C]pimelic acid, but the low concentrations of this compound precluded further investigation. This led to the suggestion of an intermediary role for this unknown biotin vitamer in the biosynthesis of biotin (Eisenberg, 1962).

The unknown vitamer has now been obtained in high concentrations from the culture medium of a biotin auxotroph of *Penicillium chrysogenum*. The production of this vitamer, as well as desthiobiotin, is controlled by the biotin concentration initially present in the culture medium. A method was developed for its purification and it is now possible to show the incorporation of radioactive pimelic acid into it. The present evidence further supports the role of this vitamer as a direct intermediate in the pathway of biotin biosynthesis.

Parts of this study were presented at the 49th Annual Meeting of the Federation of American Societies for Experimental Biology in Atlantic City, April 1965.

MATERIALS AND METHODS

Penicillium chrysogenum 62078 was grown in a Czapek–Dox medium to which was added 20mg. of pimelic acid and 0·10μg. of biotin/l. In the isotope experiments, 60μc of radioactive pimelic acid was added/l. of medium in place of the unlabelled pimelic acid. Spores from a 2-week-old culture on Sabouraud's medium were dislodged with the aid of a vibrator and a dilute solution of a wetting agent. The spores were washed twice with water by centrifugation and resuspension. Then 1l. of medium contained in a Fernbach flask was inoculated with approx. 5×10^6 spores and the culture shaken at 26° in a New Brunswick incubator–shaker at 150 oscillations/min. The mycelial mass was removed after 7 days of growth by filtration.

The short and medium columns of the Spinco model 120 amino acid analyser were operated either with or without the ninhydrin reaction system according to the procedure of Spackman, Stein & Moore (1958). Since the concentration of the unknown vitamer was frequently too low for detection with the ninhydrin reaction, samples were collected directly from the column into a fraction collector and assayed for biological activity. To determine the emergence time of the unknown vitamer in relation to the known amino acids, a stream divider was employed, analysing part of the sample with the ninhydrin reaction and part with the biological assay procedures. For large-scale separations (0·5m-mole of alanine equivalent), the preparative column (1·5cm. × 55cm.) was employed with the same buffer systems as for the short and medium columns. The salts were removed by adjusting the solution to pH2·5 and placing the material on a column (1·5cm. × 30cm.) of Dowex 50 (4X; H^+ form; 100–200 mesh).

The column was washed with 0·5N-HCl to remove the salt and the vitamer was eluted with 1·5N-HCl. The solution was evaporated to dryness and final traces of HCl were removed over NaOH. The vitamer could also be separated from salts by extraction with acetone. The solution was concentrated to a small volume *in vacuo*, the pH adjusted to 2·0 and extracted with 3vol. of acetone. The acetone extraction was repeated twice more and the extracts were then evaporated to dryness. The residue was dissolved in a minimum volume of water and the extraction procedure repeated. After four such repeated extractions the salts were almost completely removed and 70–90% of the initial biological activity was recovered.

The yeast assay for 'total' biotin and the *Neurospora crassa* assay were carried out as described by Eisenberg (1963). The disk assay procedure of Genghof, Patridge & Carpenter (1948) was also employed for the biological assay of the contents from the column chromatography.

Ascending paper chromatography was carried out on Whatman no. 1 paper except where indicated. The chromatograms were run either at 0° or room temperature (24°) to a height of 23–27cm. The solvents used were: (1) butan-1-ol–acetic acid–water (12:3:5, by vol.); (2) butan-1-ol–pyridine–water (1:1:1, by vol.); (3) butan-2-one–formic acid–2-methylpropan-2-ol–water (6:3:8:3, by vol.); (4) benzene–acetic acid–water (125:72:3, by vol.); (5) methanol–pyridine–water (20:1:5, by vol.); (6) propan-2-ol–conc. HCl–water (325:83:92, by vol.); (7) butan-1-ol–pyridine–water (80:15:14, by vol.); (8) butan-2-one–2-methylpropan-2-ol–aq. ammonia (sp.gr. 0·88)–water (3:4:1:2, by vol.). Paper electrophoresis and combined paper chromatography and electrophoresis were also carried out as described by Eisenberg (1963). The vitamers were located by bioautography, radioautography and radioscanning. Another method that was frequently employed with chromatograms containing radioactive vitamers was to punch out 6mm. disks with a paper punch along the line of ascent, the holes being made as close to each other as possible without overlapping. Each disk was first assayed for radioactivity with a high-efficiency windowless gas-flow counter and then was examined for biological activity by the disk assay procedure. This gave a more accurate determination of the R_F value and also permitted a direct comparison of the two activities on the same sample. The distribution of the radioactivity by the above procedure was in excellent agreement with the results obtained by radioscanning.

[1,7-$^{14}C_2$]Pimelic acid with a specific activity of 2·0mc/m-mole was purchased from the Nuclear Research Chemical Co., Orlando, Fla., U.S.A. The radioactivity measurements were made with the Packard Tri-Carb liquid-scintillation spectrometer with 0·2ml. of sample material, 3ml. of ethanol and 12ml. of a mixture of 0·03% bis-1,4-(5-phenyloxazol-2-yl)benzene and 0·6% 2,5-diphenyloxazole in toluene.

All chemicals were reagent grade.

RESULTS

Biotin yield and biotin vitamers. The 'total' biotin yield of the culture medium could be readily increased by the addition of pimelic acid and vigorous aeration, resulting in yields as high as 0·7–1·2mg./l. This stimulatory action of pimelic acid on this organism was first reported by Tatum (1945), but the total yields obtained in this early study were very much lower. Since the main difference in the two investigations was the assay organism used, a comparison of the two assay procedures was made. It was found that the *Neurospora* assay gave values that were consistently 10–20% of those obtained with the yeast assay procedure. An analysis of the culture medium by paper chromatography revealed the presence of three vitamers. The fastest-moving component was identified as desthiobiotin by its R_F value in two solvent systems, its electrophoretic mobility and its ability to combine with avidin. By the same criteria, the second component was identical with the unknown biotin vitamer previously found in the culture medium of *Phycomyces blakesleeanus*, and the slowest-moving component was tentatively identified as diaminopelargonic acid. Since the unknown vitamer was originally shown to be unable to support the growth of *Neurospora*, this explains the discrepancy in the two procedures provided that the unknown was present in the large quantities that are apparent on visual examination of the bioautogram. This is more readily observed with combined paper chromatography and electrophoresis, which usually reveals four vitamers with the unknown vitamer possessing the largest growth area. An estimate of the contribution of each vitamer to the total activity would be about 60–80% for the unknown, 10–20% for desthiobiotin and the remainder for the other two unknown vitamers.

During the initial studies on the 'total' biotin yield, it was observed on a number of occasions that a doubling of the biotin concentration of the medium diminished the 'total' biotin yield. The results of a study on the effect of biotin over a wide concentration range are presented in Fig. 1. Though the growth of the organism increased to a maximum with increasing biotin concentrations, the 'total' yield decreased rapidly after 5–10mμg./50ml. A similar picture was observed whether yeast or *N. crassa* was used as the assay organism. It should be noted that the maximum yield in this experiment with *N. crassa* is only one-tenth that obtained with yeast.

Incorporation studies with [1,7-$^{14}C_2$]*pimelic acid.* The filtrates from three 1l. cultures of *Penicillium chrysogenum* grown in the presence of [^{14}C]pimelic acid were combined. The pH was adjusted to 3·0 and the solution was put on to a column (7·5cm. × 20cm.) of Dowex 50 (8X; Na^+ form; 200–400 mesh). The column was first washed with 3l. of water to remove the remaining pimelic acid and also the desthiobiotin, and then with 2l. of 0·4M-sodium acetate buffer, pH 5·5, to remove many of the neutral and acidic amino acids. The column

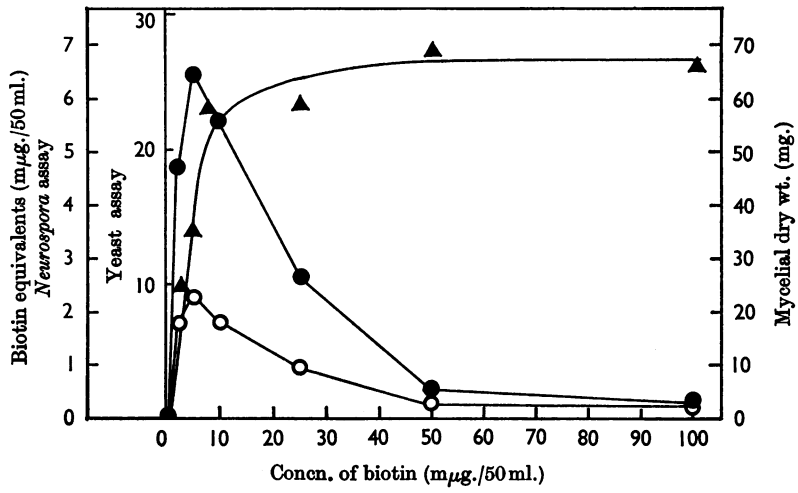

Fig. 1. Effect of biotin concentration on growth and on the vitamer yields in cultures of *Penicillium chrysogenum*. ▲, Mycelial growth; ●, 'total' biotin as measured by the yeast assay; ○, desthiobiotin as measured by the *Neurospora* assay.

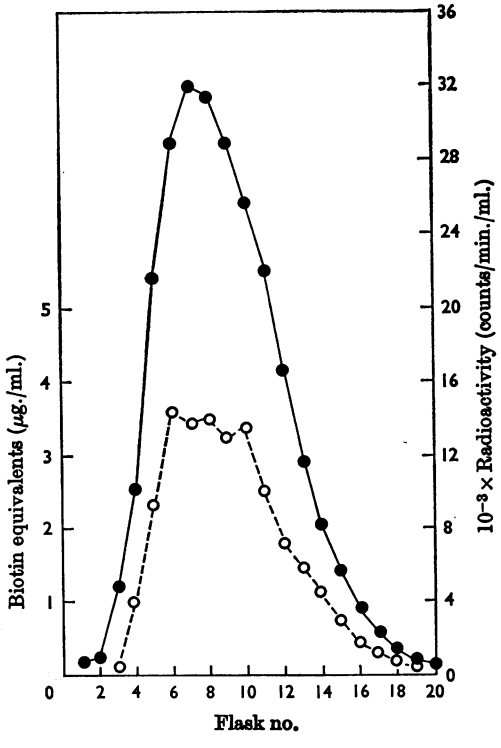

Fig. 2. Column chromatography of culture filtrates of *Penicillium chrysogenum* grown in the presence of [^{14}C]-pimelic acid. The Dowex 50 (Na^+ form) column (7·5 cm. × 20 cm.) was developed with 0·4 M-sodium acetate buffer, pH 6·5. ○, Biological activity; ●, radioactivity.

was then eluted with 0·4 M-sodium acetate buffer, pH 6·5, 100 ml. fractions being collected. The assays for both biological activity and radioactivity indicated that the two activities were closely associated (Fig. 2). Paper chromatography of samples from the peak tubes in butan-1-ol–acetic acid–water disclosed on scanning one major radioactive peak with R_F 0·62–0·64 and a few minor radioactive components. Bioautography also indicated one major product with the same R_F as above, and in addition a minor component appearing as a shoulder with R_F 0·50–0·52. There was also a barely visible compound remaining on the origin. Neither of the last two compounds was observed in the original filtrate.

The contents of flasks containing the biologically active material were combined and the salts removed by the acetone procedure described in the Materials and Methods section. An examination of this material by paper chromatography and electrophoresis at pH 3·0 and 4·0 showed only one component, with the properties of the unknown vitamer. This suggests that there was no apparent alteration of this compound during the desalting procedure. However, paper chromatography followed by electrophoresis of twice the amount of material as used above disclosed two minor vitamers in addition to the major unknown vitamer. All the vitamers were found to be radioactive by radioautography. One of the minor vitamers had the same R_F value as the major unknown vitamer but did not move in the electric field at pH 3·0, suggesting the absence of a positively charged

group. This vitamer had been observed in the original filtrate. The other minor vitamer had an R_F 0·40 and only half the electrophoretic mobility of the major unknown vitamer.

Purification of the unknown vitamer. Early studies with the amino acid analyser indicated that all the common amino acids as well as a large number of unidentifiable ninhydrin-positive compounds were present in the original culture filtrates. Since treatment on Dowex 50 columns only excluded most of the neutral and acidic amino acids, additional information was required about the chromatographic behaviour of the unknown vitamer to enable its separation from the basic amino acids. With the aid of the stream divider it was shown that the unknown vitamer appeared between lysine and histidine at pH 5·28. Fig. 3 shows a typical run under these conditions with the original filtrate and the acetone-extracted material from the [1,7-$^{14}C_2$]pimelic acid experiment. The peak biological activity appeared consistently in tubes 11–12 whereas the histidine peak appeared in tube 13. The coincidence of the two activities in both runs is evident. At pH 4·26, the relative positions of lysine and histidine did not change, but the major unknown vitamer was now observed to move beyond arginine. The arginine peak appeared at 650–680 ml. and was completely out by 720 ml., whereas the unknown vitamer peak appeared at about 760 ml.

These studies showed that the unknown vitamer could be separated from the basic amino acids if the material was first chromatographed at pH 4·26, which removed all amino acids up to and including most of the arginine. If the material recovered from this column was then rechromatographed at pH 5·28, arginine and other compounds with similar properties were left behind. This procedure was adopted for the further purification of the acetone-extracted material except that it was carried out on a preparative scale with a 1·8 cm. × 55 cm. column, and the detergent and reducing agent used with the amino acid analyser were omitted.

In Fig. 4 only the results of the final column chromatography at pH 5·28 are shown. Once again the coincidence of the biological activity and radioactivity is evident.

Some properties of the purified vitamer. The purified desalted material was chromatographed in a number of solvent systems with 10 mμg. of biotin equivalents/strip. The results in Table 1 show that, in addition to the main vitamer, additional vitamers can be resolved in three of the eight systems used. In solvent system (8) there was heavy trailing from the origin to the solvent front, but the three

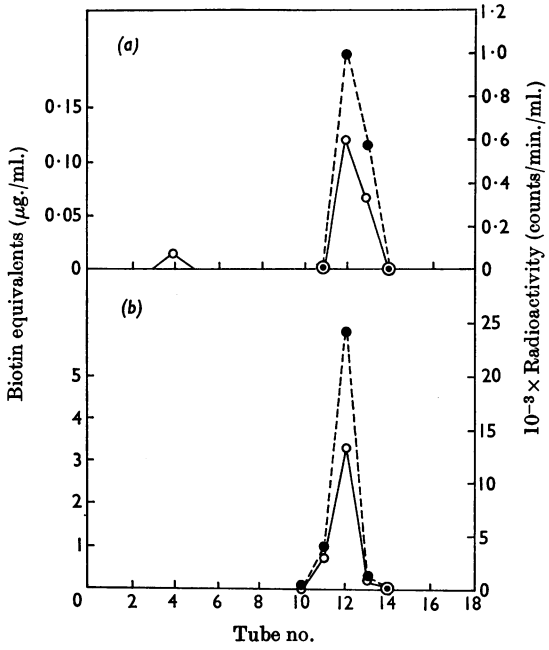

Fig. 3. Column chromatography of the unknown radioactive vitamer. The short column (0·9 cm. × 18 cm.) of the amino acid analyser was developed with 0·35 M-sodium citrate buffer, pH 5·28, at 50°. (*a*) Original filtrate; (*b*) acetone-extracted material from the column chromatography at pH 6·5. ○, Biological activity; ●, radioactivity.

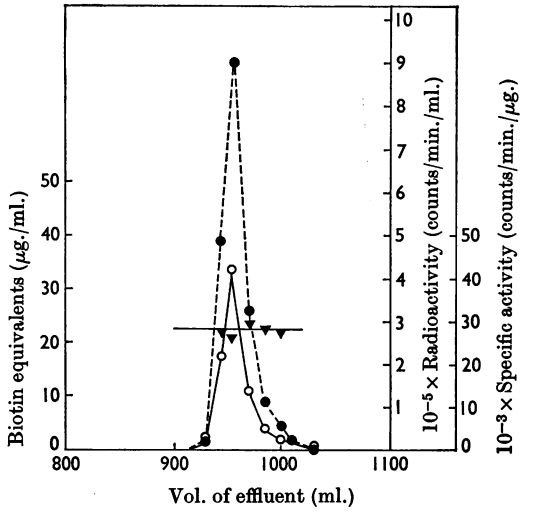

Fig. 4. Purification of the unknown radioactive vitamers by column chromatography. The preparative column (1·8 cm. × 55 cm.) of the amino acid analyser was developed with 0·38 M-sodium citrate buffer, pH 5·28, at 30° and 50°. ○, Biological activity; ●, radioactivity; ▼, specific activity.

Table 1. *Ascending paper chromatography of the purified unknown vitamer in various solvent systems*

Solvents (1) and (2) were run at 0° and the remainder at room temperature (24°).

Solvent system Component.....	R_F 1	2	3
(1) Butan-1-ol–acetic acid–water	0·62	0·51	
(2) Butan-1-ol–pyridine–water	0·43		
(3) Butan-2-one–formic acid–2-methylpropan-2-ol–water	0·70	0·54	
(4) Benzene–acetic acid–water	0·44		
(5) Methanol–pyridine–water	0·80		
(6) Propan-2-ol–conc. HCl–water	0·84		
(7) Butan-1-ol–pyridine–water	0·16		
(8) Butan-2-one–2-methylpropan-2-ol–aq. NH_3 (sp.gr. 0·88)–water	0·76	0·54	0·26

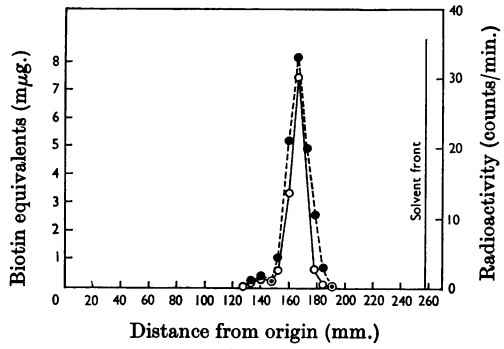

Fig. 5. Ascending paper chromatography of the unknown radioactive vitamer purified by column chromatography. The developing solvent system was butan-1-ol–acetic acid–water. Assays for radioactivity (●) and biological activity (○) were carried out by the punch-out procedure described in the text.

vitamers were clearly visible and components 2 and 3 were present in about the same concentration as component 1. On the other hand, component 2 in solvent systems (1) and (3) appeared to be minor and was not evident when Whatman no. 40, no. 41 or no. 50 paper was substituted for Whatman no. 1 paper. However, there was present some light trailing behind the main component whereas normally there is none on Whatman no. 1 paper.

Combined paper chromatography and electrophoresis at twice the concentration used above revealed the same two vitamers, both having about the same electrophoretic mobility. To ascertain the relative concentration of the minor component in terms of biotin equivalents, 35mμg. of material was chromatographed in solvent system (1) and the paper chromatogram analysed by the punch-out procedure described above. The results of this analysis are shown in Fig. 5 and it was calculated from the area under the curves that the second component constitutes only 5% of either activity.

Stability of the unknown vitamer. We had found previously that the 'total' biotin activity of the culture filtrates changes very little in the range pH 1·0–6·0. However, as the pH was increased beyond 8·0 the 'total' activity fell off very rapidly. Similar losses in activity were also observed with a desalting procedure on Dowex 50 (H^+ form) with 0·2N-ammonia as the eluting agent. With the latter procedure, paper chromatography of a radioactive sample revealed a new radioactive vitamer with R_F 0·87–0·89 but of low biological activity compared with the major unknown vitamer. This observation suggested the formation of the new vitamer from the unknown vitamer. Evidence was also available to suggest that the component with R_F 0·52 was also formed from the unknown vitamer. Thus after the final preparative column chromatography at pH 5·28 a 10mμg. sample of the peak tube showed only the major unknown vitamer in solvent system (1). However, after standing for 2 weeks in the refrigerator the same sample revealed the component with R_F 0·52 with concentrations of material as low as 3–4mμg. It thus appears that the pH may be the determining factor as to which of the two new vitamers is formed.

The greater stability of the unknown vitamer in acid medium was demonstrated when a partially purified preparation in N-sulphuric acid was autoclaved for 1hr. and lost only 50% of the total activity. In addition the purified radioactive preparation when heated for 24hr. in a sealed ampoule at 105° in 6N-hydrochloric acid showed no biologically active or radioactive compound other than the major unknown vitamer.

DISCUSSION

The present study with *Penicillium chrysogenum* has shown that the predominant vitamer in the culture medium is not desthiobiotin but rather an unknown biotin vitamer previously described by Eisenberg (1963). The initial failure to observe this vitamer could be accounted for by the inability of the vitamer to support the growth of *N. crassa*. This organism and *Saccharomyces cereviseae* have been the preferred assay organisms for a broad

spectrum of biotin analogues. Their similarity in response to these analogues suggests that the loci of the genetic blocks are either identical in both or very close together. However, this may also be a reflection of the fact that many of these analogues, which have one intact ring, are not directly on the pathway and what is being observed is a repair phenomenon. The different responses of these two organisms to the unknown vitamer, which is devoid of a ureido and tetrahydrothiophen ring, suggest that it is an early intermediate in the biosynthetic pathway and that the loci for the genetic blocks are different in the two organisms. One cannot exclude, of course, the possibility of a permeability barrier towards the unknown vitamer in *N. crassa*.

The inhibitory action of biotin certainly suggests the operation of a negative-feedback inhibition, which has been observed for intermediates in a variety of synthetic processes. A similar effect of biotin on its own further formation in a wild strain of *Escherichia coli* has also been described by Pai & Lichstein (1965*a*). In further studies Pai & Lichstein (1965*b*,*c*) presented evidence suggesting that the mechanism for this inhibition involved enzyme repression rather than end-product inhibition. The fact that the unknown vitamer and desthiobiotin are inhibited to the same degree by biotin also suggests the possibility of a co-ordinate type of repression. One difference that has been noted in the behaviour of these two organisms is the requirement for pimelic acid in order to observe the inhibition in *Penicillium chrysogenum*. This acid has no effect on the biotin yield in *E. coli* (Pai & Lichstein, 1965*a*). The above observation may account for the failure to observe the biotin effect in the early studies with *Penicillium chrysogenum* (Tatum, 1945).

The precursor relationship of pimelic acid to biotin has been previously demonstrated with the aid of isotopically labelled pimelic acid (Eisenberg, 1962; Elford & Wright, 1963). It has also been postulated that pimelic acid may condense with a C_3 unit as the first step in the reaction sequence (Eisenberg, 1963; Lezius, Ringelman & Lynen, 1963) and therefore all intermediates after this condensation with labelled pimelic acid would also become labelled. With the aid of the amino acid analyser it has been possible to design a purification procedure freeing the unknown vitamer from most of the amino acids present. During the purification of the unknown vitamer it has been possible to show the coincidence of the biological activity and the radioactivity as well as a constant specific activity in terms of biotin equivalents. The actual specific activity of the unknown vitamer cannot be calculated without knowledge of its molecular weight and its relative biological activity. These data further support the intermediary role of the unknown vitamer in the biogenesis of biotin.

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