STUDIES ON THE BIOSYNTHESIS OF BIOTIN

by

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STUDIES ON THE BIOSYNTHESIS OF BIOTIN

SECTION I

INTRODUCTION

The existence of an unidentified organic substance which stimulated yeast growth was originally noted by Wildiers in 1901, and termed "bios." This was later shown to be multiple in nature, being separated into two components, bios I and II, by Lucas in 1924. Bios II was likewise a mixture containing a charcoal adsorbable fraction, designated bios IIb. In 1935 Kögl isolated bios IIb in pure form. This compound he called "biotin."

In 1942 du Vigneaud, Hofmann, and Melville (4) proved the structure of biotin from the results of a series of degradation reactions. Their assigned structure was confirmed by Harris, Wolf, Mozingo, and Folkers (6) by a total synthesis and comparison of properties with the natural product.

Many strains of bacteria, yeast and fungi require biotin for optimum growth. In fact, of the microorganisms tested, those which do not require an exogenous supply of the vitamin possess the ability to synthesize it (8,9). It would seem, therefore, that a biotin

requirement from an exogenous source is due to a lack of synthetic ability and that all microorganisms require biotin for normal metabolism. For this reason the biosynthesis of biotin becomes of interest and importance. This biosynthetic property of many microorganisms makes an elucidation of the process necessary as an aid in the development of our knowledge of microbial metabolism. Nevertheless, very few publications have appeared on this phase of biotin research and at the present time it is impossible to draw anything approaching a complete picture of the biosynthetic reactions involved in the formation of biotin by microorganisms.

The first indication of a biotin precursor appeared when Dittmer, Melville, and du Vigneaud (2) showed desthiobiotin, a degradation product of biotin, to have an additive effect with biotin and also to be active alone when tested on a strain of Saccharomyces cerevisiae. It was then found that desthiobiotin is converted to biotin by the yeast cells. This same problem was answered in a different manner by Rogers and Shive (11) in their investigation of the biosynthesis of biotin. In studying the effects of 2-oxo-4-imidazolidine caproic acid, a competitive inhibitor of desthiobiotin, on Escherichia coli, it was concluded that the biosynthesis of biotin proceeds through desthiobiotin and that there

is apparently no alternative system readily available to the organism.

A further degradation product of biotin, namely, 7,8-diaminopelargonic acid has a yeast growth-stimulating activity of approximately ten per cent of the activity of biotin (1). The yeast tested probably converts the compound to biotin within the cell, but it is not known whether this is a normal intermediate.

Pimelic acid is the only other compound besides desthiobiotin which has an accumulation of evidence substantiating its position in the series of reactions leading to the formation of biotin by microorganisms. Before its relation to biotin had been conceived, pimelic acid was found by Mueller (1937) to be a growth stimulant for Corynebacterium diphtheriae. Five years later du Vigneaud, Dittmer, Hague, and Long (3) demonstrated the replacement of pimelic acid with biotin for this same organism. Further proof of pimelic acid as a biotin precursor was given by Eakin and Eakin (5) by finding an increase in the quantity of biotin or biotin-active substance produced by Aspergillus niger in the presence of pimelic acid.

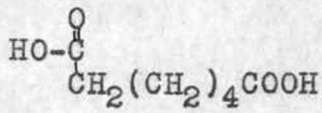
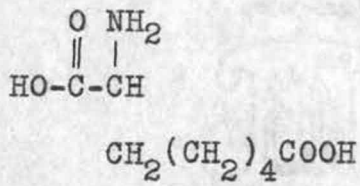
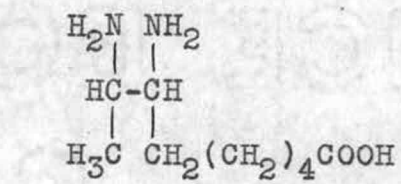
The production of a substance with the specific biological activity of desthiobiotin was demonstrated by Tatum (13) using an X-ray produced mutant strain,

Penicillium chrysogenum 62078, which cannot grow on desthiobiotin. The addition of pimelic acid to the medium increased the synthesis of the desthiobiotin-active substance ten-fold. This observation suggests that desthiobiotin, and possibly pimelic acid, are normal intermediates in the biosynthesis of biotin.

In the search for another precursor of biotin lying between pimelic acid and desthiobiotin it was observed that DL-α-aminosuberic acid has biotin activity for a strain of Saccharomyces cerevisiae (Table I). The majority of the work in this investigation involves methods of showing that α-aminosuberic acid is actually converted to biotin and that the observed stimulation of growth is not due to some other cause.

TABLE I

BIOTIN ACTIVE COMPOUNDS

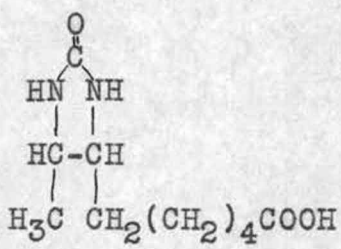
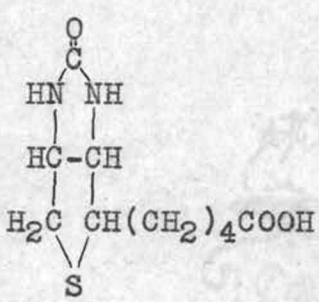
Compound	Per cent Biotin Activity for *S. cerevisiae*
 Pimelic Acid	0
 DL-α-Aminosuberic Acid	0.0001
 7,8-Diaminopelargonic Acid	10 "Natural"*

(Continued)

*Refers to a degradation product of naturally occurring biotin.

TABLE I (Continued)

BIOTIN ACTIVE COMPOUNDS

Compound	Per cent Biotin Activity for *S. cerevisiae*
 Desthiobiotin	100 "Natural" 30-35 synthetic
 Biotin	100

SECTION II

EXPERIMENTAL

Synthesis. – The α-aminosuberic acid used in these experiments was synthesized according to a method of Wood and du Vigneaud (16) in which it is an intermediate in the preparation of DL-desthiobiotin.

Saccharomyces cerevisiae FB. – One of the strains of yeast used for these tests was isolated from a cake of Fleischmann yeast, and the stock culture carried on molasses-agar slants (3.3% molasses, 0.12% $(NH_4)_2HPO_4$, 2% agar). Inocula for tests were obtained by transfer to malt-agar slants (5% Difco Malt Extract and 0.15% agar) and incubation at 30° C. for 16 to 20 hours. Changing the type of slants for the inocula resulted in lower blanks and improved biotin response curves.

Basal Medium. – The basal medium was that formulated by Snell, Eakin, and Williams (12) in their biotin assay with yeast. The pH was adjusted to 4.8-5.0.

Procedure. – Tests were carried out in six inch bacteriological test tubes supported in a wire rack.

Supplements were added to each tube and the volumes adjusted to five ml. with distilled water. Five ml. of double strength basal medium was then introduced. The tubes were covered with a clean towel and sterilized by autoclaving at fifteen pounds pressure for fifteen minutes.

The inoculum was prepared by removing carefully a loop of cells from a malt-agar slant and suspending them in ten ml. of sterile physiological saline. The cells were centrifuged, washed twice, and resuspended in saline; then diluted to an optical density of 0.220. Each tube was inoculated with one drop of the final cell suspension, which is equivalent to 0.02 mg. of wet yeast cells per tube.

The tests were incubated at 30° C. for eighteen hours, after which time the tubes were cooled for fifteen minutes to slow the growth, shaken thoroughly and measured for amount of growth turbidimetrically.

For the yeast assay, the test solutions consisted of the properly diluted samples at 1, 2, 3, and 4 ml. levels, and the standard curve which ranged from 0.000025γ to 0.00050γ of biotin per tube. The growth period was sixteen hours at 30° C.

Saccharomyces cerevisiae Y567. — Also used in testing was a strain of brewer's yeast, NRRL Y567, which was found unable to utilize desthiobiotin. The stock culture and inoculum for this yeast were carried on malt-agar slants as mentioned above.

Basal Medium. — This yeast responded well to biotin on the medium previously described and therefore the same basal medium was used.

Procedure. — Tests were performed by the same procedure as outlined above.

Lactobacillus arabinosus. — Lactobacillus arabinosus 17-5 was cultured as described in the biotin assay procedure of Wright and Skeggs (17).

Medium. — The basal medium was the same as that used for biotin assay with L. arabinosus 17-5.

Procedure. — For both the testing of compounds and assaying the assay procedure of Wright and Skeggs was used. Growth was measured turbidimetrically after about eighteen hours incubation.

Aspergillus niger. — Aspergillus niger NRRL 3 was carried on malt-agar slants of the same type as employed for yeast. Inocula were taken from cultures

incubated for 72 hours at 30° C.

Medium. — The basal medium for the yeast assay and tests described above was used.

Procedure. — Tests were performed in 50 ml. Erlenmeyer flasks. Supplements, in two ml. of water, plus ten ml. of medium were introduced. The flasks were plugged with cotton and sterilized by autoclaving.

The inoculum was prepared by adding five ml. of sterile distilled water to a 72 hour sporulated culture and gently stirring above the mycelium to obtain a uniform suspension of spores. A small quantity was withdrawn and diluted 1:5 in sterile water. Each flask was seeded with one drop of the diluted spore suspension.

After 72 hours incubation at 30° C. the cultures were autoclaved at fifteen pounds pressure for fifteen minutes, filtered, and adjusted to pH 6.8-7.0. These media were assayed with yeast and L. arabinosus.

Results. — The yeast-growth-promoting activity of DL - α -aminosuberic acid was found to be 0.0001% of the activity of biotin for FB yeast. The effect was linear over a range of 10 γ to 1000 γ per culture, and was additive in the presence of biotin (Table II). The test substance showed no activity for yeast 567 or L. arabinosus, neither of which can utilize desthiobiotin.

Of the dibasic acids adipic, pimelic, suberic, azelaic and sebacic, only suberic acid had biotin growth-promoting activity for FB yeast (Table II). These acids were all totally inactive for yeast 567 and L. arabinosus. If it is assumed that only the L- α - aminosuberic acid was active, then suberic acid was observed to have about 40% of the activity of its amino derivative.

When tested together with 2-oxo-4-imidazolidine caproic acid, which prevents conversion of desthio-biotin to biotin, α -aminosuberic acid was able to over-come the inhibitory effects to about the same degree as its desthiobiotin activity would indicate, biotin and desthiobiotin being equally active (Table III).

Homobiotin, which inbibits the functioning of biotin, was found to behave towards α -aminosuberic acid in a manner similar to an equivalent amount of

TABLE II

BIOTIN ACTIVITY OF SUBERIC ACID
AND DL- α -AMINOSUBERIC ACID

Micrograms Test Substance per Tube	MICROGRAMS BIOTIN						Micrograms Biotin per tube	Optical Density
	none		0.000025		0.000050			
	Suberic Acid	Amino-suberic Acid	Suberic Acid	Amino-suberic Acid	Suberic Acid	Amino-suberic Acid		
0	.095	.095	.160	.160	.220	.220	0	.095
10	.125	.130	.180	.175	.230	.240	0.000025	.160
30	.170	.200	.245	.260	.290	.280	0.000050	.220
100	.280	.320	.310	.340	.320	.340	0.000075	.270
300	.340	.370	.355	.380	.365	.385	0.0001	.310
1000	.375	.380	.385	.400	.380	.400	0.0002	.335
							0.0005	.380
							0.001	.415

Organism: FB yeast Incubation: 18 hours at 30° C.

Experimentally determined values represent optical density (2-log per cent transmission). Distilled water reads zero, and a reading of 2.0 represents complete opacity.

TABLE III

EFFECTS OF
2-OXO-4-IMIDAZOLIDINE CAPROIC ACID

Micrograms Inhibitor per Tube	Micrograms Desthiobiotin		Micrograms Aminosuberic Acid		
	0.000050	0.000150	30	100	300
0	.290	.315	.115	.165	.270
.3	.185	.240	.100	.125	.160
1.	.125	.180	.095	.090	.120
3.	.090	.110	.085	.085	.085
10.	.080	.085	.080	.080	.085
30.	.070	.070	.070	.070	.085
100.	.070	.060	.065	.055	.070

Blank: .055

Organism: FB yeast

Incubation: 18 hours at 30° C.

Experimental values represent optical density.

biotin (Table IV). However, in the case of this and the desthiobiotin inhibiton the results do not appear to be strictly competitive as is the case with both biotin and desthiobiotin.

The data in Table V record the influence of pimelic acid upon biotin synthesis by A. niger. Whereas this organism normally produces about 0.015γ of biotin per twelve ml. culture, the addition of one mg. of pimelic acid results in a fifteen-fold increase in the production of biotin (0.20γ), according to the yeast assay. However, the increase per twelve ml. culture produced by an equal quantity of α-aminosuberic acid is only about ten-fold.

With a suboptimal amount of pimelic acid (10γ per culture), the excess biotin production was still approximately ten-fold, whereas an equimolar amount of L- α -aminosuberic acid yielded only a two-fold increase in biotin production.

According to assays with L. arabinosus, the extra biotin found with excess pimelic and α-aminosuberic acids is approximately 0.01γ per culture. This represents only a two-fold increase; and about ten per cent of the amount recorded by the yeast assay.

TABLE IV

EFFECTS OF HOMOBIOTIN

Micrograms Inhibitor per Tube	Micrograms Biotin			Micrograms Aminosuberic Acid			
	0.000050	0.000150	0.001 Biotin	30	100	300	1000
0	.155	.310	.335	.115	.165	.270	.315
0.01	---	---	---	.080	.120	.240	---
0.03	.100	.200	---	.060	.105	.215	---
0.1	.080	.165	---	.045	.070	.195	---
0.3	.065	.150	---	.040	.045	.210	---
1.	.065	.130	.320	.035	.045	.200	.300
3.	.050	.100	.300	.035	.045	.190	.260
10.	.045	.060	.280	.035	.045	.185	.250
30.	.045	.040	.165	---	---	.160	.245
100.	---	---	.075	---	---	.140	.235

Blank: .055

Organism: FB yeast

Incubation: 18 hours at 30° C.

Experimental values represent optical density.

TABLE V

BIOTIN SYNTHESIZED
BY *ASPERGILLUS NIGER*

Addenda	Biotin per 12 ml. Culture: Yeast Assay micrograms	Biotin per 12 ml. Culture: *L. arab.* Assay micrograms
None	0.013	0.009
None	0.016	0.008
10γ Pimelic Acid	0.160	---
10γ Pimelic Acid	0.129	---
1 mg. Pimelic Acid	0.199	0.021
1 mg. Pimelic Acid	0.205	0.019
25γ DL-α-Aminosuberic Acid	0.032	---
25γ DL-α-Aminosuberic Acid	0.027	---
1 mg. DL-α-Aminosuberic Acid	0.145	0.021
1 mg. DL-α-Aminosuberic Acid	0.139	0.020

Organism: *Aspergillus niger* NRRL 3

Incubation: 72 hours at 30° C.

DISCUSSION

The growth-promoting activity of α-aminosuberic acid upon FB yeast would usually be considered to be due to its conversion to biotin, or desthiobiotin when tested as described. However, several substances which are structurally unrelated to biotin have been shown under certain conditions to give rise to biotin activity or to otherwise exert a sparing action upon the vitamin. These substances are oleic acid (L. casei) (14), aspartic acid (Torula cremoris) (7), or a combination of the two (L. arabinosus) (10), as well as several surface-active agents for lactic acid bacteria (15). It therefore seemed desirable to test aminosuberic acid by its effect upon biotin production in other organisms and its ability to counteract biotin and desthiobiotin inhibitors.

α-Aminosuberic acid is not strictly competitive in its counteraction of 2-oxo-4-imidazolidine caproic acid inhibition. This may not be unexpected in view of the probability that this amino acid passes through several intermediates before desthiobiotin is formed, so that its effect upon the inhibitor is necessarily remote. Since a qualitative competition exists between aminosuberic acid and the inhibitor, the conclusion that the former compound is converted to desthiobiotin appears justified. Similarly, homobiotin, which inhibits biotin

utilization, also inhibits low levels of aminosuberic acid.

The failure of homobiotin to significantly affect growth at high levels of aminosuberic acid (in contrast to the imidazolidone which inhibits desthiobiotin utilization) is surprising. It appears from this observation that desthiobiotin (or aminosuberic acid) may be used directly by the yeast in metabolism without first being converted to biotin, for if this conversion took place homobiotin should be inhibitory in any case.

Further evidence that desthiobiotin appears to be the principal end-product formed from aminosuberic acid, comes from the observation that latter compound is inactive for the growth of L. arabinosus or yeast 567, both of which require preformed biotin for growth. Moreover, the comparative assays on A. niger cultures with L. arabinosus and FB yeast (which responds to desthiobiotin) indicate that the bulk of the activity is due to the desthio form.

The activity of suberic acid may be due to its being aminated; or suberic acid and the amino derivative may both be changed to some closely related compound. The former hypothesis seems more likely since a comparison of structural formulae show α-aminosuberic acid and biotin to have two possible points of enzyme

attachment in common, namely, the amine group and the ω-carboxyl group. In either case, the observation that only these eight-carbon acids possess activity indicates that an eight-carbon compound is an intermediate between pimelic acid and desthiobiotin.

If α-aminosuberic acid is a normal intermediate in (desthio) biotin synthesis by microorganisms one would expect to find more of these substances in a medium in which A. niger was cultured in the presence of the amino acid rather than in the presence of pimelic acid. However, results to the contrary were obtained. This was especially pronounced when suboptimal amounts of the substances were tested. These contrasting results would seem to provide evidence for differentroutes of biotin synthesis in A. niger and in FB yeast. In A. niger, the superior activity of pimelic, and suberic (5) acids over aminosuberic acid suggests that the latter compound may be degraded back to an unsubstituted acid before being utilized. In yeast, pimelic acid is inactive, and suberic acid, although active, is surpassed by aminosuberic acid, as pointed out above.

The inactivity of both suberic acid and its amino analogue for L. arabinosus suggests that their activity

for yeast is not due to a general surface-active phenomenon, or to a sparing action such as that provided by aspartic acid.

SUMMARY

1. DL-α-aminosuberic acid has been found to have biotin growth-promoting activity, for a strain of S. cerevisiae, equivalent to 0.0001% of biotin activity.

2. Of all dibasic acids tested, suberic acid alone had biotin activity for the same strain of yeast.

3. The relative activities of pimelic and the suberic acids for FB yeast and A. niger suggest different routes of biotin synthesis in the two organisms.

4. α-Aminosuberic acid behaves in a manner comparable to its desthiobiotin or biotin equivalent when tested in the presence of their respective inhibitory analogues, except that at high levels aminosuberic acid is only slightly affected by homobiotin.

5. Evidence indicates that desthiobiotin can be utilized by FB yeast per se or in some manner in which the inhibitory effect of homobiotin is greatly reduced.

6. From comparative assays of A. niger culture medium it is concluded that desthiobiotin is the principal product arising from pimelic and aminosuberic acids.

7. It is proposed that an eight-carbon acid is an intermediate between pimelic acid and desthiobiotin for FB yeast.

SECTION V

BIBLIOGRAPHY

1. Dittmer, K. and du Vigneaud, V., Science 100 129-131 (1944).

2. Dittmer, K., Melville, D. B. and du Vigneaud, V., Science 99 203-205 (1944).

3. du Vigneaud, V., Dittmer, K., Hague, E. and Long, B., Science 96 186-187 (1942).

4. du Vigneaud, V., Hofman, K. and Melville, D. B., J. Am. Chem. Soc. 64 188-189 (1942).

5. Eakin, R. E. and Eakin, E. A., Science 96 187-188 (1942).

6. Harris, S. A., Wolf, D. E., Mozingo, R. and Folkers, K., Science 97 447-448 (1943).

7. Koser, S. A., Wright, M. H. and Dorfman, A., Proc. Soc. Exptl. Biol. Med. 51 204-205 (1942).

8. Landy, M. and Dicken, D. M., Proc. Soc. Exptl. Biol. Med. 46 449-452 (1941).

9. Landy, M., Dicken, D. M., Bicking, M. M. and Mitchell, W. R., Proc. Soc. Exptl. Biol. Med. 49 441-444 (1942).

10. Potter, R. L. and Elvehjem, C. A., J. Biol. Chem. 172 531-537 (1948).

11. Rogers, L. L. and Shive, W., J. Biol. Chem. 149 57-61 (1947).

12. Snell, E. E., Eakin, R. E. and Williams, R. J., J. Am. Chem. Soc. 62 175-178 (1940).

13. Tatum, E. L., J. Biol. Chem. 160 455-459 (1945).

14. Williams, V. R. and Fieger, E. A., Ind. Eng. Chem., Anal. Ed. 17 127-130 (1945).

15. Williams, V. R. and Williams, H. B., J. Biol. Chem. 177 745-750 (1949).

16. Wood, J. L. and du Vigneaud, V., J. Am. Chem. Soc. 67 210-212 (1945).

17. Wright, L. D. and Skeggs, H. R., Proc. Soc. Exptl. Biol. Med. 56 95-98 (1944).