Studies on Biosynthesis of Biotin by Microorganisms

Part I. Accumulation of Biotin-Vitamins by Various Microorganisms

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Received May 10, 1965

The accumulation of biotin-vitamins in the culture media of a large number of microorganisms (about 700 strains) was studied. The contents of the biotin-vitamins were quantitatively determined by microbiological assays with *Lactobacillus arabinosus* and *Saccharomyces cerevisiae*.

It was found that large amounts of biotin-vitamins were accumulated by various microorganisms such as *Streptomyces*, molds and bacteria, and that the yield of biotin-vitamins was enhanced by the addition of pimelic acid or azelaic acid to the media. It was also found that the main portion of the vitamins accumulated by many microorganisms did not support the growth of *Lactobacillus arabinosus*, while it did support that of *Saccharomyces cerevisiae*. The small amounts of true biotin were observed in the culture media of various *Streptomyces* and molds, but hardly in the culture media of bacteria.

INTRODUCTION

Biotin-vitamins were synthesized by various microorganisms,^{1,2)} but the yields in the culture media were rather low as compared with other most vitamins.

Schopfer³⁾ demonstrated, in his extensive study of biosynthesis of biotin by a mold, *Phycomyces blakesleeanus*, that over 90 per cent of the total biotin produced by the mold appeared in the culture medium. Recently, Eisenberg,^{4,5)} using the same mold, demonstrated that remarkable amounts of biotin-vitamins were accumulated in the culture medium, and that its yield was enhanced about 10- to 20-fold by the addition of pimelic acid and by the aeration of the culture. He

also showed the occurrence of an unknown biotin-vitamin which might be an intermediate of biotin biosynthesis. In a series of studies with other microorganisms, it has been demonstrated that the yield of biotin-vitamins was enhanced by the addition of pimelic acid to the medium.^{6~9)}

From the results of these studies above mentioned and of other observations with various microorganisms,^{10~14)} it has been postulated that pimelic acid is an intermediate in biosynthesis of biotin by microorganisms. Recent studies with radioactive pimelic acid suggested a di-

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rect participation of pimelic acid in biotin biosynthesis.¹⁵⁻¹⁷⁾ In addition, Lynen et al. recently proposed a hypothesis that biotin was synthesized from cysteine, pimelyl-CoA and carbamyl phosphate.¹⁸⁾

This report is a part of the study on the biosynthesis of biotin by microorganisms. The purpose of studies in this series is to clarify the mechanisms through which biotin is synthesized by microorganisms. Since low biotin yield imposes certain limitation on a study of the purpose, an attempt has been made to isolate the microorganisms which accumulate much amounts of biotin-vitamins in the culture medium. The contents of biotin-vitamins in the culture medium of a large number of microorganisms were initially examined, then the effect of pimelic acid and some organic acids on their accumulation was investigated.

MATERIALS AND METHODS

Microorganisms. Most of the strains of molds, bacteria and *Streptomyces* used in this investigation were isolated from soil and other natural materials. Some strains used were preserved in our Laboratory.

Media. a) For molds; glucose 50 g, casamino acid 5 g, peptone 5 g, KH_2PO_4 1 g, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.5 g, MnSO_4 10 mg, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ 10 mg, pimelic acid 0.5 g, in 1000 ml of tap water. Final pH was adjusted to 6.8. b) For bacteria; peptone 10 g, casamino acid 5 g, glycerin 20 g, K_2HPO_4 1 g, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.5 g, KCl 0.5 g, MnSO_4 10 mg, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ 10 mg, NaHCO_3 1 g, $\text{V} \cdot \text{B}_1$ 100 μg , $\text{V} \cdot \text{B}_2$ 200 μg , $\text{V} \cdot \text{B}_6$ 400 μg , nicotinic acid 200 μg , *p*-aminobenzoic acid 200 μg , *Ca*-pantothenate 200 μg , pimelic acid 0.5 g, in 1000 ml of tap water. Final pH was adjusted to 7.2. c) For *Streptomyces*; soluble starch 20 g, peptone 20 g, casamino acid 5 g, NaCl 1 g, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.5 g, K_2HPO_4 0.5 g, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ 10 mg, MnSO_4 10 mg, pimelic acid 0.5 g, in 1000 ml of tap water. Final pH was adjusted to 7.0.

Culturing Conditions. a) Bacteria; the bacteria

were cultivated in the medium indicated above. The medium (4.0 ml) was taken in the test tubes (16 mm $\times$ 180 mm). The test tubes were incubated for 3 days at 28°C with continuous shaking (240 reciprocation per minute). The cultures, after the growth, were centrifuged to remove the cells. The contents of biotin-vitamins in the supernatants were determined. b) Molds; the molds were cultivated in the medium described above. The medium (4.0 ml) was taken in the test tubes (16 mm $\times$ 180 mm). The test tubes were incubated for 5 days at 28°C with shaking (240 reciprocation per minute). After the growth, the cultures were centrifuged to remove the cells. The contents of biotin-vitamins in the supernatants were determined. c) *Streptomyces*; the *Streptomyces* were cultivated in the medium described above. Five ml of the medium was taken in test tubes (25 mm $\times$ 200 mm). The test tubes were incubated for 5 days at 28°C with shaking (340 reciprocation per minute). The cultures, after the growth, were centrifuged to remove the cells. The contents of biotin-vitamins in the supernatants were determined.

Microbiological Assays of Biotin-Vitamins. "Total biotin" was quantitatively determined by microbiological assay with *Saccharomyces cerevisiae* according to the method of Snell et al.¹⁹⁾ The term "total biotin" used in this paper encompasses the entire spectrum of biotin-vitamins utilized by *Saccharomyces cerevisiae*. This includes biotin, desthiobiotin, diaminobiotin, diaminopelargonic acid, biocytin, and other vitamins with activity for the growth of *Saccharomyces cerevisiae*. The term "true biotin" represents the vitamins capable of supporting the growth of *Lactobacillus arabinosus*, and includes biotin, biotin-*d*-sulfoxide, diaminobiotin and other vitamins with activity for the growth of *Lactobacillus arabinosus*. The microbiological assay of true biotin was carried out by the general assay procedure of Wright and Skeggs,²⁰⁾ except that the biotin content was determined by turbidity measurements.

RESULTS

I. Accumulation of Biotin-Vitamins in the Culture Medium of Various Microorganisms.

In order to search the microorganisms which accumulate large amounts of biotin-vitamins in the culture medium, the contents of biotin-

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TABLE I. THE ACCUMULATION OF BIOTIN-VITAMERS BY VARIOUS MOLDS

Molds tested	Biotin-vitamins accumulated (μg per ml)			
	True biotin		Total biotin	
	a)*	b)*	a)*	b)*
<i>Mucor javanicus</i>	0.04	0.05	0.25	4.50
<i>Mucor circinelloides</i>	0.10	0.14	0.40	5.00
<i>Mucor racemosus</i>	0.10	0.14	0.40	2.50
<i>Rhizopus oryzae</i>	0.16	0.26	0.19	1.50
<i>Rhizopus tonkinensis</i>	0.04	0.11	0.15	0.40
<i>Rhizopus nigricans</i>	0.07	0.28	0.13	1.00
<i>Rhizopus delemere</i>	0.18	0.30	0.19	0.64
<i>Penicillium chrysogenum</i> THOM	0.25	0.40	0.35	0.80
<i>Monascus purpureus</i>	0.02	0.03	0.25	0.60
<i>Aspergillus niger</i>	0.04	0.05	0.10	0.38
<i>Aspergillus nidulans</i>	0.04	0.04	0.25	1.00
<i>Aspergillus cellulosae</i>	0.03	0.04	0.30	0.64
1-49-S-3**	0.12	0.13	1.00	1.00
1-39**	0.25	0.26	0.26	0.27
1-33-S-5**	0.05	0.26	0.48	1.00
1-48-S-3**	0.19	0.22	1.50	1.50
1-38-S-2**	0.24	0.24	0.68	1.80
1-56**	0.09	0.31	0.15	1.30

* a): Pimelic acid was not added.

b): Pimelic acid (500 μg per ml) was added to the medium.

** Unidentified molds isolated from soil.

TABLE II. THE ACCUMULATION OF BIOTIN-VITAMERS BY *Streptomyces*

<i>Streptomyces</i> tested	Biotin-vitamins accumulated (μg per ml)			
	True biotin		Total biotin	
	a)*	b)*	a)*	b)*
<i>Streptomyces</i> sp. isolated from soil)				
A-1001-3	0.04	0.14	0.05	1.00
A-1014-2	0.06	0.18	0.13	1.50
A-1026-1	0.04	0.11	0.05	1.00
A-1031-7	0.03	0.07	0.03	0.50
A-1030-3	0.04	0.15	0.06	0.45
741-2	0.03	0.04	0.05	0.70

* a): Pimelic acid was not added.

b): Pimelic acid (500 μg per ml) was added to the medium.

TABLE III. THE ACCUMULATION OF BIOTIN-VITAMERS BY VARIOUS BACTERIA

Bacteria tested	Biotin-vitamins accumulated (μg per ml)			
	True biotin		Total biotin	
	a)*	b)*	a)*	b)*
<i>Escherichia coli</i> CROOKS	trace	trace	trace	0.07
<i>Aerobacter cloacae</i>	"	"	"	0.05
<i>Alcaligenes faecalis</i>	"	"	"	0.40
<i>Agrobacterium tumefaciens</i>	"	"	"	0.18
<i>Bacillus megaterium</i>	"	"	"	1.00
<i>Bacillus sphaericus</i>	"	"	0.07	20.00 ~200.0
<i>Corynebacterium sepedonicum</i>	"	"	trace	1.00
<i>Sarcina lutea</i>	"	"	"	0.40
<i>Pseudomonas fluorescens</i>	"	"	"	2.00
<i>Pseudomonas aeruginosa</i>	"	"	"	2.00
<i>Bacillus</i> sp.; (isolated from soil)				
BC-80	"	"	"	3.15
BC-86	"	"	"	2.00
BC-44	"	"	"	2.00
BC-51	"	"	"	1.00
K-524	"	"	"	3.00
K-597-1-13	"	"	0.17	2.95
K-681-5-2	"	"	0.08	3.60
K-681	"	"	0.05	2.00
K-681-UV-134**	"	"	0.04	20.00 ~50.0

* a): Pimelic acid was not added.

b): Pimelic acid (500 μg per ml) was added to the medium.

** Biotin-requiring mutant obtained by ultraviolet light irradiation.

vitamins in the culture media of a large number of microorganisms (about 700 strains) such as *Streptomyces*, molds and bacteria were tested. The typical strains, which accumulated biotin-vitamins in the culture medium, are listed in Tables I, II and III.

a) Accumulation of Biotin-Vitamins by Molds.

The contents of biotin-vitamins in the culture medium of a large number of strains of molds (about 250 strains) belonging to *Mucor*, *Rhizopus*, *Aspergillus*, *Penicillium*, *Fusarium* and other fungi were tested with addition of pimelic acid. From the results, it was found that a large number of strains of molds

accumulated biotin-vitamins in the culture medium. However, the amounts and biological activity of the vitamins were variable from strain to strain. Among 83 strains of *Mucor* and *Rhizopus* tested, 75 strains (about 90 per cent) accumulated true biotin in amounts of 0.01 to 0.4 μg per ml of medium, and, among 130 strains of other fungi (*Aspergillus*, *Penicillium*, *Fusarium* and unidentified molds), 40 strains accumulated true biotin in amounts of 0.01 to 0.4 μg per ml of medium. On the other hand, 33 strains, among 83 strains of *Mucor* and *Rhizopus*, and 30 strains, among 130 strains of other fungi (*Penicillium*, *Aspergillus*, *Fusarium* and unidentified molds), accumulated biotin-vitamins, except true biotin, in amounts of 1.0 to 5.0 μg per ml of medium. As shown in Table I, it was found that the yields of true biotin were much smaller than those of total biotin.

b) Accumulation of Biotin-Vitamins by *Streptomyces*

Among 100 strains of *Streptomyces*, 90 strains accumulated biotin-vitamins in their culture media. The yields of biotin-vitamins were variable from strain to strain from 0.1 to 1.5 μg per ml of medium, and the yields of true biotin varied from 0.01 to 0.20 μg per ml of medium. As shown in the Table II, in the case of *Streptomyces*, the yields of true biotin were very low as same as molds. In general, the yields of total biotin were smaller than that of molds and bacteria.

c) Accumulation of Biotin-Vitamins by Bacteria.

In contrast with molds and *Streptomyces*, in the case of bacteria, there were few strains which accumulated true biotin in the culture medium and no strain accumulated true biotin in amounts that were observed by fungi and by *Streptomyces*. However, many strains (about 15 per cent of the 400 strains tested) accumulated biotin-vitamins, except true biotin, in amounts of 1.0 to 3.0 μg per ml of medium. As shown in Table III, the yields of total biotin from culture medium of various bacteria were approximately equal to those from cul-

ture medium of molds. Particularly, *Bacillus sphaericus* accumulated the vitamins in large amounts of 20 to 200 μg per ml of the medium under the optimal condition.

II. Effect of Pimelic Acid and Some Dicarboxylic Acids.

It has been generally accepted that pimelic acid is an intermediate of biotin biosynthesis in microorganisms. In order to clarify the effect of pimelic acid on the accumulation of biotin-vitamins, pimelic acid was added to the medium and the effect of concentrations of pimelic acid on the accumulation of biotin-vitamins by some typical strains of molds, *Streptomyces* and bacteria was examined. As shown in Table IV, at the low concentration (less than 500 μg per ml) of pimelic acid, the amounts of accumulation of biotin-vitamins were increased proportionally to the concentration of pimelic acid added. Suitable concentration of pimelic acid to make a maximal accumulation of biotin-vitamins was in range of 100 to 1000 μg per ml of medium. When over 5 mg of pimelic acid was added to the medium, the amounts of accumulation were decreased. However, the yields of true biotin accumulated by the strains listed in Table IV, except *Streptomyces*, were not increased by the addition of any concentration of pimelic acid. As shown in Tables I, II, and IV, in some molds and *Streptomyces*, 2- to 5-fold increase in amounts of true biotin was observed by the addition of pimelic acid to the medium. On the other hand, it was found that the yields of total biotin of the culture medium of a large number of microorganisms were enhanced about 10- to 100-fold by the addition of pimelic acid. Especially, in the case of *Bacillus sphaericus*, 200- to 2000-fold increase in amounts of biotin-vitamins was observed when the suitable concentration of pimelic acid was added to the medium.

Effect of some organic acids, except pimelic acid, on the accumulation of biotin-vitamins by various microorganisms was investigated. The result is shown in Table V. From the

TABLE IV. EFFECT OF PIMELIC ACID CONCENTRATIONS ON THE ACCUMULATION OF BIOTIN-VITAMERS BY VARIOUS MICROORGANISMS

Pimelic acid added (μg per ml)	Microorganisms tested	Biotin-Vitamins accumulated (μg per ml)											
		<i>Mucor javanicus</i>				<i>Mucor circinelloides</i>				<i>Bacillus sphaericus</i>			
		True	Total	True	Total	True	Total	True	Total	True	Total	True	Total
0.0		0.05	0.15	0.09	0.80	trace	0.12	trace	0.17	trace	0.10	0.06	0.07
1.0		0.05	1.50	0.08	1.50	"	1.50	"	0.25	"	0.65	0.10	0.12
5.0		0.04	2.00	0.08	3.10	"	1.90	"	1.50	"	1.30	0.12	0.13
10.0		0.04	2.50	0.08	4.00	"	2.00	"	1.55	"	1.70	0.14	0.15
50.0		0.06	4.00	0.09	5.00	"	12.10	"	2.10	"	2.56	0.12	0.18
100.0		0.06	10.00	0.08	5.30	"	14.00	"	2.95	"	2.20	0.20	0.50
500.0		0.06	11.10	0.08	5.21	"	15.00	"	2.94	"	2.20	0.24	1.40
1000.0		0.06	10.00	0.08	5.00	"	14.20	"	2.95	"	2.20	0.23	1.90
5000.0		0.05	3.00	0.07	2.10	"	12.30	"	2.30	"	2.00	0.24	1.70
10000.0		0.04	3.00	0.08	1.86	"	11.20	"	1.50	"	1.50	0.10	1.40

TABLE V. EFFECT OF PIMELIC ACID AND SOME ORGANIC ACIDS ON THE ACCUMULATION OF BIOTIN-VITAMERS BY VARIOUS MICROORGANISMS

Addition (0.5 mg per ml)	Biotin-vitamins accumulated (μg per ml)					
	<i>Mucor javanicus</i>		<i>Bacillus sphaericus</i>		<i>Streptomyces</i> sp. A-1030-3	
	True	Total	True	Total	True	Total
None	0.05	0.15	trace	0.12	0.03	0.03
Malonic acid	0.06	0.14	"	0.11	0.02	0.03
Succinic acid	0.04	0.16	"	0.15	0.02	0.03
Glutaric acid	0.04	0.15	"	0.12	0.02	0.04
Adipic acid	0.04	0.15	"	0.13	0.02	0.03
Pimelic acid	0.06	11.10	"	15.30	0.23	1.80
Suberic acid	0.05	0.20	"	0.30	0.09	0.13
Azelaic acid	0.05	5.00	"	10.00	0.12	0.55
Sebacic acid	0.04	0.15	"	0.15	0.03	0.04

result, it was found that azelaic acid was effective and suberic acid was slightly effective, but the other organic acids tested were not effective. However, effect of azelaic acid was lower than that of pimelic acid.

DISCUSSION

The results presented in this paper showed that biotin-vitamins were accumulated by various microorganisms such as molds, *Streptomyces* and bacteria, and that the amounts and biological activity of the vitamins were vari-

able from strain to strain of microorganisms.

The maximal yield of total biotin reported by Schopfer³¹ was about 0.02 to 0.03 μg per ml of the culture medium of *Phycomyces blakesleeana* after 7 days of growth. Recently, Eisenberg,⁴¹ using the same mold, demonstrated that the addition of pimelic acid and the aeration of the culture increased the biotin yield 10- to 20-fold, with average yield varying between 0.35 to 0.60 μg per ml of culture medium. In the present investigation, the maximal yield of total biotin in the culture medium of *Bacillus sphaericus* was observed to be 200 μg per ml of the culture medium. In the case of various molds, *Streptomyces* and bacteria, in general, the yields of total biotin varied from 2 to 5 μg per ml of the culture medium. On the other hand, the maximal yield of true biotin in the culture medium of various molds and *Streptomyces* was about 0.1 to 0.4 μg per ml of the culture medium. Especially with all bacteria tested, the accumulation of true biotin was not observed even by the addition of pimelic acid, while the accumulation of large amounts of total biotin was observed with addition of pimelic acid.

The difference between the amounts of true and total biotin accumulated by microorganisms may be caused by that desthiobiotin is the dominant component of total biotin accu-

mulated by these microorganisms. This may be due to that the microorganisms used in the present investigation have little activity to convert desthiobiotin into biotin. The conversion of desthiobiotin to biotin has been reported with *Saccharomyces cerevisiae*,^{21,22)} and also observed by the present authors with large number of *Streptomyces* and molds, but in bacteria this activity was hardly detected (unpublished observation).

In general, the addition of pimelic acid to the medium was found to enhance the yield of total biotin about 10- to 100-fold, while the cell growth was not affected by pimelic acid. The yields of total biotin were proportional to the concentration of pimelic acid added. These facts may support the hypothesis of participation of pimelic acid in biotin biosynthesis. Azelaic acid was also effective for the accumulation of biotin-vitimers by various microorganisms. This may be due to that azelaic acid was converted to pimelic acid by these microorganisms. It has been reported by Janota-Bassalik and Wright²³⁾ and by the present authors²⁴⁾ that azelaic acid was converted to pimelic acid by some soil bacteria.

In some strains of molds such as H-49-S-3, H-48-S-3 and H-33-S-1 etc. (unidentified

molds), remarkable amounts of biotin-vitimers were observed in the culture medium without addition of pimelic acid, but the amounts of biotin-vitimers in the culture medium were not increased even by the addition of pimelic acid. Many other strains of molds and *Streptomyces* accumulated relatively large amounts of biotin-vitimers without addition of pimelic acid, but, when pimelic acid was added to the medium, more large amounts of biotin-vitimers were accumulated. These facts suggested that the former strains may synthesize enough pimelic acid which is necessary for the production of the biotin-vitimers by these molds, and that the latter strains may synthesize pimelic acid, but not enough for the maximal production of biotin-vitimers by these microorganisms. It is of interest that the yield of true biotin, accumulated by the strains such as *Mucor javanicus* and *Mucor circinelloides* etc., was not affected by the addition of pimelic acid. It may be assumed that the vitamer (may be desthiobiotin), which is necessary for the maximal production of true biotin by these molds, is synthesized enough without addition of pimelic acid.

The details of the characteristics of the biotin-vitimers accumulated by these microorganisms will be discussed in the next paper.

Acknowledgement. The authors wish to express their sincere thanks to Dr. H. Yamada, Assistant Professor of Kyoto University, for his many helpful advices in the course of this work.

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