BIOTIN BIOSYNTHESIS

I. BIOTIN YIELDS AND BIOTIN VITAMERS IN CULTURES OF *PHYCOMYCES BLAKESLEEANUS*

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ABSTRACT

EISENBERG, M. A. (Columbia University, New York, N.Y.). Biotin biosynthesis. I. Biotin yields and biotin vitamers in cultures of *Phycomyces blakesleeanus*. J. Bacteriol. **86**:673-680. 1963.—The addition of pimelic acid to a well-aerated medium resulted in a 10- to 12-fold increase in the biotin production of *Phycomyces blakesleeanus*. Azelaic acid also stimulated biotin production, but not to the same extent as did pimelic acid. A number of biotin analogues were found to be inactive. Further enhancement of the biotin yield could not be attained by replacing glucose and asparagine by other carbon and nitrogen sources. Replacement cultures, however, proved to be equally as effective as growing cultures under the same conditions. The omission of trace elements reduced the growth and biotin production. The "true" biotin was affected to a greater extent than the "total" biotin. Zinc and iron proved to be the essential trace metals. In the absence of zinc, both the growth and the total biotin production were markedly reduced. The omission of iron affected primarily the biotin production. *P. blakesleeanus* produces biotin, desthiobiotin, biotin-*d*-sulfoxide, biocytin, and an unknown biotin vitamer. The latter has been identified as an amino acid by electrophoretic analysis. It is avidin-uncombinable, and does not support the growth of *Lactobacillus arabinosus* (*L. plantarum*) or *Neurospora crassa*.

Biotin is synthesized by a variety of microorganisms (Landy and Dicken, 1941), but in exceedingly small quantities as compared with most vitamins. Schopfer (1943), in his extensive study of biotin biosynthesis by the mold, *Phycomyces blakesleeanus*, indicated this organism to be one of the best sources for this vitamin. It was found that over 90% of the total biotin produced by *P. blakesleeanus* appeared in the me-

dium. This extensive excretion of the vitamin occurred after maximal growth had been attained. The biotin yield was markedly influenced by the concentration of asparagine and vitamin B₁ in the medium, as well as by the addition of trace metals.

Other investigators, in a series of studies with other microorganisms, demonstrated that the addition of pimelic acid to the culture medium enhances the yield of biotin and its vitamers. As a result of these studies and of other observations, it has been postulated that pimelic acid is an intermediate in biotin biosynthesis (Eakin and Eakin, 1942; du Vigneaud, et al., 1942; Tatum, 1945; Wright and Cresson, 1954). Recent evidence from isotopic studies suggests a direct participation of pimelic acid in the synthesis of biotin and its vitamers (Eisenberg, 1962; Elsford and Wright, 1962). Other compounds which have been shown to stimulate biotin production are azelaic and lipoic acids (Wright, Cresson, and Driscoll, 1955; Elsford and Wright, 1962). The latter compound exerts its effect only in the presence of pimelic acid.

This report is part of a study on the mechanism of biotin biosynthesis. Since low biotin yields impose certain limitations on a study of this nature, the present investigation was undertaken to determine the optimal conditions for maximal biotin production with *P. blakesleeanus*. In addition, it was necessary to ascertain the chemical nature of the biotin vitamers produced by this organism.

MATERIALS AND METHODS

Culture conditions. *P. blakesleeanus* (ATCC 6200) was maintained on Sabouraud agar slants (Difco). Spore suspensions were prepared from cultures 2 to 3 weeks old. Sterile water was added to the slant, and the spores were shaken loose with a sterile inoculating needle. The suspension was centrifuged, and the spores were washed

twice with distilled water. They were then activated by placing the suspension in a water bath at 50 C for 15 min. A 0.5-ml amount of the suspension was utilized to inoculate 100 ml of medium of the following composition: glucose, 3.0 g; L-asparagine, 150 mg; KH_2PO_4 , 150 mg; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 50 mg; thiamine hydrochloride, 10 μg . The medium was prepared with warm tap water or with distilled water to which was added the trace element mixture of either Hammer, Stewart, and Matrone (1943) or Schopfer (1943). The growth procedure, unless otherwise indicated, consisted of permitting the inoculated cultures to remain at room temperature for 1 to 2 days to permit sporulation. The culture was then placed in a shaker, kept in an incubator at 30 C, and shaken at 70 to 120 rev/min.

Assays. The term "total" biotin as used in this text encompasses the entire spectrum of biotin vitamers utilized by *Saccharomyces cerevisiae* (ATCC 9896 or 7754) for growth. This includes biotin, desthiobiotin, biocytin, biotin-*d*- and *l*-sulfoxides, diaminobiotin, diaminopelargonic acid, and a number of unknown vitamers. The assay was carried out according to the method of Hertz (1943). *Neurospora crassa* 1A was also utilized for "total" biotin determinations, but the results were consistently lower than those obtained with the yeast assay. This strain was kindly furnished by Francis Ryan, and the assay was performed according to the procedure of Horowitz and Beadle (1943). The term "true" biotin will refer to those vitamers capable of supporting the growth of *Lactobacillus arabinosus* (ATCC 8014) and includes biotin, and biotin *d*- and *l*-sulfoxides. The general assay procedure of Wright and Skeggs (1944) was utilized, except that the biotin content was determined by turbidity measurements.

Chromatography and electrophoresis. Ascending

paper chromatography was carried out on Whatman no. 1 filter paper with *n*-butanol-water-acetic acid (60:15:25) as the developing solvent. Paper-strip electrophoresis was performed in a Spinco Durrum cell at 300 v for 3 hr. The following buffer solutions were utilized in a final concentration of 0.025 M: citrate, pH 3.0; acetate, pH 4.0 and 5.0; phthalate, pH 5.9; phosphate, pH 7.5; and glycine, pH 8.5 to 10.5. Combined chromatography and electrophoresis were carried out on Whatman no. 3 MM filter paper. The material was first chromatographed in the solvent mixture (*n*-butanol-water-acetic acid) until the solvent front reached a height of 26 to 27 cm. The paper was dried at 80 to 90 C, turned 90°, and placed into a Spinco electrophoresis rack. The paper was carefully moistened with citrate buffer (pH 3.0), and electrophoresis was carried out for 3 hr at 300 v.

The bioautographic technique was used to develop the chromatograms and the electrophoretograms with *S. cerevisiae* and *L. arabinosus* as test organisms. The paper was left in contact with the agar surface during the entire incubation period. The R_f values obtained by this procedure compared favorably with those reported in the literature.

Reagents. The lactobacillus assay medium was a Difco product. Avidin, ion-exchange resins, L-meso- α,ϵ -diaminopimelic acid, and all vitamins were purchased from the California Chemical Foundation, Los Angeles, Calif. *d*-Biotin, *dl*-desthiobiotin, and all amino acids were obtained from Mann Research Laboratories, New York, N. Y. Pimelic acid was a product of Eastman Kodak Co., Rochester, N. Y., and α -aminopimelic acid was obtained from Nutritional Biochemicals Corp., Cleveland, Ohio. Biotin-*d*- and *l*-sulfoxides were kindly furnished by D. Melville. ζ,η -Diaminopelargonic acid was prepared by H. Gillespie, and 2,5-diketopimelic acid was a gift from S. W. Tannenbaum.

RESULTS

Aeration. To determine the effect of the degree of aeration on the synthetic capacity of *P. blakesleeana*, the organism was grown in both stationary and shaken cultures. The latter were either shaken twice daily (to prevent sporangia formation) or continuously at 70 to 120 rotations per min. The data in Table 1 show a threefold increase in the biotin production as the result of

TABLE 1. Effect of aeration on biotin production*

Shaking condition	Biotin yield ($\mu\text{g}/50\text{ ml}$)	
	Total	True
None.....	6.2	0.58
Twice daily.....	6.8	0.55
Continuous, 120 rev/min.....	22.2	1.60

* Cultures were grown for 6 days at room temperature in presence of 1 mg of pimelic acid and the trace-metal mixture of Hammer et al. (1943).

continuously shaking the culture medium. This increase was the same for both the total and true biotin as measured by the yeast and *L. arabinosus* assays, respectively. It is also evident that the true biotin constitutes only a small percentage of the total biotin under these conditions. The effect of the trace metals on true biotin yield will be dealt with in more detail later.

Effect of acids. Schopfer (1943) found a twofold increase in biotin production on raising the temperature from 18 to 25 C. A similar increase was observed in going from 18 to 30 C, but when pimelic acid was present in the medium, a threefold increase was obtained (Fig. 1). This effect of pimelic acid on biotin yield was much more pronounced under optimal conditions of growth. Table 2 shows that a 10- to 12-fold increase in biotin yield was obtained with a concentration of 0.4 mg of pimelic acid per 100 ml of media. Azelaic, suberic, and oleic acids were also tested, but only azelaic acid proved to be active. The pimelic acid analogues, α -aminopimelic acid, α , ϵ -diaminopimelic acid, and 2,5-diketopimelic acid, were without effect. Lipoic acid gave variable re-

TABLE 2. *Effect of pimelic acid concentration on biotin production*

Concn	Total biotin
mg/100 ml	$\mu\text{g}/100\text{ ml}$
0.0	2.6
0.2	18.9
0.4	26.3
1.0	26.5

sults, and the maximal increase obtained was about 25%.

Effect of carbon and nitrogen sources. Sucrose and maltose were found to support the growth of *P. blakesleeanus* to the same extent as glucose, but produced no further increase in biotin. Glutamic and aspartic acids, alanine, and glycine, which had been previously shown to support the growth of this organism (Leonian and Lilly, 1940), were substituted for asparagine. Only in the glutamic and aspartic acid media were the biotin yields comparable with the asparagine medium, and then only after 10 to 11 days of incubation. In the earlier stages of growth (4 to 6 days), the asparagine medium was far superior. Utilizing a medium of Leonian and Lilly (1940), in which ammonium nitrate and glucose are supplemented with various Krebs cycle intermediates, it was found that fumaric and citric acids were the most effective, but again, neither surpassed asparagine for yield.

The possible role of pimelic acid as an intermediary in biotin biosynthesis suggested as a working hypothesis the initial condensation of pimelic acid with a three-carbon precursor such as cysteine or serine. When the growth medium was supplemented with cysteine, in a concentration of 1 to 2 mg per 100 ml of media, there was no increase in biotin production, and increasing the concentration caused a marked inhibition of growth. Cystine, however, in the lower concentrations gave about 25% increase in biotin yield, and serine in concentrations of 5 to 25 mg gave variable results.

Effect of trace elements. In a number of experiments in which tap water replaced the trace element mixture of Hammer et al. (1943), better growth and higher true biotin yields were observed. Similar results were also obtained with Schopfer's (1943) trace element mixture. To determine the effect of trace elements on the true biotin yield, Schopfer's (1943) trace element mix-

FIG. 1. *Effect of temperature and pimelic acid on total biotin yield. The cultures were grown in the presence of the trace element mixture of Hammer et al. (1943) and were shaken twice daily. Samples were removed for analysis at times indicated. Open circles and triangles, no pimelic acid; closed circles and triangles, 1 mg of pimelic acid.*

TABLE 3. *Effect of trace elements on biotin production*

Mixture	Biotin produced					
	Total	True	Dry wt	True	Total*	True*
	$\mu\text{g}/100\text{ ml}$	$\mu\text{g}/100\text{ ml}$	mg	%		
Complete.....	16.3	4.44	717	24.2	22.7	6.2
–B.....	15.2	4.53	707	29.2	21.5	6.4
–Fe.....	12.9	2.51	680	19.4	19.0	3.7
–Cu.....	13.9	3.28	687	23.6	20.2	4.7
–Mn.....	13.9	4.40	695	31.6	20.0	6.3
–Zn.....	3.7	0.73	307	19.8	12.1	2.4
–Mo.....	11.2	3.06	650	27.4	17.2	4.7
+ Tap water.....	15.7	5.44	748	34.4	21.1	7.3

* Expressed as μg per mg of dry weight.

ture was utilized, and, omitting one element at a time, the results shown in Table 3 were obtained.

The greatest change occurred when zinc was omitted. The growth was reduced to less than one-half, and the biotin yield was reduced about 80%. The omission of iron, although having little effect on growth, produced a 30% decrease in total biotin and a 44% decrease in true biotin. The omission of copper, manganese, molybdenum, or boron produced little or no change in the biotin yield.

Replacement culture. It was shown by Schopfer (1943) that *P. blakesleeanus* excretes biotin into the medium for a considerable period of time after maximal growth has been obtained. However, the rate of synthesis is reduced to about

one-half during this time interval. This observation has been confirmed in the present study. On the other hand, if one utilizes the replacement of culture technique, rates of biotin synthesis almost equivalent to those obtained with normal culture procedures can be obtained. Table 4 shows the effect of altering the pH of the replacement medium on biotin production. It can be seen that there is a synthesis of biotin during 8 days over a pH range of 4 to 7, comparable with that of a growing culture.

Biotin vitamers. When the culture medium, after 7 to 10 days of growth, was chromatographed, bioautography revealed several components with biotin activity. Table 5 shows the R_F values of these components as compared with biotin and known biotin vitamers. The component with an R_F value of 0.84 actually appeared as a fusion of two components. This was verified by the fact that, when the same material was assayed with *L. arabinosus*, this component was absent, but a component with the R_F value of biotin appeared. Since *L. arabinosus* cannot utilize desthiobiotin for growth, it would appear that the compound with an R_F value of 0.84 consists of both biotin and desthiobiotin. The presence of biotin-*d*-sulfoxide with the R_F value of 0.56 was confirmed by the use of both assay organisms. The component with an R_F value of 0.63 does not correspond to any of the known vitamers reported in the literature and does not support the growth of *L. arabinosus*. This vitamer has appeared consistently in all experiments under a variety of conditions. When the medium is concentrated and chromatographed, additional components are sometimes revealed, among which is biocytin.

Paper-strip electrophoresis at pH 3.0 usually

TABLE 4. *Effect of pH on biotin production in replacement cultures*

pH	Total biotin ($\mu\text{g}/50\text{ ml}$) at		True biotin ($\mu\text{g}/50\text{ ml}$) at 8 days
	3 days	8 days	
4.1	10.1	21.0	5.6
5.0	6.9	20.5	5.3
6.0	7.8	19.3	4.9
7.0	10.4	26.2	4.4

TABLE 5. *R_F value of media components as compared with known biotin vitamers*

Compound	R_F values	
	Yeast assay	<i>Lactobacillus arabinosus</i> assay
Media.....	0.51, 0.63,	0.56, 0.79
<i>d</i> -Biotin.....	0.53, 0.76	0.56, 0.79
Biotin- <i>d</i> -sulfoxide..	0.56	0.55
<i>dl</i> -Desthiobiotin...	0.84	Not utilized

revealed two components, one moving 7.0 to 8.0 cm toward the cathode, and a second remaining near the origin. Since biotin, desthiobiotin, and biotin-*d*-sulfoxide remain near the origin at pH 3.0, it was thought that this fast-moving component represented the unknown vitamer (R_F 0.63). That this indeed was the case is shown by the results in Fig. 2 obtained from combined chromatography and electrophoresis. Component 1 is again a composite of two compounds, representing biotin and desthiobiotin. Compound 2 corresponds to biotin-*d*-sulfoxide, and component 3, which gives only a weak spot, corresponds to biocytin. Component 4 is the unknown vitamer noted previously. In the presence of avidin, only component 4 is present. At pH 5.9, electrophoresis again indicated two components; one remaining near the origin, and containing the unknown, the second, consisting of biotin, desthiobiotin, and biotin sulfoxide, moving 4.0 to 4.5 cm toward the anode.

When attempts were made to utilize this technique to obtain the unknown component in larger yields for further study, it was discovered that more than one component was frequently present, depending upon the amount of material applied to the paper strip. This was determined by cutting out the area containing the fast-moving component from a large number of electrophoretic strips run at pH 3.0, eluting with water, and then concentrating to a small volume. This material, when chromatographed, revealed usually from one to three components by bioautography. One of these components had the R_F value of the unknown compound (R_F 0.63 to 0.67) and was present in the highest concentration. The other components were tentatively identified as biocytin and desthiobiotin. Eluates obtained from control paper strips, which were run in citrate buffer without the addition of the medium, revealed a component with an R_F value similar to desthiobiotin.

Since the unknown compound could represent an intermediate in synthesis of biotin, it was felt that the electrophoretic data might afford some clue as to its chemical nature. Although it could not be readily separated by electrophoresis from other vitamers, the fact that it was the only vitamer which did not combine with avidin enabled a more accurate determination of its mobility over a wide pH range. This was accomplished by adding avidin to the bioautographic medium (Fig. 3). The mobilities in this

FIG. 2. Bioautogram of growth media after chromatography and electrophoresis. Culture grown under optimal conditions for 9 days and medium concentrated fourfold.

figure were corrected for endosmosis with the aid of glucose. It can be seen that the unknown compound is positively charged below pH 5.9, and negatively charged above pH 9.0. The mobility of biotin is given for comparison.

Further information about the biotin vitamers was also gained from their behavior on ion-exchange chromatography. When the media were placed onto a Dowex 50 H^+ column (1.8 by 20 cm), the initial effluent and the water wash showed considerable biotin activity. Chromatographic analysis indicated the presence of biotin, desthiobiotin, and biotin-*d*-sulfoxide. The column was then developed with either 1.0 N HCl, or a mixture of ethyl alcohol, water, and concentrated ammonia (1:1.0:1), and the fractions containing biotin activity were pooled. Analysis of this mixture by chromatography and bioautography revealed three components, none of which supported the growth of *L. arabinosus*. Among these components were biocytin and the unknown vitamer. It was found that the initial effluent and water wash from a Dowex-1-acetate column con-

FIG. 3. Comparison of the electrophoretic mobility of the unknown vitamer with *d*-biotin.

tained biocytin and the unknown vitamer, while the biotin, desthiobiotin, and biotin-*d*-sulfoxide remained on the column. These compounds could be eluted with 1.0 to 1.5 *N* acetic acid. Some fractionation was attained, as the biotin-*d*-sulfoxide was eluted somewhat earlier than the other two components.

DISCUSSION

The maximal biotin yields obtained by Schopfer (1943) from the culture medium after 7 days of growth varied from 0.6 to 0.8 μg in 25 ml of media. In the present studies, the addition of pimelic acid and the aeration of the culture were found to increase the biotin yield 10- to 12-fold, with the average yield varying between 20 to 25 μg in 100 ml of media. After 10 to 14 days, the biotin yield varied from 35 to 60 μg per 100 ml of

media. Replacement culture technique showed that the mycelial mat can synthesize biotin at a rate comparable with that of the growing culture. This may prove of value in isotopic experiments. None of the pimelic acid analogues proved effective in enhancing biotin biosynthesis, thus further supporting the hypothesis of a direct role of pimelic acid in biotin synthesis.

The present attempts to further enhance the biotin yields by altering the carbon and nitrogen sources were unsuccessful. The fact that asparagine cannot be surpassed as a nitrogen source may indicate a more direct role for this amino acid in biotin biosynthesis. The small increase observed with added cystine may be attributed to a change in the percentage of the various vitamers appearing in the medium. Since yeast cannot utilize biotin-*d*-sulfoxide to the same extent as biotin (Melville, Genghof, and Lee, 1954), a decrease in the percentage of the former would appear as an increase in total biotin. A similar explanation was offered by Wright et al. (1955) to account for the discrepancy between their results and those obtained by Eakin and Eakin (1942). It was thought that the presence of a reducing agent would decrease the amount of biotin-*l*-sulfoxide which is normally formed by *Aspergillus niger*. However, no evidence was presented to verify this supposition. Although no attempt was made in the present study to examine the distribution of vitamers in the presence of cysteine, an extensive investigation was carried out with lipoic acid. A small increase in biotin has been occasionally observed with 0.5 mg of lipoic acid per 100 ml. However, chromatographic analysis of these cultures revealed the complete absence of biotin-*d*-sulfoxide. This observation would support the explanation (Wright et al., 1955) of the action of reducing agents in preventing biotin oxidation, and would also suggest biotin-*d*-sulfoxide to be a normal metabolic product.

When the trace element mixture is omitted from the culture medium, not only are growth and total biotin production reduced, but the quantitative distribution of the biotin vitamers, particularly true biotin, is also altered. The absence of zinc had the greatest effect on both growth and biotin yield, suggesting a general decreased synthetic capacity of the organism. A similar effect of zinc on the growth of *P. blakesleeanus* was observed by Ødegard (1952), and, more recently,

Wacker (1962) indicated an impairment in protein synthesis in zinc-deficient *Euglena gracilis*. The omission of iron, although not materially reducing the growth of the organism, had a more pronounced effect on the true biotin, suggesting a possible role in the formation of biotin from desthiobiotin.

P. blakesleeanus has been found in the present study to produce a number of compounds possessing biotin activity. These compounds have been shown by a variety of techniques to consist of biotin, desthiobiotin, biotin-*d*-sulfoxide, biocytin, and an unknown vitamer. Quantitative estimates of these various vitamers have been difficult to attain because of the inability at present to obtain complete separation of all of the components by any of the procedures used. It would appear that desthiobiotin comprises the largest portion of the total biotin activity, and biotin the next largest. Biotin-*d*-sulfoxide constitutes about 10 to 15%, the unknown vitamer 5 to 15%, and biocytin 1 to 2% of the total activity.

The electrophoretic-mobility study of the unknown compound revealed the presence of both an acidic and a basic group. The pKa value for these two groups has been estimated to be approximately 4.5 to 5.0 and 9.2 to 9.5, respectively. These values correspond to a carboxyl and amino group, thus identifying this compound as an amino acid. The pKa of the carboxyl group would further suggest a separation of charges in this compound. This is supported by the fact that the electrophoretic mobility of the unknown resembles β -alanine and ϵ -aminocaproic acid, much more than α -alanine. It has also been observed that the unknown compound moves more slowly toward the cathode at pH 3.0 than does diaminopelargonic acid, indicating the presence of only one free amino group. This compound does not support the growth of *L. arabinosus* in concentrations which will support the growth of yeast. It is also avidin-uncombinable, indicating the absence of the ureido structure. Its activity with *N. crassa* has been difficult to ascertain as it has not been possible to free it completely from other components. However, a purified sample containing biocytin as a contaminant was assayed for total biotin with both yeast and *N. crassa*. In addition, the amount of avidin-uncombinable material was also determined with the yeast assay. It was found that the *N. crassa* values were

about one-half of the yeast values, and the biotin-uncombinable material constituted about one-half the total biotin activity. This would indicate that the unknown, in the concentrations used, is unavailable to *N. crassa* for growth.

A compound with the same R_f value has also been reported in the culture media of *Escherichia coli* (Dhyse and Hertz, 1958) and *Corynebacterium xerosis* (Genghof, 1956), and was shown to be avidin-uncombinable. A compound with similar properties has also been found (*unpublished observation*) in the medium of a biotin-requiring mutant of *Penicillium chrysogenum*. It appears to be present in much higher concentrations than desthiobiotin, which also accumulates. Work is now in progress to try to isolate this compound from *P. chrysogenum* culture media in sufficient quantities to permit chemical characterization and study of its role in the biosynthesis of biotin.

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