STUDIES ON THE VITAMIN NUTRITION OF ALLESCHERIA BOYDII SHEAR[1]

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Allescheria boydii is a pathogenic fungus isolated from a mycetoma of yellow-white grains by Boyd and Crutchfield (1921). Its life history was described by Shear (1922).

Cury (1949) showed that biotin is an essential growth factor for this mold, the growth being proportional to the amount of biotin in the medium. Until this observation biotin had not been shown to be a growth factor for any of the filamentous pathogenic fungi (Nickerson and Williams, 1947). The only references found in the available literature on the biotin requirements of the filamentous fungi pathogenic for man were those of Schopfer and Blumer (1942, 1943). These authors verified the fact that a strain of *Trichophyton album* required biotin under certain conditions, depending on the age of the culture and the composition of the medium (nitrogen source), and that biotin was a complementary growth factor for the strain of *T. album* studied (Schopfer and Blumer, 1943). More recently, Salvin (1949) reported that biotin was necessary for the development of the yeastlike phase of *Histoplasma capsulatum* and that only one of six strains studied required biotin for mycelial growth.

In view of these facts we undertook a more detailed study of the vitamin nutrition of *A. boydii* as well as of some other conditions that could interfere with its growth.

MATERIAL AND METHODS

The culture of *A. boydii* used (strain 1699) is the same as was employed by Cury (1949).

The basal medium had the following composition per liter: glucose (anhydrous), 30 g; purified asparagine (biotin-free), 2 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; KH_2PO_4, 1.5 g; and trace elements (B, Cu, Fe, Mn, Zn, and Mo). The pH was adjusted to 5.0 before sterilization with 2 N NaOH (for details see Arêa Leão and Cury, 1949). All the vitamins and other nutrilites were pure substances obtained from different sources.

The glassware consisted of 50-ml pyrex Erlenmeyer flasks with an external diameter averaging 5 cm. All the glassware was cleaned with sulfuric acid and dichromate solution, thoroughly washed with tap water, and rinsed with distilled water.

The final volume of medium per flask was 10 ml. Five ml of double-strength

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basal medium were dispensed in each flask; the other substances to be tested were added and the volume was adjusted to 10 ml with distilled water. The flasks were then plugged with white cotton and autoclaved for 15 minutes at 120 C.

Stock cultures of *A. boydii* were carried at room temperature by monthly transfer in a medium consisting of 3 per cent glucose, 1 per cent peptone (Difco), 1 per cent malt extract (Difco), and 1.5 per cent agar. The spore suspension for the inoculum was prepared by adding 10 ml of the basal medium to a culture and scraping the surface of the culture slightly with a platinum loop. A drop of the suspension was inoculated directly into the medium in each flask, thus preventing it from running down the walls of the flask. Cultures 30 days old were used for the preparation of the inocula. After inoculation the cultures were incubated for the desired time, at the end of which they were sterilized at 115 C for 15 minutes. Then the pads were removed, washed thoroughly with

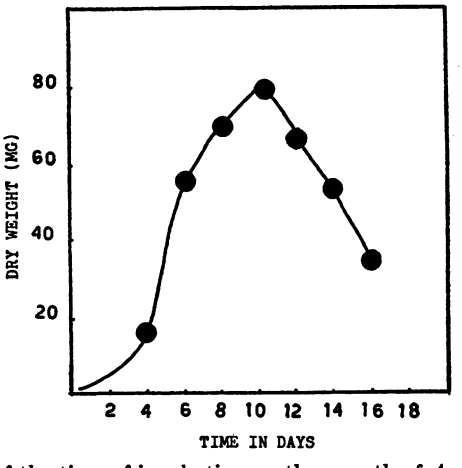

Figure 1. Influence of the time of incubation on the growth of *A. boydii*. Ten mμg biotin per flask.

distilled water, pressed out on filter paper, rolled in pellets, and dried at 110 C for 2 hours. After being cooled in a desiccator, they were weighed in an analytical balance.

All experiments were run in duplicate or triplicate and repeated at least twice. Therefore, the results (dry weight of mycelium in mg) represent the average of several determinations. Controls, i.e., flasks containing the basal medium alone, were run parallel in all experiments. In all cases, unless otherwise stated, the initial pH of the medium was 5.0 and the period of incubation 8 days at room temperature.

EXPERIMENTAL RESULTS

Influence of time. In order to ascertain the influence of the time of incubation on the growth of *A. boydii*, experiments were conducted in which biotin was added in amounts of 10 mμg per flask and the growth on successive days was measured. As can be seen in figure 1, the maximum yields were obtained after

10 days, whereas cultures harvested beyond this period (10 to 16 days) presented a decrease in weight, which is probably due to an autolytic process.

Effect of temperature. The growth of *A. boydii* was studied at different temperatures, viz., 25, 30, and 37 C, and at room temperature (ca. 27 C). The results are summarized in table 1. The optimal growth was observed at room temperature. No measurable growth occurred at 37 C. The effect of temperature is more marked when larger amounts of biotin are used.

Effect of pH. When 10 mμg of biotin were used per flask, it was observed that maximal growth occurred at pH 4 to 5. A gradual decrease in growth was ob-

TABLE 1

Effects of temperature on the growth of A. boydii

BIOTIN mμg	DRY WEIGHT (MG)		
	Temperature of incubation		
	25 C	Room temperature	30 C
0.1	3.5	3.8	3.3
1.0	36.1	33.5	34.6
10.0	63.5	67.0	51.5
100.0	65.5	75.5	54.5

TABLE 2

*Effect of the pH on the growth of A. boydii**

INITIAL pH	DRY WEIGHT (MG)	FINAL pH
2	0.0	2
3	0.0	3
4	70.7	3.6
5	67.5	3.8
6	61.6	5.6
7	57.9	6.4
8	48.9	7.4
9	48.3	8.2

* Ten mμg biotin per flask.

served when the pH was increased up to 9. No growth took place at pH 3 or below (table 2). It was proved that the mold always acidified the medium since the final pH in all cases was lower than the initial one.

Influence of vitamins and other nutrilites. In order to learn the influence of other vitamins of the B complex (thiamine, riboflavin, pyridoxine, nicotinic acid, pantothenic acid, *para*-aminobenzoic acid, choline, pteroylglutamic acid, and inositol) as well as of acid-hydrolyzed casein (vitamin-free), purine and pyrimidine bases, cysteine, glutathione, pimelic acid, and aspartic acid, experiments were conducted which are summarized in tables 3, 4, 5, and 6.

As can be seen from table 3, nicotinic acid and pyridoxine are the only vitamins that influence the growth of *A. boydii*, the former stimulating and the latter

TABLE 3

Influence of other vitamins on the growth of A. boydii

DRY WEIGHT (MG)											
	Substances added to basal medium + 10 mμg biotin										
Biotin 10 mμg	Thiamine 5 μg	Riboflavin 5 μg	Pyridoxine 5 μg	Nicotinic acid 5 μg	Calcium pantothenate 5 μg	PABA 5 μg	Choline 5 μg	Pteroylglutamic acid 1 μg	Inositol 100 μg	All* vitamins	Control
82.3	82.6	84.8	76.6	98.2	84.9	82.9	82.8	83.8	83.0	98.1	0.0

* Vitamins added in the same amounts as in each isolated experiment.

TABLE 4

Influence of nicotinic acid and pyridoxine on the growth of A. boydii

BIOTIN	NICOTINIC ACID	PYRIDOXINE	DRY WEIGHT
mμg	μg	μg	mg
10	0	0	89.9
10	0.25	0	99.3
10	0.5	0	109.4
10	1	0	113.0
10	2	0	113.1
10	3	0	115.8
10	4	0	116.0
10	5	0	117.3
10	0	5	82.3
10	5	5	114.1
0	5	0	0.0
Control	0	0	0.0

TABLE 5

Influence of casein hydrolyzate and purine and pyrimidine bases on the growth of A. boydii

BIOTIN	CASEIN HYDROLYZATE	PURINE AND PYRIMIDINE BASES*	DRY WEIGHT
mμg	mg	μg	mg
5	10	0	60.4
0	10	0	0.0
5	0	10	54.0
0	0	10	0.0
5	0	0	52.6
Control	0	0	0.0

* Adenine sulfate, guanine hydrochloride, xanthine, uracyl, and thymine added in amounts of 10 μg per flask.

depressing the growth. These effects are shown in table 4. Increasing the amount of nicotinic acid had a clearly stimulating effect. A very interesting fact observed was the neutralizing effect of nicotinic acid on the depressing action exerted by pyridoxine (table 3, row 11, and table 4).

Acid-hydrolyzed casein enhances the growth, whereas purine and pyrimidine bases (xanthine, guanine, adenine, uracyl, and thymine) do not interfere (table 5). Cysteine, glutathione, pimelic acid, and DL-aspartic acid are not effective in promoting growth and do not interfere when associated with biotin (table 6).

TABLE 6

Influence of cysteine, glutathione, pimelic acid, and aspartic acid on the growth of A. boydii

BIOTIN	CYSTEINE	GLUTATHIONE	PIMELIC ACID	ASPARTIC ACID	DRY WEIGHT
mμg	μg	μg	μg	mg	mg
1	0	0	0	0	36.8
1	20	0	0	0	36.2
0	20	0	0	0	0.0
1	0	20	0	0	36.4
0	0	20	0	0	0.0
1	0	0	10	0	37.9
0	0	0	10	0	0.0
5	0	0	0	0	61.4
5	0	0	0	2	58.8
0	0	0	0	2	0.0
Control	0	0	0	0	0.0

TABLE 7

Influence of oleic acid and sorbitan monooleate on the growth of A. boydii

BIOTIN	OLEIC ACID	SORBITAN MONOOLEATE	DRY WEIGHT
mμg	mg	mg	mg
1	0	0	24.3
1	0.5	0	25.1
0	0.5	0	0.0
1	1	0	27.9
0	1	0	1.3
1	2	0	29.9
0	2	0	1.6
1	0	1	22.2
0	0	1	0.0
1	0	5	22.6
0	0	5	0.0
1	0	10	23.5
0	0	10	2.4
Control	0	0	0.0

Temperature of incubation, 20 C.

Influence of oleic acid and sorbitan monooleate. Experiments have been conducted to study the effect of oleic acid and a polyoxyethylene derivative of sorbitan monooleate on the growth of *A. boydii*. The results obtained are summarized in table 7. Oleic acid and sorbitan monooleate, which contains oleic acid in an esterified form, did not show any growth-stimulating effect, either alone or in combination with biotin.

Biotin analogs (DL-*O-heterobiotin and* DL-*desthiobiotin*). Several papers have been published showing that biotin can be replaced by O-heterobiotin and desthiobiotin in the nutrition of microorganisms (for references see "Discussion").

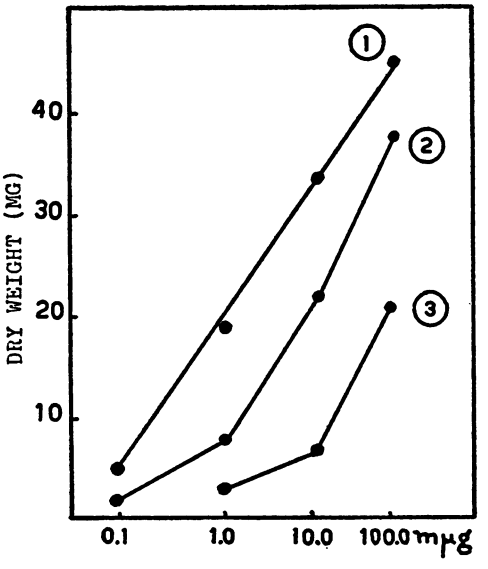

Figure 2. Growth response curves of *A. boydii* to biotin, DL-O-heterobiotin, and DL-desthiobiotin. (1) = biotin; (2) = DL-O-heterobiotin; (3) = DL-desthiobiotin. Temperature, 20 C.

TABLE 8

Comparative effect of biotin, DL-*O-heterobiotin, and* DL-*desthiobiotin*

mμg	DRY WEIGHT (MG)		
	Biotin	DL-O-heterobiotin	DL-Desthiobiotin
0.1	5.2	2.1	—
0.5	14.0	5.2	—
1	17.3	6.8	2.4
5	32.0	19.1	—
10	33.2	21.4	6.2
50	38.2	33.7	—
100	44.0	38.4	21.0
Control	0.0	0.0	0.0

Temperature of incubation, 20 C.

In the particular case of *A. boydii* both analogs can partially replace biotin (figure 2 and table 8).

DL-O-heterobiotin is comparatively more active than DL-desthiobiotin. With increasing amounts of these analogs the differences in the growth-promoting effects are less marked.

The analogs in increasing amounts did not show a response of the same intensity when associated with a fixed quantity of biotin as when used alone (table 9). Cumulative effects are observed only with amounts of 50 mμg or higher.

TABLE 9

Combined effect of biotin, DL-*O-heterobiotin, and* DL-*desthiobiotin*

BIOTIN	DL-O-HETEROBIOTIN	DL-DESTHIOBIOTIN	DRY WEIGHT
mμg	mμg	mμg	mg
5	0	0	32.0
5	0.5	0	34.2
5	1	0	34.2
5	5	0	34.1
5	10	0	34.7
5	50	0	45.6
5	100	0	47.6
5	0	1	33.2
5	0	5	33.7
5	0	10	35.3
5	0	50	40.6
5	0	100	41.6
5	0	500	42.0
Control	0	0	0.0

Temperature of incubation, 20 C.

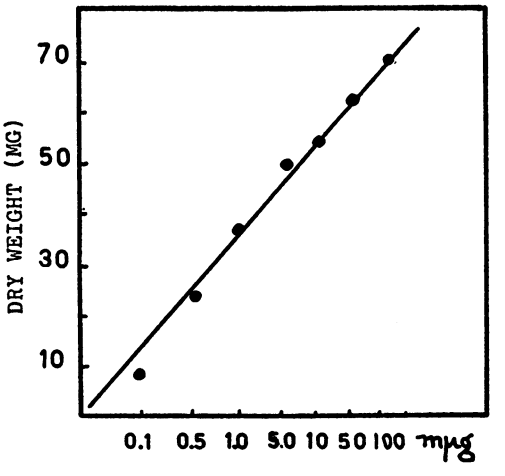

Figure 3. Growth curve of *A. boydii* as a function of the amount of biotin. Temperature, 25 C.

Growth curve with biotin. The growth reponse of *A. boydii* to biotin is proportional to the amount of this vitamin present in the medium. A minimum measurable growth response was obtained with 0.05 mμg of biotin per flask. However, a linear response curve was obtained with amounts of 0.1 to 100 mμg of biotin (figure 3).

DISCUSSION

Relatively few papers are published dealing with the nutrition and growth factor requirements of pathogenic fungi. As emphasized by Nickerson (1948), better knowledge of these requirements is needed for the various groups of pathogenic fungi. Such data may bring light to many problems relating to the mode of infection, pathogenicity, and natural habitat of these microorganisms.

In the present paper the vitamin requirements of *A. boydii*, as well as other conditions that would interfere with growth, were studied. None of the vitamins of the B complex is able to substitute biotin in the growth of this mold. However, nicotinic acid when added to the basal medium containing biotin showed an appreciable stimulatory effect, which, within certain limits, is proportional to the amount of nicotinic acid added. On the contrary, pyridoxine showed a depressive action. It is interesting to report that the depressive effect of pyridoxine is neutralized by nicotinic acid (tables 3 and 4). So far as we know, this antagonistic action has not been reported in the literature. The mechanism of this antagonism deserves a more detailed investigation.

Acid-hydrolyzed casein showed a growth-stimulating effect. Since this hydrolyzate was vitamin-free, as proved by microbiological assays, the growth-stimulating action might be attributed to the amino acids present. This point will be clarified by a study of the nitrogen nutrition of this fungus which is in progress.

We tried to determine whether cysteine and glutathione, which contain organic sulfur as a sulfhydril group, could interfere in some way with the growth of *A. boydii*. However, our results showed that both compounds were inactive.

Pimelic acid has been shown to be a growth stimulant for *Corynebacterium diphtheriae* (Mueller, 1937). Subsequently, du Vigneaud *et al.* (1942) proved that pimelic acid could be substituted for biotin in the nutrition of the Allen strain of *C. diphtheriae*. Eakin and Eakin (1942) observed that biotin synthesis by *Aspergillus niger* was stimulated by the addition of pimelic acid to the medium. These facts suggested that pimelic acid would be synthesized into biotin by these microorganisms. On the other hand, Wright (1942) observed that pimelic acid was unable to support growth of *Lactobacillus casei* when added to the medium in place of biotin. Moreover, Robbins and Ma (1942), studying 13 fungi that suffered from biotin deficiency, showed that those organisms were unable to synthesize biotin from pimelic acid. The same fact holds true for the yeasts (Koser *et al.*, 1942; du Vigneaud *et al.*, 1942). Our results led us to conclude that *A. boydii* cannot synthesize biotin from pimelic acid (table 6).

Koser *et al.* (1942) observed that aspartic acid has a marked effect on the growth of *Torula cremoris* when supplied in place of biotin, and Perlman (1948*a*) mentioned that with *Memnoniella echinata* and *Stachybotrys atra* the addition of aspartic acid to the medium reduced the biotin requirements markedly. However, we observed that with *A. boydii* aspartic acid was unable to substitute for biotin. No increase in growth was found when aspartic acid was added to the medium containing biotin (table 6).

Williams and Fieger (1945, 1946) have reported that oleic acid has biotinlike activity for *L. casei* and that this action is affected by certain external factors.

Other reports on the same subject showed that oleic acid and sorbitan monooleate could replace biotin or stimulate the growth of some microorganisms requiring this vitamin (Shull and Peterson, 1948; Shull, Thoma, and Peterson, 1949; Hodson, 1949). The same relationship was not found for *A. boydii* under the conditions of our experiments (table 7).

It has been shown that desthiobiotin can replace biotin for some microorganisms, mostly yeasts and fungi (Melville *et al.*, 1943; Dittmer *et al.*, 1944; Lilly and Leonian, 1944; Leonian and Lilly, 1945; Tatum, 1945; Stokes and Gunness, 1945; Perlman, 1948*b*). This analog has been shown to act as an inhibitor to both biotin and O-heterobiotin for *L. casei* (Dittmer and du Vigneaud, 1944; Lilly and Leonian, 1944; Tatum, 1945; Rubin, Drekter, and Moyer, 1945; Rubin, Flower, *et al.*, 1945). However, desthiobiotin is inactive for *Lactobacillus arabinosus*, *Lactobacillus pentosus*, and *Rhizobium trifolii* either when alone or when biotin is present (Lilly and Leonian, 1944; Krueger and Peterson, 1948). For *M. echinata* and *S. atra* desthiobiotin is inactive, but combined with biotin it has the same growth-promoting activity as an amount of biotin equal to the total weight of the mixture (Perlman, 1948*a*).

The biotin activity of O-heterobiotin has been demonstrated for *Lactobacillus arabinosus*, *L. casei*, *Saccharomyces cerevisae*, *R. trifolii* (Winnick *et al.*, 1945; Pilgrim *et al.*, 1945; Rubin, Flower, *et al.*, 1945; Duschinsky *et al.*, 1945), for clostridia (Perlman, 1948*b*; Shull and Peterson, 1948), for *M. echinata* and *S. atra* (Perlman, 1948*a*), and for *L. pentosus* (Krueger and Peterson, 1948).

In the present experiments with *A. boydii* we were able to show that desthiobiotin and O-heterobiotin can partially replace biotin for growth, and that O-heterobiotin appeared to be more active than desthiobiotin (figure 2 and table 8). When added to biotin, these analogs did not show an additive response (table 9). Cumulative effects were observed with amounts of 50 mμg or higher. These facts suggest that *A. boydii* is able to use the analogs only in the absence of biotin. A more detailed study of the role of biotin and biotin analogs in the growth of this mold is in progress.

An assay method for biotin based on the present study will be reported in a forthcoming paper.

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SUMMARY

The growth of *Allescheria boydii* in a chemically defined medium as a function of the amount of biotin, the pH, the time, and the temperature, as well as the influence produced by vitamins and other compounds, was studied. Ten days was the maximal time of growth and the optimum temperature was observed

to be ca. 27 C (room temperature). The optimum pH is 4 to 5, no growth occurring at pH 3 or below.

Tests using a suboptimal amount of biotin supplemented with each of the B vitamins (thiamine, riboflavin, pyridoxine, nicotinic acid, pantothenic acid, *para*-aminobenzoic acid, pteroylglutamic acid, inositol, and choline) were performed. Only nicotinic acid and pyridoxine had an effect, the former stimulating and the latter decreasing the growth. The depressive action of pyridoxine is neutralized by nicotinic acid.

Acid-hydrolyzed casein increased slightly the growth, and purine and pyrimidine bases (xanthine, adenine, guanine, uracyl, and thymine) did not interfere.

Cysteine, glutathione, pimelic acid, aspartic acid, oleic acid, and sorbitan monooleate were not effective to promote growth and did not interfere when associated with biotin.

Biotin analogs (desthiobiotin and O-heterobiotin) were able to replace biotin partially in the growth of *A. boydii*, and when associated with biotin they did not show cumulative effect unless high amounts were used.

The growth of *A. boydii* is a function of graded amounts of biotin.

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