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Studies on the Nutrition of *Candida*

II. Effect of Amino Acids on the Growth of *Candida albicans*

SHIRO MIYASHITA, TOSHIO MIWATANI AND TSUNESABURO FUJINO

Department of Bacteriology, Research Institute for Microbial Diseases, Osaka University

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SUMMARY

While studying the effect of amino acids on the growth of *Candida albicans*, six amino acids, that is, aspartic acid, glutamic acid, arginine, proline, serine and α-alanine, were found to have a biotin-sparing effect. Thus, even in the presence of suboptimal amounts of biotin, the growth of this organism was markedly increased by the addition of these amino acids to a basal medium. Although aspartic acid, glutamic acid and arginine were found to be utilized as a sole source of both carbon and nitrogen for growth, the growth enhancing effect of proline, α-alanine and serine was observed only in the presence of glucose. Kinetic studies have further revealed that a typical diauxie growth of the organism takes place in a medium containing glucose, ammonium salts and either serine or α-alanine. The significance of these observations is discussed in terms of the mechanism involved in the effect of these two amino acids.

INTRODUCTION

It was shown previously (Miyashita *et al.*, 1958) that *Candida albicans* was one of six species of *Candida* which are able to grow in Glucose-Simmons medium containing biotin for which the organisms are exacting. Thus no exogenous supply of any amino acids is required by *Candida albicans* for growth, indicating that all amino acids can be synthesized by this organism in the presence of biotin, a substance known to be involved as a co-factor in the metabolism of some amino acids. We have recently shown in preliminary experiments that, when the basal medium is supplemented by a vitamin-free casein hydrolysate, the growth of *Candida albicans* is markedly enhanced. This occurs even in the presence of small amounts of biotin which merely supports the poor growth of this particular organism in the basal medium. A series of studies on this phenomenon have further revealed that six out of the eighteen amino acids tested have a so-called "biotin-sparing effect" (Koser, *et al.*, 1942). Characteristic patterns of growth of this organism are seen in a medium containing glucose and one of the amino acids having this effect.

In this paper these results are described and some of the mechanism involved in the effect of these amino acids on patterns of the growth of *Candida albicans* are discussed.

Materials and Methods

Organism: *Candida albicans* FIA-1010 (RIMD), originally isolated from the sputum of a case of pulmonary tuberculosis, was used. For each experiment, a

24 hour culture of this organism on Sabouraud glucose agar medium was washed twice with sterile saline and the washed cell suspension used as a inoculum.

Medium: The medium used was Glucose-Simmons media to which appropriate amounts of biotin were added. The amino acids tested in this experiment were glycine, *dl*-α-alanine, *dl*-serine, *l*-cystine, *dl*-threonine, *l*-methionine, *dl*-valine, *l*-leucine, *dl*-isoleucine, *dl*-phenylalanine, *l*-tyrosine, *l*-tryptophane, *l*-histidine, *l*-arginine, *l*-glutamic acid, *l*-aspartic acid and *l*-proline.

Cultural procedure: A) Still culture. The cell suspension was inoculated into 7 ml of medium in each specially designed test tube and then incubated at 37°C. B) Shaking culture. The cell suspension was inoculated either in 100 ml of medium dispensed in a 500 ml shaking flask or into 10 ml of medium in a T-shaped shaking tube which was incubated at 35°C on a reciprocating shaker adjusted to approximate 100 3-inches strokes per min.

Estimation of growth: Growth was measured by the optical density at 550 mμ in a Coleman junior-type spectrophotometer.

Analytical methods: An aliquot of culture supernatant was employed for the quantitative determination of amino acids, ammonia and glucose as follows: Free amino acids in the supernatant were determined by colorimetric analysis using the ninhydrin reagent according to the method of Moore and Stein modified by Troll and Cannan (1953), ammonia, by the Conway method (1950) and Glucose, by the colorimetric method of Somogyi (1945).

RESULTS

Correlation between biotin and some amino acids in the growth of Candida albicans.

Preliminary experiments have shown that the growth of *Candida albicans* is markedly enhanced when a vitamin-free casein hydrolysate is added to Glucose-Simmons medium containig sufficient biotin. Such a growth enhancing effect of the casein hydrolysate was also observable when the concentration of biotin was reduced so that there was only very poor growth, indicating that the casein hydrolysate has the biotin-sparing effect for certain amino acids in the hydrolysate. To study this, the effect eighteen individual amino acids was examined.

A small amount of washed cells was resuspended into a basal media containing 0, 0.001, 0.01, 0.1 and 1 mμg/ml of crystalline biotin respectively with or without 1 mg/ml of various *l*-amino acids (or 2 mg/ml of *dl*-amino acids). These suspensions were then each dispensed in 7 ml in specially designed test tubes an incubated at 37°C and the growth was measured after 48 hours incubation at an optical density of 550 mμ in the Coleman junior-type spectrophotometer. In the basal medium the biotin content is critical for growth of *Candida albicans*. Growth increased with increasing quantities of biotin to a maximum at a concentration of 0.1 mμg/ml. Out of eighteen amino acids tested, only six, that is, aspartic acid, glutamic acid, arginine, proline, serine and α-alanine were found to have a "biotin-sparing effect". The results with aspartic acid and with histidine are presented in Fig. 1. (The results with other amino acids which had a similar effect are omitted from this figure.) The growth enhancing effect of aspartic acid was striking. As is shown in the figure, even in

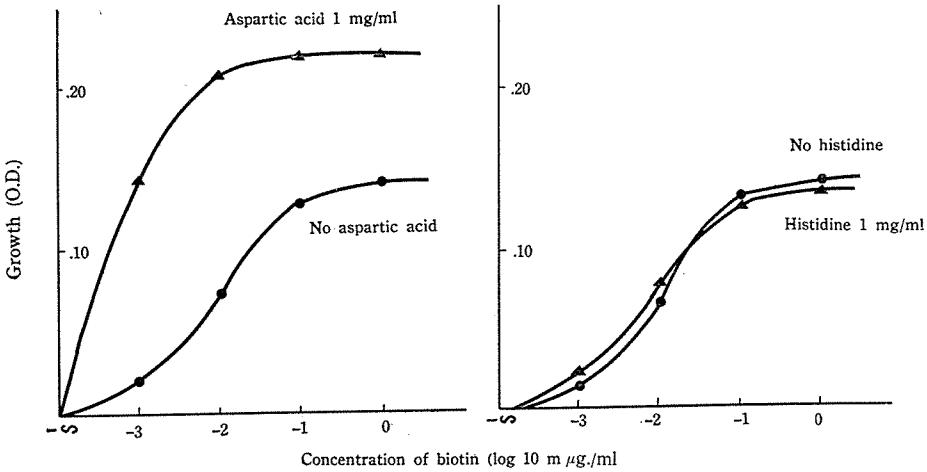


Fig. 1. Comparison of effects of aspartic acid and of histidine on the growth of *Candida albicans* FIA-1010 in relation to the concentration of biotin. The organism was grown at 37°C for 48 hours in still culture.

the presence of biotin at the concentration of 0.001 mμg/ml., the growth obtained in aspartic acid containing medium is more than the maximal growth obtained in the basal medium supplemented by a sufficient amount of biotin. With histidine, one of the amino acids with no biotin-sparing effect, no significant difference in growth was seen in its presence and absence. These results show

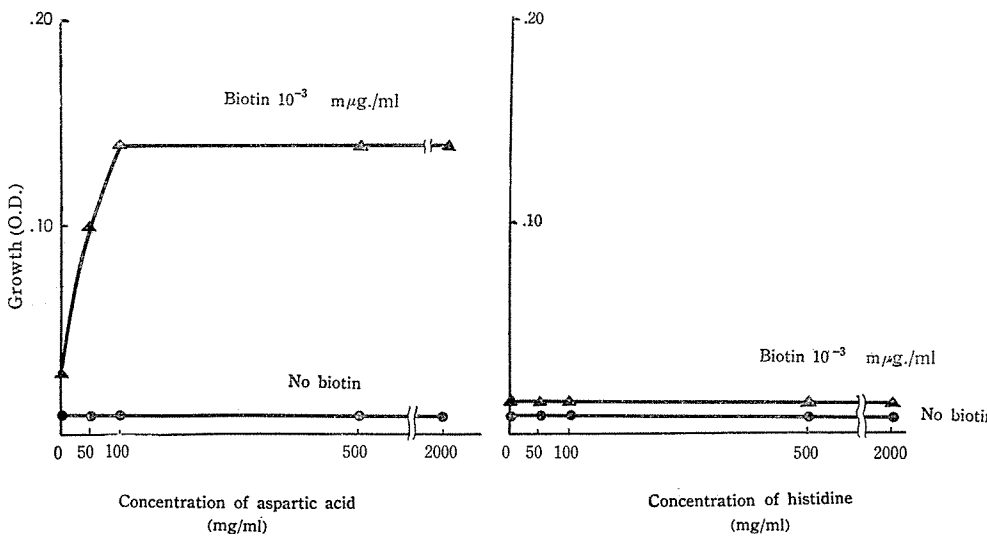


Fig. 2 Comparison of effects of aspartic acid and histidine on the growth of *Candida albicans* in the presence of suboptimal amounts of biotin in relation to the concentration of the amino acids. The organism was grown at 37°C for 48 hours in still culture.

that the growth enhancing effect of aspartic acid is displayed at a suboptimal concentration of biotin better than in the presence of optimal amounts of this particular vitamin. Further experiments showed that growth at a suboptimal concentration of biotin increases as a function of the concentration of aspartic acid in the medium. As is seen in Fig. 2, growth markedly increases with increasing aspartic acid concentration reaching a maximum at a concentration of 100 μg/ml. No such effect is observable with histidine even at a concentration of 2000 μg/ml.

These results suggest that biotin is closely associated with the metabolism of the particular amino acids with biotin-sparing effects and has an important role of these amino acids in the growth of *Candida albicans*. Although these amino acids have a common feature in having a biotin-sparing effect, they are quite different from each other in terms of their effects on the growth of the organism. Thus the effect of these amino acids on the growth was observed, in the presence of suboptimal amount of biotin, in the three different media prepared by omitting glucose, ammonium salt or both from the basal medium.

As is seen in Table 1, in the presence of glucose, all amino acids tested appear to be utilized as a sole source of nitrogen, while the amount of growth obtained with these six amino acids is much greater than that with amino acids that show no biotin-sparing effect. Of these six amino acids, however, only three, that is, aspartic acid, glutamic acid and arginine, were found to enhance growth in the absence of glucose. It is, therefore, considered that these three amino acids are utilized as sole sources of both carbon and nitrogen in the growth of the organism. No significant effect of ammonium salt on the growth of the organism was observed when the media was supplemented by certain available amino acids.

Table 1. Effect of amino acids on the growth of *Candida albicans* FIA-1010

*Medium	Incubation (hrs)	$(NH_4)_2HPO_4$	Glucose	*l*-aspartic acid	*l*-glutamic acid	*l*-arginine	*l*-proline	*dl*-serine	*dl*-α-alanine	*l*-histidine	*l*-methionine	*l*-tryptophane	No addition
A	48		0.540	0.05	0.08	0.100	0	0	0	0	0	0	0
	60			0.15	0.16	0.230	0	0	0	0	0	0	0
B	48			0.015	0.05	0.023	0	0	0	0	0	0	0
	60			0.132	0.132	0.080	0	0	0	0	0	0	0
C	24	0.015		0.022	0.043	0.075	0.023	0.007	0.008	0.005	0.010	0.009	0
	48	0.540		0.640	0.740	0.720	0.650	0.780	0.750	0.110	0.290	0.270	0.008

Note: (1) * Glucose-Simmons medium was used as a basal medium modified as follows:

A-medium: Glucose was omitted.

B-medium: Glucose and ammonium salt were omitted.

C-medium: Ammonium salt was omitted.

(2) Concentration of amino acids; *l*-form: 1 mg/ml, *dl*-form: 2 mg/ml.

(3) *Candida albicans* FIA-1010 was grown at 37°C in still culture and the figures in the table represent O. D. of cultures at 550 mμ in a Coleman junior-type spectrophotomer.

Growth of Candida albicans and its relation to amino acids in the media.

From the preceding results, it is considered that the six amino acids with biotin-sparing effects can be divided into two groups, that is, 1) the amino acids which are utilized as a source of both nitrogen and carbon (aspartic acid, glutamic acid and arginine), and 2) the amino acids utilized only as a source of nitrogen in the presence of glucose. Thus, when the organism is grown in a medium containing no ammonium salt but sufficient amounts of amino acids belonging to the second group, the growth increases with the concentration of glucose in the medium. With proline, there appears to be a lag time which is lengthened with decreasing amounts of glucose (Fig. 3). However, no change in lag time is seen when two amino acids, serine and α-alanine, are used as the sole sources of nitrogen.

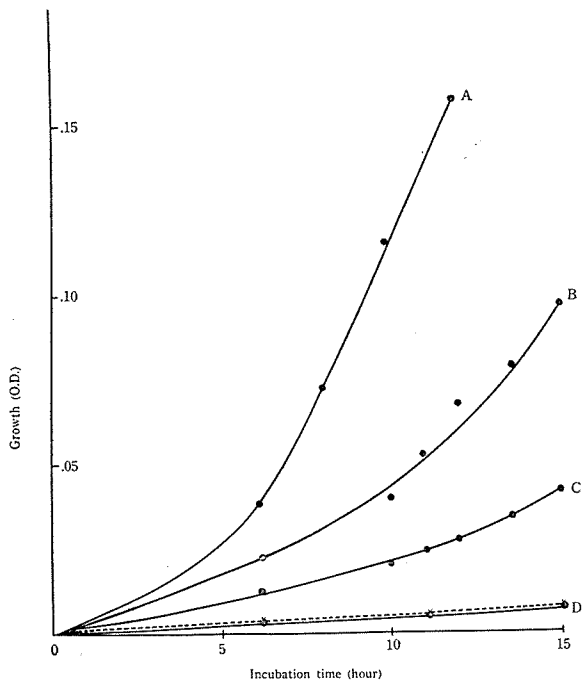


Fig. 3. Effect of glucose content on the pattern of growth of *Candida albicans* in C-medium supplemented by proline.

Candida albicans was grown at 35°C with shaking (approximately at 100 3-inches strokes per minute).

——————: C-medium + proline (1 mg/ml)
glucose concentration:
A : 10
B : 1.0
C : 0.1
D : 0
} μg/ml

.....: C-medium + glucose (10 μg/ml).

Growth of *C. albicans* in a medium containing sufficient amounts of glucose and of the amino acids with biotin-sparing effect as the sole sources of nitrogen are

presented in Fig. 4. Amino acids can therefore be devided into two further groups in terms of their effect on growth. Thus, there is about a six hours a lag with both α-alanine and serine, while no lag is seen with the other four amino acids.

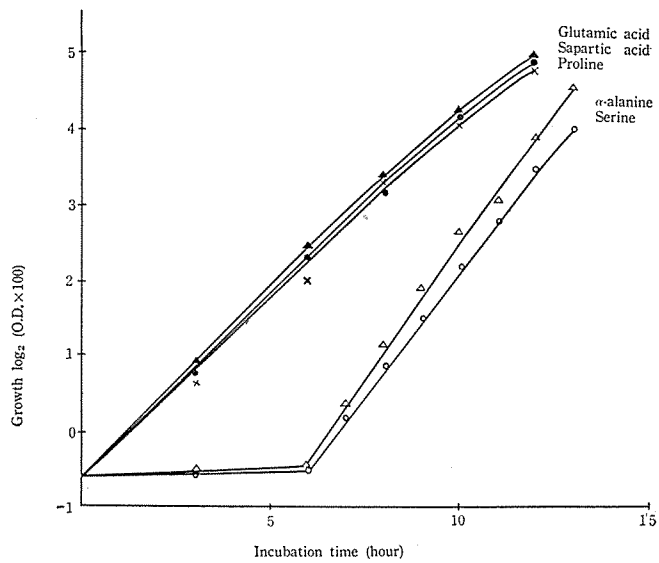


Fig. 4. Effect of amino acids on the growth of *Candida albicans*.
Candida albicans was grown at 35°C with shaking approximately at 100 3-inches strokes per min.

A similar difference between these two groups of amino acids has also been shown when they are added to a medium containing limited amount of glucose but sufficient ammonium salts. Fig. 5 show this difference. With proline, aspartic acid, glutamic acid and of arginine, the growth of the organism proceeded almost linearly as a function of the incubation time. However, with α-alanine and serine, the organism grew almost linearly during about 4 hours incubation and then growth gradually slowed down and finally, after about 1.5 hours lag time, the organism again continued to grow at a linear rate. No second phase or growth was observable in the basal medium with or without the amino acids having no biotin-sparing effect.

Considering the findings reported by Monod *et al.* (1942), the diauxie growth curve thus obtained with α-alanine and serine indicates that, in the second phase of growth, these amino acids must be utilized as a sole source of energy, by the organism suggesting the formation of adaptive enzymes which are responsible for decomposing these amino acids. Quantitative determinations of the growth of the organism in varying concentrations of glucose and amino acids have further revealed that this may be in the case.

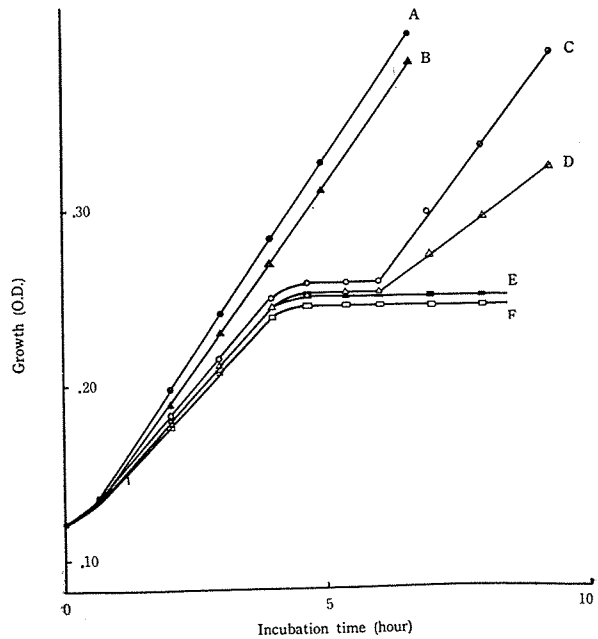


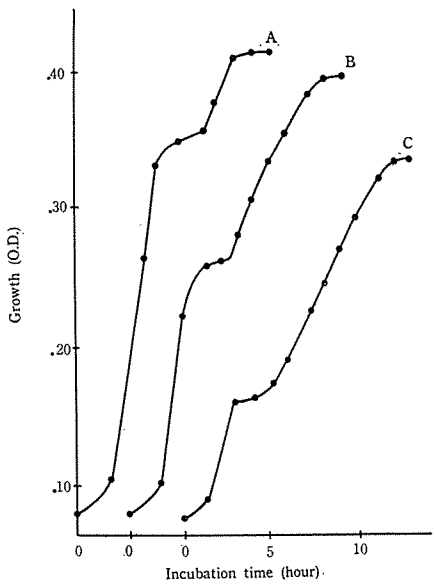
Fig. 5. Growth of *Candida albicans* in GS medium* containing sufficient biotin and relation to supplemented by amino acids.

Note: A : Glutamic acid 1 mg/ml
B : Proline 1 mg/ml
C : α-alanine 2 mg/ml
D : Serine 2 mg/ml
E : Methionine 1 mg/ml
F : Basal medium
(Glucose-Simmons medium)

* : The glucose concentration of the medium used in this experiment was 0.05%.

Candida albicans was grown at 35°C with shaking approximately at 100 3-inches strokes per min.

In these experiments, the total concentration of α-alanine and glucose was maintained at 2 mg per milliliter of basal medium and the relative concentrations of glucose and α-alanine was varied in the following order: (A) 3 : 1; (B) 2 : 2; (C) 1 : 3. Fig. 6 shows that growth in the first phase of the diauxie growth curve was equivalent to the glucose concentration in the culture media, while in the second phase it was equivalent to the α-alanine concentration. It is therefore suggested that, in the first phase of the diauxie growth curve, glucose is utilized, but in the second phase, α-alanine serves as the sole source of energy. To substantiate this speculation, further quantitative experiments were performed.



Note: A—α-alanine 500 μg + glucose 1500 μg/ml
B— " 1000 μg + " 1000 μg/ml
C— " 1500 μg + " 500 μg/ml

The culture was grown in a T-shaped shaking flask containing 10 ml medium and were incubated at 35°C on a reciprocating shaker adjusted to approximately 100 3-inches per min.

Fig. 6. Diauxie growth of *Candida albicans* and its relation to the relative concentration of glucose and α-alanine.

Quatitative determination of glucose, ammonia and amino acid in culture medium during diauxie growth.

A culture was grown in a 500 ml shaking flask containing 100 ml of Glucose (400 μg/ml)-Simmons medium supplemented by α-alanine (600 μg/ml) and biotin (10 mμg/ml). At one hour intervals, 6 ml of the culture were harvested from the shaking flask and centrifuged at 4,000 rpm for 15 minutes. The supernatant thus obtained was employed for quantitative determination of glucose, amino acids and ammonia. Free amino acids in the supernatant were determined by colorimetric analysis using the ninhydrin reagent. Results are shown in Fig. 7. Glucose was completely utilized by the end of the first phase of the diauxie growth of the organism. Free amino acids decreased markedly in concentration in the culture supernatant during the interphase of the growth cycle. The amount of ammonia was found to gradually decrease in the first phase of the growth and increase reciprocally with decrease in free amino acid content. These results indicate that in the second phase of the diauxie growth of *Candida albicans* α-alanine is utilized as the sole exogenous source of carbon and energy rather than of nitrogen and suggests that the enzymes which are responsible for the utilization of this amino acid, must be synthesized adaptively during the interphase of the growth.

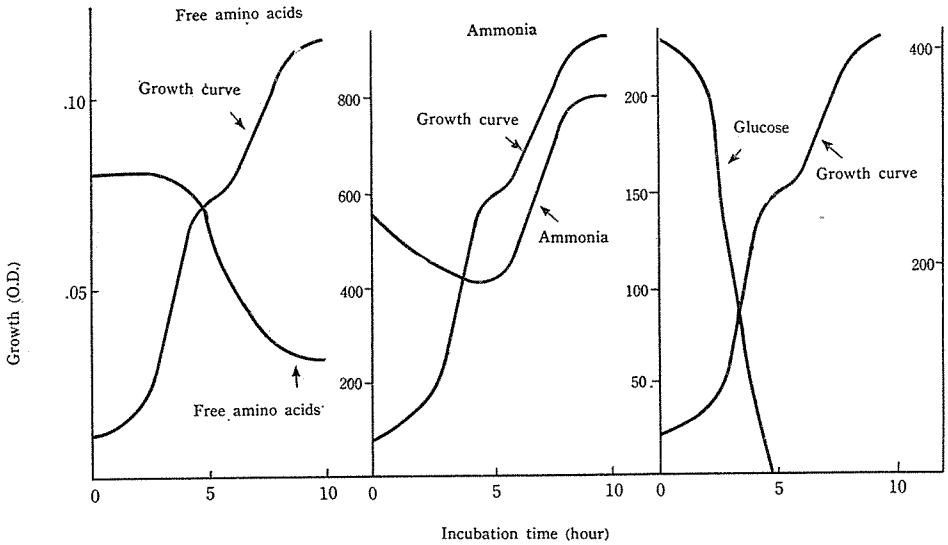


Fig. 7. Changes in concentration of free amino acids, ammonia and of glucose in the cultural supernatant of *Candida albicans* during diauxie growth.

The culture was grown in a one liter shaking flask containing 100 ml medium and were incubated at 35°C with shaking at 100 3-inches strokes per min. Right Ordinate: μg/ml.

DISCUSSION

In 1951, Broquist *et al.* showed that, when the aspartic acid content was raised to 50-100 μg/ml. the requirement of biotin by *Lactobacillus* was markedly reduced. The phenomenon had been described as the so-called "biotin-sparing effect" of the particular amino acid by S. A. Koser, *et al.* (1942). Our previously described nutritional studies (Miyashita *et al.*, 1958) demonstrated that *Candida albicans* did not grow in Glucose-Simmons medium unless some biotin were added to the basal medium. Thus *C. albicans* is exacting only for biotin and no amino acid was found to be required by the organism for normal growth. The observations obtained in the present study, however, have shown that certain amino acids have an additive effect in enhancing the growth of the organism only in the presence of biotin. Furthermore, when these amino acids were added to the basal medium, the amount of biotin (0.1 mμg/ml) required to support maximal growth of *C. albicans* was markedly decreased to such a degree that, at a concentration of 0.001 mμg/ml. only poor growth could take place without these amino acids. Although this phenomenon was considered similar to that described by Broquist *et al.*, there were found to be at least six amino acids having so-called "biotin-sparing effect" on the growth of *C. albicans;* glutamic acid, aspartic acid, arginine, proline, serine and α-alanine.

It is well known that biotin is closely related to nitrogen metabolisms, especially, to that of some amino acids in microorganisms. For example, Winzler *et al.* (1944) demonstrated that washed cells of biotin-deficient yeast (*Saccharomyces cerevisiae*) could not assimilate ammonia unless biotin was added. It is therefore assumed that the vitamin plays an essential part in the metabolic sequences which

are responsible for amino acid synthesis from ammonia. Also, from the investigations of Lichstein and Umbreit (1947) and Lichstein and Christman (1948), it was suggested that biotin might be a part of the structure of the prosthetic group of aspartase, serine and threonine deaminase. Although the intrinsic part played by biotin in the metabolism of glutamic acid, arginine, proline and α-alanine is not yet clear, the present results clearly suggest that biotin must be involved in some way in the metabolic sequences of these amino acids in *C. albicans*, as in that of serine and aspartic acid in the deamination of which biotin has in other organisms been proved to act as a co-factor.

The fact that *C. albicans* is exacting only for biotin indicates that all amino acids are synthesized from ammonium salts and glucose in the presence of the vitamin. In other words, the vitamin must be involved in the synthesis of some amino acids by the organism. Therefore, one way of explaining the "biotin-sparing effect" of these six amino acids during the growth of *C. albicans*, is that biotin is closely associated with the enzymatic synthesis of these amino acids. Addition of these ready-made amino acids could thus result in decrease in the amount of biotin necessary for their synthesis. However, since *C. albicans* can not grow in the absence of biotin even when these amino acids are added to the basal medium, the effect of these amino acids in the organism may also be partially due to another mode of action of biotin such as that has been described by several investigators (Ochoa *et al.*, 1947; Shive and Rogers, 1947; Lardy *et al.*, 1949 etc.) in other organism. Although these six amino acids all show the "biotin-sparing effect", their nutritional role in the growth of *C. albicans* appears to differ as indicated by growth experiments using GS medium with or without the added glucose and ammonium salts. Thus, while all these amino acids can be utilized as the sole source of nitrogen in the presence of glucose and biotin, three including glutamic acid, aspartic acid and arginine were found to be utilized as the sole source of both nitrogen and carbon.

Kinetic studies on the effect of these amino acids on patterns of growth of the organism have shown that diauxie growth takes place in a medium contain a sufficient amount of ammonium salt but limited glucose when the medium is supplemented by α-alanine or serine. In this case, these amino acids appear to be utilized as a source of carbon and energy rather than as a source of nitrogen.

The growth in the first phase of the diauxie growth was always equivalent to the quantity of glucose, while that in the second phase to the amount of α-alanine or serine.

The suggestion drawn from these observations is that both α-alanine and serine may serve as the sole source of energy in the second phase of growth. This suggestion was justified from a quantitative estimation of the glucose, ammonia and amino acid content during the whole growth cycle.

Diauxie growth was first described by Monod (1942) in the growth of *Bacillus subtilis* in the medium containing a limited amount of various carbohydrates. More recently, Kinjo (1955) has reported that a similar phenomenon occurs in the growth of *Aerobacter aerogenes* and *Vibrio sp.* when the medium is supplemented by different amino acids and also suggested that the amino acids in the second phase

must be utilized adaptively as a source of nitrogen. The present observation with *C. albicans* clearly indicates that both the amino acids, α-alanine and serine, are dissimilated in the second phase of diauxie growth of the organism, serving as the sole source of energy.

Since a significant decrease in the concentration of these amino acids in the culture medium only begins after the complete exhaustion of glucose by the organism it is suggested that the enzymes which are responsible for the dissimilation of these amino acids, must be synthesized adaptively, resulting in the appearance of a interphase before the second growth cycle occurs. Although the intrinsic relationship between biotin and these amino acids in the growth of *C. albicans* is still uncertain, the present study clearly demonstrates that the amino acids, which have a biotin-sparing effect, appear to behave differently in terms of their effects on the growth of *C. albicans*. The true interpretation of these effect, however, must await further biochemical studies on the role of biotin in the metabolism of these amino acids in the organism. It may be that these amino acids are involved in the biosynthesis of biotin in the organism.

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