BIOTIN DEFICIENCY AND THE SPARING EFFECT OF NORDIHYDROGUAIARETIC ACID IN *NEUROSPORA CRASSA*

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The requirement of wild-type *Neurospora crassa* for biotin in growth and survival was examined. Extensional growth rates during biotin depletion were measured. With egg-white avidin to sequester trace biotin contamination in purified medium, the requirement was absolute. The presence of the free radical scavenger, nordihydroguaiaretic acid (NDGA), enhanced growth rate during either moderate or severe biotin limitation. Conidia did not undergo biotinless death, even in the presence of avidin. Apparently, endogenous biotin concentration was sufficient for survival. Since biotin is a cofactor for fatty acid synthesis and biotin deficiency depletes the cellular lipid content, the results of these experiments are consistent with the hypothesis that nutritional deficiencies in lipid and membrane synthesis leading to synthesis of abnormal membranes promote excessive lipid peroxidation and free radical damage and, consequently, cellular senescence. Inhibition of these processes by NDGA therefore enhances the cellular growth rate with limiting biotin.

Nutritional auxotrophs requiring metabolites in lipid synthesis and, therefore, membrane synthesis, form abnormal membranes and die when cultured either with limiting amounts or without the necessary nutrient (1). The phenomenon, called unbalanced growth, and cellular death can be inhibited by inhibition of either nucleic acid or protein synthesis, either by a second mutation for an additional nutritional requirement in nucleic acid or protein synthesis, or by drugs that inhibit protein synthesis.

The common denominator in unbalanced growth leading to abnormal membranes may be excessive lipid peroxidation and free radical damage, processes that contribute to cellular senescence and death (1). Thus, the antioxidant, nordihydroguaiaretic acid (NDGA), inhibits inositol less death of conidia of *Neurospora crassa* and various antioxidants enhance the growth rate on limiting concentrations of inositol (2). Electron spin resonance spectroscopy reveals an excessive

concentration of free radicals in mycelia grown on limiting inositol, and growth with NDGA decreases the concentration (3). Furthermore, structural and functional abnormalities of mitochondrial and plasma membranes and excessive lipid peroxidation that occur during growth with limiting inositol are alleviated by NDGA (1, 4).

MIZUNAGA *et al.* (5) observed that biotin-deficient yeast cells, in the presence of aspartic acid, underwent unbalanced growth and death; whereas, fatty acid supplements with aspartate promoted growth and prevented cellular death (The requirement for aspartate is due to a biotin cofactor requirement for its synthesis from pyruvate). Similarly, HODSON (6) reported that growth of wild-type *N. crassa* in biotin-deficient medium was promoted by a mixture of aspartic and oleic acids. Furthermore, the degree of deficiency of cellular lipid content of *N. crassa* is proportional to the degree of biotin deficiency in the growth medium (7).

These conceptual and experimental developments promoted the present investigation of biotin deficiency in *N. crassa*. The prediction that NDGA should spare the biotin requirement is verified.

MATERIALS AND METHODS

Media and chemicals. VOGEL's minimal medium (8), without biotin, containing 2% enzyme-grade sucrose (Schwarz/Mann, Orangeburg, N. Y.) was treated with activated charcoal to remove traces of contaminating biotin by the method of RYAN *et al.* (9). Difco Bacto-Agar (U.S.P.) was washed with water and hot acetone (9). All glassware was washed with chromate-sulfuric acid solution and glass-distilled water.

For control media, *d*-biotin (CalBiochem, Los Angeles, Calif.) was added at 5 $\mu\text{g/l}$ before autoclaving. This concentration is the least necessary for maximal growth rate (9). Aliquots of a freshly prepared ethanolic solution of NDGA (Aldrich Chemical Co., Milwaukee, Wis.) (2) or an aqueous solution of avidin, Type I (Sigma Chemical Co., St. Louis, Mo.) were added to the medium after it was autoclaved and cooled to 55°. The nominal activity of the avidin was 10 units/mg protein where a unit is the quantity that will bind 1 μg of *d*-biotin.

Growth. Wild-type (1256, 74A8) conidia were transferred to the center of a 9-cm petri dish containing control agar medium. After overnight incubation at 29°, hyphal tips on agar plugs, 5 mm in diameter and 5 mm in height, were transferred to race tubes, 1.8 cm in diameter and 30 cm in length (9), containing 20 ml of agar medium and incubated in continuous fluorescent light at 29°.

RESULTS

In the absence of biotin supplement to purified medium, growth proceeded at a sub-normal steady-state rate for about 35 hr due to residual biotin in the

Fig. 1. Extensional growth rates of wild-type *N. crassa* during biotin depletion and the sparing effect of nordihydroguaiaretic acid.

Purified VOGEL's minimal agar was used. Rates are the average of duplicate tubes at 29°. Conidia of wild-type 74A8 were germinated on agar plates with 5 µg biotin/l. Hyphal tips with agar plugs (METHODS) were used to inoculate race tubes. (1) with 5 µg biotin/l; (2) without biotin; (3) without biotin plus NDGA (10 µg/ml); (4) without biotin plus avidin (0.01 unit/ml); (5) without biotin plus avidin and NDGA.

inoculum, and subsequently declined in rate during the next 45 hr to a new steady state of 0.4 mm·hr⁻¹ (Fig. 1). Avidin inhibited the initial steady-state growth rate, led to an earlier decline of growth rate, and complete inhibition of growth after 120 hr. Evidently, avidin sequestered the residual trace amount of biotin in the purified medium.

NDGA in the biotin-deficient medium, either with or without avidin, enhanced the initial steady-state growth rate and delayed the time before subsequent onset of deceleration of growth rate. NDGA also significantly enhanced the final steady-state growth rate in medium without avidin.

In other experiments, NDGA at the same concentration neither inhibited nor stimulated the normal steady-state growth rate on the control medium with 5 µg biotin/l. Avidin alone (0.1 unit/ml) inhibited the growth rate 50%, but with avidin and NDGA, growth was inhibited 32%. Thus, with either severe or slight biotin deficiency, NDGA had a "sparing" effect.

Incidentally, we confirmed ZALOKAR's observations (10) that biotin deficiency

inhibits conidiogenesis and carotenoid formation. Therefore, it is not possible to prepare biotin-deficient conidia.

Conidia were collected from normal cultures, washed three times with water, plated at 10^3 /plate on purified biotin-free agar medium alone, or supplemented with avidin (0.1 unit/ml), or with avidin plus aspartate (200 μ g/ml), incubated at 30° for 4 days, overlaid with control agar medium containing 5 μ g biotin/l, and incubated 4 days at 30°. All of the treatments gave luxuriant growth. Apparently, the endogenous biotin in conidia was sufficient to allow survival and prevent biotinless death.

DISCUSSION

Nutritional deficiencies of inositol and biotin in *N. crassa* appear to be analogous in that both inhibit lipid and carotenoid syntheses, and that the antioxidant NDGA enhances the growth with limiting concentrations of the vitamins.

One important difference between the inositol and biotin requirement is that the concentrations necessary for half-maximal growth rate are 2 and 2×10^{-5} μ g/ml, respectively (9). Hence, it is far more difficult to establish the absolute requirement of biotin for growth and conidial survival because of contamination of the medium with trace amounts of biotin and the endogenous biotin in conidia. With purified medium containing avidin, the growth requirement of wild-type for biotin appeared to be absolute.

The phenomenon of biotinless death appears to be related to the amount of biotin required for germination and growth. STRIGINI and MORPURGO (11) reported that the concentration of biotin necessary for germination of conidia of wild-type *Aspergillus nidulans* was 1/10 to 1/100 the concentration required by the biotinless mutant *bi-1*. Only the *bi* mutant and not wild-type *A. nidulans* undergoes biotinless death.

Although conidia of *N. crassa* do not undergo biotinless death, the deterioration of growth rate of mycelia during biotin depletion probably is caused, in part, by the interdependent processes; membrane deterioration, lipid peroxidation, and free radical damage. In analogy to inositolless senescence (1, 2, 4), the sparing effect of NDGA during biotin depletion would be by the inhibition of these processes as a free radical scavenger.

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