AMINO ACID INTERACTIONS IN *NEUROSPORA CRASSA*

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Received for publication December 8, 1960

ABSTRACT

SOBOREN, JOSEPHINE (University of California, Los Angeles), AND JOSEPH F. NYC. Amino acid interactions in *Neurospora crassa*. J. Bacteriol. **82**:20–25. 1961.—A systematic study of the effects of the naturally occurring amino acids on the growth of a wild-type strain of *Neurospora crassa* focused attention upon L-tryptophan, which exhibits a strong growth inhibitory effect. Further investigation disclosed that other tryptophan metabolites, anthranilic acid, indole, kynurenine, and 3-hydroxykynurenine also inhibit growth. The proposed antimetabolic role of these aromatic compounds explains the poor growth response of certain tryptophan-requiring strains of *N. crassa* to tryptophan supplements. The growth of normal and mutant strains of *N. crassa* on media supplemented with tryptophan is influenced by the presence of other amino acids.

This work was undertaken to obtain a better understanding of the variables which affect amino acid balance in *Neurospora crassa*. The growth requirements of this organism make it ideal for studies concerning substrate interactions at different concentration levels. *N. crassa* has additional advantages over many other species because it lends itself experimentally to biochemical, genetic approaches. In previous work with this organism, the main emphasis has been on the metabolic antagonisms that exist between two or more amino acids rather than on the need for a metabolic balance between these nutrients. A brief review of the amino acid antagonisms existing in *N. crassa* was included in a recent publication (Brockman, De Busk, and Wagner, 1959).

A systematic study in our laboratory on the effects of various naturally occurring amino acids on the growth of the normal wild-type strain focused attention upon L-tryptophan. This natural metabolite exhibits a strong inhibitory effect upon the growth of the organism when added to the medium. Further studies based on this observation were concerned with the inhibitory role of tryptophan and also with the response of *N. crassa* to substances that are metabolically in equilibrium with this amino acid. A significant finding resulting from these investigations was the observation that the tryptophan inhibitions were dependent upon the presence or absence of other amino acids. These studies were also extended to a mutant strain of *N. crassa* that lacks the ability to make tryptophan. The latter studies parallel in part earlier work (Shanmuga Sundaram and Sarma, 1954) on a strain of *N. crassa* mutant 39401 which has partially lost its ability to make aromatic precursors of tryptophan.

MATERIALS AND METHODS

Cultures. The strains of *N. crassa* employed were the normal wild-type strain 1A and a tryptophan-requiring mutant strain C-83 previously described (Mitchell and Lein, 1948). Mutant C-83 apparently cannot form tryptophan from indole-3-glycerol phosphate, its immediate precursor. Tracer studies (Partridge, Bonner, and Yanofsky, 1951) have shown that the genetic block concerned with tryptophan synthesis in strain C-83 is complete since the mutant cannot make tryptophan in quantities which are experimentally detectable. Growth of this organism is therefore completely dependent upon exogenous tryptophan. It is not restored to a normal growth by tryptophan supplements.

A single key experiment was undertaken with a second tryptophan-requiring mutant, strain 39401, in an effort to determine if the inhibition response of this organism to various amino acids was stereospecific. This information was essential to reconcile our results on strain C-83 with previous work on strain 39401 by Shanmuga Sundaram and Sarma (1954). This nicotinic acid dependent strain of *N. crassa* (39401), previously described (Mitchell and Nyc, 1948), is able to utilize indole, tryptophan, kynurenine, 3-hydroxykynurenine, 3-hydroxyanthranilic acid, and

nicotinic acid. Although the phenotypic behavior of the organism suggests that its mutation results in an inability to convert anthranilic acid to indole-3-glycerol phosphate, a genetic analysis (Haskins and Mitchell, 1952) provided evidence that the primary mutation was concerned with a biosynthetic step involving a precursor of anthranilic acid. Haskins and Mitchell also showed that the phenotypic behavior of 39401 was influenced by modifying genes and that this organism could be adapted to grow on quinic acid, tyrosine, phenylalanine, and anthranilic acid. Tracer studies showed that even the unadapted strain could synthesize tryptophan from anthranilic acid and other precursors.

Procedure for growing molds. The normal strain 1A was cultured in 125-ml Erlenmeyer flasks on 20 ml of Fries minimal medium (Beadle and Tatum, 1945) buffered at pH 5.6 for 72 hr at 25 C. Mutant strains C-83 and 39401 were cultured 96 hr under the same conditions in a medium that was always supplemented with 1 μmole of L-tryptophan per flask. Additional supplementations were made as described below. All growth responses are expressed as the dry weight of the mycelium after harvest.

Supplements. The anthranilic acid used was a product of Eastman Organic Chemicals. The 3-hydroxy-DL-kynurenine that was obtained from Mann Research Laboratories was recrystallized from water. All other chemicals employed as supplements to the minimal medium were from the California Foundation for Biochemical Research. Supplements of L-amino acids and other water soluble compounds to wild-type 1A and mutant 39401 were made at levels of 2.5, 5, 10, and 25 μmoles per flask. Amino acid supplements to strain C-83 were made at the levels 1, 5, 10, 20, and 40 μmoles per flask. All stock solutions for the supplements were at 25 μmoles per ml. The insoluble amino acids L-cystine and L-tyrosine were added as dry samples to the culture flasks. Since indole may be lost on autoclaving, it was filter sterilized in a solution of 50% ethanol before being added to autoclaved media. The sparingly soluble samples of 3-hydroxyanthranilic acid and 3-hydroxy-DL-kynurenine were weighed into small porcelain combustion boats, which were dropped into the hot culture flasks immediately on completion of the autoclaving procedure, thus effecting sterilization of the samples without prolonged heating and possible decomposition. Except for the few special procedures noted above, all other culture media were sterilized in an autoclave for 15 min at 121 C.

RESULTS

Growth of wild-type N. crassa on the naturally occurring amino acids. Eighteen L-amino acids were screened individually to determine their growth effect on wild-type *N. crassa* over a concentration range of 2.5 to 25 μmoles per flask. The amino acids investigated were isoleucine, lysine, methionine, phenylalanine, glutamic acid, aspartic acid, glycine, histidine, arginine, alanine, valine, cystine, proline, serine, threonine, tyrosine, tryptophan, and leucine. At the concentration of 10 μmoles per flask, arbitrarily chosen for comparison, all of the above amino acids except tryptophan, isoleucine, glycine, threonine, and cystine give less than 10% inhibition of growth. Of the inhibitory amino acids, tryptophan proved the most effective (Table 1). On this basis L-tryptophan was selected for further studies. Subsequent experiments were planned to study the synergistic effect of tryptophan in combination with varying amounts of other individual amino acids on the growth of strain 1A. In these studies the concentration of tryptophan was always 5 μmoles per flask. This is the lowest concentration which will cause a near maximal growth inhibitory effect. A systematic screening of all the L-amino acids over the range 2.5 to 25 μmoles per flask for their ability to overcome the growth inhibition caused by 5 μmoles of tryptophan disclosed that tyrosine, phenylalanine, methionine, leucine, alanine, and isoleucine could partially

TABLE 1. *Inhibitory effect of amino acids on growth of Neurospora crassa strain 1A*

Supplement	Growth of mold (mg)* on increasing amounts of amino acids, μmoles per 20 ml				
	0	2.5	5	10	25
L-Tryptophan	45	18	17	13	12
L-Lysine		35	35	31	30
L-Isoleucine		38	32	30	29
L-Threonine		43	39	25	31
L-Cystine		49	41	29	25
Glycine		42	39	30	16
L-Histidine		45	46	41	27

* Each value represents the average of triplicate samples.

TABLE 2. *Stimulatory effect of individual amino acids on growth of cultures of Neurospora crassa strain 1A inhibited by 5 μmoles of tryptophan**

Supplement	Growth of mold (mg)† on increasing amounts of amino acids, μmoles per 20 ml				
	0	2.5	5	10	25
L-Phenylananine	22	23	29	38	41
L-Tyrosine		35	36	41	49
L-Methionine		30	33	37	40
L-Leucine		32	31	35	33
L-Isoleucine			25	26	29
L-Alanine			30	26	34

* Growth on unsupplemented minimal medium is 46 mg.

† Each value represents the average of triplicate samples.

TABLE 3. *Inhibitory effect of tryptophan metabolites on growth of Neurospora crassa strain 1A*

Supplement	Growth of mold (mg)* on increasing amounts of tryptophan metabolites, μmoles per 20 ml				
	0	2.5	5	10	25
Anthranilic acid	49	29	13	1	0
Indole		39	25	4	0
L-Kynurenine		27	18	9	4
DL-Hydroxykynurenine	42	37	37	33	

* Each value represents the average of triplicate samples.

restore the growth of wild type to that obtained on minimal medium (Table 2).

Growth of wild-type N. crassa on tryptophan metabolites. The growth effect on strain 1A of various tryptophan cycle intermediates (Haskins and Mitchell, 1948) and of other tryptophan metabolites not involved in the cycle was determined by supplementation over the concentration range 2.5 to 25 μmoles per flask. Quinic acid, shikimic acid, quinolinic acid, 3-hydroxyanthranilic acid, and nicotinamide had a negligible growth inhibitory effect over this concentration range. It is significant that none of these compounds is involved in the tryptophan cycle postulated by Haskins and Mitchell (1948). L-Kynurenine, indole, and anthranilic acid were found to inhibit growth of the wild-type strain (Table 3). At concentrations up to 5 μmoles per 20 ml the inhibition by these materials was comparable to tryptophan; at higher levels these substances were more effective depressors of growth than tryptophan. DL-Hydroxykynurenine has a moderate inhibitory effect on growth (Table 3).

TABLE 4. *Stimulatory effect of individual amino acids on growth of Neurospora crassa strain C-83 supplemented with 1 μmole tryptophan*

Supplement	Growth of mold (mg)* on increasing amounts of amino acids, μmoles per 20 ml					
	0	2.5	5	10	20	40
L-Phenylalanine	4	11	15	20	0	0
L-Tyrosine		7	10	14	21	11
L-Methionine		8	11	15	23	0
L-Leucine		7	11	16	23	0
L-Valine		6	9	12	16	18
L-Isoleucine		7	8	10	14	16
L-Threonine		6	8	10	15	17
L-Alanine		5	7	10	13	21
L-Serine		5	6	9	12	14

* Each value represents the average of duplicate samples.

TABLE 5. *Supplement balance related to growth response of Neurospora crassa strain C-83*

Supplement, μmoles per 20 ml		Growth of strain C-83, mg per 20 ml*
L-Tryptophan	L-Leucine	
0	0	0
0.5	0	2.7
0.5	5	8.0
0.5	10	10.9
0.5	15	0
0.5	25	0
1.0	0	4.0
1.0	5	10.6
1.0	10	15.1
1.0	15	21.3
1.0	25	8.2
1.0	40	0
2.5	0	9.7
2.5	40	38.4

* Each value represents the average of duplicate samples.

Growth of strain C-83 on the naturally occurring amino acids. N. crassa strain C-83 when supplemented with 1 μmole of tryptophan per 20 ml of media gives a growth response that is less than 10% of that obtained with the normal

wild-type strain. The increased growth response of this organism to greater tryptophan supplements is approximately linear up to 5 μmoles per 20 ml at which level the weight of the mycelium (about 20 mg per culture) is still less than one-half of that expected for normal strains.

Studies were undertaken to determine the individual effect of 17 naturally occurring amino acids on the growth of strain C-83 when it was cultured on a suboptimal tryptophan supplement of 1 μmole per 20 ml culture. The amino acids, histidine, glutamic acid, aspartic acid, glycine, proline, lysine, and arginine, were found to have a negligible effect on the growth obtained with 1 μmole of tryptophan. Cystine was the only amino acid found inhibitory over most of the concentration range tested. Phenylalanine, leucine, methionine, isoleucine, serine, tyrosine, valine, threonine, and alanine were found to stimulate the growth of strain C-83 (Table 4). Valine, alanine, threonine, isoleucine, and serine stimulated the growth of strain C-83 over the entire concentration range considered, but tyrosine, leucine, methionine, and phenylalanine stimulated only at lower concentrations, drastically inhibiting growth at higher concentrations. The growth response of strain C-83 when cultured on tryptophan supplemented with leucine was found to be dependent on the relative concentrations of the two supplements (Table 5).

Growth of strain 39401 on L*-tryptophan in the presence of other* L*- and* D*-amino acid supplements.* Shanmuga Sundaram and Sarma (1954) found that several of the amino acids listed in Table 4 inhibited the utilization of tryptophan by strain 39401. When there appeared to be a discrepancy between the response of strain C-83 and strain 39401 to a particular amino acid it was noted that earlier workers had employed a DL-amino acid. Alanine, threonine, and valine were arbitrarily selected to ascertain whether this apparent discrepancy might be explained by the metabolic stereospecificity of the supplements. In the present study the L-isomers of these amino acids were found to stimulate the growth of strain 39401, cultured on 1 μmole of tryptophan, at all levels investigated. The corresponding D-isomers have no appreciable effect on growth.

DISCUSSION

In *N. crassa* it has been shown (Haskins and Mitchell, 1948) that tryptophan disappears rapidly from a culture medium that has been inoculated with wild-type or tryptophan-requiring strains. A large part of the tryptophan of the medium disappears before there is appreciable growth of mold by conversion to other compounds, notably the fluorescent products of tryptophan decomposition. At least one-fourth of the tryptophan degraded by the mold is converted to substances lacking biological activity for the tryptophan series of *N. crassa* mutants. Both wild-type and tryptophan-requiring mutants produce a blue fluorescence during the course of growth on tryptophan supplements. This fluorescence reaches a maximal intensity and then diminishes except in mutants, such as strain C-83, which are unable to utilize anthranilic acid, in which case the fluorescence persists. On the basis of these studies Haskins and Mitchell suggested that some of the intermediates of tryptophan breakdown are also involved in a synthesis of tryptophan, thus forming an internal tryptophan cycle in this organism.

The original cycle included anthranilic acid, indole, tryptophan, kynurenine, and possibly three other biologically active fluorescent substances whose structures remain unknown. Recent work (Yanofsky and Rachmeler, 1958) indicates that indole is not directly involved in tryptophan synthesis, but may act as an exogenous substitute for indole-3-glycerol phosphate, which is the naturally occurring intermediate in the cycle. The position of anthranilic acid in the cycle with respect to the aromatic precursors entering this metabolic pathway has been questioned (Partridge et al., 1951). The elucidation of the pathway from anthranilic acid to tryptophan through indole-3-glycerol phosphate as an obligatory intermediate suggests that anthranilic acid is on the main pathway of tryptophan synthesis in *N. crassa* (Partridge et al., 1951).

Of interest is the present finding that a normal strain of *N. crassa* is inhibited by all of the substances known to participate in the tryptophan cycle. With the exception of 3-hydroxykynurenine, an immediate product of the cycle, none of the other known tryptophan metabolites which was tested inhibits growth appreciably. This suggests that when substances metabolically associated with the tryptophan cycle are given in large amounts as exogenous supplements they are interconverted into various products at a rate that

is incompatible with normal growth. The supplementation of tryptophanless mutants with required metabolites can lead to undesirable inhibitions by the very agents intended to stimulate growth. This may explain the poor growth response to tryptophan of mutants such as C-83 which are blocked within the tryptophan cycle. The supplementary antagonism may be exaggerated in these organisms because they accumulate intermediates of the tryptophan cycle as a result of their mutations (Haskins and Mitchell, 1948).

The same amino acids which negate tryptophan inhibition in a normal strain also increase the growth response of a tryptophanless mutant to tryptophan supplements. A comparison of the relative efficiency of these amino acids with respect to their expression in these two assay systems shows a high correlation. This is further evidence that the poor growth of strain C-83 on a tryptophan supplement is due to an antagonism by the same products that inhibit the normal strain when it is grown in the presence of tryptophan. The data in Table 5 show that the growth response of strain C-83 to tryptophan and leucine is dependent on the molar ratio of these metabolites. Amino acid balance studies of this type are being continued in an effort to elucidate the metabolic significance of these synergisms in terms of specific enzyme functions.

The inhibitory effect of various amino acids upon the enzymatic turnover of tryptophan metabolites has been investigated in two laboratories. Jakoby and Bonner (1953) noted that the inhibitory effect of many amino acids upon the enzyme kynureninase is due to the combination of the amine with pyridoxal phosphate, thereby removing this cofactor. Shanmuga Sundaram and Sarma (1954) studied the effect of individual amino acids on the utilization of L-tryptophan by a mutant (39401) of *N. crassa*, and suggested that certain amino acids inhibit the various enzyme systems which are taking part in the conversion of tryptophan to nicotinic acid. This suggestion was based on experiments in which six of the growth-stimulating amino acids for strain C-83 listed in Table 4, phenylalanine, methionine, leucine, isoleucine, threonine, and serine, were found to inhibit the growth response of strain 39401 to tryptophan supplements. These authors may have been working at different substrate levels than those employed in the present studies and therefore were not aware of the potential stimulatory role of these amino acids. The organism used by Shanmuga Sundaram and Sarma, strain 39401, is an incompletely blocked mutant known to change its phenotypic behavior by adaptive response to certain substrates. Some of the discrepancy between the results with strains 39401 and C-83 may be inherent in the strains themselves. The substitution of DL-amino acids for the natural isomers in these studies does not appear to be a serious variable as far as qualitative results are concerned. Shunmuga Sundaram and Sarma (1955) also studied the effect of some protein hydrolyzates on the utilization of tryptophan and its metabolites by strain 39401. The protein hydrolyzates were found to inhibit the utilization of tryptophan, kynurenine, and 3-hydroxykynurenine, whereas they had no influence on the utilization of 3-hydroxyanthranilic acid and nicotinic acid. These authors suggested that certain amino acids in the hydrolyzate inhibit the enzyme systems responsible for the conversion of tryptophan to 3-hydroxyanthranilic acid.

Previous studies (Jakoby and Bonner, 1953; Shanmuga Sundaram and Sarma, 1954, 1955) were concerned primarily with the inhibitory role of amino acids on tryptophan metabolism. The present studies are based on the observation that certain amino acids can negate the growth-inhibiting effect of tryptophan metabolites on molds. Future studies will be made to determine the metabolic significance of these findings in *N. crassa*.

ACKNOWLEDGMENTS

The authors wish to thank Herschel K. Mitchell of the California Institute of Technology for kindly supplying strains C-83 and 39401.

This study was supported in part by a grant (RG-5794) from the National Institutes of Health, and by the Cancer Research Fund of the University of California.

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