GROWTH INHIBITION OF NEUROSPORA BY CANAVANINE, AND ITS REVERSAL*

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Canavanine, an amino acid from jack beans, was discovered by Kitagawa and coworkers in 1929 (1, 2). The substance is not combined in the proteins of the seed, but occurs in the free state, and makes up 2.5 per cent of the dry weight of jack beans (3). In a series of papers available to the authors for the most part in abstract only, the Japanese workers have reported extensive investigations into the chemistry and physiology of the substance. The structure of canavanine was established by Gulland and Morris (4) and by Kitagawa and Takani (5) as $NH_2 \cdot C(:NH) \cdot NH \cdot O \cdot CH_2 \cdot CH_2 \cdot CHNH_2 \cdot COOH$. Natural canavanine is of the L configuration (6).

Canavanine is split by a liver enzyme to yield urea and canaline, $NH_2 \cdot O \cdot CH_2 \cdot CH_2 \cdot CHNH_2 \cdot COOH$ (3). More recent evidence has indicated that the canavanine-splitting enzyme may be identical with arginase (7). It has been claimed by Ogawa ((8) and elsewhere) that canavanine is essential for young rats. The amino acid is non-toxic to mice, but produces symptoms of intoxication when injected into dogs in a dose of 200 to 400 mg. per kilo of body weight (9).

In experiments designed to test the effectiveness of canavanine in supporting the growth of certain amino acid-requiring mutants of *Neurospora* we found instead that the substance exerts a strong inhibitory effect on the growth of the mold. Further investigation has revealed a number of interesting aspects of this phenomenon, among which are the high degree of toxicity of the substance for the mold, the complete reversibility of the inhibition under certain conditions, and the existence of a genetic factor determining sensitivity or tolerance.

Materials and Methods

The strains of mold used in this study are 1A, 4A, and 25a. All are wild type strains of *Neurospora crassa*, derived originally from single

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ascospores, and grow normally on the usual minimal medium containing sugar, salts, and biotin (10). Growth was measured as the dry weight of mold produced in 72 hours at 25° in 20 ml. of medium contained in 125 ml. Erlenmeyer flasks.

Canavanine flavianate was isolated from jack bean meal by the method of Gulland and Morris (4). The flavianate was decomposed according to the procedure of Cadden (6), and canavanine was obtained as the sulfate. Electrometric titration and elementary analysis of the salt indicate the constitutional formula $(C_5H_{12}N_4O_3)_2 \cdot H_2SO_4$.

Calculated.	C 26.65,	H 5.75,	N 24.90,	sulfate S 7.11;	equivalent wt.	225
Found.	" 27.21,	" 6.31,	" 24.50,	" " 7.05;	"	"	229

Results

In contrast to other natural amino acids which have been shown to produce inhibition of growth in *Neurospora* (11–13), canavanine is effective against the wild type, as well as against mutant strains. Different degrees of sensitivity are exhibited by different wild strains, however. Following the initial observation that canavanine retards the growth of strain 1A, a number of other wild types were tested. It was found that three grades of resistance to the action of the amino acid can be distinguished: a high degree of resistance, shown by strain 4A; medium resistance, shown by strain 1A; and low resistance, shown by strain 25a. In Fig. 1 are plotted typical experiments showing the growth of these strains as a function of canavanine concentration. It is seen that growth of strain 25a is abolished by concentrations of canavanine sulfate exceeding 1.25 γ per ml. (5.55×10^{-6} M with respect to canavanine). With strain 1A a 10-fold greater concentration is required to bring about a 55 per cent inhibition of growth, while strain 4A is inhibited to the extent of only 15 per cent by the highest concentration tested.

Although the responses of strains 25a and 1A to canavanine are quite reproducible, strain 4A has not shown the same degree of tolerance to the substance in all experiments. In some tests it has behaved very much as strain 1A (medium resistance), while in others it has shown absolute resistance. The results of a preliminary experiment have indicated that the age of the culture is probably a factor in determining the response of strain 4A, resistance increasing with age.

In the present experiments the standard incubation period of 72 hours was used. If the culture flasks are allowed to incubate for longer periods, growth of strain 25a will eventually begin, even in the presence of relatively high canavanine concentrations. Whether this is caused by a loss of canavanine through spontaneous decomposition to desaminocanavanine (14), or by a change in the mold, we are not yet prepared to say.

Reversal of Canavanine Inhibition—On the assumption that canavanine interferes with the production or utilization of an essential metabolite, the growth of strain 1A was measured in the presence of an inhibiting concentration of canavanine (50 γ of canavanine sulfate per ml.) plus various supplements. In a preliminary experiment it was found that a mixture of water-soluble vitamins has only a slight effect on the inhibition, while hydrolyzed casein in a concentration of 1.25 mg. per ml. of medium completely reverses it. Two mixtures of amino acids were then tested, one containing Rose's essential amino acids, the other the "non-essential" amino acids. Canavanine inhibition was abolished by the mixture of

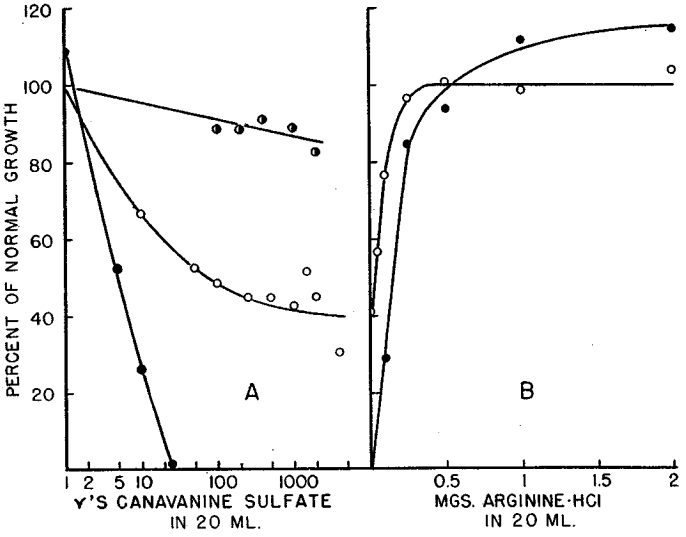

FIG. 1. *A*, inhibition of the growth of *Neurospora* by canavanine. ◑, strain 4A; ○, strain 1A; ●, strain 25a. *B*, reversal of inhibition by arginine. Canavanine sulfate concentration, 0.25 mg. per 20 ml. in the strain 25a curve (●) and 1 mg. per 20 ml. in the strain 1A curve (○).

essential amino acids (final concentration, 0.05 mg. of each per ml.), while the non-essential mixture produced a small effect (final concentration, 0.1 mg. of each per ml.).

The ten amino acids making up the essential mixture were then tested singly, with the results indicated in Table I. It is seen that complete reversal of the inhibition was obtained only with arginine; lysine and methionine were moderately effective (60 per cent reversal), while the remaining amino acids showed small activities.

When the same series of amino acids was tested on strain 25a, it was found again that the inhibition is completely relieved by arginine. Lysine

is also effective in this strain, with an activity equal to 0.25 that of arginine on a molecular basis. None of the other amino acids showed any activity (Table I). Typical recovery curves are plotted in Fig. 1. It is calculated that the effect of 1 molecule of canavanine is neutralized by about 3 molecules of arginine in strain 25a and by 0.3 molecules of arginine in strain 1A.

Experiments were next carried out to determine the relative effectiveness of optical isomers of arginine and lysine in overcoming canavanine inhibition. The results show that L-arginine and L-lysine are twice as active as the racemic mixtures in protecting strain 25a from the inhibition (Table II). It is concluded that only the natural enantiomorphs are active in

TABLE I

Effect of Essential Amino Acids on Canavanine Inhibition

Concentration of canavanine, M/4500 (50 γ of canavanine sulfate per ml.) in the strain 1A series and M/10,500 (21.4 γ of canavanine sulfate per ml.) in the strain 25a series. Concentration of other amino acids, 0.1 mg. per ml. in the strain 1A series and M/400 in the strain 25a series.

Supplement	Strain 1A, growth	Strain 25a, growth
	mg.	*mg.*
None	72	53
Canavanine	14	0
" + L-arginine·HCl	80	54
" + DL-lysine·HCl	49	46
" + DL-leucine	27	0
" + DL-isoleucine	33	0
" + L-methionine	47	0
" + DL-valine	36	0
" + DL-phenylalanine	27	0
" + L-tryptophan	27	0
" + DL-threonine	27	0
" + L-histidine·HCl·H_2O	25	0

this respect. This is somewhat surprising in view of the fact that *arginineless* mutants of *Neurospora* utilize DL-arginine for growth just as readily as L-arginine (15).

In another series of experiments, the effect on the growth of strain 25a of simultaneously varying the canavanine and arginine concentrations was measured. The results are presented in Table III. It will be noted that the degree of inhibition is independent of the absolute concentrations of the two amino acids, but is determined solely by the ratio of the concentrations. Inhibition quotients (ratio of canavanine concentration to arginine concentration) calculated from these data are close to 1.2 for complete inhibition and 0.3 for complete reversal.

Several miscellaneous guanidine derivatives have been tested for relief of canavanine inhibition, including guanidine, creatine, carbamidoarginine, guanidine valeric acid, guanidine butyric acid, and benzoylargininamide. The last two compounds were kindly supplied by Professor Carl Niemann. None of these substances was found to be active by itself. If the

TABLE II

Effect of Optical Isomers of Arginine and Lysine on Canavanine Inhibition

The values represent growth of strain 25a in mg. Canavanine concentration, M/10,500 throughout.

Arginine or lysine concentration	L-Arginine	DL-Arginine	L-Lysine	DL-Lysine
M/800	70.0	64.0	62.5	47.5
M/1600	64.0	60.5	48.0	18.5
M/3200	58.5	42.0	21.0	0.0
M/6400	42.0	5.0	0.5	0.0
M/12,800	4.5	0.0	0.0	0.0
M/25,600	0.0	0.0	0.0	0.0

TABLE III

Growth of Strain 25a on Independently Varying Concentrations of Arginine and Canavanine

The values represent growth in mg.

Arginine concentration	Canavanine concentration						
	0	M/42,000	M/21,000	M/10,500	M/5250	M/2625	M/1313
M/200		49.5	52.0	50.0	50.0	47.0	59.0
M/400	54.0	49.0	58.0	52.5	58.5	51.0	45.5
M/800		49.5	48.5	47.0	46.5	45.0	17.5
M/1600		50.5	49.5	54.5	50.0	24.0	2.0
M/3200		49.5	54.5	54.0	31.5	4.0	0.0
M/6400		56.0	53.0	37.5	9.5	0.0	0.0
M/12,800		50.5	34.0	4.0	0.0	0.0	0.0
M/25,600		33.0	5.0	0.0	0.0	0.0	0.0
M/51,200		4.0	0.0	0.0	0.0	0.0	0.0
0	50.0	0.0	0.0	0.0	0.0	0.0	0.0

medium is supplemented with just enough arginine to provide for a small amount of growth, then the further addition of carbamidoarginine produces a marked increment in the growth and guanidine a slight increase. The activity of carbamidoarginine is probably ascribable to its conversion to arginine by the mycelium, since experiments with *argininеless* mutants have shown that it supports the growth of these strains.

Experiments have been carried out to determine whether changes in temperature and pH affect the inhibition. Flasks containing an inhibitory concentration of canavanine (9.5×10^{-5} M) in basal medium were inoculated with strain 25a and incubated at 20°, 25°, 30°, and 35°, respectively. No growth was observed in any of the flasks after 78 hours. In another series of flasks, medium buffered at six pH values in the range 4.8 to 6.9 and containing canavanine in a concentration of 4.75×10^{-5} M was inoculated with strain 25a and incubated at 25° for 72 hours. Again, no relief of the inhibition was observed.

Genetics of Canavanine Tolerance—The differences between various wild type strains with respect to canavanine tolerance made it of interest to investigate the inheritance of this character. Strain 25a was crossed with strain 4A and the spores from eighteen asci were isolated in order and transferred to agar slants. The resulting cultures were then tested for canavanine tolerance. In thirteen of the asci canavanine tolerance segregated in a manner indicating that tolerance and sensitivity are determined by alternative forms of a single gene. The remaining five asci, however, could not be so simply interpreted and it is evident that a more extensive series of crosses will be necessary in order to establish the mode of inheritance of this pair of characters.

Dr. H. J. Teas has analyzed a number of other crosses of resistant and sensitive strains, with results essentially similar to ours.

DISCUSSION

Perhaps the most remarkable feature of the inhibition by canavanine is the extraordinarily high degree of toxicity displayed by this compound, an amino acid of the natural series. Not only is canavanine effective in very low concentrations (order of 10^{-6} M), but the neutralization quotients 0.3 and 1.2 obtained above contrast markedly with the values 100 to 10,000 usually found for metabolic antagonists (16). It is of interest to note that the ability to synthesize arginine and lysine, the only effective antidotes so far discovered, is not protective; the strains used in this study are wild types capable of synthesizing all of the amino acids necessary for their normal growth from sugar and inorganic salts. This suggests that the relative immunity to canavanine intoxication displayed by strain 4A results from a mechanism for detoxifying or otherwise disposing of the compound, a mechanism presumably possessed by strain 25a in a much less active form, if at all. This would be analogous to the case described by Woolley (17), who found that certain pyrithiamine-fast microorganisms possess a system for destroying pyrithiamine, whereas sensitive strains do not.

Little can be said at present as to the mechanism of the inhibition.

Although the data suggest a competition between arginine and canavanine for an enzyme surface, it is difficult on this basis to explain the anticanavanine action of lysine. The present observations would appear to be related in some way to the lysine-arginine antagonism discovered by Doermann (11). One possible mechanism seems to be definitely excluded by our data; namely, the interference of canavanine with arginine synthesis. On this basis, one would expect to provide complete protection against canavanine by supplying sufficient arginine in the medium for normal growth requirements. Studies of *argininеless* mutants (15) have shown that arginine in a concentration of M/2000 is sufficient for good growth. As can be seen in Table III, however, M/1600 arginine is not protective against the inhibition.

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SUMMARY

L-Canavanine from jack beans is a powerful inhibitor of the growth of certain wild type strains of Neurospora. Growth of the most sensitive strain is abolished by canavanine in a concentration of 5.55×10^{-6} M. Growth of a second strain is partly inhibited by canavanine, while a third strain is almost completely resistant. Resistance and sensitivity appear to be genetically determined. The inhibition is reversed by L-arginine and, in one of the strains, by L-lysine. The antagonism between canavanine and arginine is of the "competitive" type, approximately 3 molecules of arginine being required to neutralize the effect of 1 molecule of canavanine in the most sensitive strain. Lysine is about 0.25 as active as arginine for this strain. Although the mechanism of the inhibition is not known, the data exclude the possibility of interference by canavanine with arginine synthesis.

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