BIOCHEMISTRY AND GENETICS OF CANAVANINE RESISTANCE IN *NEUROSPORA*

Thesis by

James Barrie Logan

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To

Adolph Coors,

Hal and Carla,

and

Greg and Marty, wherever you are.

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ABSTRACT

The pattern of inheritance of resistance to growth inhibition by canavanine in Neurospora crassa is shown to result from interactions between a major gene and several modifiers. The major gene controls the production of a constitutive enzyme that destroys canavanine. The modifiers affect the rate of uptake of the analog from the medium. Strains which lack the enzyme activity are canavanine sensitive; strains which possess it are resistant, but the level of resistance is dependent on the rate of uptake.

The canavanine degrading enzyme was partially purified and its properties studied. The detoxification reaction was shown to be a cleavage of canavanine yielding hydroxyguanidine.

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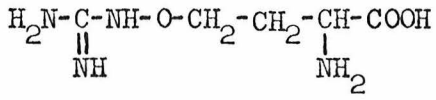
INTRODUCTION

Canavanine (α-amino-γ-guanidinoxybutyric acid) is one of the few known examples of a natural compound containing the guanidinoxy function (1).



Canavanine

This amino acid was found originally in seeds of the jack bean (Canavalia ensiformis) by Kitagawa and Tomiyama (2). Using chromatographic techniques and a specific color reagent, Fearon and Bell (1) detected it in the seeds of many species of the Leguminosae, but they failed to find it in other plant families or in animal tissues. Subsequently, it was shown to be restricted to the legume subfamily, the Papilionoideae, and its distribution is considered to have taxonomic significance (3).

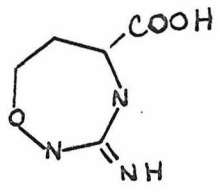
As with many other groups of secondary plant products, questions concerning the function of nonprotein amino acids automatically arise. Quantitative determinations of canavanine in the Papilionoideae revealed that in some species the free amino acid represents about 4 per cent of the dry weight of the seed (4). At the same time it was noted that the nitrogen content of the amino acid itself is approximately twice as great as that of storage protein. These facts have led to the suggestion that the primary role of canavanine in seeds is

one of nitrogen storage (5); a suggestion consistent with its disappearance during germination (6).

Canavanine does not yield a positive Sakaguchi reaction, a test for mono-substituted guanidines, and can therefore be easily distinguished from arginine. However, the most striking reaction of canavanine involves a variant of activated sodium nitroprusside reagent. Freshly prepared solutions are unreactive, but after exposure to daylight the reagent is converted to pentacyanoammonioferrate (III), $[Fe(CN)_5NH_3]^{2-}$, which is apparently the active compound (7). The reaction with this agent is specific for guanidinoxy compounds.

On prolonged treatment with nitrous acid, canavanine is capable of yielding about 75 per cent of its total nitrogen as N_2. The O-guanidinyl group is very much less basic than the guanidinyl group. This fact is reflected in the isoelectric point of canavanine (pH 8.2) as contrasted with one of pH 10.8 for arginine (8).

Canavanine can form two condensation products with itself. The first compound, desaminocanavanine, was described by Kitagawa and Tsukamoto (9) in the series of original reports concerning canavanine.

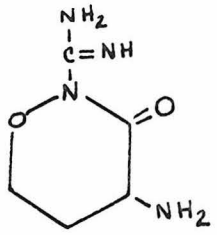


Desaminocanavanine

Desaminocanavanine readily forms under mild conditions in aqueous or alcoholic solutions of canavanine with the loss of 1 mole of ammonia.

This product exhibits a positive Sakaguchi reaction, and is negative to ninhydrin reagent and to diacetyl reagent.

More recently another condensation product of canavanine has been described (10). This compound, 4-amino-2-amidinoperhydro-1,2-oxazine-3-one, is also formed by heating aqueous or alcoholic solutions of canavanine.



4-amino-2-amidinoperhydro-1,2-oxazine-3-one

It gives a negative Sakaguchi reaction and a positive reaction with diacetyl reagent.

The enzymatic reactions in which canavanine is known to participate are as follows: (a) reductive cleavage to homoserine and guanidine by Streptococcus faecalis (11); (b) hydrolysis to homoserine and hydroxyguanidine by bacterial preparations (13); (c) hydrolysis to canaline and urea by arginase (2); (d) hydrolysis to O-ureido-homoserine and ammonia by the arginine desiminase of S. faecalis (12); (e) oxidation by L-amino acid oxidase of Neurospora crassa, presumably to the corresponding keto acid and ammonia (14); (f) oxidation to β-guanidinoxypropionamide, carbon dioxide and water by the arginine decarboxyoxidase of Streptomyces griseus (15); (g) condensation with fumaric acid to yield canavaninosuccinic acid catalyzed by the argino-succinase of several organisms (16); (h) condensation with β-alanine

to yield β-alanyl-canavanine catalyzed by chick pectoral muscle carnosine synthetase (17); (i) condensation with tRNA to yield canavanyl-tRNA catalyzed by the arginyl-RNA synthetase of rat liver (18); (j) transamidination with ornithine (or other amidine acceptors) to yield canaline and arginine (or other guanidine compounds) by preparations from kidney and Streptomyces griseus (19).

These reactions are shown in Figure 1 [after Kalyankar, Ikawa and Snell (13)]. Reactions a and b are the only ones which specifically require canavanine. In nature, histidine is the normal substrate for carnosine synthetase (Reaction h), although chick muscle preparations exhibit a very broad specificity. L-amino acid oxidase (Reaction e) is a relatively nonspecific enzyme. The other six reactions are ones for which arginine is a much more effective and probably, therefore, the primary substrate.

As might be expected from this documentation of reactions, canavanine is a potent antagonist of arginine. It was first shown to inhibit the growth of Neurospora crassa (20) and certain strains of bacteria (21) in 1948. Since that time it has been shown to be a growth inhibitor of yeast (22), a Tobacco fungus (23), algae (22), Walker carcinoma 256 cells (24), several plants (25,26,27), and chick heart cells grown in tissue culture (28). These inhibitions were shown to be reversed by arginine in most cases.

Canavanine acts in a manner similar to other amino acid analogs in inhibiting growth. It has been shown to interfere with protein synthesis in various bacteria (29,30). Incorporation of the analog

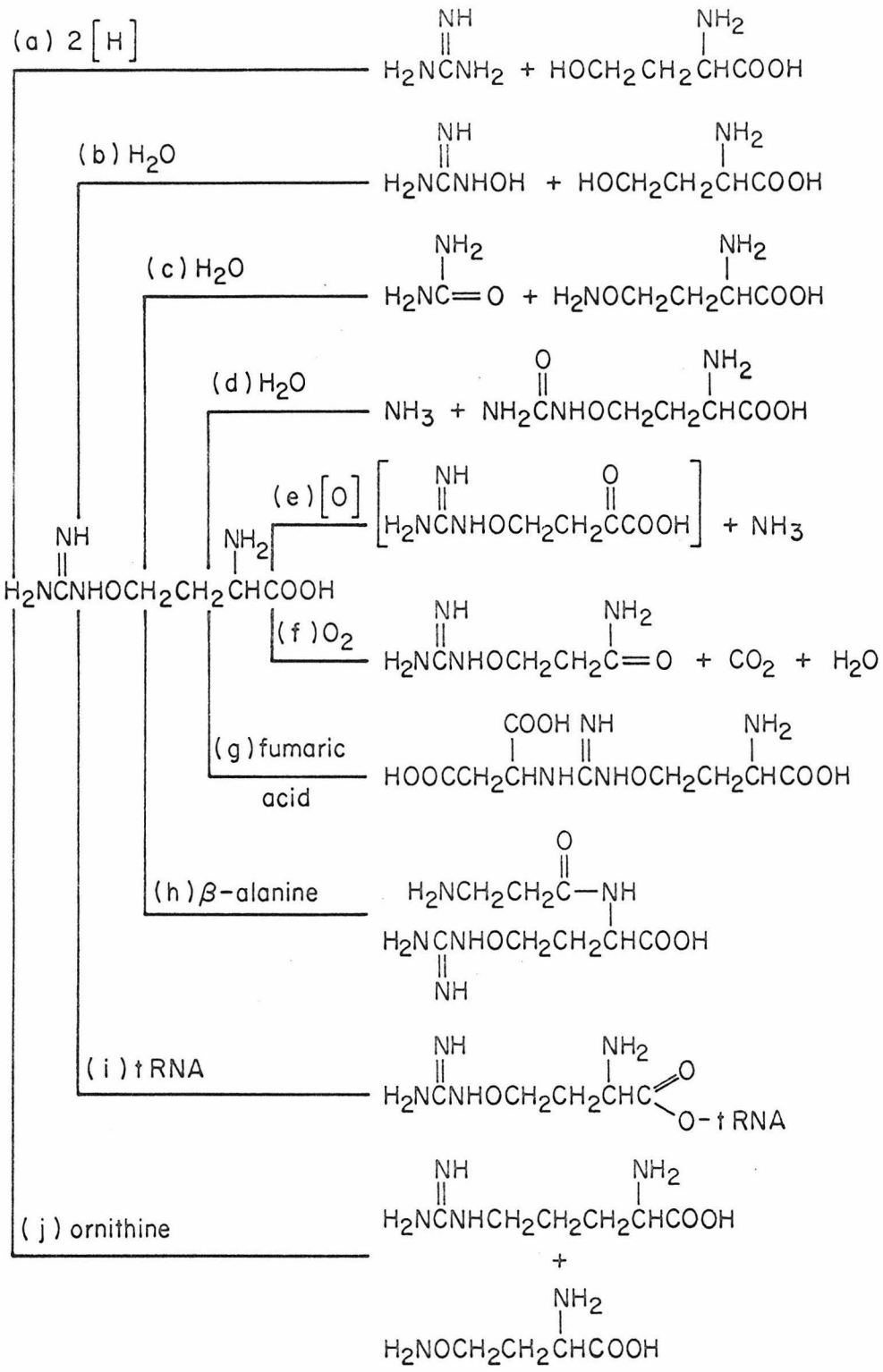


FIGURE 1

KNOWN REACTIONS OF CANAVANINE

into protein has been demonstrated in Staph. aureus (31), E. coli (32), Walker carcinoma 256 cells (24), and rat liver ribosomal preparations (18). The resulting protein molecules are likely to exhibit altered biological properties, since there is a considerable difference in the ionization of the oxyguanidino and guanidino groups.

The ability of canavanine to act as substrate for, or inhibitor of, arginine-tRNA synthetase has been tested, for enzyme preparations from rat liver (18) and E. coli (31). In the rat liver system, the inhibition was shown to be of a competitive nature and the analog was incorporated into both tRNA and protein.

Synthesis of DNA ceased when canavanine was added to arginine-dependent strains of Staph. aureus (29). Schachtele and Rogers (32) have examined the effect of canavanine on DNA synthesis more closely in E. coli where canavanine has been shown to cause an exponential loss in viability under conditions of low intracellular arginine. "Canavanine death" appeared dependent upon incorporation of the analog into cellular protein, thus inhibiting all transcription, preventing the initiation of DNA replication, and disrupting the organization of the genome in the cell. These authors argue that there are specialized sites on the bacterial cell membrane which function in organizing replication and transcription of the bacterial genome, and that canavanyl-proteins cause cell death by interfering with this function.

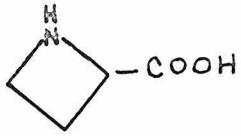
Occasionally various microbial mutants demonstrate a resistance to toxic analogs, including canavanine. There are various kinds of protective mechanisms that may be envisioned, including the existence

in the resistant strain of amino acid activating enzyme molecules possessing markedly reduced affinities for the toxic substances. Such a mechanism has been demonstrated. Lewis has studied the basis of ethionine-resistance in Coprinus lagopus and has suggested that an altered methionine activating enzyme is involved (33). A p-fluorophenylalanine-resistant mutant of E. coli has been shown to possess an altered phenylalanine-tRNA synthetase which is unable to activate the analog (34).

The production by a resistant mutant of key enzymes required for endogenous amino acid biosynthesis that are no longer subject to either repression or end-product inhibition by the analog would also provide a protective mechanism. Maas (35), from a consideration of E. coli mutants resistant to canavanine, has concluded that, besides being incorporated into protein, this analog mimics arginine in effecting repression of the biosynthetic enzymes. E. coli K-12 strains resistant to canavanine fell into two classes, both of which represented mutations of the arginine regulator gene (R_{arg}). R_1 strains were altered in such a way that the regulatory machinery was no longer responsive to either arginine or canavanine (i.e., they were derepressed mutants), and the R_2 mutants remained repressible by arginine but were no longer repressed by the analog. Presumably these mutants owed their resistance to the production of arginine in excess of growth requirements, and the enhanced intracellular concentration of amino acid allowed successful competition with the analog for incorporation into protein.

Growth inhibiting effects may also be reduced if an organism possesses a transport system with decreased affinity for the toxic substance. A canavanine resistant mutant of E. coli W possessing a defective transport system failed to accumulate arginine, lysine or ornithine and, by inference, canavanine. Thus the analog was excluded from the sites at which toxicity is normally exerted (36). In yeast a canavanine-resistant mutant has been described in which the uptake of L-arginine is specifically impaired (54).

The final mode of resistance to be discussed involves a degradative mechanism for converting the toxic molecule to a harmless compound. An interesting example of such a mechanism is seen in an Agrobacterium species which rapidly degrades azetidine-2-carboxylic acid, a potent analog of proline.



Azetidine-2-carboxylic acid

The reaction is a hydrolytic cleavage to yield α-hydroxy-γ-aminobutyric acid (37). This organism does not itself synthesize azetidine-2-carboxylic acid, and its proline-tRNA synthetase is capable of activating the analog (38). The lack of growth inhibition of this organism by azetidine-2-carboxylic acid then must depend upon the prior destruction of the imino acid by the hydrolytic enzyme before any significant quantities reach the intracellular site of the activating enzyme.

Canavanine resistance in Neurospora seems to be more complex than any of the protective mechanisms discussed above, since there

appear to be two levels of resistance; full resistance and intermediate resistance (20). Moreover, the inhibition of growth by canavanine in sensitive strains can be completely reversed in a competitive way by lysine as well as arginine.

The mechanism of canavanine resistance in Neurospora has been the topic of several investigations. In the original report on the subject, Horowitz and Srb demonstrated the growth inhibitory property of canavanine in wild-type strains and its reversal by arginine and lysine (20). Using mutant strains, Teas (39,40) showed that canavanine could fulfill the amino acid requirement of certain resistant strains that grow if supplied with homoserine, but did not satisfy the amino acid requirement of resistant strains that did not grow if supplied with homoserine. From these results he concluded that canavanine must function in promoting growth by replacement of homoserine. However, the inability of canavanine to support the growth of strain 51504, a resistant strain able to grow on homoserine, suggested that the active product of canavanine metabolism was not homoserine itself but some "homoserine-like" product. It was proposed that canavanine resistance and canavanine sensitivity were due to the resistant strains having an enzyme which splits canavanine, the sensitive strains lacking this enzyme (41). Arginase was rejected as a candidate for this canavanine splitting enzyme since canaline, the product of the action of arginase on canavanine, was found to inhibit the growth of both canavanine-resistant and canavanine-sensitive strains.

The genetics of canavanine resistance in Neurospora has been known to be complex for some time. Horowitz and Srb reported the analysis of the spores of eighteen ordered asci from a cross of a fully resistant strain with a sensitive strain. In thirteen of the asci canavanine tolerance segregated in a manner indicating that tolerance and sensitivity were determined by alternative forms of a single gene. The remaining five asci, however, could not be so simply interpreted. Teas (40) reported essentially similar results and noted that crosses with strains of intermediate resistance were particularly difficult to interpret. Lockhart and Garner (42) crossed strains that were reisolates of the threonineless mutants used by Teas. On the basis of results from five ordered asci, they argued for the existence of two nonallelic genes governing canavanine resistance and sensitivity.

The uptake of basic amino acids has been carefully examined in Neurospora (43). The data indicated the existence of a common concentrating mechanism for arginine, lysine, and canavanine. This finding is in agreement with the independent findings of Konobu (44). Arginine and lysine reciprocally inhibited each others uptake, and canavanine was an effective competitor for uptake of both amino acids. The transport mechanism had an affinity for arginine which was twice as great as that for lysine and four times greater than that for canavanine. However, these authors ruled out this common uptake system as the agent determining canavanine resistance since resistant strains did not accumulate basic amino acids at a slower rate than sensitive strains. It was also found that canavanine was equally competitive for arginine and lysine

uptake in all strains tested. On the other hand, the observed reversal of canavanine inhibition by both arginine and lysine is at least partially explained by the presence of a common transport mechanism.

In 1963 an abstract appeared in Genetics in which Bauerle and Garner summarized their conclusions about the mode of canavanine resistance in Neurospora (45). They argued that resistance could not be explained by any of the following mechanisms: (a) decreased uptake rates in resistant strains; (b) loss of normal end-product control of arginine biosynthetic enzymes in resistant strains; (c) production of biosynthetic enzymes of arginine that are no longer subject to repression in resistant strains; or (d) an ability of resistant strains to detoxify canavanine.

This was the state of the problem when I became interested in it. From the conclusions of Bauerle and Garner it seemed that the most profitable area of metabolism to examine next was the initial step in protein biosynthesis, amino acid activation. Altered amino acid-tRNA synthetases had been implicated in ethionine resistance in Coprinus and p-fluorophenylalanine resistance in E. coli. A comparison of arginyl-tRNA synthetases in canavanine-resistant and canavanine-sensitive strains of Neurospora had not been previously performed. Since the mechanism of canavanine resistance was important for the interpretation of certain other data from Dr. Horowitz' laboratory, such a comparison of synthetases was the starting point for the following study.

MATERIALS AND METHODS

Strains of Neurospora

Two wild-type strains of Neurospora crassa (67), 4A and 25a, were used in most experiments. Strain 4A is fully resistant to growth inhibition by canavanine, while strain 25a is sensitive (20).

The homoserineless strain 51504 was kindly provided by Helen Macleod from the Neurospora stocks of the Caltech Division of Biology. This mutant has a growth requirement for homoserine or methionine plus threonine (39).

Limited use was made of several other strains. Strains 80702Ra (cys-2), 39816 (cys-10), 1A, 69-1113a, Sing 2A, 262A, 16a, and 19A were obtained from the stocks of the Caltech Division of Biology. Strains 33933 (lys-1), 4545 (lys-3), and 15069 (lys-4) were provided by the Fungal Genetics Stock Culture Center (Hanover, New Hampshire). Strain P110 was obtained from Joyce Maxwell.

Chemicals

DL-canavanine-C^{14}-guanido (31.6 mc/mmole) was obtained from Calbiochem and Schwarz Bioresearch, Inc. L-arginine-UL-C^{14} (220 mc/mmole) and guanidine-C^{14}-nitrate (1-5 mc/mmole) were obtained from New England Nuclear Corp. DL-homoserine-4-C^{14} (11.5 mc/mmole) was obtained from Schwarz Bioresearch, Inc. Cyanamide-C^{14} (20 mc/mmole) was obtained from Volk Radiochemical Company. L-canavanine-H^3 was the gift of Dr. E. F. Jansen.

Hydroxyguanidine was prepared by the method of Kalyankar, Ikawa and Snell (13). Aspartic semialdehyde was prepared by the method of Black and Wright (70). The allyglycine used in this synthesis was obtained from K and K Laboratories and the ozone generator was loaned by Dr. A. J. Haagen-Smit. Homoserine lactone was prepared by the method of Armstrong (65). O-acetyl-homoserine was prepared by the method of Wiebers and Garner (71).

Culture of Neurospora

All Neurospora strains were maintained in stock cultures on agar slants of Horowitz complete medium (68).

Cultures were grown exponentially according to the methods of Luck (69), and Davis and Harold (52).

Stationary cultures were grown at 25°C in 125 ml Erlenmeyer flasks containing 20 ml of Vogel's minimal salt solution (53) supplemented with 2 per cent sucrose.

Fractionation of Mycelium

The distribution of C^{14}-canavanine in various fractions of the mycelium was determined by the procedure of Roberts et al. (72). The mycelium was first extracted twice with 2 ml of ice cold 5 per cent trichloroacetic acid (TCA) for 30 minutes and washed with 2 ml of cold 5 per cent TCA. The wash and the extract were pooled. The mycelium was then extracted with 4 ml of 75 per cent ethanol at 45°C for 30 minutes, followed by extraction with 4 ml of 75 per cent ethanol:ether

(1:1) at 45°C for 30 minutes. The ethanol and ethanol-ether fractions were combined and the residue heated to remove the ether. Two ml of 5 per cent TCA were added and the tubes were placed in a water bath at 95°C for 1 hour. The hot TCA was removed and the mycelium was washed with 1 ml of 5 per cent TCA at room temperature. The wash and the hot TCA fraction were pooled. The residue was washed successively with water, ethanol, and ether, and the washes were discarded. The residue was dried and then extracted with 1 ml of 3 per cent sodium hydroxide. The cold TCA fraction contains small metabolites, while the hot TCA extract contains nucleic acids, including amino acyl- and peptidyl-tRNA. The ethanol-ether fraction contains mostly lipids. The protein of the mycelium is extracted by the sodium hydroxide treatment.

Preparation of Amino Acyl-tRNA Synthetase

Enzyme preparations were made by the method of Barnett and Jacobson (77). The enzyme was kept frozen at -20°C and aliquots were thawed for assay.

Preparation of RNA

Ten grams of mycelium grown in exponential culture were harvested and ground in sand with 2 ml of 10 per cent sodium dodecyl sulphate. Ten ml of water-saturated redistilled phenol and 10 ml of 0.01 M sodium acetate buffer, pH 5.1, were added and the mixture shaken vigorously for 20 minutes. The extract was centrifuged, the aqueous

layer removed, and the extraction with phenol was repeated 4 times. An equal volume of ether was added to the aqueous layer and the mixture shaken for 20 minutes. The aqueous layer was removed and extracted with ether two more times. The salt concentration of the aqueous layer was adjusted to 0.2 M sodium acetate and two volumes of ethanol were added. The precipitate was collected, dissolved in 0.5 M Tris buffer, pH 8.8, and incubated at 37°C for 45 minutes. This treatment removes amino acids attached to tRNA. The RNA was again precipitated by adjusting the salt concentration to 0.2 M sodium acetate and adding 2 volumes of ethanol. The precipitate was dissolved in water and dialyzed against cold water overnight. The solution was then frozen and aliquots were thawed for use in the assay of amino acyl-tRNA synthetases.

Color Reagents and Stains

All protein determinations were made by the method of Lowry et al. (73).

Pentacyanoammonioferrate (PCAF) reagent was synthesized by the method of Herington (7). Guanidinoxy compounds react characteristically with this reagent.

Nitroprusside-alkaline ferricyanide reagent and α-naphthol-diacetyl reagent were prepared as described by Elliott (74). These reagents were used to detect guanidine and its derivatives on paper chromatograms.

Amino acids were detected on paper using the cadmium-ninhydrin method of Blackburn (75).

Assay of Canavanine Destruction

Duplicate 5 µl aliquots of the solution to be tested were each spotted on a 1.5 x 20 cm strip of Whatman No. 1 filter paper. The origin was 5.5 cm from one end. Hydroxyguanidine and guanidine separated from canavanine when the strips were developed by descending chromatography in methanol:1 N acetic acid (99:1). Chromatography was stopped as soon as the solvent reached the end of the paper and the strips were air dried. The strips were then cut at a point 15 cm from the solvent-front end of the chromatogram. This section was then cut in half. The half-section nearest the solvent front contained approximately 98 per cent of the hydroxyguanidine produced by canavanine cleavage. The half-section including the origin contained approximately 95 per cent of the remaining canavanine. These half-sections were rolled into cylinders, placed in 10 ml scintillation fluid, and counted in a Nuclear Chicago Series 720 liquid scintillation system.

Large Scale Preparation of Canavanine Cleavage Products

An incubation mixture contained 20 mg (0.82 mmoles) of L-C^{12}-canavanine·H_2SO_4, 5 µc of DL-C^{14}-canavanine, 2 ml of Fraction III protein (10 mg/ml) or Fraction IV protein (3 mg/ml) and 0.1 M sodium pyrophosphate buffer, pH 8.5, to make a final volume of 10 ml. The incubation was carried out under oxygen at 37°C for 12 hours. Under these

conditions 25 to 40 per cent of the canavanine is degraded as measured by hydroxyguanidine production. The reaction was stopped by the addition of 10 ml of ethanol or 2 N hydrochloric acid. After centrifugation, the volume of the supernatant was reduced to zero by vacuum desiccation. The residue was dissolved in 1 ml of water.

Crosses

Crosses between Neurospora strains were made by coinoculation of Westergaard-Mitchell crossing medium (76) with conidial suspensions. Twenty-five ml of the agar-supplemented medium were contained in each sterile petri dish.

RESULTS

Growth Inhibition by Canavanine

Canavanine is a potent growth inhibitor in Neurospora. At levels of canavanine greater than 1 μg/ml (3.6 μM) the growth of strain 25a is abolished. However, the results of a typical growth experiment (Figure 2) make clear that there are differing degrees of sensitivity. The difference in growth response of the three wild-type strains is quite marked; these responses have been termed sensitivity, intermediate resistance, and full resistance (20). The strain of intermediate resistance (69-1113a) shows 53 per cent inhibition of growth at a concentration of canavanine 100 times that which abolished growth in the sensitive strain (25a). The fully resistant strain (4A) is inhibited only to the extent of 16 per cent at the highest concentration tested.

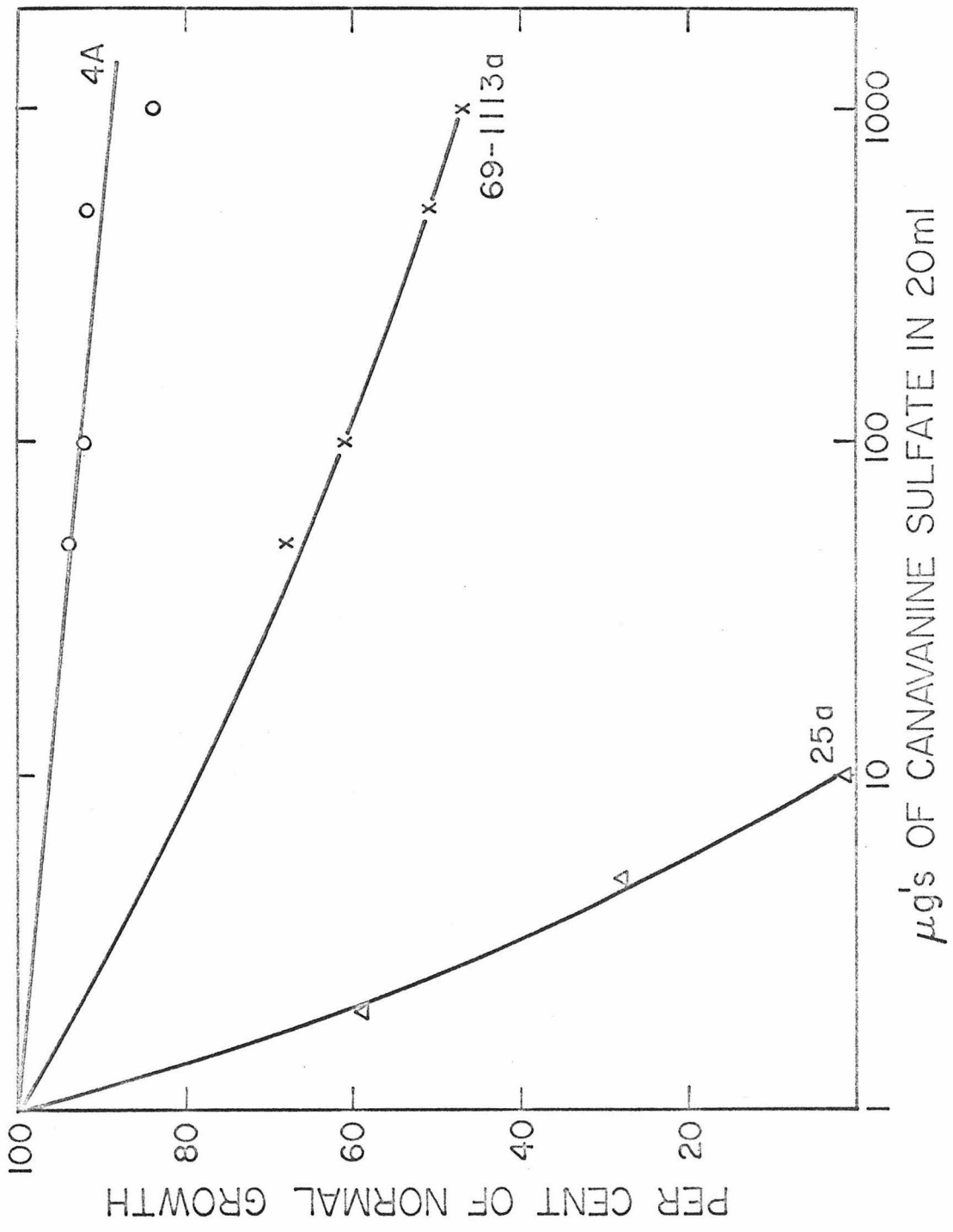
Mechanism of Growth Inhibition

Incorporation of Canavanine into Proteins. The mode of inhibition of growth by canavanine has been the source of considerable speculation. As early as 1948, Volcani and Snell proposed that canavanine inhibited cell growth in bacteria by interfering in the synthesis of proteins (21). Since that time canavanine has been shown to be incorporated into the proteins of a number of organisms (see Introduction). In all cases tested, canavanine substituted for arginine. In view of the large difference in the ionization of the guanidino

FIGURE 2

INHIBITION OF GROWTH BY CANAVANINE

Growth was measured as dry weight of mold produced in 72 hours at 25°C in 20 ml of medium contained in 125 ml Erlenmeyer flasks. The mycelial pads were dried at 100°C overnight.


PER CENT OF NORMAL GROWTH
100
80
60
40
20
1
10
100
1000
μg's OF CANAVANINE SULFATE IN 20ml
4A
69-1113a
25a

and the oxyguanidino groups, proteins containing canavanine would very likely have some altered properties.

The production of defective proteins by the incorporation of canavanine appears to be the mechanism of growth inhibition in Neurospora. In 1963 Bauerle and Garner reported that after growing cells were exposed to canavanine, a high replacement of arginine residues by canavanine occurred in the protein fraction of a canavanine-sensitive strain, while arginine replacement was negligible in resistant strains (45).

This finding was confirmed by M. Fling in Horowitz' laboratory (personal communication). After incubating in the presence of canavanine, cultures were harvested, and the protein fraction isolated and hydrolyzed. Canavanine was isolated and measured. In a sensitive strain (25a) a level of 3-4 mg canavanine/380 mg protein was found. In a resistant strain (4A) the level was 0.5 mg/400 mg protein. A molar ratio of 15 canavanine:72 arginine was found in 25a and 1.6 canavanine:68 arginine in 4A. Fling and Horowitz also showed that the presence of canavanine prevents induction of tyrosinase in a sensitive strain, but not in a resistant strain. This suggests that incorporation of canavanine into the enzyme results in loss of activity.

The foregoing results were checked with radioactive canavanine (DL-canavanine-guanido-C^{14}), which has recently become available. The results were in essential agreement with those cited above. Mycelial mats were given pulses of radioactive canavanine and then fractionated using the procedure of Roberts et al. (Methods). A comparison of the

distribution of label in the cell showed a striking difference between strain 25a (can-s) and strain 4A (can-r) (Table 1). Twelve per cent of the label taken up by the culture appeared in the protein fraction of the sensitive strain, while in the resistant strain this fraction contained only 2 per cent of the label. Of the counts taken up, 97 per cent were in the pool of small metabolites in the resistant strain. When the data were converted to moles of canavanine per mg mycelium and per mg protein, the strain difference was even more pronounced (Table 2). The amount of canavanine in protein per mg mycelium in the sensitive strain was nearly 13 times that in the resistant strain. The amount of canavanine in protein per mg of protein was 8 times as large in the sensitive strain as in the resistant strain.

From the foregoing data it is concluded that Neurospora may show resistance to canavanine by preventing the incorporation of the analog into proteins. Growth inhibition by canavanine is presumed to be due to the altered properties of canavanine-containing proteins produced in sensitive strains.

Arginyl-tRNA Synthetase. Bauerle and Garner (45) reported that canavanine resistance and sensitivity in Neurospora could not be explained by detoxification, by a difference in uptake, by a difference in repression of arginine biosynthetic enzymes, or by a difference in normal end-product control of the functions of arginine biosynthetic enzymes (see Introduction). Given these results, the most likely prospect for the mediator of canavanine resistance seemed to be the

TABLES 1 and 2

DISTRIBUTION OF C^{14}-CANAVANINE

and

CANAVANINE IN PROTEIN

Exponential cultures were grown by the procedure of Luck (69). Ten ml aliquots were removed from the culture and collected on a Buchner funnel with gentle suction. The mycelial mat was peeled off and resuspended in 10 ml fresh medium for one hour. One μc (31.6 mμmoles) DL-C^{14}-canavanine was added and each flask was placed on a shaker for 30 minutes. The mycelium was harvested on a Buchner funnel, washed with 50 ml cold water, and plunged into 2 ml cold 5% TCA. The tissue was then fractionated using the procedure of Roberts et al. Radioactivity was measured on a Nuclear Chicago low background counter.

TABLE 1

DISTRIBUTION OF C^{14}-CANAVANINE

	Per cent of label taken up	
	25a (can-s)	4A (can-r)
small metabolites	82.6	96.6
lipids	1.4	0.0
nucleic acids	3.7	0.9
proteins	12.2	2.3

TABLE 2

CANAVANINE IN PROTEIN

	25a (can-s)	4A (can-r)	25a/4A
$\frac{\mu\mu\text{moles canavanine in protein}}{\text{mg dry weight of mycelium}}$	60.4	4.8	12.6
$\frac{\mu\mu\text{moles canavanine}}{\text{mg protein}}$	282	34.6	8.2

arginyl-tRNA synthetase. Failure of the synthetase of a sensitive strain to discriminate between arginine and canavanine could lead to the incorporation of canavanine into its protein. Possession of an arginyl-tRNA synthetase with more narrow limits of substrate specificity could prevent the activation of canavanine and confer the property of canavanine resistance.

When this hypothesis was tested, it was found not to be valid. Canavanine is in fact activated by the arginyl-tRNA synthetase of Neurospora, but the degree of activation is the same for synthetases extracted from resistant and sensitive strains (Table 3). Moreover the competition between arginine and canavanine for activation is also very similar in the two strains. At a concentration of arginine one-fourth that of canavanine, the extract from a sensitive strain showed a 64 per cent inhibition of canavanyl-tRNA synthesizing activity, while the extract from the resistant strain was inhibited 68 per cent. The data in Figure 3 again demonstrate that the specificities of the arginyl-tRNA synthetases in the two strains are very similar. Canavanine competes for arginine activation and the K_i's determined from the data in Figure 3 were the same for both strains.

The finding that canavanine could be incorporated into tRNA demonstrates that arginyl-tRNA synthetase, like the other arginine-metabolizing enzymes, cannot fully distinguish this analog from the normal substrate. Since the latter stages of protein synthesis show no specificity for the amino acid moiety (46), canavanine incorporation

TABLE 3

CANAVANINE ACTIVATION

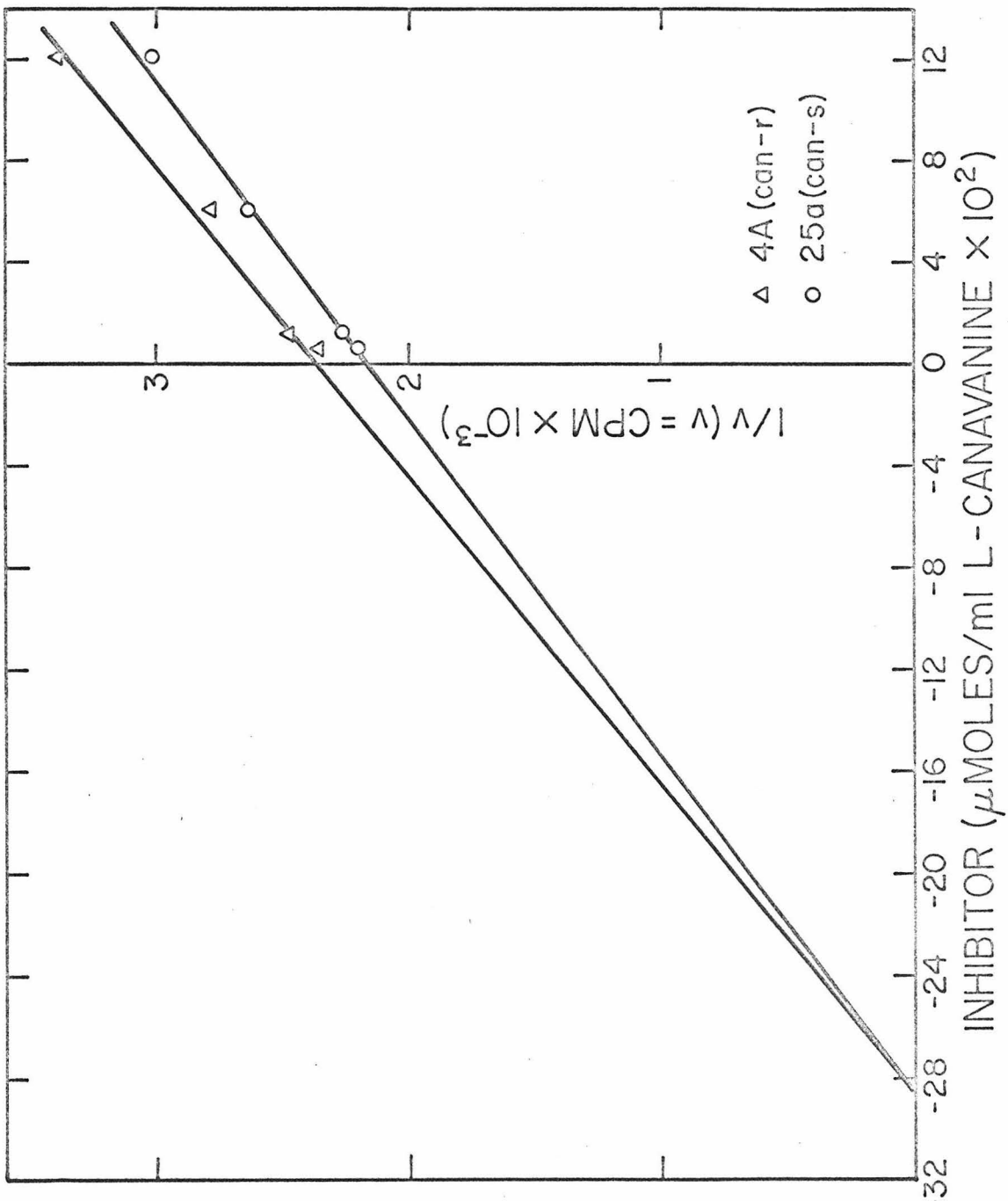
Strain	Specific activity cpm/mg protein	Per cent inhibition by arginine
25a (can-s)	10,641	64.5
4A (can-r)	9,545	67.9

Each incubation mixture contained in one ml: 764 µg RNA, 1 µmole ATP, 1 µmole each of the common amino acids except for arginine, 40 µmoles potassium maleate buffer, pH 6.9, 4 µmoles magnesium chloride, 0.5 µmoles EDTA, 5 µmoles dithiothreitol, 40 µmoles KCl, 1 µc (31.6 mµmoles) DL-C^{14}-canavanine, 0.704 mg 4A synthetase or 0.562 mg 25a synthetase. In the competition experiment 8 mµmoles L-C^{12}-arginine were added to the above. Incubation was at 37°C for 5 minutes. Fifty µl aliquots were placed on Whatman No. 3MM filter paper disks which were then placed in cold 10% TCA. The disks were washed in succession with 66% ethanol containing 0.5 M NaCl, 10% TCA, 5% TCA, and ethanol:ether (1:1). After drying, the disks were counted in a Nuclear Chicago Scintillation Counter.

FIGURE 3

COMPETITION FOR ARGININE ACTIVATION

The incubation mixtures were the same as those described in Table 3 except 10 mμmoles L-C^{14}-arginine were substituted for the radioactive canavanine. Nonradioactive L-canavanine was added in the indicated proportions to each incubation mixture. Incubation was for 14 minutes at 37°C. Further manipulations were as described in Table 3.


1/v (v = CPM x 10⁻³)
INHIBITOR (μMOLES/ml L-CANAVANINE x 10²)
Δ 4A (can-r)
o 25a (can-s)
3
2
1
0
-4
-8
-12
-16
-20
-24
-28
-32
4
8
12

into canavanyl-tRNA also supports the conclusion that canavanine is incorporated into proteins.

The great similarity in specificity for canavanine shown by the arginyl-tRNA synthetases from resistant and sensitive strains makes it unlikely that resistance can be ascribed to a synthetase with enhanced ability to discriminate between arginine and canavanine.

Detoxification of Canavanine

When the demonstration of an altered arginyl-tRNA synthetase proved unsuccessful, several of the original conclusions of Bauerle and Garner were re-examined. Specifically, the metabolism and the uptake of canavanine were investigated. These results will now be discussed, for they are somewhat at variance with those of Bauerle and Garner.

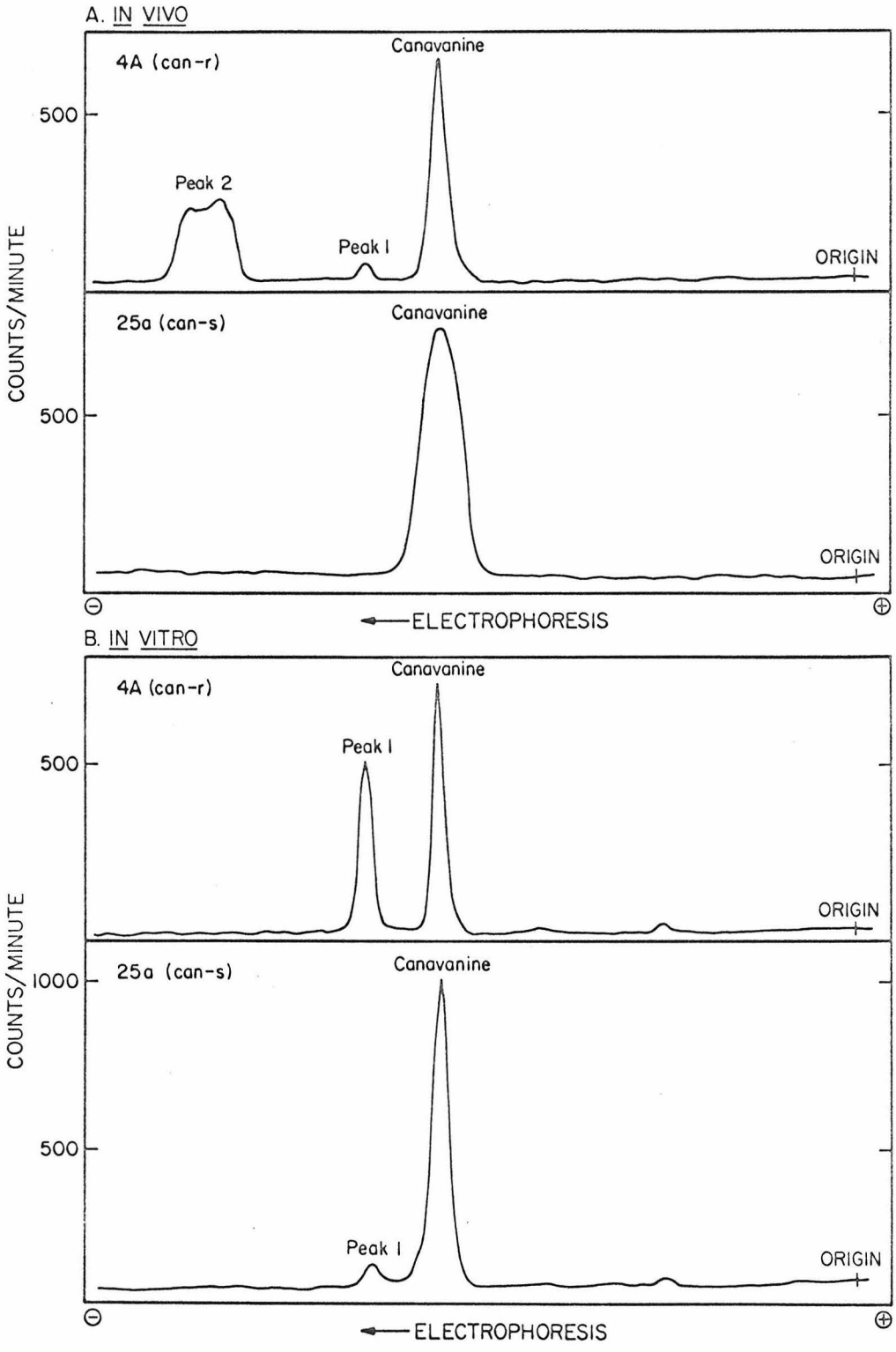
Comparison of Metabolism of Canavanine in Resistant and Sensitive Strains. An electrophoretic analysis of the radioactive metabolite pool of a pad of Neurospora that has been pulsed with C^{14}-labeled canavanine reveals a striking difference between the pattern obtained from a resistant strain and that from a sensitive strain. Figure 4A shows the kinds of patterns that are routinely observed. The resistant strain (4A) yields multiple peaks of radioactivity corresponding to canavanine plus two unknown compounds. The sensitive strain yields only a single peak which corresponds to canavanine. These facts

FIGURE 4

METABOLISM OF CANAVANINE IN RESISTANT AND SENSITIVE STRAINS

A. in vivo--Three-day-old stationary cultures were pulsed for one hour with 0.5 μc DL-C^{14}-canavanine, and then extracted with 5 ml of cold 5% TCA. The TCA was removed from the extract with ether. Fifty μl aliquots of the concentrated extracts were applied to strips of Whatman No. 1 filter paper. After electrophoresis (6.7% formic acid, pH 1.6, 50 volts/cm, 1.25 hours) the pattern of radioactive peaks was recorded using a Nuclear Chicago Actigraph II, Model C100A.

B. in vitro--One hundred μl of each crude enzyme extract (see Text) were added to an incubation mixture containing 15.8 mμmoles (0.5 μc) DL-C^{14}-canavanine and 350 μl of 0.1 M sodium pyrophosphate buffer, pH 8.5; total volume 0.5 ml. After one hour at 37°C, the reaction was stopped by the addition of 0.5 ml 5% TCA. After centrifugation, the supernatant was extracted with ether to remove the TCA, and 25 μl aliquots were applied to strips of Whatman No. 1 filter paper and subjected to electrophoresis and analysis as described above.


A. IN VIVO
4A (can-r)
Canavanine
Peak 2
Peak I
ORIGIN
25a (can-s)
Canavanine
ORIGIN
500
500
COUNTS/MINUTE
⊖
⊕
ELECTROPHORESIS
B. IN VITRO
4A (can-r)
Canavanine
Peak I
ORIGIN
25a (can-s)
Canavanine
Peak I
ORIGIN
500
1000
500
COUNTS/MINUTE
⊖
⊕
ELECTROPHORESIS

suggest that the property of canavanine resistance may reside in the ability to metabolize canavanine.

When the activities of crude, cell-free extracts of the two strains are examined, a similar sort of difference is observed. Six-day-old standing pads were pressed dry and ground with sand in two volumes of 0.1 M sodium pyrophosphate buffer, pH 8.5. After centrifugation the supernatants were incubated with radioactive canavanine for one hour. The resulting reaction mixtures were then examined for the pattern of radioactivity after electrophoresis (Figure 4B). Controls using boiled extracts in the incubation mixture yield only a canavanine peak.

The extract from the resistant strain (4A) produces a much larger peak 1 than the sensitive strain (25a) extract, suggesting again an increased capacity to modify canavanine as the mode of resistance. However, the pattern of radioactivity after electrophoresis differs between the in vivo and the in vitro experiments with strain 4A. In vivo, peak 2 is the major product, while in vitro peak 1 is predominant.

Two points need to be emphasized about the radioactive canavanine used in these experiments. First, the compound is a racemic mixture, DL-canavanine, so that an enzyme could act on essentially all of the L-canavanine present and the remaining D-canavanine would still produce a considerable canavanine peak on the Actigraph tracing. The second point is that the C^{14} label is located in the carbon of the guanidino group. Therefore any peaks observed after electrophoresis must contain this carbon atom.

It can be said in summary that a difference between resistant and sensitive strains can be demonstrated both with intact pads and with extracts. The difference is in the ability of resistant strains to modify canavanine, but the major product appears to differ between *in vitro* and *in vivo* experiments. The identification of these products was now undertaken.

Identification of Peak 1. The chromatographic properties of peak 1 were determined on samples prepared by incubating radioactive canavanine with crude extracts of strain 4A in the manner described for Figure 4B. The chromatographic mobility of peak 1 was shown to be identical with that of authentic hydroxyguanidine ($H_2N\text{-}\underset{\underset{NH}{\|}}{C}\text{-}NHOH$), in three solvent systems: (1) ethanol:water:glacial acetic acid; (2) ethanol:1 N ammonium hydroxide; and (3) n-butanol:formic acid: water. The R_F values of the relevant compounds are shown in Table 4.

Peak 1 also has the same mobility as authentic hydroxyguanidine in electrophoresis (6.7% formic acid, pH 1.6). Electrophoretic distances from the origin are listed in Table 5. Figure 5A is a photograph showing the correspondence between an Actigraph tracing and the actual electropherogram. Figure 5B is a similar picture showing a chromatogram developed with ethanol:1 N ammonium hydroxide (77:23).

Amounts of peak 1 compound large enough to be detected using chemical reagents can be obtained by using larger reaction mixtures, partially purified enzyme and a Dowex 50(H^+) column (Methods). The column separates peak 1 compound from any residual canavanine or

TABLE 4

R_F VALUES IN SEVERAL SOLVENT SYSTEMS

Compound	R_F		
	Ethanol-water-acetic acid	Ethanol-ammonia	n-Butanol-formic acid-water
canavanine	0.08	0.23	0.02
hydroxyguanidine	0.68	0.40	0.22
peak 1	0.68	0.40	0.22
guanidine	0.63	0.60	0.26
peak 2	0.63	0.60	0.26

Radioactive material and authentic substances were mixed and chromatographed, and also chromatographed separately, in determining R_F values. Radioactive peaks were located using a Nuclear Chicago Actigraph II, while the authentic compounds were detected using α-naphthol-diacetyl reagent.

Whatman No. 1 filter paper was used with ethanol-water-acetic acid and Whatman No. 3MM with ethanol-ammonium and butanol-formic acid-water. The solvent systems are as follows: ethanol:water: glacial acetic acid (77:23:1), ethanol:1 N ammonium hydroxide (77:23), n-butanol:formic acid:H_2O (75:15:10). Descending chromatography was used in all cases.

TABLE 5

ELECTROPHORETIC COMPARISONS OF PEAKS 1 AND 2 WITH AUTHENTIC COMPOUNDS

Compound	Distance from origin (cm)
canavanine	50.0
arginine	52.7
hydroxyguanidine	60.5
peak 1	60.5
guanidine	76.0
peak 2	76.0

Electrophoresis (6.7% formic acid, pH 1.6) was carried out on a Gilson Model D electrophoretor at 50 volts/cm for 1.5 hours. Peaks 1 and 2 were positioned by means of a Nuclear Chicago Actigraph II and the authentic compounds were detected with nitroprusside-alkaline ferricyanide spray.

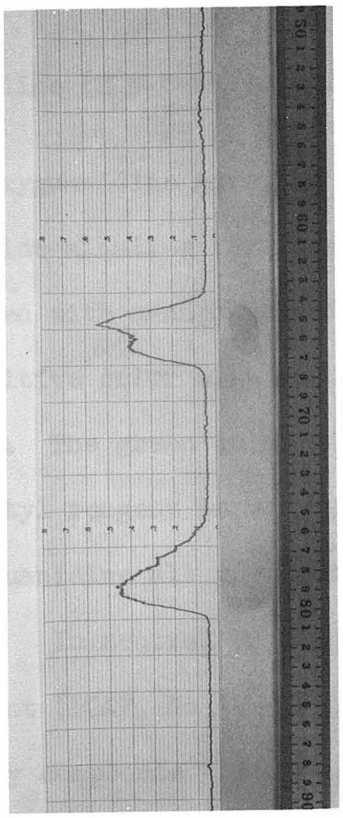
FIGURE 5

CORRESPONDENCE OF ACTIGRAPH TRACINGS AND CHROMATOGRAMS

A. 15.8 mμmoles of radioactive canavanine (0.5 μc) were incubated under oxygen with Fraction III protein (see Properties of Canavanine Cleaving Enzyme) for one hour. Aliquots of this incubation mixture were spotted together with authentic canavanine and hydroxyguanidine on a strip of Whatman No. 1 filter paper. Electrophoresis (6.7% formic acid, pH 1.6, 50 volts/cm) was for 1.5 hours. Canavanine and hydroxyguanidine were detected using nitroprusside-alkaline ferricyanide reagent.

B. Descending chromatography of a similar incubation mixture was performed using ethanol:1 N ammonium hydroxide (77:23) as solvent. The paper was Whatman No. 1. Development was for approximately eight hours. Nitroprusside-alkaline ferricyanide was again used to visualize canavanine, hydroxyguanidine, and guanidine.

A



⊕

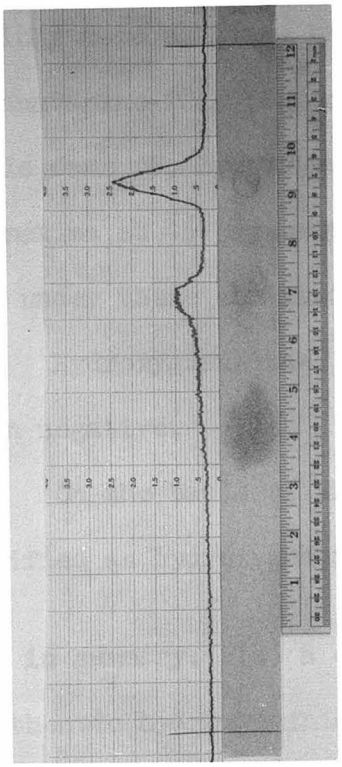
ELECTROPHORESIS

canavanine

hydroxyguanidine

⊖

B



← origin

canavanine

hydroxyguanidine

guanidine

CHROMATOGRAPHY

← solvent front

guanidine ($H_2N-\underset{\underset{NH}{\|}}{C}-NH_2$). After chromatography, this peak-1 material and hydroxyguanidine gave the same purple color when sprayed with nitroprusside-alkaline ferricyanide reagent and the same green color when sprayed with α-naphthol-diacetyl reagent. The latter reagent gives a positive color with guanidine and its mono- and disubstituted derivatives. The green color given by hydroxyguanidine with α-naphthol-diacetyl reagent is very characteristic (13). Canavanine, arginine and guanidine all give pink colors.

In neutral or slightly acid solution, pentacyanoammonioferrate reagent (PCAF, $Na_3[Fe(CN)_5NH_3]$) reacts characteristically with guanidinoxy compounds (1). Guanidinoxy compounds react only within the range pH 5-7.5, whereas alkyl-substituted guanidines react only in solutions more alkaline than pH 8. At pH 7, where it is most stable, the canavanine-PCAF pigment has a single absorption maximum at 517 mμ. At this same pH, hydroxyguanidine and peak-1 material both have absorption maxima at 467 mμ. Guanidine, arginine, and citrulline give no color under these conditions.

Hydroxyguanidine, peak-1 compound, and guanidine are all ninhydrin negative.

On the basis of the above results, the peak-1 compound is identified as hydroxyguanidine, $H_2N-\underset{\underset{NH}{\|}}{C}-NHOH$. The identifying tests were, in summary: (a) a chromatographic mobility identical with that of authentic hydroxyguanidine in three different solvent systems; (b) an electrophoretic mobility identical with that of authentic

hydroxyguanidine; (c) the presence of a guanido function as demonstrated by its positive reaction with the α-naphthol-diacetyl reagent (47); (d) a color identical with that of authentic hydroxyguanidine when sprayed with nitroprusside-alkaline ferricyanide and α-naphthol-diacetyl reagents; (e) the presence of a guanidinoxy linkage as indicated by its reaction with pentacyanoammonioferrate reagent (1); (f) a PCAF-complex absorption spectrum identical with that of authentic hydroxyguanidine.

Identification of Peak 2. The free metabolite pool of strain 4A that has been pulsed with radioactive canavanine contains a large amount of peak-2 compound. When the chromatographic behavior of this peak was compared with that of several likely compounds it was found that it shared the same mobility as guanidine in three different solvent systems. These were the same solvent systems used in the identification of peak 1. The appropriate R_F values are given in Table 4.

The electrophoretic mobility was also identical for peak 2 and guanidine (Table 5). As can be seen, through the technique of paper electrophoresis, hydroxyguanidine can readily be separated from guanidine, and canavanine from arginine by virtue of the decreased basicity of the O-guanidinyl group (canavanine is isoelectric at pH 8.2, as contrasted with a pH of 10.8 for arginine).

The peak-2 compound is identified as guanidine on the basis of this chromatographic and electrophoretic evidence. Further confirmatory evidence was not sought because experiments to be described in

the next section indicated that the production of peak-2 compound was not directly related to the mechanism of canavanine resistance.

Metabolism of Guanidine and Hydroxyguanidine. Canavanine-resistant strains differ from canavanine-sensitive strains in their capacity to produce both guanidine and hydroxyguanidine from canavanine. The production of these two products can be visualized in three different ways, as follows: (a) a reductive cleavage of canavanine yielding guanidine, which is then hydroxylated to produce hydroxyguanidine; (b) hydrolytic cleavage of canavanine yielding hydroxyguanidine which is then reduced to guanidine; (c) two distinct pathways of canavanine degradation, one yielding guanidine and the other hydroxyguanidine.

Experiments using radioactive guanidine and hydroxyguanidine were designed to distinguish among these possibilities. After pulses with the appropriate radioactive compound, the free metabolite pools of whole pads were examined electrophoretically for labeled metabolic products. As can be seen in Figure 6A, neither the resistant strain nor the sensitive strain can further metabolize guanidine. It remains as free guanidine. Hydroxyguanidine, on the other hand, is efficiently converted to guanidine in both the resistant and sensitive strains. These results are shown in Figure 6B.

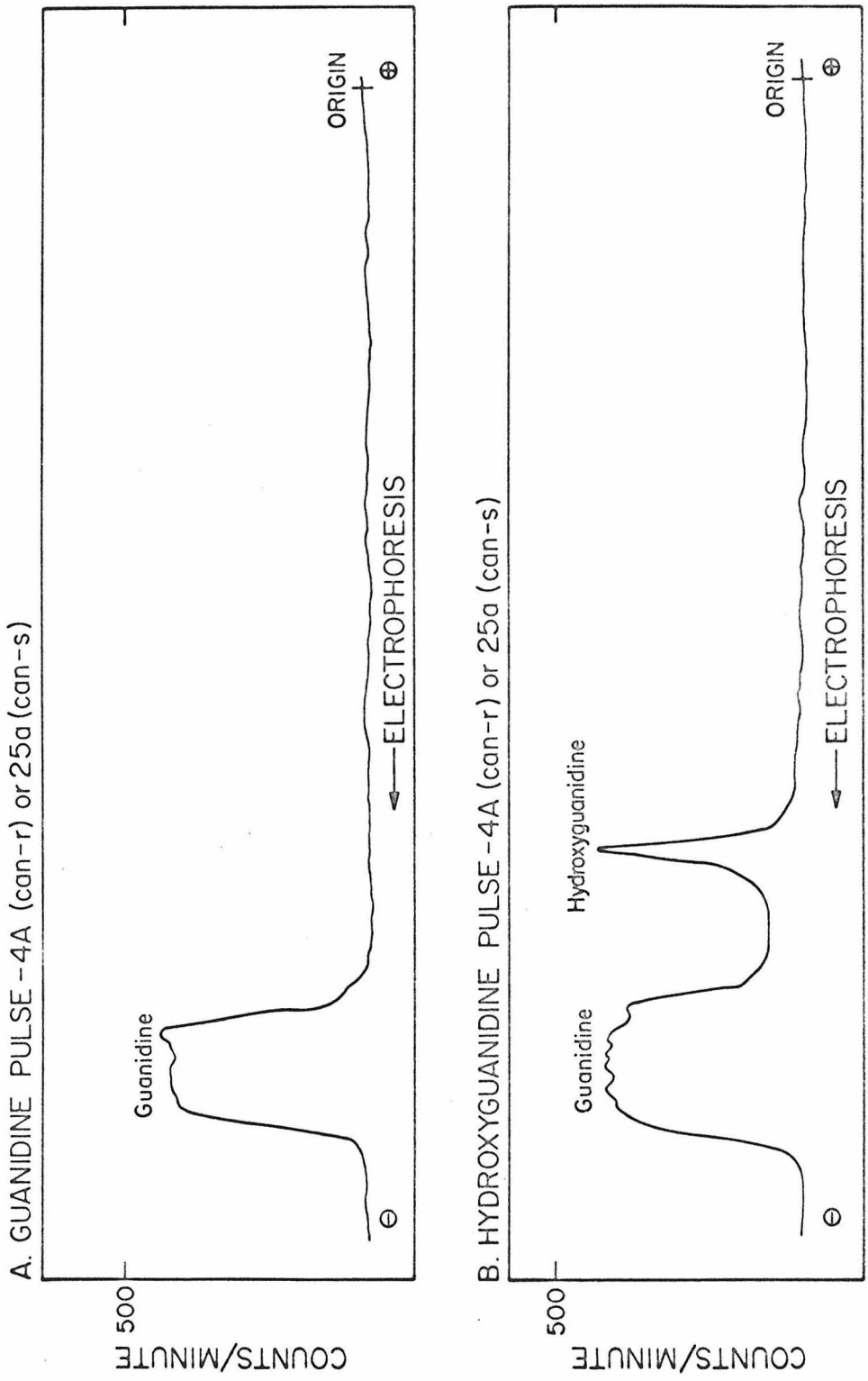
From these data it seems most likely that there is a single pathway in resistant strains in which canavanine is cleaved to yield hydroxyguanidine, which is then rapidly reduced *in vivo* to guanidine. It is the cleavage part of this pathway that is essentially missing in

FIGURE 6

METABOLISM OF GUANIDINE AND HYDROXYGUANIDINE

A. Guanidine pulse. A three-day-old stationary culture each of 4A and 25a was washed with water, the excess water pressed out, and placed in 20 ml of 0.1 M sodium phosphate buffer, pH 6.0, containing 2.5 μc C^{14}-guanidine nitrate in a 250 ml Erlenmeyer flask. The flask was placed on a rotary shaker for one hour, after which the pad was collected on a Buchner funnel and washed quickly with 100 ml cold water. The pad was removed from the funnel and placed in 5 ml cold 5% TCA. After one hour, the extraction mixture was centrifuged at 10,000 x g for 10 min. The supernatant was saved and the pad washed with 2 ml cold 5% TCA. The combined wash and supernatant were extracted with ether to remove the TCA. The aqueous phase was placed in a vacuum desiccator and the volume was reduced to zero. The residue was dissolved in 0.2 ml water and a 25 μl aliquot was spotted on a strip of Whatman No. 1 filter paper. The strips were subjected to electrophoresis (6.7% formic acid, pH 1.6, 50 volts/cm) for 1.25 hours. The Nuclear Chicago Actigraph II recorded the pattern of radioactivity after electrophoresis.

B. Hydroxyguanidine pulse. The manipulations were exactly the same as those employed for the guanidine pulse. Three-day-old stationary cultures of each 4A and 25a were given a one hour pulse with 2.5 μc C^{14}-hydroxyguanidine in 20 ml 0.1 M sodium phosphate buffer, pH 6.0 on a shaker.


A. GUANIDINE PULSE-4A (can-r) or 25a (can-s)
COUNTS/MINUTE
500
Guanidine
ORIGIN
⊖
⊕
ELECTROPHORESIS
B. HYDROXYGUANIDINE PULSE-4A (can-r) or 25a (can-s)
COUNTS/MINUTE
500
Guanidine
Hydroxyguanidine
ORIGIN
⊖
⊕
ELECTROPHORESIS

sensitive strains. However, we cannot entirely rule out the possibility that there is an additional minor pathway present which produces guanidine from canavanine directly.

It is presumed that the reason reduction of hydroxyguanidine does not take place *in vitro* (see Figure 4) is that this reaction is somewhat complex, and some vital cofactor, perhaps providing reducing power, may be missing and cannot be generated in the extracts. Attempts were not made to restore this activity *in vitro*. It should be noted, however, that the addition of reduced riboflavin phosphate greatly stimulated the reduction of hydroxyguanidine catalyzed by homogenates of guinea pig liver (48).

It was of some interest to ask, what is the normal function of the enzyme which catalyzes the reduction of hydroxyguanidine? It was thought that hydroxylamine reductase might also use hydroxyguanidine as a substrate. There are three species of NADP hydroxylamine reductases in *Neurospora*, peaks A, B, and C (in order of relative sedimentation in a sucrose gradient) (49, 50, 51). Peak A is identical with sulfite reductase. Cys 2, cys 4, and cys 10 (me 4) mutant strains lack this hydroxylamine/sulfite reductase. Peak B activity is adaptive, not being formed when *Neurospora* is grown on medium containing NH_4 as its sole nitrogen source. Peak C is found only in small amounts.

Mutant strains 80702Ra (cys 2) and 39816 (cys 10) were grown on medium containing ammonium as the sole source of nitrogen, and both strains were found capable of reducing hydroxyguanidine to guanidine.

However, by comparison with strain 4A, the capacity to reduce hydroxyguanidine was more limited in strain 39816.

These data do not answer the original question, but one can conclude that the hydroxylamine/sulfite reductase (peak A) does not seem to play a critical role in the reduction of hydroxyguanidine. Peak B activity does not seem to be a likely candidate either. However, further experiments are necessary to fully determine the relationship, if any, between hydroxylamine reductases and hydroxyguanidine reduction.

Nontoxicity of Hydroxyguanidine, Guanidine, and Homoserine. In the preceding sections the enzymatic cleavage of canavanine to yield hydroxyguanidine has been described. To add further support to the conclusion that this reaction is primarily responsible for conferring canavanine resistance, the effect of hydroxyguanidine on growth was determined. The effect of homoserine was of some interest, since the only known reaction of canavanine with hydroxyguanidine as a product also yields homoserine. Guanidine was tested for growth inhibition since it is rapidly produced *in vivo* by the reduction of hydroxyguanidine.

Homoserine gave some inhibition (19%) of the growth of strain 25a, but had no effect on strain 4A (Table 6). Hydroxyguanidine produced no inhibition in either strain, while guanidine was slightly stimulatory for both strains. These compounds were tested at concentrations at which canavanine abolishes the growth of strain 25a and inhibits the growth of strain 4A by 15 per cent.

TABLE 6

NONTOXICITY OF VARIOUS COMPOUNDS

Supplement 0.05 mg/ml	25a (can-s)		4A (can-r)	
	Dry weight of mycelium mg	Per cent normal growth	Dry weight of mycelium mg	Per cent normal growth
none	64	100	55	100
guanidine	73	114	65	112
hydroxyguanidine	64	100	61	105
homoserine	52	81	59	102

Growth was measured as dry weight of mold produced in 89 hours at 25°C in 20 ml of medium contained in 125 ml Erlenmeyer flasks. The mycelial pads were dried at 100°C overnight. Each recorded weight is the average of duplicates.

Purification and Properties of Canavanine Cleaving Enzyme

Consideration of the identity of the canavanine cleavage product or products other than hydroxyguanidine is deferred until a later section. Using the production of hydroxyguanidine as an activity assay, the canavanine cleaving enzyme has been partially purified and some of its properties determined. These results are described below.

Purification of Enzyme. A summary of the data from a typical enzyme preparation is shown in Table 7. Strain 4A (can-r) was grown in exponential culture by the method of Davis and Harold (52), using Vogel's minimal salt solution (53), supplemented with 2 per cent sucrose. Mycelium was collected on cheesecloth, washed once in cold distilled water, pressed dry, and immediately frozen in liquid nitrogen. All steps of the purification were carried out at 4°C.

Extraction--The frozen mycelium was disrupted by grinding with mortar and pestle while frozen in liquid nitrogen. The resulting powder was suspended in 10 volumes of cold 0.1 M sodium pyrophosphate buffer, pH 8.5, containing 0.01 moles per liter β-mercaptoethanol, and stirred for 30 minutes. The suspension was centrifuged at 17,000 x g for 30 minutes, and the precipitate discarded.

Streptomycin Sulfate Treatment--The supernatant was made to 2 per cent streptomycin sulfate and stirred for 20 minutes. The suspension was centrifuged at 25,000 x g for 30 minutes and the precipitate discarded.

Ammonium Sulfate Fractionation--The supernatant was made to 40 per cent saturation with solid ammonium sulfate, stirred for 30 minutes, and left standing for one hour. Following centrifugation at 12,000 x g, the precipitate was discarded and the supernatant made to 80 per cent saturation with ammonium sulfate crystals. The suspension was stirred for 30 minutes and left standing overnight. The precipitate was collected by centrifugation at 12,000 x g for 30 minutes and dissolved in 0.002 M β-mercaptoethanol, 0.02 M sodium pyrophosphate buffer, pH 8.8. Dialysis against two 4-liter volumes of this same 0.02 M buffer for 48 hours was followed by dialysis for 4 hours against 4 liters of cold distilled water.

Chromatography on DEAE-Sephadex--Approximately 20 ml of the dialyzed solution containing 200 mg of protein were applied to a column (2.6 x 20 cm) of DEAE-Sephadex (A-50) previously equilibrated with 0.002 M β-mercaptoethanol, 0.02 M sodium pyrophosphate buffer, pH 8.8. The same buffer was now passed through the column by gravity flow and 5.5 ml fractions were collected. No canavanine cleaving activity appeared in the eluate. When the eluate showed baseline levels of absorption at 280 mμ, the eluting buffer was changed to 0.01 M β-mercaptoethanol, 0.1 M sodium pyrophosphate, pH 8.5, and most of the canavanine cleaving activity eluted from the column in a sharp peak (see Figure 7). The peak tubes were pooled and the solution from these tubes was concentrated using a Diaflo UM-10 ultrafiltration membrane (Amicon Corporation). The final protein concentration was

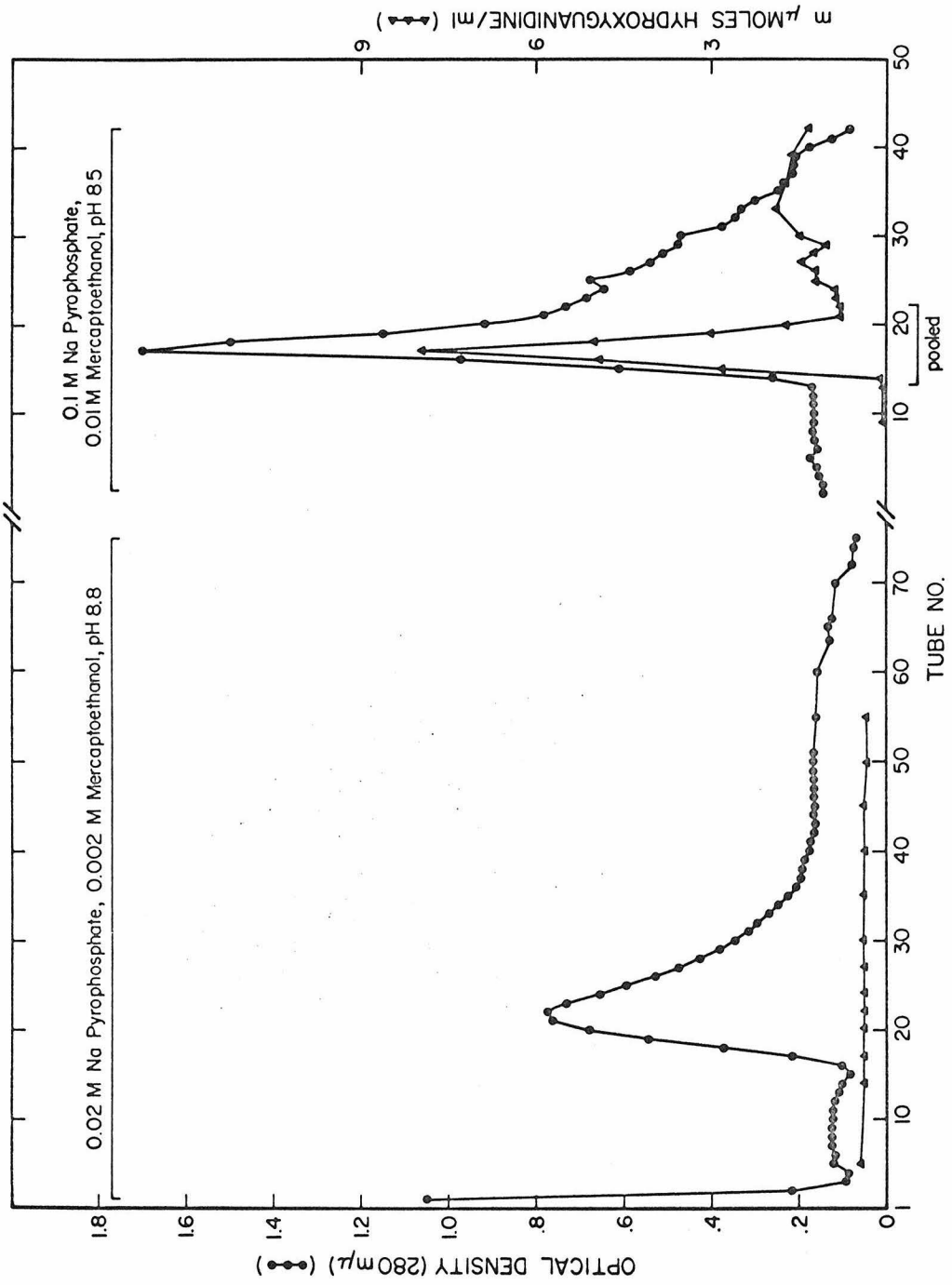


FIGURE 7

CHROMATOGRAPHY ON DEAE-SEPHADEX

TABLE 7

SUMMARY OF ENZYME PURIFICATION

	Fraction	Volume ml	Total activity units	Total protein mg	Specific activity units/mg	Yield %
I.	Crude extract	130	85,900	1416	61	
II.	Streptomycin sulfate	127	83,300	809	103	97
III.	Ammonium sulfate fractionation	22	67,000	210	318	78
IV.	DEAE-Sephadex	14	18,400	40	454	21

The values in the table are for 13 g (wet weight) of mycelium. A unit is defined as that amount of enzyme which forms 1 μμmole of hydroxyguanidine per minute at 37°C. Specific activity equals units per mg of protein.

3 mg per ml. When a 50 µl aliquot of this preparation was subjected to disc-gel electrophoresis and stained with 1 per cent amido Schwarz, one major band and 6 minor bands could be detected. The enzyme solution has been preserved in a frozen state without detectable loss of activity for several months.

Kinetics of Enzyme Reaction. Canavanine cleavage as a function of time, enzyme concentration, and substrate concentration was determined. At a concentration of 31.6 mµmoles/ml DL-C^{14}-canavanine and 1.2 mg/ml Fraction IV protein, the production of hydroxyguanidine is linear for 30 minutes at 37°C (Figure 8). After that time the rate decreases as the concentration of L-canavanine becomes limiting. At 70 minutes, approximately 80 per cent of the available L-canavanine has been destroyed.

The initial rate of hydroxyguanidine production is proportional to enzyme concentration (Figure 9). Increasing aliquots of Fraction III protein were used in this experiment.

As shown in Figure 10, at an enzyme concentration of 500 µg Fraction III protein per 0.5 ml, hydroxyguanidine production is linear with increasing L-canavanine concentration up to 0.6 µmoles/0.5 ml. A double reciprocal plot of these data indicates that at high substrate concentrations there may be substrate activation.

pH Optimum. The effect of pH on canavanine cleaving activity was determined in the range pH 5.5-8.0 with sodium phosphate buffers (Figure 11). The optimum pH for the reaction is between pH 6.4 and

FIGURE 8

EFFECT OF TIME ON CANAVANINE CLEAVING ACTIVITY

The incubation mixture contained 31.6 mμmoles (1.0 μc) DL-C^{14}-canavanine, 1.2 mg Fraction IV protein, and 0.1 M sodium pyrophosphate buffer, pH 8.5, in a final volume of 1.0 ml. Incubation was at 37°C. Fifty μl aliquots were removed at five minute intervals and placed in an equal volume of cold ethanol. After centrifugation the supernatant solutions were assayed for canavanine destruction (Methods).

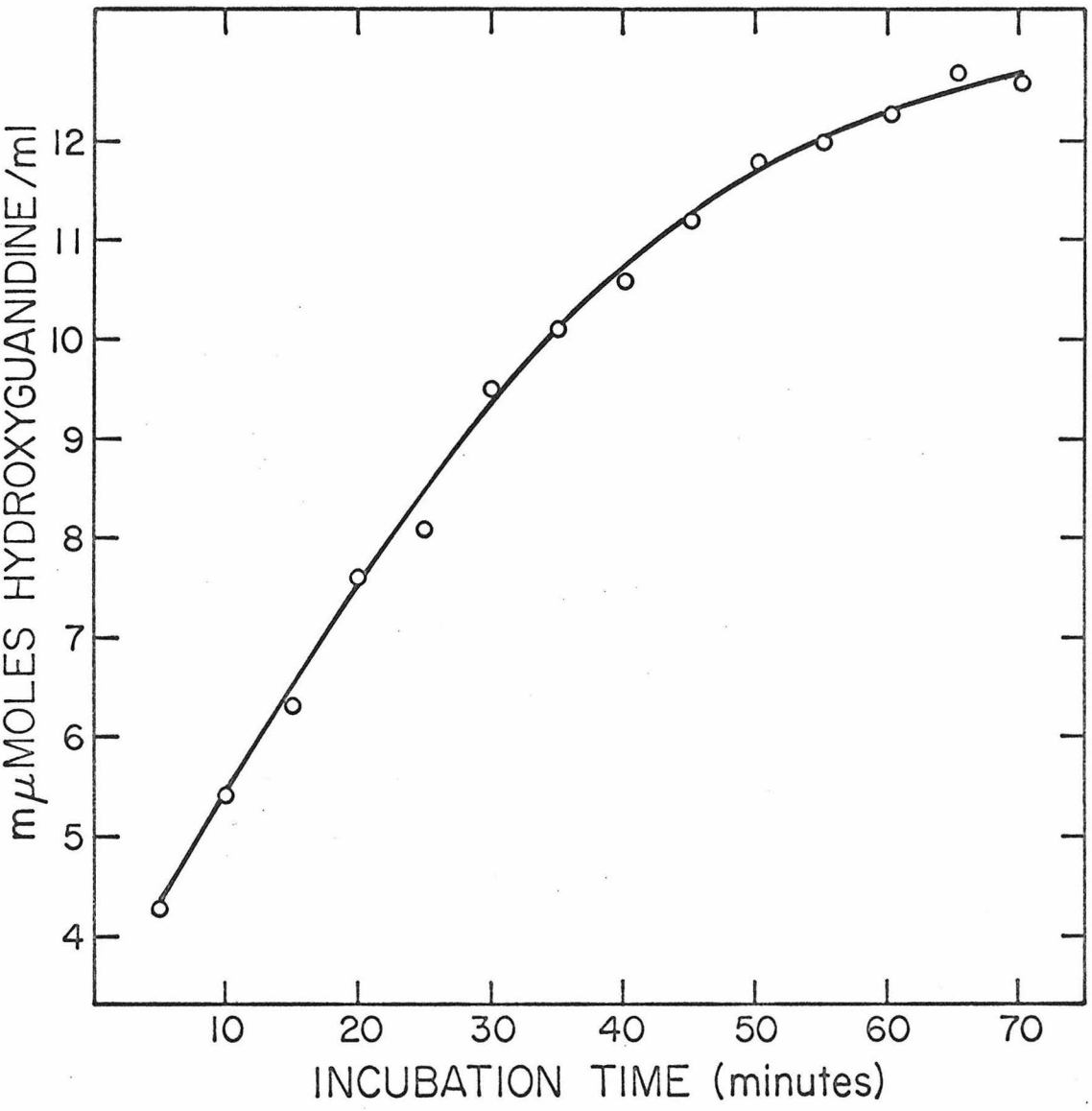

mμMOLES HYDROXYGUANIDINE/ml
4
5
6
7
8
9
10
11
12
10
20
30
40
50
60
70
INCUBATION TIME (minutes)

FIGURE 9

THE RELATION OF THE REACTION RATE TO ENZYME CONCENTRATION

In addition to the indicated amount of Fraction III protein, each incubation mixture contained 7.9 mμmoles DL-C^{14}-canavanine, 1.25 μmoles EDTA, and enough 0.1 M sodium phosphate buffer, pH 6.5, to make a final volume of 0.25 ml. The mixtures were incubated at 38°C for 25 minutes, when the reaction was stopped by the addition of an equal volume of 1 N hydrochloric acid. After centrifugation, duplicate 5 μl aliquots of the supernatant were assayed for canavanine destruction (Methods).

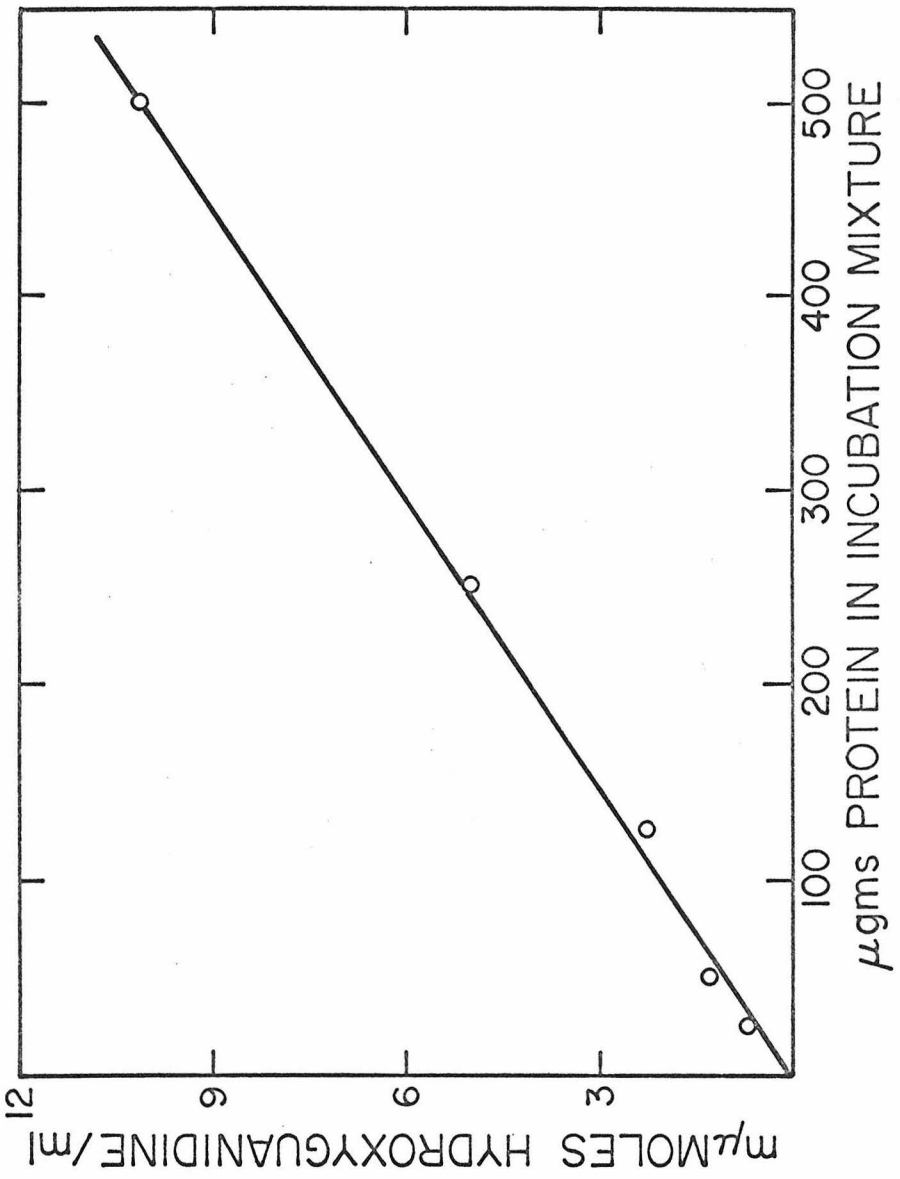

mμMOLES HYDROXYGUANIDINE/ml
3
6
9
12
100
200
300
400
500
μgms PROTEIN IN INCUBATION MIXTURE

FIGURE 10

THE EFFECT OF SUBSTRATE CONCENTRATION ON CANAVANINE CLEAVING ACTIVITY

In addition to the indicated amount of L-canavanine·H_2SO_4, each incubation mixture contained 0.5 μc DL-C^{14}-canavanine, 2.5 μmoles EDTA, 500 μg Fraction III protein and 0.1 M sodium phosphate buffer, pH 6.5, to give a final volume of 0.5 ml. After incubation at 43°C for 25 minutes, a 50 μl aliquot was removed and placed in an equal volume of 1 N hydrochloric acid. After centrifugation the supernatant was assayed for canavanine destruction (Methods).

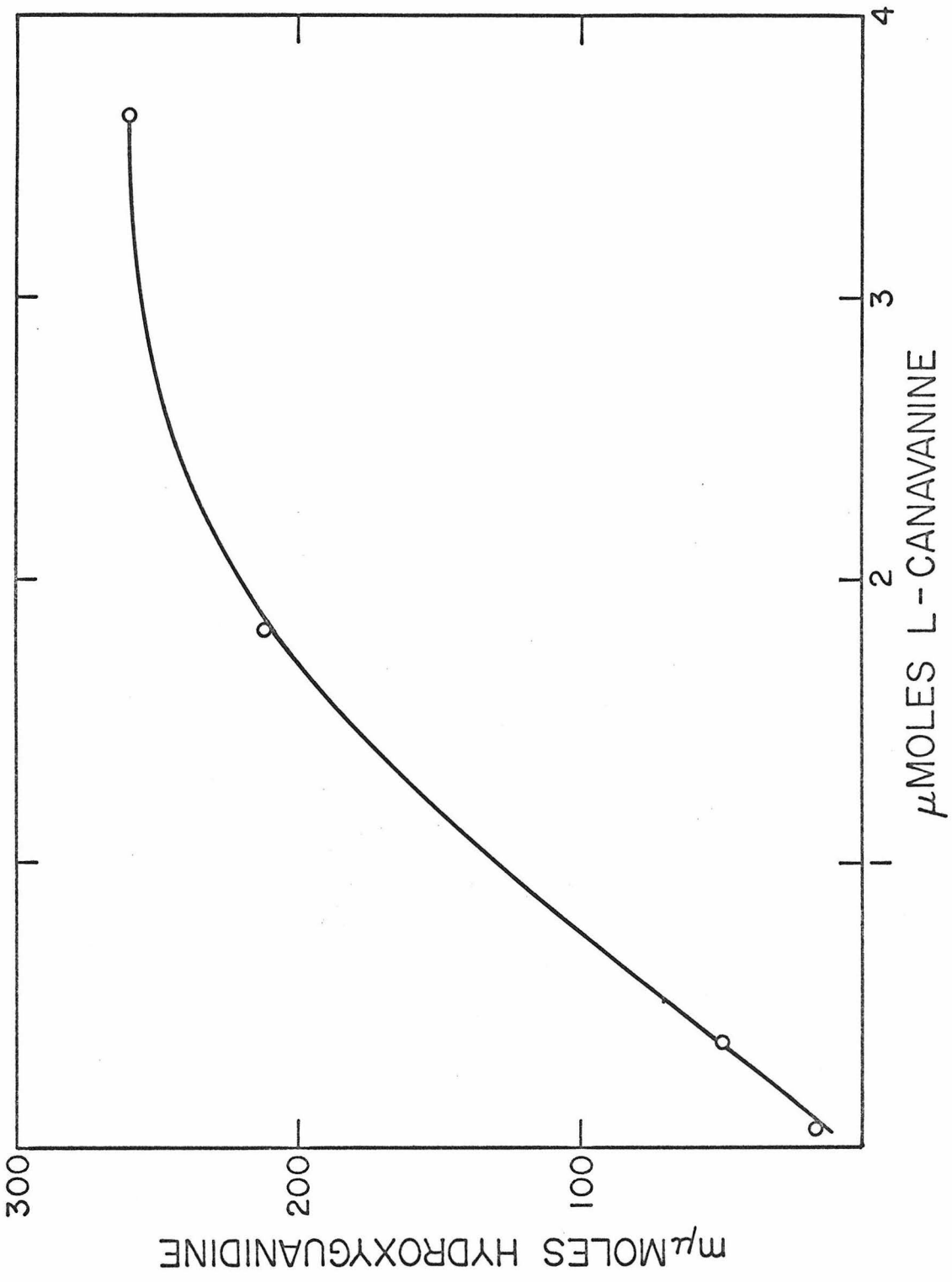
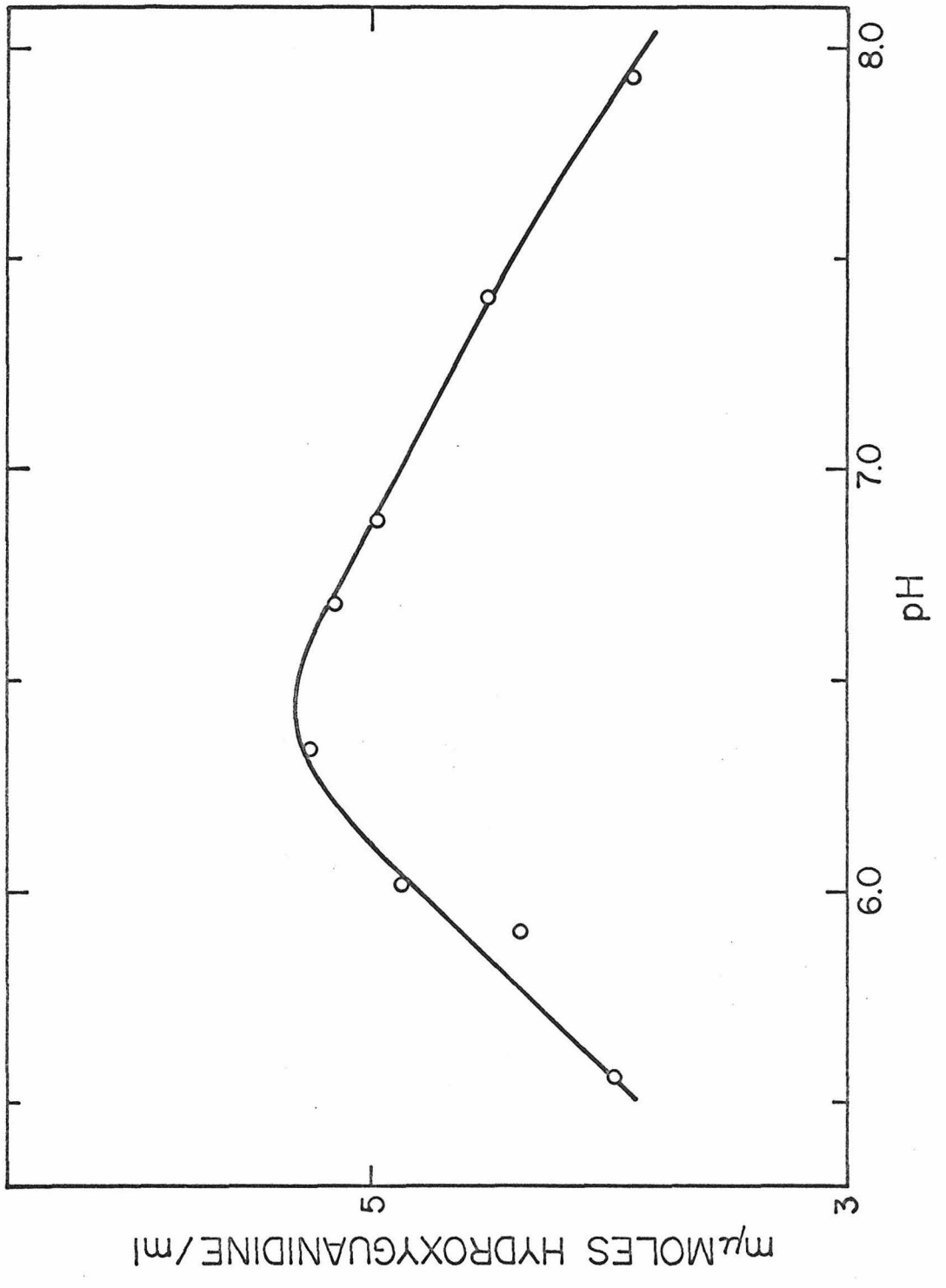

mμMOLES HYDROXYGUANIDINE
300
200
100
μMOLES L-CANAVANINE
1
2
3
4

FIGURE 11

RELATION OF REACTION RATE TO pH

Each incubation mixture contained 15.8 mμmoles (0.50 μc) DL-C^{14}-canavanine, 0.5 μmoles EDTA, enzyme solution equivalent to 300 μg Fraction III protein, and 0.1 M sodium phosphate buffer to make a final volume of 0.50 ml. The incubations were carried out at 37°C for 25 minutes. The reaction was stopped by the addition of an equal volume of 1 N hydrochloric acid. After centrifugation duplicate 5 μl aliquots of the supernatant were assayed for canavanine destruction (Methods).


mμMOLES HYDROXYGUANIDINE/ml
5
3
6.0
7.0
8.0
pH

6.5. However, enzyme activity is not very strongly pH dependent. Quite significant activities were observed at both pH 5.5 and pH 8.5 as well as at pH values nearer the optimum.

Activities comparable to those in phosphate buffers were obtained in 0.05 M Tris buffers, pH 6.9-8.7, and 0.1 M sodium pyrophosphate buffers, pH 6.5 and 8.5. No activity was observed in 0.05 M glycine-sodium hydroxide buffers, pH 8.6-10.2.

In most of the experiments on properties of the enzyme, the assay solutions were buffered at pH 6.5. Because of the chelating properties of pyrophosphate buffers and because of its ease of removal by ethanol precipitation, 0.1 M sodium pyrophosphate buffer, pH 8.5, was used in a large number of other experiments.

Heat Stability. The rate of canavanine cleavage as measured by hydroxyguanidine production increases in a linear fashion with increasing assay temperature up to about 45°C (Figure 12). At temperatures over 50°C there is rapid thermal denaturation and at 62°C there is no measurable activity of the enzyme.

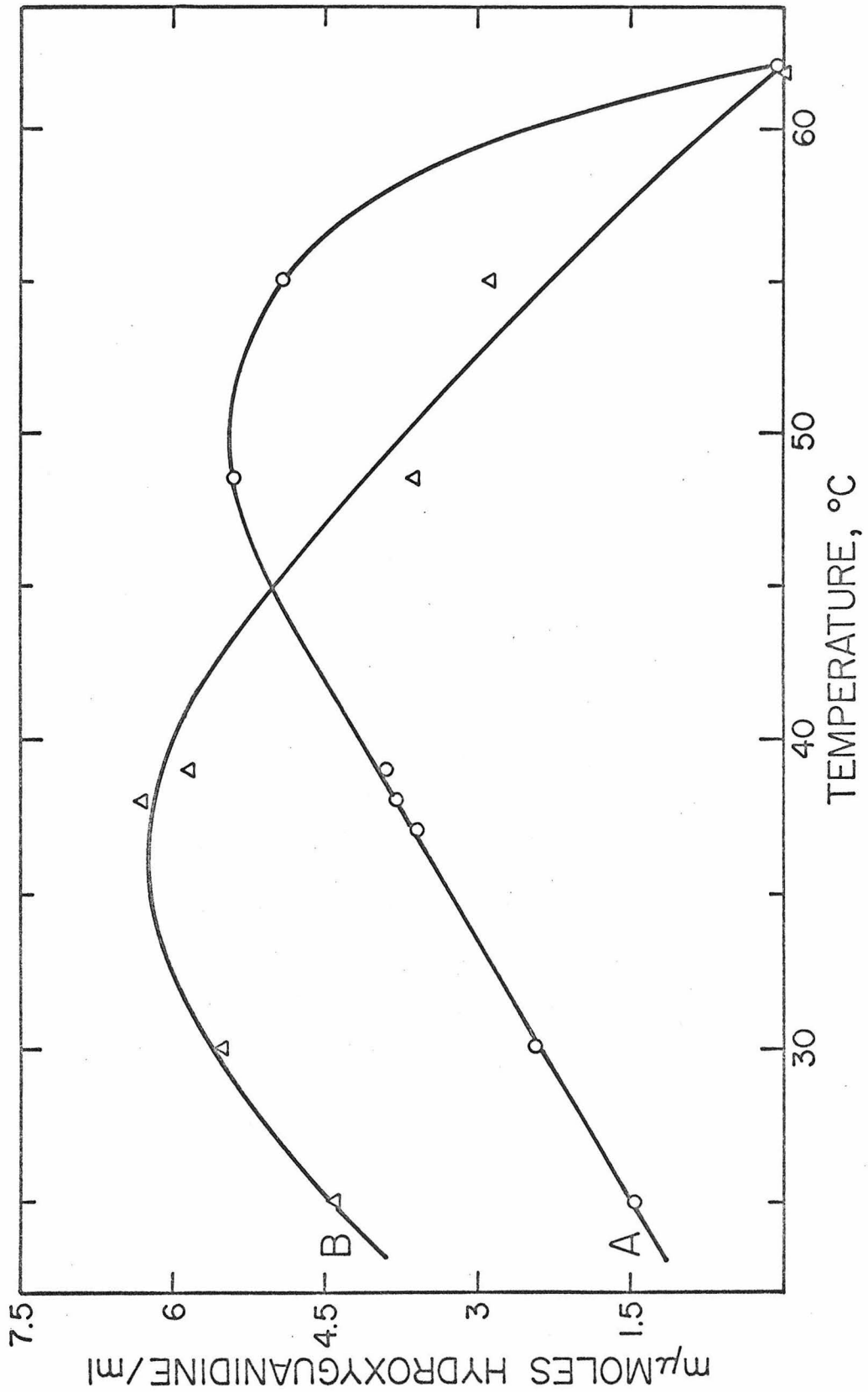
Preheating of enzyme preparations at moderate temperatures prior to assay gave a considerable stimulation of activity (Figure 12). Preincubation at 38°C for 30 minutes produced a 70 per cent increase in enzyme activity.

The enzyme seems relatively resistant to heat. It survived 30 minutes preheating at 48.5°C with no loss of activity and preheating at 55°C with less than 20 per cent inactivation.

FIGURE 12

EFFECT OF TEMPERATURE ON REACTION RATE AND ENZYME STABILITY

Each incubation mixture contained 15.8 mμmoles (0.50 μc) DL-C^{14}-canavanine, 12.5 μmoles EDTA, 400 μg Fraction III protein, and 0.1 M sodium phosphate buffer, pH 6.5, to make a final volume of 0.50 ml. The reaction was stopped after 25 minutes by the addition of an equal volume of 1 N hydrochloric acid. After centrifugation duplicate 5 μl aliquots of the supernatant were assayed for canavanine destruction (Methods). Curve A shows the amount of products formed in 25 minutes at indicated temperatures. Curve B shows the amount of products formed in 25 minutes at 37°C after 30 minutes preincubation of enzyme alone at the indicated temperature.


7.5
6
4.5
3
1.5
mμMOLES HYDROXYGUANIDINE/ml
B
A
30
40
50
60
TEMPERATURE, °C

Lack of Cofactor Requirement. During the course of this work several attempts were made to find a cofactor which would stimulate the reaction rate. An increased rate was desired so that better yields of reaction products could be obtained at high substrate concentrations. Also such a substance might provide a clue to the mechanism of canavanine cleavage and thereby suggest possible reaction products other than hydroxyguanidine. Several of the common cofactors were tried under standard assay conditions and in no case was there any effect when compared with control assays. All compounds tested were neither stimulatory nor inhibitory. The compounds tested were as follows: ATP, NAD, NADH, NADP, NADPH, CoA, Acetyl CoA, FAD and pyridoxal phosphate.

Inhibition of Activity. The production of hydroxyguanidine by the cleavage of canavanine is sensitive to inhibition by several metal ions (Table 8). Of those tested, Ca(II), Fe(II), and Mg(II) are the most effective, giving per cents of inhibition of 27, 24, and 19 respectively at 10^{-4} M concentration. Hg(II) was slightly stimulatory.

The presence of trace metal contaminants in the reagents or the enzyme preparation may explain the stimulatory effect in Tris buffer of the metal chelating agent EDTA (Table 9). This effect is most pronounced at high pH values at which there is little activity in Tris alone. Addition of EDTA to incubation mixtures buffered at a high pH value with Tris produces activities comparable to those obtained at the optimal pH 6.5. EDTA produces very little activation with phosphate

TABLE 8

EFFECT OF INHIBITORS ON CANAVANINE CLEAVING ACTIVITY

Inhibitor	Concentration M	Per cent of control activity
none		100
Ca (II)	10^{-4}	73
Cu (II)	10^{-4}	93
Co (II)	10^{-4}	91
Fe (II)	10^{-4}	76
Mg (II)	10^{-4}	81
Mn (II)	10^{-4}	103
Hg (II)	10^{-4}	116
KCN	10^{-4}	102
Zn (II)	10^{-4}	105
homoserine	3.16×10^{-7}	79
hydroxyguanidine	3.16×10^{-7}	75
homoserine + hydroxyguanidine	3.16×10^{-7} each	49

Incubation mixtures with metal ions contained, in a total volume of 0.5 ml, 15.8 mμmoles DL-C^{14}-canavanine, 400 μg Fraction III protein, 0.05 μmoles metal ion and 0.05 M Tris buffer, pH 6.88. Incubation mixtures with homoserine and hydroxyguanidine contained, in addition to the same amount of canavanine and enzyme as above, EDTA at a concentration of 10^{-3} M and 0.1 M sodium phosphate buffer, pH 6.88. The reaction was stopped after 25 minutes at 37°C by the addition of hydrochloric acid.

TABLE 9

EFFECT OF EDTA ON CANAVANINE CLEAVING ACTIVITY

Buffer with 10^{-3} M EDTA	Per cent of control activity
0.05 M Tris, pH 6.88	162
0.05 M Tris, pH 7.71	183
0.05 M Tris, pH 8.31	361
0.1 M phosphate, pH 8.0	109
0.1 M pyrophosphate, pH 8.5	96

Abbreviations used are: EDTA (ethylenediamine-tetra-acetic acid), Tris [tris(hydroxymethyl)methyl-amine], phosphate (sodium phosphate), and pyrophosphate (sodium pyrophosphate). The pH values given are those at assay temperature, 37°C. Control activities were determined in the indicated buffer without added EDTA. Standard assay conditions were used. Fraction III protein was employed and the reaction stopped at 25 minutes by the addition of hydrochloric acid.

or pyrophosphate buffers, presumably because these buffers can bind trace amounts of toxic metal ions.

At concentrations 10 times that of canavanine, homoserine and hydroxyguanidine give significant inhibition of canavanine cleaving activity. Either of these compounds could conceivably control this activity *in vivo* by competition or product inhibition.

Effect of Oxygen. Equilibrating an incubation mixture with pure oxygen had a pronounced stimulatory effect on the rate of canavanine cleavage (Figure 13); under appropriate conditions doubling the rate (Table 10). This fact was routinely utilized in the latter part of this work to make large scale preparations of reaction products (Methods). Incubation under nitrogen drastically reduced hydroxyguanidine to one third the control rate (Table 10). The residual cleaving activity under nitrogen may be due to oxygen dissolved in the reagents and the enzyme preparations.

The rate of oxygen consumption during the cleavage of canavanine was recorded using a Model 53 Biological Oxygen Monitor (Yellow Springs Instrument Company), a polarographic oxygen electrode. At pH 8.5, 30°C, the measured rate of oxygen consumed was 20.4 mμmoles O_2/hr. The amount of hydroxyguanidine produced in the same reaction mixture was 17.1 mμmoles/hr. From these data it would appear that one molecule of oxygen is consumed for every molecule of canavanine destroyed. However, confidence in this conclusion is tempered by the following facts: (1) this was a single measurement and has not been

FIGURE 13

PRODUCTION OF HYDROXYGUANIDINE UNDER OXYGEN

Two identical incubation mixtures were prepared containing 15.8 mμmoles DL-C^{14}-canavanine, 500 μg Fraction III protein and 0.1 M sodium pyrophosphate buffer, pH 8.5 in 0.5 ml. Oxygen was bubbled slowly through one mixture and the other was left open to the air. Incubation temperature was 37°C. Fifty μl aliquots were removed at the indicated times and placed in an equal volume of cold ethanol. Canavanine destruction was measured by standard procedures (Methods).

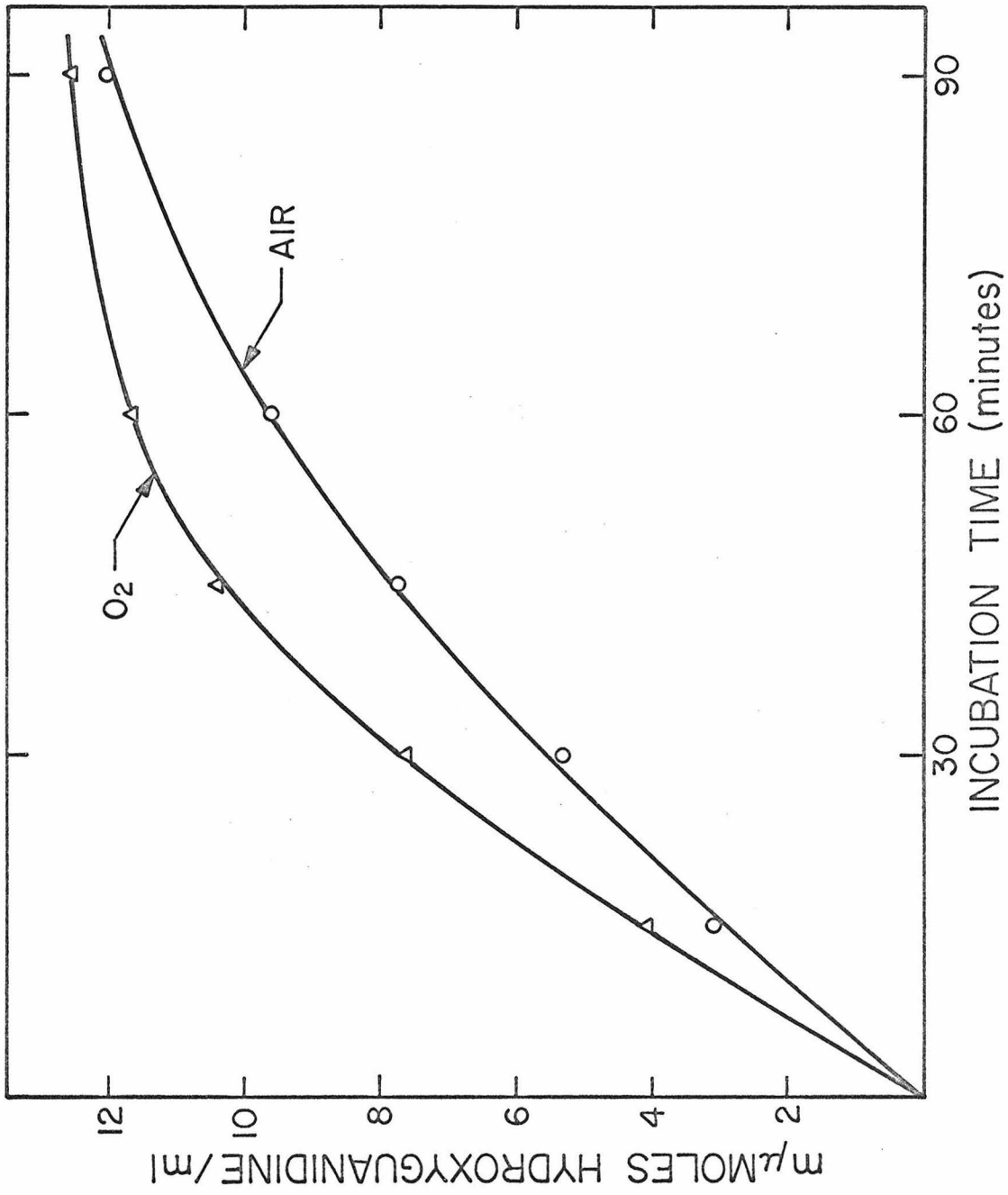

mμMOLES HYDROXYGUANIDINE /ml
2
4
6
8
10
12
30
60
90
INCUBATION TIME (minutes)
AIR
O2

TABLE 10

EFFECT OF OXYGEN AND NITROGEN ON CANAVANINE CLEAVING ACTIVITY

Gas	Hydroxyguanidine formed, μμmoles	Per cent of control assay
air	1,140	100
nitrogen	379	33
oxygen	2,280	200

Incubation mixtures contained 31.6 mμmoles DL-C^{14}-canavanine, 500 μg Fraction III protein, and 0.1 M sodium pyrophosphate buffer, pH 8.5 in 1.0 ml total volume. The reaction was run in 20 ml beakers whose tops were covered with a rubber stopper containing a gas inlet and a chimney outlet. The gas inlet of one beaker was connected to a tank of oxygen and that of another to a tank of nitrogen. The third beaker was left in equilibrium with the air as the control assay. The reaction mixtures were placed on a Dubnoff shaker-water bath and incubated at 35°C for 20 minutes. The reaction was stopped by the addition of an equal volume of cold ethanol. Canavanine destruction was determined by standard procedures (Methods).

confirmed; (2) the measured rate of oxygen consumption is very near the limit of resolution of the oxygen monitor.

Genetics of Resistance

In their paper which first noted the effect of canavanine on Neurospora crassa, Horowitz and Srb described strains with three levels of resistance: one with high resistance, one with intermediate resistance, and one with low resistance (sensitivity) (20) (see Figure 2). The occurrence of partial resistance suggests that the explanation for the action of canavanine in Neurospora crassa is more complicated than merely the presence or absence of canavanine cleaving activity. To elucidate more fully the nature of canavanine resistance, the inheritance of the canavanine character as well as biochemical and physiological differences among strains differing in canavanine susceptibility have been studied.

As noted in the Horowitz and Srb paper, the distinction between full and intermediate resistance is not always easy to make. In some experiments the growth response of strain 4A, the fully resistant strain most often used in the present study, was very much like that of a strain of intermediate resistance. In other experiments it showed complete resistance. On the other hand, the behavior of sensitive and true intermediate strains was quite reproducible. It was found that using a large inoculum of young conidia made the response of strain 4A more reproducible and more typical of high resistance. In the experiments which follow, a strain which gave 80 per cent or more normal

growth at a concentration of 0.05 mg/ml canavanine sulfate in the growth medium was designated fully resistant. Intermediate strains were those which gave any growth at all up to the 80 per cent level. The growth of sensitive strains was abolished by canavanine sulfate concentrations greater than 7.5×10^{-4} mg/ml.

Genetics of Canavanine Effect. (a) Origin of Strains. Two wild-type strains, 4A (can-r) and 25a (can-s), were crossed and asci dissected. Analysis of the segregation of the canavanine character was obscured in some of these asci by presence of a factor producing slow growth on minimal medium. It seemed possible after years of being maintained by periodic transfers to fresh slants of complete medium that the strains had become heterokaryotic. Therefore, an ascus which showed both good growth of all isolates and segregation of all three levels of resistance was selected from this cross for further genetic analysis. Growth of the eight strains derived from single ascospores of this one ascus was tested in minimal medium and in medium supplemented with canavanine (Table 11).

(b) Crosses. The following crosses were made: 3-221A(R) x 3-225a(S); 3-221A(R) x 3-227a(S); 3-223A(I) x 3-225a(S); and 3-223A(I) x 3-227a(S) (Methods). The final cross gave only unripe spores and, therefore, data from it are unavailable. Asci from the remaining three crosses were dissected and the levels of resistance of the spore pairs were determined by growth tests in minimal medium and in medium supplemented with L-canavanine. The data for each of these crosses

TABLE 11

CANAVANINE SUSCEPTIBILITY OF NEUROSPORA STRAINS

Strain	Mating type	Dry weight of mycelium (mg) supplement in minimal medium		Per cent of normal growth	Canavanine susceptibility
		None	0.05 mg/ml L-canavanine		
3-221	A	45	45	100	R
3-222	A	44	40	90	R
3-223	A	56	12	22	I
3-224	A	56	27	48	I
3-225	a	56	0	0	S
3-226	a	48	0	0	S
3-227	a	48	0	0	S
3-228	a	47	0	0	S

Growth was measured as dry weight of mold produced in 72 hours at 25°C in 20 ml of medium contained in 125 ml Erlenmeyer flasks. The mycelial pads were dried at 100°C overnight. R = full resistance; I = intermediate resistance; S = sensitivity.

are given in Tables 12-14. If, for the moment, full and intermediate resistance are considered together as the resistant phenotype, then the most striking fact to emerge from these crosses was that sensitivity and resistance segregated two to two in every ascus. From these results it was concluded that the determination of the sensitive versus nonsensitive phenotype is governed by a single gene. The frequency of second division segregation of sensitivity (21/33) was the same as that found by Teas (11/17), who concluded that a canavanine resistance (cnr) gene is linked closely with aur on the right arm of the first linkage group (39). These linkage data were confirmed by Perkins (55). It seems likely that the gene determining sensitivity is the same as the cnr gene.

However, it is clear that more than one gene is involved in the total expression of resistance to canavanine since an intermediate phenotype appears in the progeny of a cross between resistant and sensitive strains. This would not be expected if the phenotypes R, S and I were governed by a set of alleles at a single locus. Lockhart and Garner had previously come to the same conclusion (42), and postulated that two genes determined the reaction of Neurospora to canavanine.

The data in Tables 12-14 argue against an explanation based on only two genes. However, to understand this point, it is helpful first to consider the two gene case. The simplest way to account for the results of the original cross of 4A x 25a assumes a two factor scheme of the following type:

TABLE 12

TYPES OF ASCI RESULTING FROM CROSS OF 3-221A X 3-225a (R X S)

Spore pair		No. asci	Spore pair		No. asci
1	S	2	1	S	1
2	S		2	S	
3	I		3	I	
4	I		4	R	
1	S	5	1	S	3
2	I		2	I	
3	S		3	S	
4	I		4	R	
1	S	1	1	S	1
2	R		2	S	
3	S		3	R	
4	R		4	R	
			Total No. of Asci = 13		

	No. Asci
S segregating 1st division	4
S segregating 2nd division	9

R = full resistance

I = intermediate resistance

S = sensitivity

TABLE 13

TYPES OF ASCI RESULTING FROM CROSS OF 3-221A X 3-227a (R X S)

Spore pair		No. asci	Spore pair		No. asci
1	S	4	1	S	1
2	S		2	R	
3	I		3	S	
4	I		4	R	
1	S	5	1	S	2
2	I		2	S	
3	S		3	I	
4	I		4	R	

Total No. of Asci = 12

	No. Asci
S segregating 1st division	6
S segregating 2nd division	6

R = full resistance

I = intermediate resistance

S = sensitivity

TABLE 14

TYPES OF ASCI RESULTING FROM CROSS OF 3-223A X 3-225a (I X S)

Spore pair		No. asci	Spore pair		No. asci
1	S	2	1	S	6
2	S		2	I	
3	I		3	S	
4	I		4	I	
			Total No. of Asci = 8		

	No. Asci
S segregating 1st division	2
S segregating 2nd division	6

R = full resistance

I = intermediate resistance

S = sensitivity

phenotype	genotype
resistant (R)	Bd
intermediate (I)	BD
sensitive (S)	bd,bD

The ascus from this cross selected for further study showed segregation for all three levels of resistance. Assuming two genes, this may be diagrammed as follows:

P_1 4A (Bd) x 25a (bD)

F_1

phenotype	genotype
R	Bd
I	BD
S_1	bD
S_2	bd

The resulting sensitive strains differ in genotype; one is parental (bD) and the other is recombinant (bd). It would be possible to generate the intermediate phenotype from a cross with the resistant strain using one of these sensitive strains but not using the other.

$$R\ (Bd) \times S_1\ (bD) \Longrightarrow I\ (BD)$$
$$R\ (Bd) \times S_2\ (bd) \not\Longrightarrow I\ (BD)$$

The actual data are shown in Tables 12 and 13, and it can be seen that the intermediate phenotype appears in the progeny of both crosses between the resistant strain and sensitive strain. This fact is inconsistent with the two factor scheme described above, and

therefore it is concluded that the level of canavanine resistance is governed by more than two genes.

Let us now consider the three gene case. Assume canavanine resistance to be determined by the genes B, D and E, which were present in the parent strains in the following distribution: 4A = Bde and 25a = bDE. This scheme may be summarized as follows:

phenotype	genotype
R	Bde
I	BDE, BDe, BdE
S	bDE, bDe, bdE, bde

These designations are not arbitrary. They have some basis in experimental fact, as will be made clear in the following sections.

An ascus which segregated all three levels of resistance was selected from the original cross of 4A (Bde) x 25a (bDE). The four types of R I S S asci which can result from such a cross are listed below:

Possible R I S S Asci

phenotype	genotype			
	(1)	(2)	(3)	(4)
R	Bde	Bde	Bde	Bde
I	BDE	BDE	BDe	BdE
S	bDE	bDe	bDE	bDE
S	bde	bdE	bdE	bDe

The results of crosses of resistant, intermediate, and sensitive spores within the ascus exclude the three gene hypothesis. Ascus

(1) does not explain the result, because in the cross involving R x S (bde), no progeny of intermediate resistance would be expected. As we have seen, in both of the actual crosses involving R x S, progeny of intermediate phenotype were observed.

Genotype sets (2), (3) and (4) would yield all the observed ascus types. However, they would not produce the observed relative frequency of ascus types. The progeny of both crosses of R x S showed a preponderance of asci which segregated only I and S as compared with those segregating only R and S. In one cross the ratio was 7:2 and in the other, 9:1. The genotype sets (2), (3) and (4) predict a ratio of 1:1 or less in one or the other of these crosses. Therefore, it is concluded that more than three genes are required to explain the observations.

There is another possibility, however: misclassification of fully resistant strains as intermediate strains would reduce the R R S S ascus class and enlarge the I I S S class, and thereby produce an aberrant, high ratio (IISS:RRSS). The result would be an unnecessary multiplication of the factors required to explain the data.

Inheritance of Canavanine Cleaving Activity. Six ordered asci from the crosses described above were tested for the presence or absence of canavanine cleaving activity. All fully and partially resistant strains possessed activity. All sensitive strains lacked it. This conclusion has been confirmed by the examination of several wild type

and auxotrophic strains of Neurospora crassa from various origins throughout the course of this work.

It thus appears that sensitivity corresponds to lack of cleaving activity. If this is true then the segregation of the canavanine character is a measure of the segregation of enzyme activity. In that case the cnr gene described and mapped by Teas may control canavanine cleaving activity.

Cleaving Activity in Fully and Partially Resistant Strains. The spectrum of differing degrees of resistance that is observed among the strains might be explained if there were a corresponding spectrum in the amount of cleaving activity. By this view, if strains were ordered by decreasing resistance, there would be a parallel decreasing order of measurable enzyme activity. A fully resistant strain would have enough cleaving activity to destroy any canavanine before inhibition of growth. This explanation does not seem to hold, for when such an ordering of strains was performed, no correlation with cleaving activity was observed other than the previously mentioned lack of activity in all sensitive strains (Table 15). Some partially resistant strains show considerable amounts of cleaving activity while some fully resistant strains have relatively modest amounts. The mean cleaving activity in fully resistant strains was 525 μμmoles hydroxyguanidine formed per hour per mg protein, with a standard deviation of ± 23. Strains of intermediate resistance had a mean activity of 520 ± 22 μμmoles hydroxyguanidine formed per hour per mg protein.

TABLE 15

LEVEL OF CANAVANINE CLEAVING ACTIVITY IN VARIOUS STRAINS

Three-day-old standing pads were harvested and pressed dry. Two pads were ground together in 0.5 ml 0.1 M sodium pyrophosphate buffer, pH 8.5. Fifty µl of this extract were incubated with 7.9 mµmoles DL-C^{14}-canavanine and buffer in a final volume of 0.25 ml at 37°C for one hour. The reaction was stopped by the addition of an equal volume of hydrochloric acid. Canavanine destruction was measured using standard procedures (Methods). The number in parentheses is the percentage of normal growth of that strain when grown on canavanine at a level of 0.05 mg/ml canavanine sulfate. The strains are listed in decreasing order of resistance.

TABLE 15

LEVEL OF CANAVANINE CLEAVING ACTIVITY IN VARIOUS STRAINS

Canavanine susceptibility (% normal growth)	Specific activity μμmoles hydroxy-guanidine/hr /mg protein	Canavanine susceptibility (% normal growth)	Specific activity μμmoles hydroxy-guanidine/hr /mg protein
R (103)	444	S	33
R (100)	516	S	17
R (100)	512	S	0
R (97)	555	S	29
R (92)	598	S	46
I (73)	438	S	7
I (65)	414	S	22
I (49)	433	S	18
I (48)	625	S	9
I (45)	577	S	37
I (39)	456	S	44
I (37)	516	S	24
I (27)	587		
I (22)	641		
I (15)	509		
I (13)	520		

The difference between fully and partially resistant strains might be explained on the basis of the observed inhibition of canavanine cleavage by hydroxyguanidine. If this inhibition occurs *in vivo* it would decrease the effective canavanine destroying capacity and thereby decrease the potential resistance of the strain. Fully resistant strains remove accumulated hydroxyguanidine *in vivo* by reducing it to guanidine. Perhaps partially resistant strains lack the ability to reduce hydroxyguanidine. This idea was tested and found to be incorrect. When intact mycelial pads of partially resistant strains were pulsed with radioactive canavanine, they showed the same production of guanidine as fully resistant controls.

Uptake of Canavanine. Several studies in *E. coli*, yeast, and *Neurospora* have shown that certain mutants are resistant to growth inhibition by amino acid analogs by virtue of an impaired ability to concentrate the analog from the medium (see Introduction). In those cases where canavanine was the examined analog, two kinds of defects in amino acid permeability were found. The first involves a recessive mutation in yeast in which the uptake of L-arginine is specifically impaired (54). The second case is in *E. coli* where the accumulation of a considerable number of compounds is affected (38).

Because of this experience in other systems, it seemed important to measure the comparative rate of entry of canavanine into the mycelium of selected strains in the system under consideration in this report. Since basic amino acids are taken up very rapidly from the medium (56), it was necessary to find conditions under which the uptake

of both canavanine and arginine were linear for a sufficient amount of time to permit rate measurements to be made. An estimate of the rate of amino acid entry was obtained by incubating mycelial pads in media containing the labeled amino acid, taking aliquots of the medium at regular intervals and measuring the loss of radioactivity using a planchet counter. Concentrations of the amino acids were determined such that uptake was linear for at least fifteen minutes in the case of canavanine and at least ten minutes in the case of arginine.

The rates of uptake of both canavanine and arginine were then measured in a number of strains derived from the crosses previously described. Using the conditions determined in the preliminary experiments described above, mycelial pads were pulsed with the radioactive compound, the metabolite pool was extracted, and the accumulated radioactivity measured. The data from these experiments are summarized in Table 16. It can be seen that uptake of both canavanine and arginine was low for all fully resistant strains. Uptake of both amino acids was high for all partially resistant strains. From these facts it appears that the combination of low permeability to canavanine plus canavanine cleaving activity imparts full resistance. The intermediate phenotype apparently results when high permeability permits a flood of incoming canavanine to exceed the detoxifying capacity of the canavanine cleaving enzyme.

Sensitive strains showed both high and low uptake rates for both amino acids. Obviously, however, the low rates that were

TABLE 16

UPTAKE OF CANAVANINE AND ARGININE IN VARIOUS STRAINS

Canavanine uptake--The strains were grown in stationary culture at 25°C for three days in 20 ml of minimal medium contained in 125 ml Erlenmeyer flasks. One μc (31.6 mμmoles) DL-C^{14}-canavanine was added directly to the medium and the flask placed on a rotary shaker for 10 minutes. The pad was collected on a Buchner funnel, washed with 100 ml cold water, and plunged into 5 ml cold 5% TCA. After centrifugation duplicate 25 μl aliquots of the supernatant were counted on a Nuclear Chicago low background counter. The results are recorded as counts per minute per dry weight of mycelium.

Arginine uptake--The specific activity of C^{14}-arginine was diluted by the addition of C^{12}-arginine. One μc (25.8 μmoles) was added to the medium and the flask shaken for two minutes. The pad was then treated exactly as above.

TABLE 16

UPTAKE OF CANAVANINE AND ARGININE IN VARIOUS STRAINS

Origin of strain	Strain	Canavanine susceptibility	Canavanine cleaving activity	Uptake, cpm/mg	
				Canavanine in 10 minutes	Arginine in 2 minutes
Cross 1 4A X 25a	3-221	R	+	480	198
	3-223	I	+	2940	790
	3-225	S	-	3070	992
	3-227	S	-	2160	514
Cross 2 3-221 X 3-225	7-211	I	+	2730	1620
	7-213	S	-	2880	1972
	7-215	R	+	580	122
	7-218	S	-	2420	130
	7-541	S	-	2500	822
	7-543	S	-	1740	410
	7-545	I	+	2900	994
	7-547	I	+	3020	978

TABLE 16 (continued)

Origin of strain	Strain	Canavanine susceptibility	Canavanine cleaving activity	Uptake, cpm/mg	
				Canavanine in 10 minutes	Arginine in 2 minutes
Cross 3 3-221 X 3-227	4-112	I	+	2440	2000
	4-113	I	+	3520	1078
	4-115	S	-	2980	730
	4-117	S	-	1480	824
	4-421	S	-	560	838
	4-423	S	-	3280	808
	4-425	R	+	400	426
	4-427	I	+	2970	1034
Cross 4 3-223 X 3-225	6-1031	I	+	3500	2516
	6-1034	S	-	3000	1456
	6-1035	S	-	4700	1288
	6-1037	I	+	3500	1600

measured do not represent negligible rates of canavanine transport since there was not a concomitant loss of canavanine sensitivity.

Bauerle and Garner (43) have reported that canavanine is taken up from the medium in Neurospora by the same mechanism which concentrates arginine and lysine. Pall (personal communication) has confirmed that there is an uptake system specific for basic amino acids. In the light of these facts it is surprising that one strain (7-218) showed a low rate of uptake for arginine and a high rate for canavanine. More work needs to be done, especially with various competitors, before any firm conclusions can be drawn concerning the specificity of the uptake system involved with canavanine resistance.

The results in Table 16 indicate that the inheritance of slow canavanine uptake is complex. Several asci do not show simple segregation for uptake. Moreover, in every tested cross between a strain of fast uptake and one of slow uptake, some asci failed to segregate slow-uptake progeny. If one assumes two genes controlling canavanine uptake, such asci are not possible from both crosses involving slow-uptake strain, 3-221. The parent strains for these crosses were derived from different spore pairs of a single ascus and therefore in a scheme involving two genes for transport, fast-uptake strains 3-225 and 3-227 must differ in genotype. In a cross with strain 3-221, it would be possible to produce an ascus which failed to segregate slow-uptake progeny with one of these strains, but not with the other. It appears that at least three genes controlling canavanine transport must be assumed to explain these observations.

Prevalence of Resistance. In 1960 Perkins (55) reported that he had examined for canavanine resistance ten different Neurospora crassa strains of various origins, including wild types 74A, and Abbott 4A. He found only one sensitive strain, al(Y602). This strain originated from a cross in which one of the parents was the known sensitive strain 25a.

The level of canavanine resistance was determined in 14 strains in the present work. Only one, 25a, was found to be sensitive. These results support Perkins' conclusion that sensitivity rather than resistance is the unusual condition. The strains that were found to be fully or partially resistant to canavanine are as follows: 1A, 69-1113a, 4A, Sing 2a, 262A, 16a, 19A, 80702a (cys-2), 39816 (cys-10 or Me 4), P110, 33933 (lys-1), 4545 (lys-3), and 15069 (lys-4).

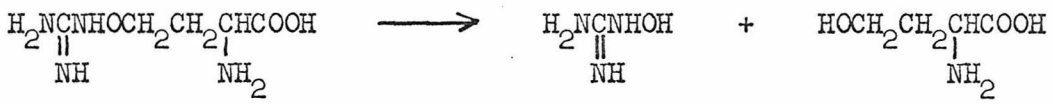
Other Reaction Products

As previously mentioned, the radioactive canavanine used in these studies is labeled only in the carbon of the guanidino group. By following the fate of this label it has been possible to demonstrate that hydroxyguanidine is a product of the degradation of canavanine. Different methods must be used in the search for other reaction products.

Of all the reaction pathways for canavanine destruction that suggest themselves, a simple hydrolytic cleavage seems the most likely prospect for the following reasons: (a) the production of hydroxyguanidine can easily be accounted for, using such a mechanism.

Hydroxyguanidine is a direct and straightforward product of canavanine hydrolysis. (b) The enzymatic degradation of canavanine shows no requirement for any cofactor of any kind as might be expected from more complex reaction mechanisms (see Purification and Properties of Canavanine Cleaving Enzyme). In a solution containing only buffer, an extensively dialyzed enzyme preparation that has been eluted from a DEAE column can rapidly degrade canavanine. (c) The hydrolytic cleavage of canavanine is a known reaction (13). A pseudomonad capable of utilizing canavanine as a sole source of carbon and nitrogen was found to cleave canavanine to yield hydroxyguanidine and homoserine.

Homoserine and hydroxyguanidine are the expected products from a hydrolytic cleavage of canavanine.



The attempt to demonstrate the production of homoserine during the enzymatic degradation of canavanine in resistant strains of Neurospora has, however, proved unsuccessful. The evidence arguing against homoserine as a reaction product is given below:

Homoserine not a Reaction Product. (a) Paper Chromatography. The procedures used to prepare large amounts of canavanine cleavage products are described in Methods. Partially purified enzyme preparations were incubated under oxygen for long periods of time with 10 or 20 mg L-canavanine·H_2SO_4. Under these conditions 25-40% of the

canavanine was degraded as measured by hydroxyguanidine production. Assuming stoichiometric production of hydroxyguanidine and homoserine, aliquots which should have contained from 10 μg to 400 μg of homoserine were applied to paper chromatograms. These are quantities easily detected by ninhydrin reagent. After developing in the first solvent, the strip containing the reaction products was sewn onto a new piece of filter paper and developed at right angles in the second solvent system. The various separation systems used are listed in Table 17. Chromatograms were also developed in just a single dimension with each of the separation systems listed. All chromatograms were dipped in cadmium-ninhydrin reagent and the color developed in the dark in an ammonia-free atmosphere. The results of all the analyses of reaction products by paper chromatography were equally negative. A spot of the appropriate intensity failed to appear in every case at the expected position for homoserine as determined by the concurrent running of standards.

(b) Column Chromatography. It is very easy to separate acidic, neutral and basic amino acids from each other using a Dowex 50 (H^+) column (63). If a mixture of amino acids is placed on such a column, elution with 1 N hydrochloric acid will remove neutral and acidic amino acids, and the basic amino acids are removed by further elution with 4 N hydrochloric acid.

A Dowex 50 (H^+,X-8) column (0.8 x 22.0 cm) was found to separate a standard mixture of homoserine and canavanine readily. A reaction mixture presumed to contain approximately 5 mg canavanine and

TABLE 17

SYSTEMS FOR TWO DIMENSIONAL CHROMATOGRAPHY

	Separation technique	
	First dimension	Second dimension
I	77% ethanol, 12 hours, descending	electrophoresis, 6.7% formic acid, pH 1.6, 58 volts/cm
II	pyridine:water (80:20) 12 hours, descending	electrophoresis, 6.7% formic acid, pH 1.6, 58 volts/cm
III	n-butanol:formic acid:water (75:15:10) 12 hours, descending	electrophoresis, 6.7% formic acid, pH 1.6, 58 volts/cm
IV	electrophoresis, 6.7% formic acid, pH 1.6, 58 volts/cm	77% ethanol, 12 hours, descending

Whatman No. 3MM filter paper was used throughout. Electrophoresis was performed on a Gilson Model DW electrophoretor.

5 mg homoserine was placed on this column. Three-milliliter fractions were collected as the column was eluted first with water, followed by 1 N hydrochloric acid and finally 4 N hydrochloric acid. Fractions were pooled and subjected to electrophoresis. In no fraction could a ninhydrin positive material with the electrophoretic mobility of homoserine or its lactone be detected.

(c) Tritiated Canavanine. Tritiated canavanine was incubated with a crude extract of mycelium from strain 4A using standard assay conditions. After precipitation of the enzyme, the supernatant was subjected to two dimensional chromatography using System I (Table 17). In one set of experiments the position of homoserine was determined by running standards alongside the experimental solution in each solvent, developing the standards of ninhydrin, and drawing a grid. In another set of experiments, carrier homoserine was added to the sample, and after development in both solvents, a light ninhydrin detector spray was used to locate the position of homoserine. In both sets of experiments, the indicated homoserine portion of the chromatogram was eluted and the radioactivity determined in a scintillation counter. The radioactivity measured was always at or near background levels.

(d) Homoserineless Mutant. Strain 51504 is a canavanine-resistant strain of Neurospora which has a growth requirement for homoserine or threonine plus methionine. In experiments with the radioactive compound, a pad of this strain grown in stationary culture was able to take up canavanine from the medium and degrade it to yield hydroxyguanidine and guanidine. As reported by Teas, this mutant

cannot use canavanine as a source of homoserine (39). This result has been confirmed and the data are shown in Table 18. At a level of 1 mg per flask, homoserine can support full growth. In combination with 1 mg threonine, microgram quantities of homoserine stimulate growth. If homoserine were an initial product of canavanine cleavage, then canavanine should enhance the growth of mutant strain 51504. It does not.

Observed Chromatographic Pattern. As mentioned in the previous section, two dimensional chromatography of a canavanine degradation reaction mixture does not produce a ninhydrin positive spot in the expected position for homoserine. However, in addition to unreacted canavanine, a second intense ninhydrin positive spot is observed at a position other than that of homoserine. Figure 14 shows a typical chromatogram developed in the first direction with 77 per cent ethanol and then at right angles in electrophoresis (6.7% formic acid, pH 1.6, 58 volts/cm). Seventy-seven per cent ethanol was used because serine and homoserine, which do not separate well in electrophoresis, are easily separated in this solvent. Hydrophobic amino acids run rapidly in the ethanol solvent while hydrophilic amino acids run slowly. Hydroxy amino acids give compact spots with intermediate R_F's. Acidic and basic amino acids remain near the origin and tend to streak. Electrophoresis separates essentially on the basis of charge, with basic amino acids traveling the greatest distance from the origin and acidic amino acids the least when 6.7 per cent formic acid is the buffer.

TABLE 18

GROWTH RESPONSE OF STRAIN 51504 TO CANAVANINE AND OTHER AMINO ACIDS

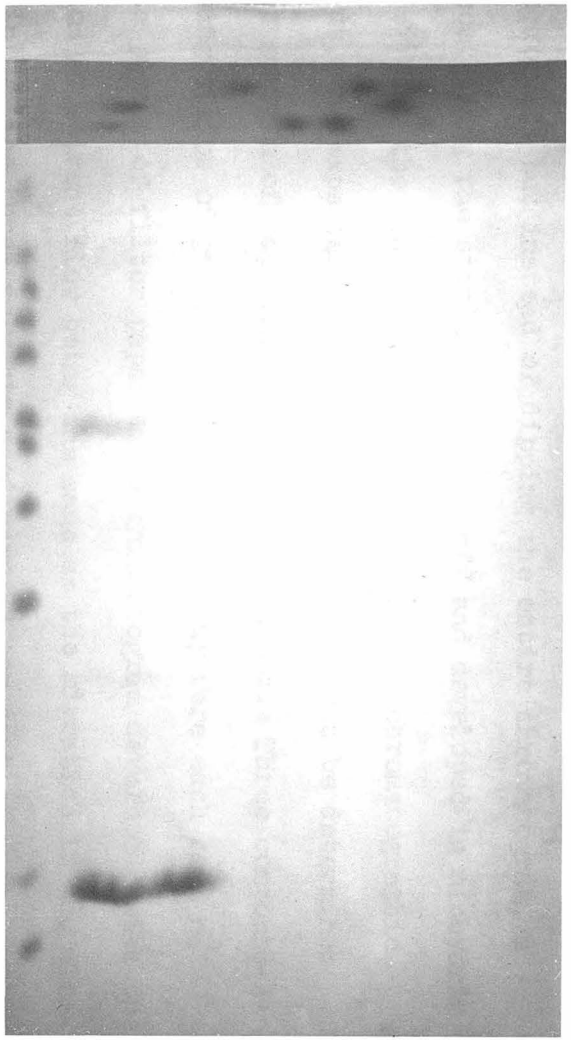
Amino acid supplement per flask	Dry weight of mycelium (mg)
None	3
1 mg L-canavanine	3
1 mg DL-homoserine	65
1 mg L-methionine	4
1 mg L-threonine	26
1 mg L-threonine + 1 mg L-methionine	69
1 mg L-threonine + 1 mg L-methionine + 800 µg L-canavanine	50
1 mg L-threonine + 5 µg DL-homoserine	28
1 mg L-threonine + 10 µg DL-homoserine	28
1 mg L-threonine + 20 µg DL-homoserine	42
1 mg L-threonine + 50 µg DL-homoserine	61
1 mg L-threonine + 40 µg L-canavanine	23
1 mg L-threonine + 80 µg L-canavanine	22
1 mg L-threonine + 400 µg L-canavanine	26

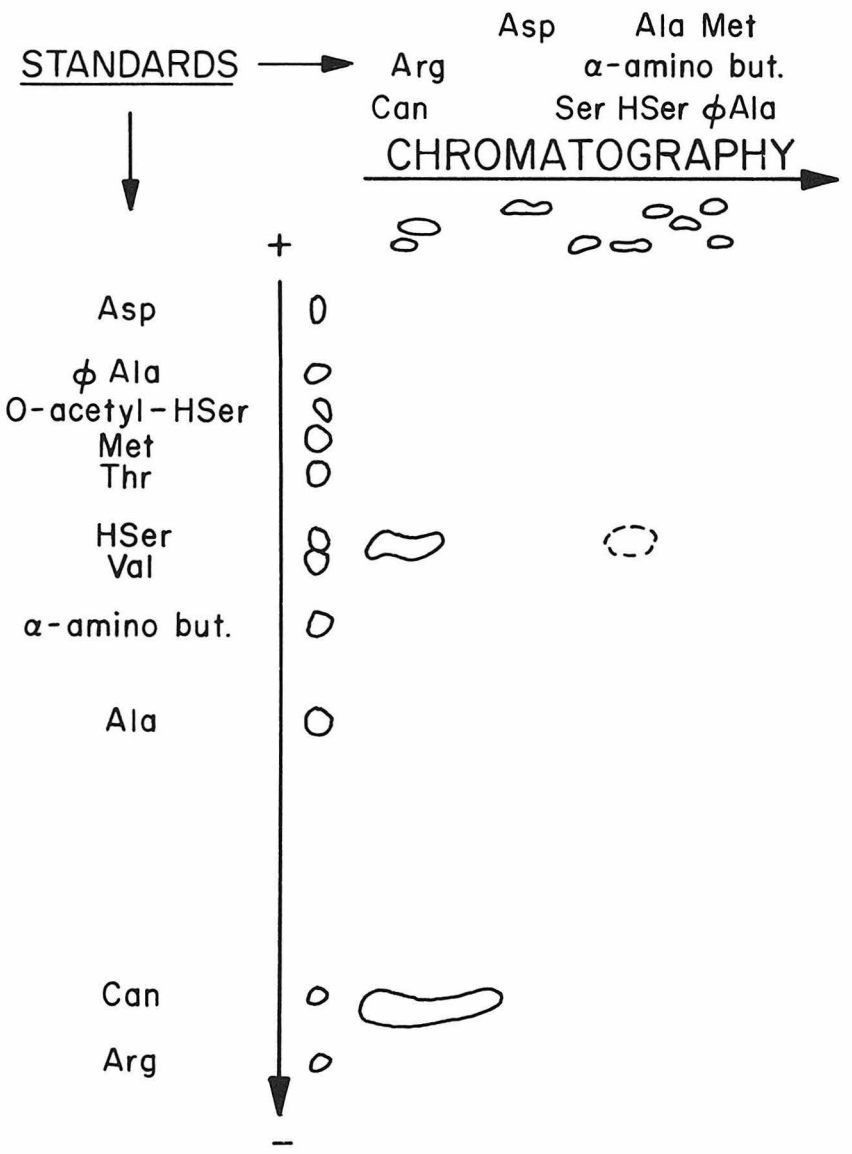
Growth was measured as the dry weight of mold produced in 89 hours at 25°C in 20 ml of medium contained in 125 ml Erlenmeyer flasks. The mycelial pads were dried at 100°C overnight.

FIGURE 14

TWO DIMENSIONAL CHROMATOGRAPHY OF REACTION MIXTURE

An aliquot of a standard preparative reaction mixture (Methods) was spotted on Whatman No. 3MM filter paper and, together with standards, developed descending for twelve hours with 77% ethanol. The portion of the paper containing the reaction mixture was cut off and sewn at right angles onto a new filter paper. The chromatogram was developed in the second direction by electrophoresis (6.7% formic acid, pH 1.6, 58 volts/cm, 105 minutes). After development with cadmium-ninhydrin reagent, a photograph was taken. The expected migration of homoserine is indicated by the dotted lines on the diagram.




STANDARDS
Asp
Ala Met
Arg
α-amino but.
Can
Ser HSer φAla
CHROMATOGRAPHY
+
Asp
φ Ala
O-acetyl-HSer
Met
Thr
HSer
Val
α-amino but.
Ala
Can
Arg
−

As can be seen in Figure 14, the unknown ninhydrin positive compound (compound X) remains near the origin during chromatography and travels with homoserine during electrophoresis. This chromatographic pattern of compound X is very reproducible. Preparations made in a variety of ways have repeatedly given the same pattern when subjected to two dimensional chromatography in separation System I (1. 77 per cent ethanol, 2. electrophoresis). A very similar pattern is also obtained when preparations are developed in System II (1. pyridine:water, 2. electrophoresis) or in System III (1. butanol: formic acid:water, 2. electrophoresis). In these separation systems compound X again stays near the origin during chromatography and travels with homoserine during electrophoresis.

If two dimensional chromatography of System I is used as a preparative rather than an analytical technique, the properties of compound X can be studied. Several such two dimensional separations were performed; but instead of dipping the entire chromatogram, a strip containing the standards was cut off and developed in ninhydrin reagent. With the aid of the electrophoretic and chromatographic standards, the expected position of compound X could be determined and this portion of each chromatogram was cut out. Three compound-X-containing strips of paper prepared in this way were each sewn on separate pieces of filter paper and a chromatogram developed, with the direction of solvent flow being the same as the migration in the electric field. The three solvents used were 77 per cent ethanol, pyridine:water (80:20), and butanol:acetic acid:water (75:15:10), and in

each of these solvents compound X had the same mobility as homoserine. Figure 15 shows a typical chromatogram.

Compound X was eluted from a fourth strip with water. The eluate was treated with dilute hydrochloric acid and the resulting solution was analyzed using paper electrophoresis (6.7% formic acid, pH 1.6, 58 volts/cm). In addition to homoserine, a compound was formed that had the same yellowish-brown color with cadmium-ninhydrin reagent as homoserine lactone and also migrated on paper identically with an authentic sample of homoserine lactone.

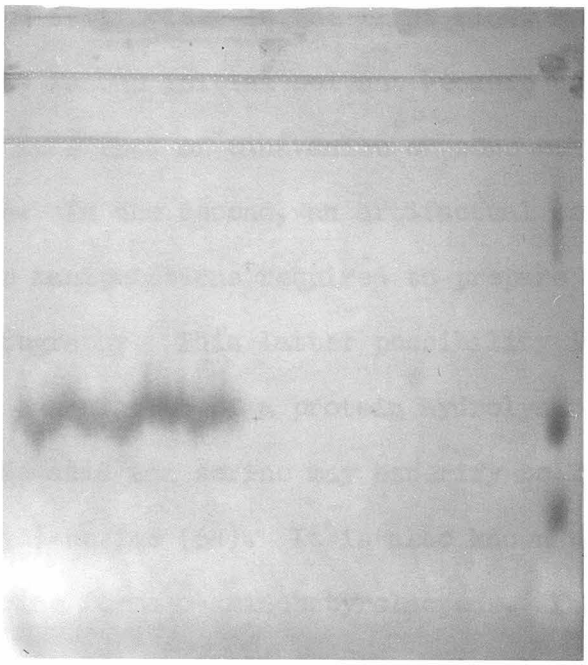
In summary, it can be said that after two dimensional chromatography, homoserine is almost certainly present on the chromatogram. This identification was made on the basis of the compound's chromatographic migration, given above for three solvents and for paper electrophoresis. The identification was confirmed by the production of homoserine lactone. Homoserine is present on the chromatogram but it failed to travel the expected distance in the initial solvent.

There are two possible explanations for this aberrant chromatographic behavior. The first is that not homoserine but some closely related compound present in the reaction mixture is somehow converted to homoserine under the conditions employed during chromatography. The second is that homoserine is present in the reaction mixture but does not appear in its proper chromatographic position due to some artifact. Since data in the preceding section argued against the presence of homoserine in the reaction mixture, it is very important to rule out this second possibility.

FIGURE 15

CHROMATOGRAM OF ISOLATED COMPOUND X

An aliquot of a standard preparative reaction mixture (Methods) was spotted on Whatman No. 3MM filter paper and, together with standards, developed descending for twelve hours with 77% ethanol. The portion of the paper containing the reaction mixture was cut off and sewn at right angles onto a new filter paper. The chromatogram was developed in the second direction by electrophoresis (6.7% formic acid, pH 1.6, 58 volts/cm, 105 minutes). With the aid of standards run simultaneously, a 5 cm wide section containing compound X was cut from the electrophoretogram and sewn onto a fresh piece of filter paper. Chromatography in the same direction as electrophoresis was carried out in 77% ethanol (12 hours descending).



STANDARDS

canavanine
asparagine

homoserine

α amino butyric
acid

① 77% ethanol

② electrophoresis

③ 77% ethanol

Experiments were designed to test for the presence of two kinds of artifacts. In the first kind, homoserine would fail to move properly in the initial solvent because it was physically retarded by the great excess of canavanine or some other substance in the reaction mixture. In the second, an artifactual compound would be formed during the manipulations required to prepare a reaction mixture for chromatography. This latter possibility is suggested by the fact that during evaporation of a protein hydrolysate in a vacuum desiccator, glutamic acid and serine may esterify to form the compound O-(γ-glutamyl)-serine (64). It is also known that in acidic solution homoserine forms α-aminobutyrolactone. In concentrated neutral or slightly acidic solutions, homoserine lactone reacts with itself to form a diketopiperazine (65). Since vacuum desiccation was routinely used in this work to concentrate samples for chromatography, it seemed important to rule out the possibility that free homoserine was being destroyed by reactions with itself, with canavanine, or with any other compound of the reaction mixture during experimental manipulations.

The idea that homoserine is produced but not detected due to an artifact was rejected on the basis of the following experiments:

(a) Attempt to reconstruct artifact. A small amount (5 μg) of homoserine was added to the standard aliquot of a reaction mixture prepared for chromatography. Development in System I produced both a distinct homoserine spot and a compound X spot. In another set of experiments, canavanine and homoserine were weighed out in a ratio of 20:1 and 200:1 and were passed through all the manipulations of a

standard reaction mixture. They were incubated together in buffer at 37°C for one hour, the buffer precipitated with ethanol, and the supernatant volume reduced to zero in a vacuum desiccator. The residue was dissolved in a small amount of water, developed in System I, and the chromatogram dipped in cadmium-ninhydrin reagent. Although there was some streaking of homoserine during chromatography when the ratio was 200:1, a distinct homoserine spot could be detected with ninhydrin reagent in each case.

(b) Reverse order of chromatography. If there were some artifact which prevents the separation of homoserine and canavanine during chromatography in ethanol:water, it clearly does not operate during electrophoresis since the compounds migrate separately in this dimension. By this view, high voltage electrophoresis must be powerful enough to separate canavanine and homoserine, perhaps through hydrolysis of a hypothetical canavanyl-homoserine compound in the formic acid buffer. If this were the case, simply reversing the order of events (i.e., performing electrophoresis first and chromatography second; System IV, Table 17) should produce a homoserine spot when analyzing a reaction mixture. When this is done, there is a most surprising and confusing result. No ninhydrin spot of the appropriate intensity is found at all. No spot is found when a simple one dimensional electropherogram is examined and none is found when the paper is developed in the second dimension with ethanol:water. No spot is found on the anode side or the cathode side of the origin.

(c) Dowex 50 column. If the only problem in demonstrating homoserine chromatographically were the presence of excess canavanine, then it should be easy to remove this canavanine using a Dowex 50 (H^+,X-8) column as described in the section entitled Homoserine not a Reaction Product. As described, a reaction mixture so treated gives no evidence of any homoserine. It should be noted in passing that O-(γ-glutamyl)-serine is hydrolyzed when applied to just such a Dowex 50 column which is then developed with hydrochloric acid.

Having rejected the possibility that homoserine remains undetected due to an artifact of chromatography, an effort was made to test the possibility that the reaction mixture contains a labile homoserine derivative. Experiments were designed to try to find some procedure that might generate homoserine. These attempts have been unsuccessful. Each experiment involved treating an aliquot of the reaction mixture in a particular way and testing chromatographically for the production of homoserine. The various procedures tried are listed below:

(a) Hydrolysis by formic acid--As mentioned before, initial electrophoresis fails to produce any ninhydrin positive material with a mobility of homoserine when any portion of the electropherogram is developed at right angles with ethanol:water.

(b) Incubation in 77 per cent ethanol overnight.

(c) Chromatography in 77 per cent ethanol--Two dimensional chromatography in this solvent did not produce a ninhydrin positive spot running off the diagonal.

(d) Heating gently in dilute hydrochloric acid.

(e) Hydrolysis under nitrogen in 6 N hydrochloric acid at 105°C overnight.

Fate of Homoserine in Reaction Mixture. Because of the unresolved questions concerning the role of homoserine in the cleavage reaction, it seemed important to determine what would happen to homoserine if it were present in the reaction mixture. Is free homoserine rapidly converted to some other compound or form? To explore this possibility experiments using radioactive homoserine were designed.

Labeled homoserine was incubated with partially purified canavanine-cleaving enzyme under conditions routinely used to destroy canavanine, and the resulting mixture analyzed by chromatography and electrophoresis. As can be seen in Figure 16, a second peak of radioactivity appears in addition to that of homoserine. The chromatographic properties of this second peak are as follows: It migrates faster than homoserine in 77 per cent ethanol, and it migrates toward the anode in electrophoresis at pH 1.6, indicating a high acidic compound.

Production from homoserine of this acidic peak has the following properties:

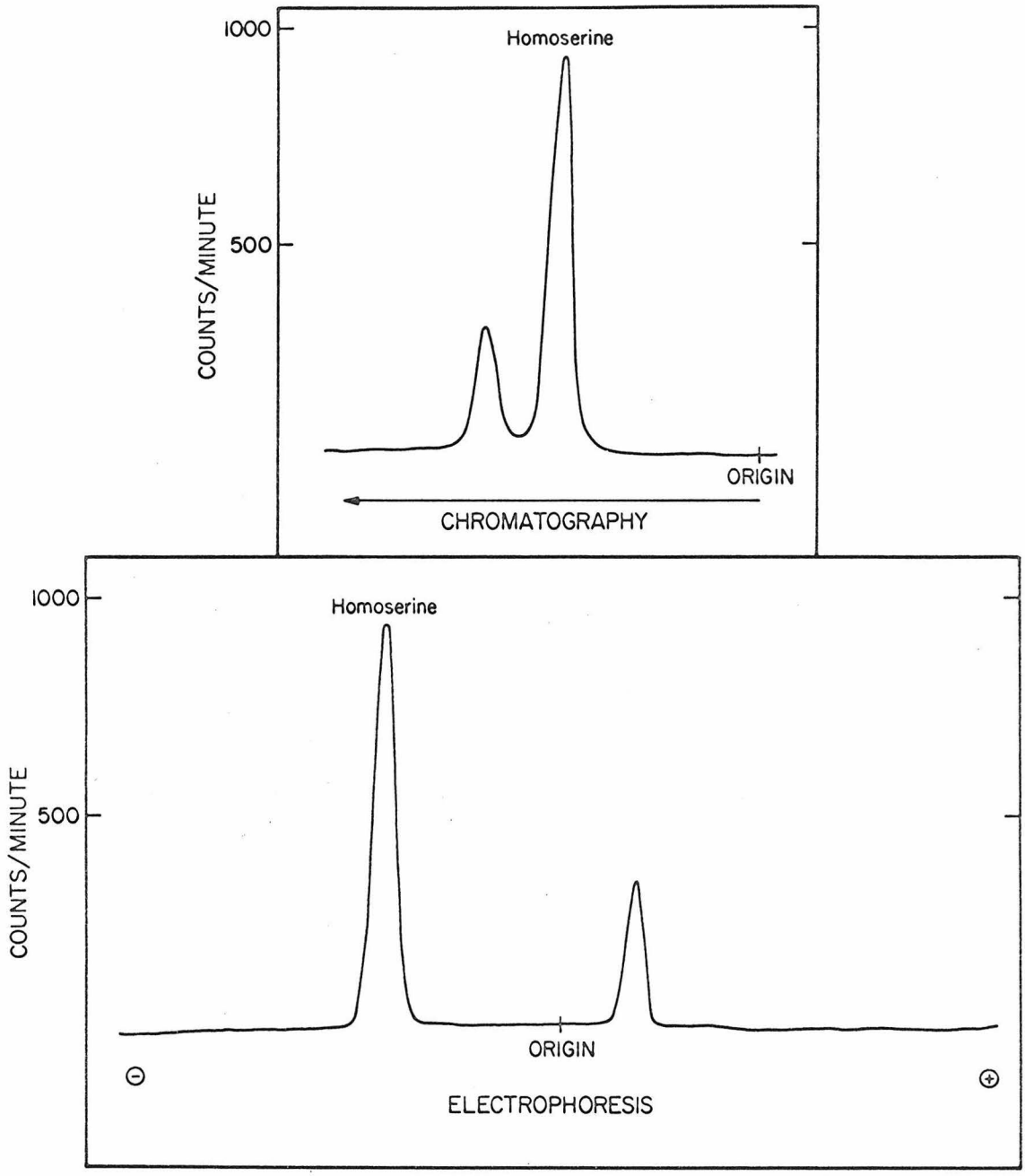
(a) Production increases with time of incubation, (b) with increasing enzyme concentration, and (c) is enhanced by performing the incubation under oxygen.

(d) The reaction is inhibited by canavanine. At a molar concentration of canavanine 1/10 that of homoserine, the peak is

FIGURE 16

MODIFICATION OF THE MIGRATION OF HOMOSERINE

An incubation mixture containing 87.0 mμmoles DL-homoserine-4-C^{14} and 300 μg Fraction III protein in 0.1 M sodium pyrophosphate buffer, pH 8.5, was incubated at 37°C for one hour. The reaction was stopped and the buffer precipitated by the addition of an equal volume of cold ethanol. An aliquot of the supernatant was chromatographed (12 hours descending) with authentic homoserine using 77% ethanol as a solvent. Electrophoresis (6.7% formic acid, pH 1.6, 50 volts/cm, 1.25 hours) was also performed. An Actigraph tracing of the resulting distribution of radioactivity was made. The paper was Whatman No. 1. Homoserine was detected using ninhydrin reagent.


1000
500
COUNTS/MINUTE
Homoserine
ORIGIN
CHROMATOGRAPHY
1000
500
COUNTS/MINUTE
Homoserine
ORIGIN
⊖
⊕
ELECTROPHORESIS

perceptibly diminished after one hour of incubation. With a hundred-fold excess of canavanine, there is no peak.

(e) The production of the acidic peak occurs in the absence of any phosphate. An aliquot of Fraction IV protein was extensively dialyzed against 0.05 M Tris buffer, pH 7.2, to remove any phosphate. This enzyme preparation was then used in an incubation mixture containing only Tris buffer and radioactive homoserine. The acidic peak was quite readily produced. From this experiment it appears unlikely that O-phosphohomoserine is the acidic compound.

The evidence clearly indicates that there is an enzymatic modification of homoserine under normal canavanine-cleaving assay conditions. However, the relevance of the modification to the problem of cleavage products of canavanine is not so clear. If this reaction were responsible for obscuring homoserine produced by the canavanine cleavage reaction, then one would expect it to take place in the presence of an excess of canavanine, but it does not. In addition different chromatographic properties would be expected from the modified homoserine to explain the aberrant behavior of canavanine cleavage products in System I (1. 77 per cent ethanol, 2. electrophoresis). One would expect a slow rather than fast migration with respect to homoserine in 77 per cent ethanol.

Homoserine Derivatives. The chromatographic pattern of the products of canavanine cleavage has been described in a previous section. In separation System I a ninhydrin positive material (compound X) remains near the origin during chromatography in ethanol and travels

with homoserine during electrophoresis. Further examination of this material after two dimensional chromatography suggests that it is homoserine, although it did not migrate as homoserine in the initial solvent. As suggested earlier, one possible explanation is that a labile derivative of homoserine is formed in the reaction mixture and during chromatography homoserine is generated.

Several derivatives of homoserine have been tested and all of them migrate farther in 77 per cent ethanol than compound X. The chromatographic position relative to homoserine (R_H) of the compounds tested are listed below. Also included are a number of compounds which at certain stages of this work seemed possible cleavage products of canavanine. The values given are for descending chromatography in 77 per cent ethanol for 12 hours.

	R_H
compound X	0.3
homoserine lactone	1.45
O-acetylhomoserine	1.41
aspartic semialdehyde	0.93
aspartic acid	0.63
asparagine	0.46
α-amino butyric acid	1.39
threonine	0.95

Other derivatives of homoserine have been considered. Although none of them has been firmly ruled out, there is some evidence which argues against the presence of most of them in the canavanine cleavage

reaction mixture. The derivatives and the nature of the evidence are listed below:

(a) O-acetyl- and N-acetylhomoserine--The addition of neither coenzyme A nor acetyl-coenzyme A stimulated the rate of canavanine cleavage. O-acetylhomoserine has the wrong chromatographic properties to be compound X (see above).

(b) Succinyl derivatives--O-succinylhomoserine is labile to alkali and the pH of the reaction mixture, 8.5, probably would cause cleavage of the compound (66). N-succinylhomoserine remains a possibility and more information concerning its properties needs to be obtained.

(c) Phosphate derivatives--Electropherograms of the products of canavanine cleavage were sprayed with ammonium molybdate reagent and irradiated with ultraviolet light. No phosphate-containing compound could be detected.

DISCUSSION

The complexity of the genetics of resistance to canavanine in Neurospora is shown in this thesis to result from interactions between a major gene and several modifiers. The major gene controls the presence of the activity of a constitutive enzyme that destroys canavanine. The modifiers affect the rate of uptake of the analog from the medium. Strains which lack activity of the enzyme are canavanine-sensitive; strains which possess it are resistant, but the level of resistance is dependent on the rate of uptake.

Unlike mammalian systems, resistance to harmful substances by detoxification is not very common among microorganisms. Resistance is often due to an impaired transport system. When resistance to an analog is involved, there may be overproduction of the normal metabolite or production of key biosynthetic enzymes that are no longer subject to either repression or end-product inhibition by the analog.

However, some cases of detoxification have been observed in microorganisms. In an Agrobacterium, resistance to growth inhibition by azetidine-2-carboxylic acid, the lower imino acid homolog of proline, is due to a hydrolytic ring fission yielding a harmless compound (39). Drug resistant mutants of bacteria have been described which enzymatically inactivate each of the following antibiotics: chloramphenicol (57), mitomycins (58), penicillins (59), streptomycin (57), and kanamycin (57). The chemical nature of the drug detoxification has been demonstrated only in the case of penicillin where the enzyme penicillinase is known to hydrolyze a β-lactam bond.

The reaction mechanism of canavanine degradation in Neurospora has not been fully elucidated, primarily because the identity of all reaction products has not been determined. One of the products was shown to be hydroxyguanidine. This fact, plus the lack of any complex requirement for activity, suggested a hydrolytic cleavage of canavanine. However, it was not possible to demonstrate the production of homoserine, an expected product of such a hydrolysis.

Oxygen greatly stimulates the degradation of canavanine and appears to be consumed at a rate equal to the loss of canavanine, thus suggesting an oxidative cleavage. The liver microsomes of mammals can perform a number of cleavage reactions involving oxygen, often with the additional requirement of NADPH. An example is the dealkylation of aromatic ethers.

$$\phi\text{-O-R} \xrightarrow{O_2,\ \text{NADPH}} \phi\text{-OH} + \text{O=R}$$

A reaction mechanism of this type does not appear likely, since (1) the addition of NADPH does not stimulate the reaction, and (2) the expected product, aspartic semialdehyde, could not be found in the reaction mixture. Other oxidative reaction mechanisms which require cofactors and/or result in the production of aspartic semialdehyde or aspartic acid appear equally unlikely.

Two oxygen consuming reactions in which canavanine is utilized as a substrate have been described (see Introduction). One is catalyzed by the arginine decarboxyoxidase of Streptomyces griseus and the other, by the L-amino acid oxidase of Neurospora. Canavanine-sensitive

strains possess L-amino acid oxidase activity and when the enzymes from sensitive and resistant strains were compared, no difference was seen in their activity towards canavanine over a wide range of conditions (60). Preparations of the canavanine cleavage enzyme show no L-amino acid oxidase activity. These facts rule out L-amino acid oxidase as the canavanine cleaving enzyme. Since the product of canavanine cleavage is ninhydrin positive, other oxygen-requiring enzymatic reactions which produce non-amino acids are also excluded.

However, the role of oxygen may not be a direct one. There is still some residual canavanine-degrading activity under nitrogen, i.e. in the absence of oxygen. This suggests that oxygen may act to remove an inhibitory product and thereby enhance activity.

The identity of the four-carbon fragment produced by canavanine cleavage remains unknown, but one further possibility bears mentioning. Homoserine, though absent initially, appears to be generated by the manipulations required for chromatographic analysis of a reaction mixture. This fact implies that the unknown reaction product is a somewhat labile derivative of homoserine. An inability of strain 51504 to convert such a derivative to homoserine would explain why canavanine cannot support the growth of this mutant.

A more complete understanding of the reaction mechanism of canavanine degradation might shed some light on another intriguing problem. Canavanine is not a normal metabolite in Neurospora, and yet the enzymatic apparatus exists to metabolize it. Therefore, questions automatically arise concerning the normal function of the enzyme

involved in canavanine cleavage. The analog participates in a number of reactions in which arginine is the primary substrate (see Introduction), and therefore one might suspect that the canavanine-cleaving enzyme normally functions in arginine metabolism. This does not appear to be the case, since arginine is not a substrate for the cleavage enzyme. The situation is very similar in the case of resistance to azetidine-2-carboxylic acid in bacteria, where the enzyme which detoxifies the analog does not utilize the natural metabolite, L-proline, as a substrate.

The only arginine-specific pathway for arginine degradation in Neurospora is mediated by the enzyme arginase (61). Canavanine is split by arginase to give urea and canaline. Teas has reported that canaline is toxic to Neurospora strains regardless of canavanine sensitivity (40). Because of these facts arginase has been ruled out as a factor determining canavanine resistance.

The normal role of the cleaving enzyme might be in lysine metabolism. Support for this idea comes from the work of Mora. Mutants selected as resistant to canavanine were found also to be resistant to the lysine analog, L-thiosine (s-β-aminoethyl cysteine) (J. Mora, personal communication). The reverse was true as well; mutants selected as thiosine resistant were also canavanine resistant. From the analysis of a cross between these mutants, it was tentatively concluded that the mutations are allelic. I have checked three mutants blocked in lysine biosynthesis (lys-1, lys-2 and lys-4), and all of them possessed canavanine cleaving activity. Therefore, the lysine biosynthetic enzymes

mediating the steps blocked in these mutants are not likely prospects as the cleaving enzyme. However, further work needs to be done before any conclusion can be drawn concerning the relationship, if any, between lysine metabolism and canavanine resistance.

It can be argued that there is no normal function of this enzyme; that it is specific only for canavanine. It may have had a function at some earlier time in the evolution of Neurospora, so the argument goes, but that function has since been superseded. The organism now carries what is essentially superfluous information in the form of the canavanine cleavage gene. How else can one explain why strain 25a, which lacks the enzyme, is not an auxotroph?

Two comments can be made in response to this argument. First, the presence of the enzyme is quite widespread. In fact, only strain 25a and progeny from crosses involving strain 25a have been shown to lack canavanine cleaving activity. It seems that if the enzyme played no essential role in cell metabolism, there would be no selective advantage to its presence. Therefore, one might expect to find a larger number of strains which lacked cleaving activity. Second, it is not necessary to assume that strain 25a has lost all activity of the enzyme. The enzyme of this strain might have an altered specificity such that it could no longer detoxify canavanine. At the same time it could perform its normal function in the same manner as enzymes from resistant strains. This is analogous to those amino acyl-tRNA synthetases which have lost the ability to activate an analog but retained activity toward the natural amino acid.

Whatever the normal function, if any, of the enzyme involved, the possession of canavanine cleaving activity confers resistance to growth inhibition by the analog. However, the level of resistance is subject to an additional control. Regulation of the accumulation of canavanine from the medium acts as a fine control determining the degree of resistance. Strains with cleaving activity and high uptake show intermediate resistance, while strains with cleaving activity and low uptake are fully resistant. It is suggested that the canavanine-degrading capacity of strains of intermediate resistance is exceeded at high concentrations of the amino acid and therefore some molecules of the analog are incorporated into protein, thus inhibiting growth.

This proposal is supported by a few simple calculations. At a concentration of DL-canavanine of 1.58 μM, the growth of strain 3-223(I) is inhibited by almost 20 per cent, while that of strain 3-221(R) is inhibited not at all. At this same level of canavanine in the medium, the uptake of the analog by strain 3-223(I) is 24 μμmoles/min/mg dry weight, while its rate of canavanine destruction is only 3.1 μμmoles/min/mg dry weight. On the other hand the fully resistant strain has a rate of canavanine destruction equal to the rate of uptake (3.9 μμmoles/min/mg dry weight). Of course, all such calculations need to be qualified by warnings about the danger of equating rates determined in vitro to in vivo rates. However, it is encouraging that the numbers are in the right direction.

The genetic control of this difference in canavanine permeability is quite complex. The limited genetic data available suggest

that at least three genes are involved. Multigenic control of amino acid uptake is neither unusual nor unexpected. In Neurospora, three loci have been described which affect the rate of transport of neutral amino acids (62). This multiplicity of genetic control is probably a reflection of the essential complexity of the mechanism by which a molecule enters the mycelium. Grenson has suggested that such a mechanism might include a succession of operations of the following kind: recognition of the molecule, transport through the permeability barrier, energetic interactions, and release of the molecule inside the mycelium (54). In addition, more than one transport system may be involved in the uptake of any given amino acid (56).

In conclusion, a few comments are in order about the apparent conflict between the results of this study and those of Bauerle and Garner (45). These workers suggested that neither detoxification nor reduced transport of the analog was the basis for canavanine resistance in Neurospora. Their work was not done on the same strains as mine, and since resistance to an antimetabolite may be produced by a variety of mechanisms, it is entirely possible that their strains simply have a different mode of resistance. However, these strains were threonine-less auxotrophs, the resistant ones of which were capable of growth with canavanine as the sole supplement, while sensitive ones were not. This fact suggests the presence of a canavanine-degrading mechanism in the resistant strains.

The report rejecting detoxification appeared only in abstract form, and therefore it is not known how carefully or by what techniques

canavanine destruction was sought. The C^{14}-labeled canavanine, which was so useful in the present study was not available at that time. The use of radioactivity provides a degree of sensitivity and a versatility that may not be attainable by other methods. This point may be illustrated by some early experiments of mine in which canavanine degrading activity was sought in extracts using PCAF reagent. I was unable to demonstrate the destruction of canavanine by this method, presumably because of interfering color production by hydroxyguanidine.

Unavailability of the radioactive compound also made it difficult for Bauerle and Garner to measure canavanine uptake directly. Instead, they demonstrated that the analog competes for arginine and lysine transport and used the accumulation of these amino acids as an indirect measure of the ability to concentrate canavanine. They did not observe a difference in arginine uptake between strains of full and intermediate resistance. The conflict between this observation and my own might be explained by the different culture conditions employed. Their measurements were made with germinated conidia while mine were made with three-day-old stationary cultures.

My experience has been that with a given strain the uptake of arginine is much slower in cultures in exponential growth than in standing cultures. Perhaps the pool of free arginine is very large in exponential cultures and exerts control over the release of arginine from the transport system. If the release of arginine became the rate limiting step in uptake, then any difference in affinity at the initial recognition site would be obscured. In this case a strain of

intermediate resistance would accumulate arginine, and by inference canavanine, at a rate below its full potential but similar to that of a fully resistant strain.

Culture conditions might affect arginine permeability in another way. There are at least two transport systems which accumulate neutral amino acids (56). One of these is not found in young, rapidly growing cultures. They are both found in old mycelial cultures. Let us assume a multiplicity of transport systems for basic amino acids. Let one system have a high affinity for arginine and be present in stationary cultures only. Let the other have a low affinity for arginine and be present in both exponential and stationary cultures. If the system with high affinity were the one impaired in resistant strains, then Bauerle and Garner would not have detected it by their methods.

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