Specificity of the Neurotoxin from *Lathyrus sativus* as an Amino Acid Antagonist†

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ABSTRACT: This paper reports evidence that a neurotoxin from the pulse *Lathyrus sativus* behaves as a potent amino acid antagonist in certain microorganisms. The toxin was isolated in 1963 from *Lathyrus sativus* seeds. Consumption of these seeds as a major calorie source has long been associated with outbreaks of the paralytic human disease known as neurolathyrism. The toxin was assigned the structure β-*N*-oxalyl-L-α,β-diaminopropionic acid. This paper describes work to confirm the structure of the toxin and to determine its specificity as an amino acid antagonist. The toxin depresses growth of wild type or of amino acid requiring strains of *Saccharomyces cerevisiae*. Growth depression is alleviated by supplementing the medium with L-glutamate or, less effectively, with L-aspartate. Sensitivity of various yeast strains to the toxin is inversely related to the size of the amino acid pool, of which glutamate is a major component. The toxin behaves as a competitive inhibitor of the transport of L-glutamate and L-aspartate into resting yeast cells. It does not inhibit the incorporation of these amino acids into aminoacyl-tRNA or into protein.

Consumption of *Lathyrus* peas has been associated for more than 2000 years with a crippling human disease now known as neurolathyrism. (For recent reviews, see Sarma and Padmanaban, 1969, and Rao *et al.*, 1969.) Three research groups in India (Murti *et al.*, 1964; Roy *et al.*, 1963; Rao *et al.*, 1964) have reported the isolation of a neurotoxin from *Lathyrus sativus* seeds. The toxin produces muscle tremors, paralysis of the legs, and convulsions when injected into animals with an immature or damaged blood–brain barrier (Rao and Sarma, 1967) or when introduced intrathecally into adult monkeys (Rao *et al.*, 1967). The toxin was assigned the structure β-*N*-oxalyl-L-α,β-diaminopropionic acid (Rao *et al.*, 1964; Murti *et al.*, 1964), and its possible role as the cause of human neurolathyrism was suggested.

Conclusive evidence that human neurolathyrism is due solely to β-*N*-oxalyl-L-α,β-diaminopropionic acid is lacking. However, the compound warrants investigation because of its demonstrated neurotoxic properties.

The structural similarity of the toxin to common amino acids suggested to us that its neurotoxicity may stem from a possible role as an amino acid antagonist. This paper describes work to confirm the structure of the toxin and to determine the specificity of the toxin as an amino acid antagonist.

Materials and Methods

Synthetic Toxin. All microbiological experiments described in this paper were carried out with highly purified synthetic toxin prepared from the copper chelate of L-α,β-diaminopropionic acid and ethyl oxalyl chloride. L-α,β-Diaminopropionic acid (Calbiochem, La Jolla, Calif.) was converted to a copper chelate as described by Rao *et al.*, 1964. Ethyl oxalyl chloride was synthesized in about 85% yield with minor modifications of the method of Wilds and Shunk (1948) from a 9% molar excess of potassium ethyl oxalate (Eastman Chemical Co., Rochester, N. Y.) and oxalyl chloride. A tenfold molar excess of ethyl oxalyl chloride in diethyl ether was dripped into a cold (0–5°) solution of the copper chelate of L-α,β-diaminopropionic acid in 35% (v/v) methanol–water adjusted to pH 8.5 and buffered with excess magnesium oxide. After stirring for 50 min at 0° and 30 min at 30–40°, the reaction mixture was filtered and the aqueous phase (containing the product) separated. The procedure for saponification of the ester, precipitation of copper, and purification on ion-exchange resin was that of Rao *et al.*, 1964. The yield of crude product (mp 160–182°) was about 55%. It gave a single ninhydrin-positive spot in thin-layer chromatography (silica gel H, solvent I). (For composition of thin-layer chromatography solvents, see Table I.) It was free of α-*N*-oxalyl-L-α,β-diaminopropionic acid as indicated by homogeneity in high-voltage electrophoresis at pH 3.6 (Bell and O'Donovan, 1966; Bell and Tirimanna, 1965). After recrystallization from hot, distilled water, a product was obtained in 4–10% yield which melted at 204.5–206° dec. The molar ninhydrin yield (Rosen, 1957) was 98.5% that of glycine standard. *Anal.* Calcd for $C_5H_8O_5N_2$: C, 34.09; H, 4.57; N, 15.90. Found: C, 34.25; H, 4.52; N, 15.64.

Natural Toxin. In experiments to confirm that the toxin is a derivative of an L-amino acid, we used a sample of natural toxin isolated in our laboratory from *L. sativus* seeds (Rao *et al.*, 1964) and purified by repeated recrystallization from hot distilled water. It melted at 206–209° dec and was equivalent in biological activity (yeast assay with *Sacharomyces cerevisiae* S 288C-27 and X 2394-11C) to highly purified synthetic toxin. *Anal.* Calcd for $C_5H_8O_5N_2$: C, 34.09; H, 4.57; N, 15.90. Found: C, 34.39; H, 4.68, N, 15.81. It gave a single ninhydrin-positive spot in thin-layer chromatography (solvent I) and in high-voltage electrophoresis at pH 3.6.

All other chemical experiments were carried out with a partially purified sample of natural toxin isolated in the laboratories of Dr. P. S. Sarma, Indian Institute of Science, Bangalore, India. This sample melted at 196–198° dec and was 66–77% as active in biological assays as the synthetic

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TABLE I: R_F Values × 10^2 of 2,4-Dinitrophenyl (N_2Ph) Derivatives from Degradation of Synthetic and Natural Toxin.[a]

Derivative	Solvent I	Solvent II	Solvent III	Solvent IV	Solvent V
N_2Ph derivative of toxin					
Natural	84	15	6	40	
Synthetic	86	14	6	40	
N_2Ph derivative of diaminopropionic acid					
Natural	74	21	25	46	
Synthetic	72	18	26	44	
N_2Ph-serine					
Standard		9	56		67
Natural		9 (16)[b]	50 (58)		69 (66)
Synthetic		9 (17)	50 (55)		68 (66)

[a] Toxin from synthetic and natural sources was treated with fluorodinitrobenzene and degraded as shown in Figure 2. Thin-layer chromatography of the N_2Ph isolates from natural and synthetic toxin were carried out on 0.25-mm layers of silica gel H in the following solvents: I, 95% ethanol–water (63:37 v/v); II, upper phase from combining toluene–pyridine–2-chloroethanol–0.8 N ammonium hydroxide (10:3:6:6 v/v); III, chloroform–1.5 N acetic acid–1-propanol (10:6:10 v/v); IV, 1-butanol–glacial acetic acid–water (3:1:1 v/v); V, 1-butanol–glacial acetic acid–water (12:1:1 v/v). [b] The R_F values of an unidentified minor product detectable after diazotization and hydrolysis are shown in parentheses. The unknown did not correspond in R_F value (solvent II) to known photodecomposition products of N_2Ph-serine (Pollara and Von Korff, 1960; Russell, 1963) prepared by exposing N_2Ph-serine standard in 1% aqueous bicarbonate to light for 3 days.

product. The molar ninhydrin yield was 74% of that of glycine standard.

Preparation and Purification of Derivatives. Trimethylsilyl derivatives of natural and synthetic toxin were prepared with minor modifications of the procedure of Chambas and Horning (1967), by heating the samples for 5 hr at 80° in a mixture of bis(trimethylsilyl)acetamide–*N*-trimethylsilylimidazole–trimethylchlorosilane (10:10:1 v/v). *N*-Trifluoroacetyl-*n*-butyl derivatives of natural and synthetic toxin were prepared according to the procedure of Gehrke and Stalling (1967). The derivatives were purified by gas–liquid chromatography in a Varian Model 1520-C gas chromatograph (Varian Instrument Co., Walnut Creek, Calif.) with thermal conductivity detectors and linear temperature programmer. Operating conditions for isolation of the trimethylsilyl derivatives were as follows: column, 10 ft × 0.25 in. glass packed with 65–80 mesh hexamethyldisilazane-treated Chromosorb G (high performance) coated with 3% OV-17; helium, 50 cm^3/min; injector, 225°; detector, 225°; oven, 100° at time of injection with temperature increased at 6°/min; recorder speed, 20 in./hr. Conditions for isolation of *N*-trifluoroacetyl-*n*-butyl derivatives were the same, except as follows: injector, 245°; detector, 240°; oven, 100° at time of injection and increased at 4°/min.

Degradation of Toxin. Toxin from natural or synthetic sources was treated with excess fluorodinitrobenzene in aqueous solution at pH 8–9 (Clark, 1964; Sanger, 1945). Unreacted reagent was removed by diethyl ether extraction. The product was extracted with diethyl ether after adjusting the pH of the reaction mixture to 1.

The yellow oily product obtained after evaporation of solvent was hydrolyzed in 4 N HCl for 2 hr at 100° in a sealed tube. The cooled hydrolysate was first extracted with diethyl ether to remove oxalic acid, then evaporated to dryness under reduced pressure.

The yellow oil obtained by evaporation of the hydrolysate was treated with nitrous acid (Schneider, 1937) to convert any free amine present to a diazonium salt. The diazonium salt was hydrolyzed by stirring at 30°, pH 3, for about 1 hr (Schneider, 1937). Evaporation of an ethyl acetate extract of the acidic reaction mixture yielded a yellow oil which could be separated into two products by thin-layer chromatography (see Table I). The ratio of major to minor product was about 3:1. The major product corresponded to N_2Ph-serine standard (Sigma Chemical Co., St. Louis, Mo.) in R_F value and was isolated by preparative thin-layer chromatography on 0.5-mm layers of silica gel H in solvent II.

Tryptophanase Assay. Tryptophanase assays were carried out by the procedure of Newton and Snell (1964) with reaction volume reduced to 0.1 ml. Tryptophanase was isolated from *Escherichia coli* B/1t7A as the apoenzyme (Morino and Snell, 1970); 0.24 unit of enzyme was preincubated with 10 nmoles of pyridoxal phosphate and 10 μmoles of potassium phosphate buffer (pH 7.8) for 20 min at 37° to permit reassociation of apoenzyme with cofactor. The reaction was started by the addition of substrate and was incubated for 30 min with gentle shaking at 37°. Substrates were 15 μmoles of L-α,β-diaminopropionic acid, HCl standard (Calbiochem, La Jolla, Ca.), 5.6 μmoles of natural toxin, or 0.5 μmole of α,β-diaminopropionic acid isolated from the natural toxin after hydrolysis as described by Rao *et al.* (1964). The reaction was stopped by the addition of alkali, and 2,4-dinitrophenylhydrazones formed as described by Newton and Snell (1964). The mixture was then acidified, the 2,4-dinitrophenylhydrazones extracted with ether, and aliquots of the ether extract chromatographed on 0.5-mm layers of silica gel H in benzene–acetic acid (80:20 v/v) against the 2,4-dinitrophenylhydrazone of pyruvic acid standard.

Nutritional Studies with Microorganisms. Wild-type and amino acid requiring strains of *S. cerevisiae* were provided by Dr. Richard Snow, Department of Genetics, University of California, Davis, Calif., and Dr. Robert K. Mortimer, Donner Laboratory, University of California, Berkeley, Calif. Of 13 strains tested for sensitivity to the toxin, four were studied in detail: *S. cerevisiae* aX 2180-1A, wild type; *S. cerevisiae* αS 288C-27, is_1; *S. cerevisiae* αX 2394-11C, is_3,

ur_3, ad_{2-1}, $1e_{1-12}$, ly_{1-1}, (hi_{5-2}?), (hi_6?), $ar_{4-2/17}$, tr_{5-48}; *S. cerevisiae* αS 2582B, is_2, le_2, tr_5, hi_8, ly_2. (Gene symbols are as described in Mortimer and Hawthorne, 1969.)

Yeasts were maintained on slants prepared from 2% peptone, 2% glucose, 1% yeast extract, and 2% agar. In assays to test the specificity of the neurotoxin as an amino acid antagonist, yeast cells were grown in a chemically defined liquid medium (Bacto Yeast nitrogen base without amino acids, Difco Laboratories, Detroit, Mich.). The medium is that of Wickerham (1951) with minor modifications (Difco Manual, 1953). It was supplemented with 2% glucose and with adequate amounts of the amino acids and nucleic acid bases required by each yeast strain for growth (T. Mehta and B. E. Haskell, 1972, unpublished data). Unless indicated otherwise, supplements (micrograms per milliliter of medium) were as follows: *S. cerevisiae* S 288C-27, isoleucine, 40; *S. cerevisiae* x 2394-11C, isoleucine, 40; tryptophan, 20; arginine, 20; histidine, 20; leucine, 20; lysine, 20; valine, 100; adenine, 40; uracil, 40; *S. cerevisiae* S 2582B, tryptophan, 20; valine, 20; leucine, 20; lysine, 20; histidine, 20; isoleucine, 10. Amino acids were combined with sufficient water to bring the final volume of the medium to 10 ml in 25 × 150 mm culture tubes and were heated at 121° for 15 min. The medium and toxin were filter sterilized and added aseptically just before inoculating. The inoculum consisted of 1 drop (0.05 ml) of a suspension (0.22 mg of dry cells per ml) of log phase cells per tube. Careful control of inoculum size proved critical for reproducible results. The tubes were incubated at 30° with shaking (150 excursions per minute; displacement, 1.5 in.) for 18–60 hr. Growth was determined by measuring turbidity in a Bausch and Lomb Spectronic 20 colorimeter equipped with a 1-in. adaptor at 650 mμ.

All chemicals used in media preparation were reagent grade. Except for *N*-acetyl-L-aspartic acid which was synthesized (Barker, 1953), biochemicals were purchased from commercial sources and were the purest grade available.

Amino Acid Uptake, tRNA Charging, and Protein Synthesis in Resting Yeast Cells. Experiments to determine the effect of the toxin on amino acid uptake, tRNA charging, and protein synthesis were carried out with resting cells of *S. cerevisiae* S 288C-27 (Halvorson and Cohen, 1958; Ames, 1964). For details, see caption to Figure 4. Log phase cells were incubated in buffer lacking vitamins which S 288C-27 requires for growth but supplemented with glucose and with 1.6 mM isoleucine, the only amino acid which this yeast cannot synthesize. Preliminary experiments with [^{14}C]isoleucine established that the toxin has no effect on the rate of uptake of 1.6 mM isoleucine into resting yeast cells. Cell suspensions routinely were preincubated with toxin for 5 min. The effect on the rate of amino acid uptake was the same, whether toxin was preincubated with cells for 5 min or was added simultaneously with the amino acid whose uptake into the cell was to be tested. Amino acids uniformly labeled with carbon-14 were purchased from New England Nuclear Corp., Boston, Mass.

tRNA Charging and Protein Synthesis in a Cell-Free System from Yeast. S-30 and S-100 fractions were isolated from log phase cells of *S. cerevisiae* S 288C-27 with minor modifications (L. Ferenczy, K. Raghu, and D. Gottlieb, 1971, personal communication) of the procedure of Matthaei and Nirenberg (1961). tRNA-charging assays were carried out as described by Shearn and Horowitz (1969). Details of the assay are described in footnote *a*, Table VI.

Cell-free protein synthesis was carried out with minor modifications (L. Ferenczy, K. Raghu, and D. Gottlieb, 1971, personal communication) of the mobile assay system described by Coleman (1969). For details of assay procedure, see footnote *a*, Table VII.

Results

Confirmation of Structure. Prior to carrying out biological studies, we confirmed that the toxin has the structure proposed by other investigators (Rao *et al.*, 1964; Murti *et al.*, 1964): β-*N*-oxalyl-L-α,β-diaminopropionic acid. To facilitate the comparison of a partially purified sample of natural toxin with highly purified synthetic toxin, both toxin samples were converted to volatile derivatives and purified by preparative gas–liquid chromatography. Gas–liquid chromatography tracings of *N*-trifluoroacetyl-*n*-butyl derivatives from natural and synthetic toxins showed a single major peak at 191° which separated cleanly from solvents and from small amounts of volatile derivatives of hydrolysis products of the toxin. Infrared spectra of the *N*-trifluoroacetyl-*n*-butyl derivative collected at 191° from natural (A) and synthetic (B) toxin are compared in Figure 1. Similarity of the spectra indicates that natural toxin and synthetic toxin are the same compound.

When toxin from natural and synthetic sources was converted to a trimethylsilyl derivative, gas–liquid chromatography tracings of both preparations yielded a single major peak emerging at 210°. The effluent emerging at 210° in the preparation from natural toxin was collected and analyzed in a mass spectrometer (Varian Model M-66, Varian Instrument Co., Palo Alto, Calif.). The fragmentation pattern (*m/e* 374, 359, 257, 229, 147, 117, 75, 73 (base peak)) was consistent with the assumption that the toxin is an oxalyl derivative of α,β-diaminopropionic acid. However, these data do not indicate whether the toxin is an α-*N*-oxalyl or a β-*N*-oxalyl derivative. The largest fragment detectable in mass spectrometry (*m/e* 374)—probably a tris(trimethylsilyl) derivative of 5,6-diketo-2-piperazine carboxylate—could be formed from either one.

To determine whether the toxin is an α-*N*-oxalyl or a β-*N*-oxalyl derivative, we therefore degraded it according to the scheme shown in Figure 2. Samples of synthetic and natural toxin were treated with fluorodinitrobenzene, hydrolyzed to remove the oxalyl moiety, treated with nitrous acid to diazotize any free amine present, and hydrolyzed to convert the diazonium salt to a hydroxyl group. This sequence of reactions would be expected to convert a β-*N*-oxalyl derivative of α,β-diaminopropionic acid to N_2Ph-serine.

Both dinitrophenylation and hydrolysis of the amide bond yielded a product which was chromatographically homogeneous. (See Table I for a comparison of R_F values of N_2Ph derivatives from natural and synthetic toxin.) After diazotization and hydrolysis, two products which exhibited similar chromatographic behavior were obtained from both synthetic and natural toxin. The major product corresponded in R_F value to N_2Ph-serine standard. (See Table I.) The minor product is unidentified. The amount varied with hydrolysis conditions, more being formed at high temperatures.

The major product of the degradation of synthetic and natural toxins was isolated by preparative thin-layer chromatography and analyzed in a nuclear magnetic resonance spectrometer (Varian A-60, Varian Instrument Co., Palo Alto, Calif.). Figure 3 shows that the nuclear magnetic resonance (nmr) spectra of N_2Ph isolates from natural and synthetic toxin are identical to the nmr spectrum of N_2Ph-serine standard.

To confirm that the toxin is a derivative of the L isomer of α,β-diaminopropionic acid, a highly purified sample of

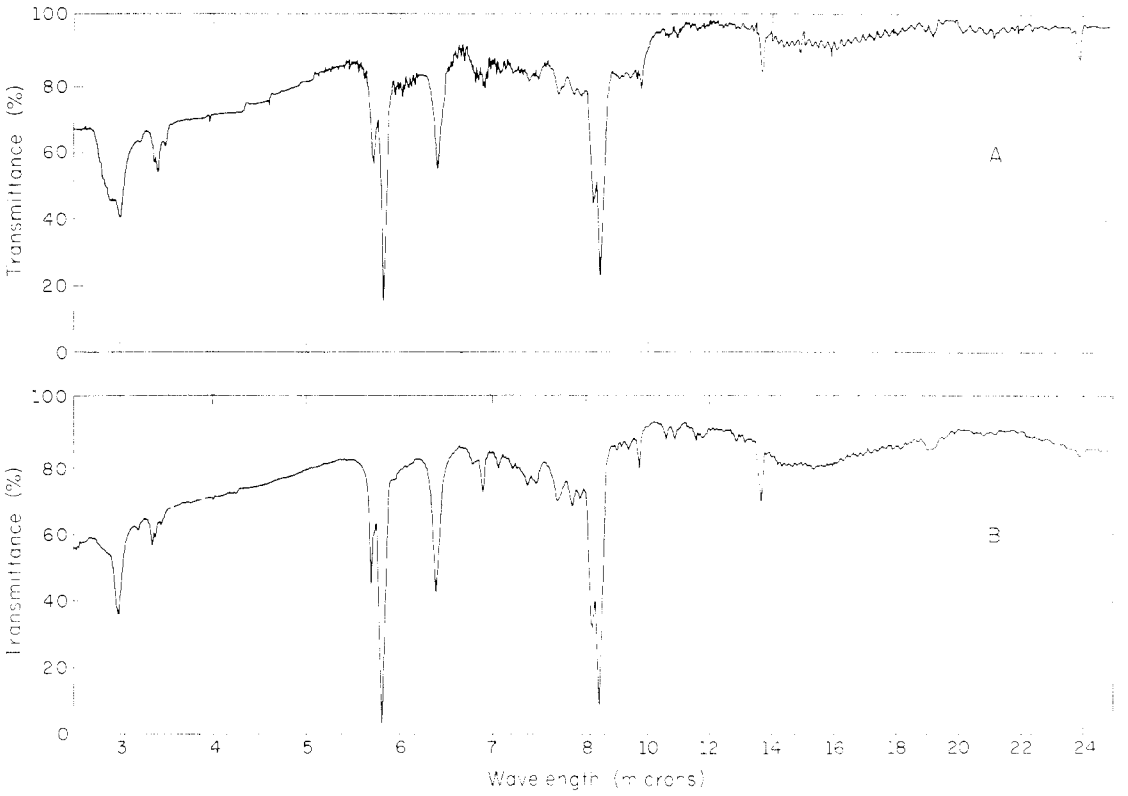

FIGURE 1: Infrared spectra of *N*-trifluoroacetyl-*n*-butyl derivatives of neurotoxin from synthetic and natural sources. Samples of neurotoxin from synthetic (A) and natural (B) sources were converted to *N*-trifluoroacetyl derivatives and purified by gas–liquid chromatography. Effluent from the major peak (191°) was collected and analyzed as a KBr pellet in a Perkin-Elmer 337 grating infrared spectrophotometer. Structure of the 191° compound is unknown. When analyzed in a mass spectrometer, the derivative yielded a complex fragmentation pattern with no molecular ion corresponding to the anticipated molecular weight of the parent derivative, 384. The fragmentation pattern was identical for derivatives from synthetic and from natural toxin.

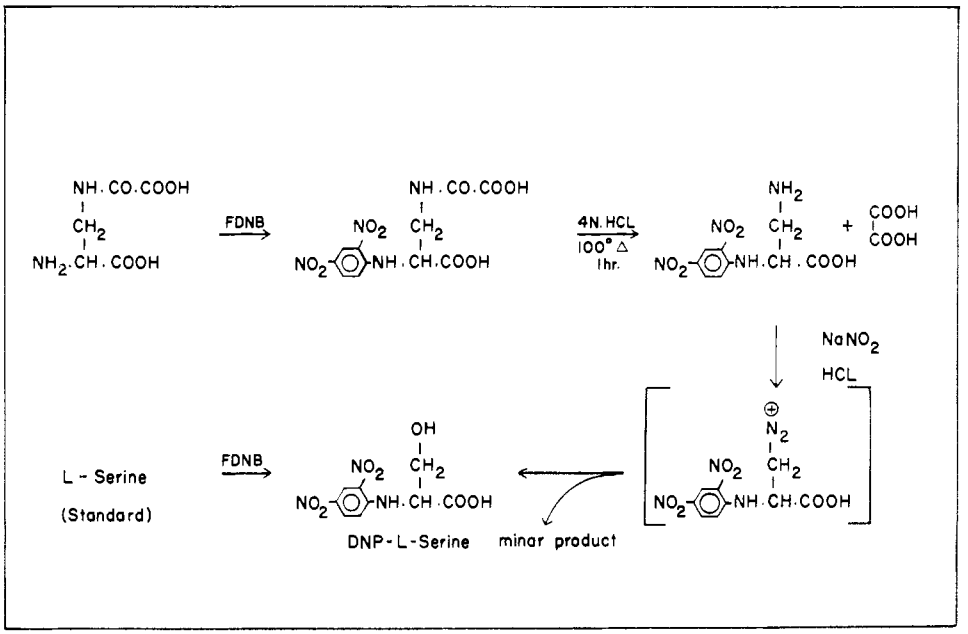

FIGURE 2: Scheme for degrading natural and synthetic neurotoxin samples to 2,4-dinitrophenylserine.

natural toxin isolated in our laboratory was tested as a substrate for tryptophanase, a pyridoxal phosphate enzyme which acts only on the L isomers of amino acids (Morino and Snell, 1970). L-α,β-Diaminopropionic acid is a substrate for this enzyme (Morino and Snell, 1970). Tryptophanase converted either the natural toxin or α,β-diaminopropionic acid isolated from the natural toxin after hydrolysis (Rao *et al.*, 1964) to a product whose 2,4-dinitrophenylhydrazone corresponded in

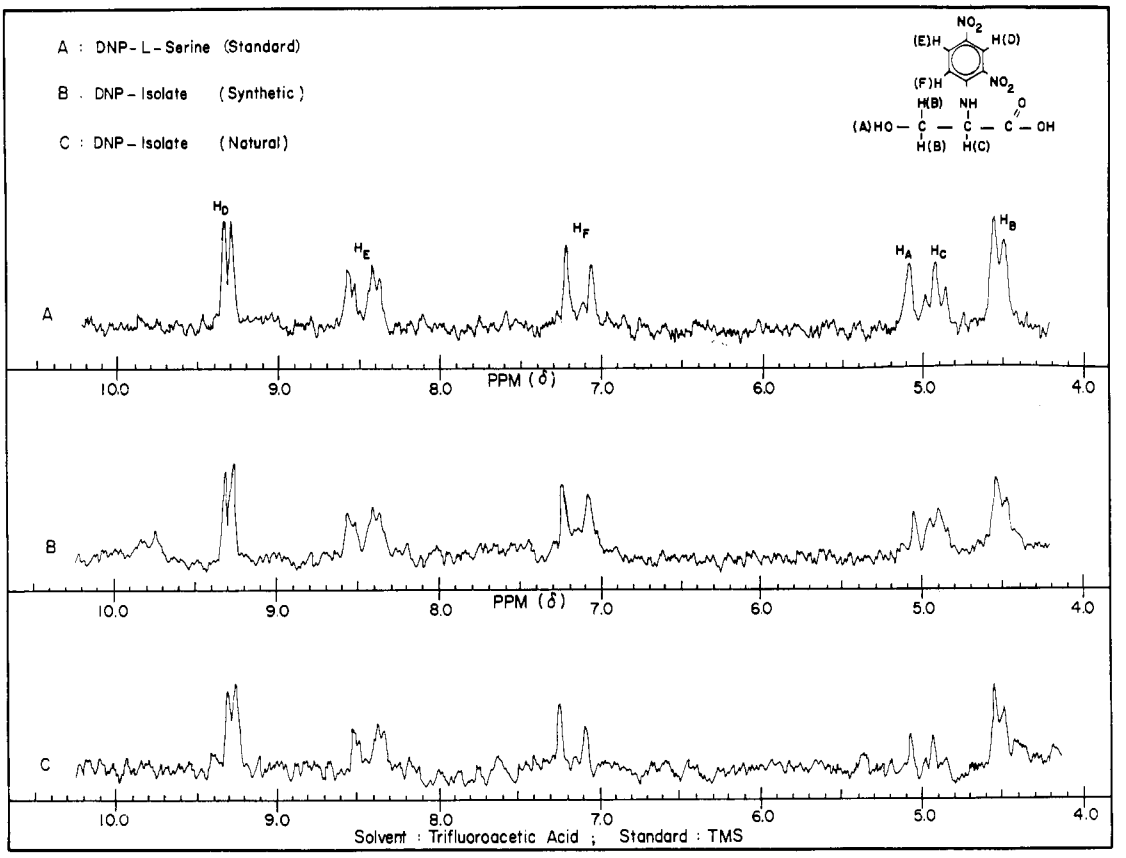

FIGURE 3: Nuclear magnetic resonance spectra of N_2Ph-serine derived from L-serine standard, from synthetic toxin, and from natural toxin. Neurotoxin from synthetic and natural sources was degraded according to the scheme shown in Figure 2. The major degradation product of natural and synthetic toxin corresponded in R_F value to N_2Ph-serine standard in thin-layer chromatography. This material was isolated by preparative thin-layer chromatography and analyzed in a Varian A-60 nuclear magnetic resonance spectrometer in trifluoroacetic acid with tetramethylsilane as internal standard. The spectrum of N_2Ph-serine standard is shown in A, the N_2Ph isolate from synthetic toxin in B, and the N_2Ph isolate from natural toxin in C. Assignment of protons is based on a comparison with the known spectrum of N_2Ph-serine standard (Fujiwara *et al.*, 1962; Fujiwara and Arata, 1964) and on standard methods for the interpretation of nmr spectra (Dyer, 1965).

R_F value to that of pyruvic acid standard in thin-layer chromatography (R_F value $\times$ 100 equaled 46).

Characteristic peaks in infrared spectra of highly purified toxins from synthetic and natural sources were the same as those in the published spectra of β-*N*-oxalyl-L-α,β-diaminopropionic acid (Rao *et al.*, 1964).

Nutritional Studies. EFFECT ON YEAST GROWTH. Highly purified synthetic neurotoxin depresses yeast growth when added to a nutritionally complete, chemically defined medium. Results of a typical experiment are shown in Table II. The neurotoxin depressed growth of *S. cerevisiae* S 288C-27, an isoleucine-requiring strain which lacks threonine deaminase, when added to a medium containing 40 μg of isoleucine per ml (Table II, line 2), about twice the amount of isoleucine required by this strain for full growth (T. Mehta and B. E. Haskell, 1971, unpublished data). Reversal of growth depression occurs when either of the dicarboxylic acids structurally related to the toxin is added to the medium. Neither glutamic acid nor aspartic acid is required by this strain for growth. The growth stimulus obtained when glutamate or aspartate is added to the complete medium is slight (Table II, lines 5 and 6).

Other common amino acids, glucose, nucleic acid bases, citrate, succinate, malate, fumarate, and α-ketobutyric acid had little or no effect in reversing growth depression.

Results similar to those in Table II were obtained with 12 other strains of *S. cerevisiae* (both wild-type and amino acid requiring strains) and with 2 strains of *Neurospora crassa* The toxin invariably depressed growth when added to a chemically defined complete medium. Although any amino acid which stimulated growth to some extent counteracted the effects of the toxin, complete reversal of growth depression was obtained only by the addition of glutamate or aspartate to the medium.

Several strains of *Escherichia coli* and of *Lactobacilli* were tested for sensitivity to the toxin with negative results. The toxin did not depress growth, either in a complete or in an amino acid limiting medium. Reasons for the resistance of these strains to the toxin is not known.

SPECIFICITY OF THE TOXIN. Table III shows that structural integrity of the toxin is necessary for growth depression in yeast. The same molar concentration of the hydrolysis products of the toxin (L-α,β-diaminopropionic acid and oxalic acid)—either alone or combined—do not depress yeast growth. Results in Table III are from an experiment with *S. cerevisiae* S 288C-27. However, they are typical of those obtained with other yeast strains and *Neurospora*.

Compounds structurally related to L-glutamic acid and L-aspartic acid had little or no effect in reversing growth depression. A preliminary experiment established that for yeast S 288C-27 the minimum amount of glutamic acid needed to reverse growth depression caused by the addition of 0.25 μg/ml of neurotoxin (1.42 μM) to the medium was 0.25 mM. When the same molar concentration of other structurally related

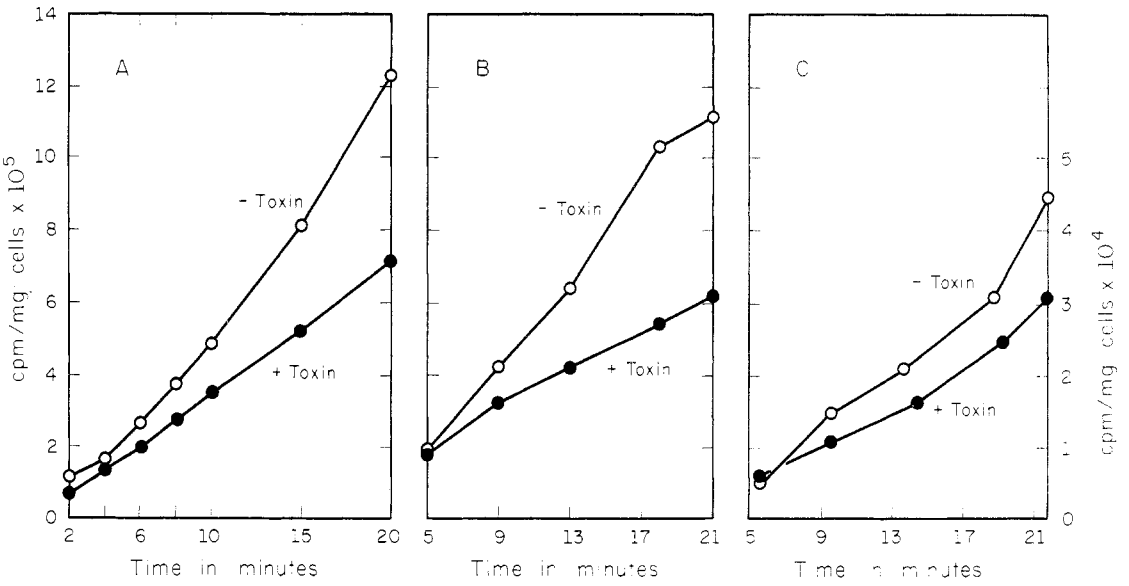

FIGURE 4: Effect of the toxin from *Lathyrus sativus* on the uptake of [^{14}C]glutamate into resting yeast cells (A), into aminoacyl-tRNA (B), and into protein (C). Log phase cells of *S. cerevisiae* S 288C-27 (1.5 mg dry weight of cells in a final volume of 0.5 ml) were incubated with shaking at 30° in 0.1 M succinate–phosphate buffer, pH 4.5, containing 1% glucose and 1.6 mM isoleucine with or without 0.226 mM toxin. The treatment was started by adding [^{14}C]glutamate (3.15 μCi; 0.450 mM). Aliquots of the reaction mixture were withdrawn at intervals and glutamate uptake, incorporation into aminoacyl-tRNA and into protein determined (Ames, 1964). Samples were combined with scintillation fluid consisting of 0.5% 2,5-diphenyloxazole in toluene and counted in a Packard Tri-Carb scintillation counter Model 3320. Efficiency of count, as determined by the channels ratio method (Davidson and Feigelson, 1957), was greater than 90%.

compounds was tested for ability to neutralize the toxin, it could be shown that L-glutamic acid is more effective than any other compound tested in reversing growth inhibition. The relative growth response, expressed as a percent of that obtained in the presence of 0.25 mM glutamic acid, was as follows: for L-aspartic acid, 60; for D-glutamic acid, 17; for D-aspartic acid, 7; for *N*-acetylglutamic acid, 9; and for *N*-acetylaspartic acid, 8. L-Asparagine, L-glutamine, DL-homocysteic acid, glutathione, α-ketoglutaric acid, and oxaloacetic acid had no effect in reversing growth depression.

SENSITIVITY OF VARIOUS YEASTS TO TOXIN. Marked differences were apparent in the amount of toxin required for 50% growth inhibition in four strains of *S. cerevisiae*, as shown in Table IV. The molar ratio of toxin to glutamate required for 50% growth inhibition varies more than 20-fold between the most sensitive and the least sensitive strains. None of the four strains requires glutamic acid for growth. (For nutritional requirements, see Materials and Methods.)

It seemed possible that the resistance of various yeast strains to the toxin might depend upon the size of the intracellular

TABLE II: Effect of Toxin on Yeast Growth in Various Media.[a]

Additions to Basal Medium (μg/ml of Medium)				
Toxin	L-Isoleucine	L-Glutamic Acid	L-Aspartic Acid	Relative Growth Response
	40			100
0.2	40			44
0.2	40	40		110
0.2	40		40	105
	40	40		112
	40		40	103

[a] *S. cerevisiae* S 288C-27, an isoleucine auxotroph, was grown in a chemically defined basal medium consisting of ammonium sulfate, glucose, salts, and vitamins (see Materials and Methods) with the additions indicated above. Growth was estimated turbidimetrically. The growth response is expressed as a percent of that obtained in a nutritionally complete medium; that is, the basal medium supplemented with excess (40 μg per ml) isoleucine.

TABLE III: Effect of β-*N*-Oxalyl-α,β-diaminopropionic Acid and Its Hydrolysis Products on Growth.[a]

Medium	Relative Growth Response
Complete	100
Complete plus toxin	8
Complete plus oxalic acid	96
Complete plus α,β-diaminopropionic acid	95
Complete plus α,β-diaminopropionic acid and oxalic acid	107

[a] *S. cerevisiae* S 288C-27 was grown in a chemically defined complete medium containing 40 μg/ml of isoleucine with or without the addition of equimolar amounts (1.42 μM) of toxin (β-*N*-oxalyl-L-α,β-diaminopropionic acid) or its hydrolysis products (oxalic acid and α,β-diaminopropionic acid). Growth was measured turbidimetrically. The growth response is expressed as a per cent of that in the complete medium without toxin.

TABLE IV: Relationship of Toxin Sensitivity to Size of the Intracellular Amino Acid Pool in Various Yeasts.

Yeast Strain	Amount of Toxin Which Produces 50% Growth Inhibition[a]		Intracellular Amino Acid Pools[b] (μmoles/100 mg of Dry Cells)			
	ng/ml	Molar Ratio of Toxin to Glutamate	Total	Glu	Asp	Glu + Asp
X 2180-1A	99.8	$(1.28 \times 10^{-2}):1$	16.7	9.5	1.1	10.6
X 2394-11C	133.0	$(5.94 \times 10^{-2}):1$	44.5	8.9	1.9	10.8
S 288C-27	245.1	$(6.70 \times 10^{-2}):1$	56.2	14.3	3.1	17.4
S 2582B	485.0	$(27.4 \times 10^{-2}):1$	104.2	43.2	2.1	45.3

[a] To determine the amount of toxin which produces 50% growth inhibition, cells were grown in chemically defined, nutritionally complete media (see Materials and Methods) containing varying amounts of toxin but no glutamic acid. None of the yeasts requires glutamic acid for growth. For nutritional requirements, see Materials and Methods. The molar ratio of glutamate to toxin which results in 50% growth inhibition was determined by supplementing media containing the minimum amount of toxin necessary to suppress growth with varying amounts of L-glutamic acid. [b] Free amino acid was extracted from log phase cells of each yeast as described by Halvorson and Spiegelman (1952). Total intracellular amino acids were estimated by the quantitative ninhydrin method of Rosen (1957). Quantitation of individual amino acids was carried out on a Technicon TSM amino acid analyzer.

amino acid pool. Glutamate is reported to be a major component of intracellular amino acid pools in yeast (Halvorson and Spiegelman, 1952). When the free amino acids were extracted from log phase yeast cells of each strain and analyzed, sensitivity of various yeast strains to the toxin proved to be inversely related to the size of the intracellular amino acid pool (see Table IV). The yeasts most sensitive to the toxin have both the smallest amino acid pools and the smallest intracellular concentrations of glutamate and aspartate.

Experiments with Resting Cells. Nutritional experiments have a disadvantage in that any substance which stimulates growth appears to counteract the effects of the toxin. To confirm the specificity of the toxin as an amino acid antagonist, experiments therefore were carried out with log phase resting cells. The effect of the toxin upon transport of amino acids into the cell, upon the incorporation into aminoacyl-tRNA and into protein was determined.

The neurotoxin inhibited total uptake of [^{14}C]glutamate into resting yeast cells in 20 min by about 50% when the molar ratio of glutamic acid to toxin was 2:1 (see Figure 4A). Similar results were observed with [^{14}C]aspartate was substituted for glutamate. The toxin had no effect upon the uptake of several other amino acids which had shown slight effects in neutralizing the toxin in yeast growth experiments. These included histidine, lysine, valine, isoleucine, and leucine.

The toxin also depressed the incorporation of [^{14}C]glutamic acid into aminoacyl-tRNA (Figure 4B) and into protein (Figure 4C). Similar results were obtained with [^{14}C]aspartate.

No effect of the neurotoxin on glutamate and aspartate uptake, on tRNA charging, or on protein synthesis was observed when similar experiments were carried out with *Streptococcus faecalis* ATCC 8043 or with *Escherichia coli* A-19, a methionine-requiring mutant.

The hydrolysis products of the neurotoxin, L-α,β-diaminopropionic acid (0.226 mM) and oxalic acid (0.226 mM), had no effect upon the uptake of [^{14}C]glutamic (0.452 mM) acid into yeast cells, upon its incorporation into aminoacyl-tRNA or upon its incorporation into protein.

Figure 5 shows that the neurotoxin behaves as a competitive inhibitor of the transport of [^{14}C]glutamic acid and [^{14}C]-aspartic acid into the yeast cell. The K_m for glutamic acid transport is 1.69×10^{-4} M; for aspartic acid transport, 1.33×10^{-4} M. K_i values for the toxin as an inhibitor of glutamate and aspartate transport are 0.99×10^{-4} and 0.93×10^{-4}, respectively.

Table V shows that the effect of the toxin on incorporation of glutamic acid into aminoacyl-tRNA and into protein

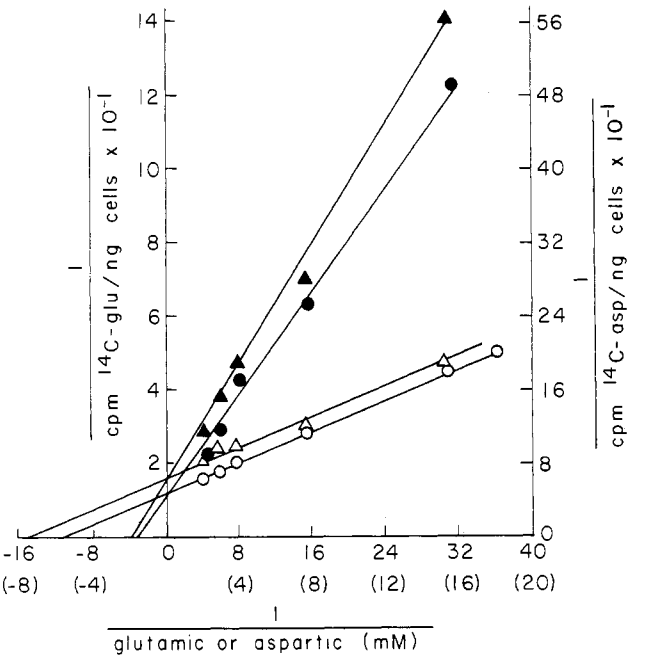

FIGURE 5: The effect of varying concentrations of glutamate or aspartate on the transport of these amino acids into yeast cells in the presence of toxin. Experiments to determine the effect of toxin (0.226 mM) on the transport of varying amounts of L-glutamate and L-aspartate into the yeast cell were carried out as described in the caption to Figure 4. The Lineweaver–Burk plots above show that the toxin behaves as a competitive inhibitor of the transport of L-glutamate and L-aspartate into resting cells of *S. cerevisiae* S288C-27. Identification of curves is as follows: glutamate alone, open circles; glutamate plus toxin, closed circles; aspartate alone, open triangles; aspartate plus toxin, closed triangles. Concentrations of aspartate are shown in parentheses.

TABLE V: Incorporation of [^{14}C]Glutamic Acid into Aminoacyl-tRNA and into Protein Expressed as a Per Cent of Total [^{14}C]-Glutamate Uptake into Cell.[a]

	Time after Addition of [^{14}C]Glutamate (min)				
	5.5	9.5	13.5	18.5	20.5
tRNA-AA					
− toxin	87.26 ± 6.20	93.27 ± 4.30	90.16 ± 9.90	93.28 ± 3.80	92.31 ± 1.40
+ toxin	85.28 ± 17.50	84.36 ± 16.80	86.18 ± 8.90	92.96 ± 10.10	93.39 ± 9.20
Protein					
− toxin	5.40 ± 2.70	4.80 ± 0.44	3.53 ± 0.90	3.37 ± 0.25	3.57 ± 0.45
+ toxin	8.60 ± 4.74	5.16 ± 1.49	4.88 ± 0.68	4.86 ± 0.24	4.38 ± 1.36

[a] Data shown in Figure 4 are recalculated to express results as a per cent of the total [^{14}C]glutamate uptake into resting cells of *S. cerevisiae* S 288C-27. Results are the mean ± the standard error for three determinations.

probably is a consequence of reduced uptake into the yeast cell. When counts incorporated into aminoacyl-tRNA and into protein are expressed as a per cent of the total counts taken up into the yeast cell, there is no statistically significant differences between toxin-treated and toxin-untreated cells. To confirm that the toxin affected only amino acid uptake and not tRNA charging and protein synthesis, experiments were carried out with cell-free tRNA charging and protein-synthesizing systems isolated from the same yeast, *S. cerevisiae* S 288C-27.

Cell-Free tRNA Charging. Table VI shows that the neurotoxin does not depress the incorporation of either [^{14}C]-glutamate or [^{14}C]aspartate into the corresponding aminoacyl-tRNA. In experiments with or without toxin, the glutamate and aspartate concentrations were the minimum needed to saturate the glutamyl- or aspartyl-tRNA present in the reaction mixture. Thus, a competitive effect of the toxin on incorporation of either amino acid into aminoacyl-tRNA should have been readily detectable. The molar ratio of neurotoxin to glutamate was 3.1:1; the molar ratio of neurotoxin to aspartate, 2.9:1.

Cell-Free Protein Synthesis. The effect of the toxin on the incorporation of labeled amino acids into protein in a mobile, cell-free, protein-synthesizing system is shown in Table VII.

TABLE VI: Effect of Toxin on the Incorporation of [^{14}C]-Glutamate and [^{14}C]Aspartate into an Amino Acid–tRNA Complex in a Cell-Free System from Yeast.[a]

Treatment	Incorporation into Aminoacyl-tRNA (cpm/mg of S-100 Protein)	
	Glutamyl-tRNA	Aspartyl-tRNA
Boiled enzyme	260	550
Complete system	25,283	65,133
Complete system plus toxin	24,033	63,600

[a] Experiments to determine the effect of toxin on cell-free tRNA charging were carried out as described by Shearn and Horowitz (1969) with an aminoacyl-tRNA synthetase preparation (S-100 fraction) isolated from log phase cells of *S. cerevisiae* S 288C-27. In experiments with [^{14}C]aspartic acid, the reaction mixture contained: 100 mM Tris buffer (pH 7.3), 50 mM magnesium acetate, 2.5 mM ATP, 5.0 mM reduced glutathione, 5 mM dithiothreitol, 50 μg of yeast tRNA (Lot 7001, Schwarz-Mann, Orangeburg, N. Y.), 75 μg of S-100 protein, and 97.2 μM aspartic acid (0.492 μCi) with or without 284.5 μM toxin in a final volume of 125 μl. In experiments with [^{14}C]glutamic acid (92.0 μM, 0.429 μCi) substituted for aspartic acid, the reaction mixture was modified as follows: magnesium decreased to 10 mM; ATP, increased to 5 mM; phosphoenolpyruvate, 20 mM added. The aminoacyl-tRNAs were isolated on filter paper disks, washed, combined with scintillation fluid, and counted. The efficiency of count for carbon-14 was 92%.

TABLE VII: Effect of Neurotoxin in the Incorporation of [^{14}C]-Amino Acids into Protein in a Cell-Free System from Yeast.[a]

	dpm of [^{14}C]Amino Acid Incorporated into Protein per mg of S-30 Protein
Reaction stopped at zero time	145
Minus ATP and GTP	617
Complete system	11,500
Complete system plus toxin	11,730

[a] Cell-free protein synthesis was carried out with minor modifications of the procedure of Coleman (1969) with an S-30 fraction isolated from *S. cerevisiae* S 288C-27. The following mixture was equilibrated with a Sephadex G-25 (superfine) column, with or without the addition of 1.42 mM neurotoxin: 100 mM Tris buffer (pH 7.3) containing 1 mM magnesium acetate, 50 mM ammonium chloride, 15 mM mercaptoethanol, 1 mM glutathione, and 50 mM ammonium chloride; 3 mM ATP 0.3 mM GTP, and 0.21 mM (0.5 μCi) of a mixture of 21 amino acids (0.02 μmole each). Fifteen of the amino acids, including aspartic acid and glutamic acid, were uniformly labeled with carbon-14. S-30 protein (0.22 mg) was washed through the column with the Tris buffer mixture described above at a flow rate of 4 ml/hr. The eluate was collected as a single fraction and heated in 10% trichloroacetic acid, and the heat-stable precipitate isolated by filtration and counted. The efficiency of count was about 85%.

Protein synthesis occurs as an S-30 fraction from *S. cerevisiae* S 288C-27 is washed through a Sephadex column equilibrated with amino acids, energy sources, buffer, and metal ions. Toxin, when added, was equilibrated with the column along with the amino acids. Preliminary experiments established that the rate of transport of the toxin through the column was the same as that of the amino acid substrates. The toxin did not depress the incorporation of labeled amino acids into protein when the molar ratio of toxin to total amino acids was 6.76 to 1. (The molar ratio of toxin to glutamate or aspartate was 142:1.)

Discussion

Structure Confirmation. Prior to undertaking biological studies with the neurotoxin from *Lathyrus sativus*, we considered it desirable to confirm that the structure assigned by other investigators (Rao *et al.*, 1964; Murti *et al.*, 1964) is correct. In particular, it seemed important to confirm that the toxin is a β-*N*-oxalyl derivative of α,β-diaminopropionic acid. Both α-*N*-oxalyl and β-*N*-oxalyl derivatives of α,β-diaminopropionic acid are reported to occur naturally (Roy and Rao, 1968). Bell and O'Donovan (1966) also report that the α-*N*-oxalyl isomer is formed by rearrangement of the β-*N*-oxalyl isomer under mild conditions. Toxicity of the α-*N*-oxalyl isomer has not been evaluated.

Evidence that the *Lathyrus sativus* toxin is a β-*N*-oxalyl derivative of α,β-diaminopropionic acid (Rao *et al.*, 1964; Murti *et al.*, 1964) depended upon color reactions characteristic of compounds with a free α-amino group (Kalyankar and Snell, 1957; Larsen and Kjaer, 1960). Although an enzymatic synthesis of the toxin has been described (Malathi, 1967), the same color tests mentioned above were used to demonstrate the structure of the product.

This paper provides spectral evidence that natural and synthetic toxins are the same compound. Both are demonstrated to be β-*N*-oxalyl derivatives of α,β-diaminopropionic acid by a degradation scheme whose anticipated product is N_2Ph-serine. That the major product obtained by degrading natural and synthetic toxins is N_2Ph-serine is shown by the nmr spectra which are identical to that of N_2Ph-serine standard. Enzymatic conversion of the toxin to pyruvic acid with tryptophanase confirms that the toxin is a derivative of an L-amino acid. These data supply additional evidence that the neurotoxin from *Lathyrus sativus* has the structure β-*N*-oxalyl-L-α,β-diaminopropionic acid. They justify the use of highly purified synthetic toxin in the biological experiments described here.

Specificity as an Amino Acid Antagonist. Experiments with mutant and wild-type yeasts demonstrate that the neurotoxin from *Lathyrus sativus* behaves as a highly specific competitive antagonist of L-glutamic acid and L-aspartic acid. These amino acids protect yeast cells from growth depression when added to culture media. They compete with the toxin for transport into the yeast cell. When yeast strains are ranked according to their resistance to the toxin, those with the largest intracellular pools of glutamate and aspartate are the most resistant to the growth-depressing effects of the toxin.

The behavior of the *Lathyrus sativus* toxin as a glutamate and an aspartate antagonist is not unexpected in view of its structural resemblance to these two compounds. Surprisingly, however, there has been no previous demonstration that the *Lathyrus sativus* toxin behaves as an antagonist of dicarboxylic amino acids. On the contrary, studies both in another laboratory (Jacob *et al.*, 1967) and in our own (T. Mehta and B. E. Haskell, 1972, unpublished data) have shown that the neurotoxin inhibits common glutamate- and aspartate-metabolizing enzymes either very weakly—*i.e.*, at toxin concentrations which greatly exceed those of substrate—or not at all. We have observed that the neurotoxin has no effect on the activity of glutamic acid decarboxylase, glutamine synthetase, glutamic-oxaloacetic transaminase, glutamic acid dehydrogenase, and aspartic transcarbamylase when the substrate concentration is limiting and the molar concentration of toxin is approximately equal to that of substrate.

Rao *et al.* (1964) first demonstrated that the *Lathyrus sativus* toxin inhibits microbial growth. These investigators showed that the amount of toxin added to media is linearly related to growth inhibition in strains of *E. coli*, *S. aureus*, *Candida albicans*, and *Neurospora crassa*. Details of culture conditions were not reported.

Under our experimental conditions, the *Lathyrus sativus* toxin behaves as an unusually potent amino acid antagonist. Its affinity for the yeast transport system for dicarboxylic acids is greater than that of either aspartate or glutamate (K_i equals about 1×10^{-4} M as compared to K_m values of 1.69×10^{-4} M for glutamate and 1.33×10^{-4} M for aspartate). It inhibits growth by 50% at molar concentrations which constitute only about 33.3–1.25% those of glutamate (see Table IV). These results are in contrast to those with the other common amino acid antagonists where the concentrations needed for inhibition often exceed that of the natural amino acid by 10- to 100-fold (Ames, 1964; Surdin *et al.*, 1965; Rabinowitz *et al.*, 1954; Beerstecher, 1954; Beerstecher and Shive, 1947; Harding and Shive, 1948).

The *Lathyrus sativus* toxin appears to be highly specific, both with regard to the amino acids affected and with regard to the site of action. The primary, if not the only, effect of the toxin appears to be to block uptake of glutamate and aspartate into the yeast cell. It does not affect the incorporation of glutamate or of aspartate into aminoacyl-tRNA. It does not inhibit the incorporation of aspartate or glutamate into protein.

If the toxin inhibits glutamate or aspartate metabolism in resting cells of *S. cerevisiae* S288C-27, we found no evidence of this in experiments in which ^{14}C-labeled metabolites of glutamate or aspartate were extracted from the cells (Reid *et al.*, 1970) after 20-min incubation with or without toxin, separated by electrophoresis (Hammerschlag *et al.*, 1971), and counted. In toxin-treated or toxin-untreated cells, more than 90% of the glutamate and about 70% of the aspartate were recovered unchanged. The pattern of aspartate metabolites recovered from toxin-treated and toxin-untreated cells did not differ.

We have not excluded the possibility that the *Lathyrus sativus* toxin may inhibit biosynthesis of glutamate and aspartate. However, our attempts to alleviate the growth-depressing effects of the toxin by supplying a precursor of aspartate or glutamate gave negative results. Assuming that no permeability barrier exists to these compounds, one might expect that glutamine, asparagine, α-ketoglutarate or oxaloacetate would be at least partially effective in alleviating toxicity. These compounds had no effect.

We conclude that the effects of the *Lathyrus sativus* toxin are most readily interpretable as a competitive inhibition of the permease for dicarboxylic amino acids. Adding glutamate and aspartate to media for yeasts which do not require these compounds for growth (see Materials and Methods) may protect cells from the toxin simply by blocking entry of the toxin into the yeast cell. Similarly, high intracellular concen-

trations of glutamate and aspartate may have a protective effect because they slow entry of the toxin into the cell. It was observed that the sensitivity of various yeast strains to the *Lathyrus sativus* toxin is least in the cells with the largest intracellular pools of glutamate and aspartate (Table IV). Joiris and Grenson (1969) have shown that high intracellular concentrations of glutamate serve as feedback inhibitors of the permease for dicarboxylic amino acids in *Saccharomyces cerevisiae*.

The possible role of a metabolite of the toxin in depressing yeast growth needs investigating. However, it is clear from our present data that hydrolysis products of the neurotoxin—α,β-diaminopropionic acid and oxalic acid—are not toxic to the yeast cell. They do not depress yeast growth and they do not inhibit uptake of radioactive glutamate or aspartate into resting yeast cells. In this respect, the yeast system resembles chick experiments in which it has been demonstrated that integrity of the amide bond is necessary for toxicity (Rao *et al.*, 1964).

Amino Acid Antagonism and Neurotoxicity. A possible relationship of the glutamate-antagonizing properties of the *L. sativus* toxin to its neuroxtoxicity deserves comment. Although considerable evidence exists that glutamate is the neurotransmitter at the neuromuscular junction in crustaceans and in insects (Takeuchi and Takeuchi, 1964, 1965; Robbins, 1959; Van Harreveld and Mendelson, 1959; Usherwood *et al.*, 1968; Faeder and O'Brien, 1970), it is not known whether glutamate or aspartate behave as neurotransmitters in animals and in man. Aspartate, glutamate, and other structurally related compounds depolarize spinal neurons in the cat when applied iontophoretically to the external surface (Curtis *et al.*, 1960; Curtis and Watkins, 1960). However, Curtis and his associates consider that the neuroexcitatory properties of glutamate and similar compounds are nonspecific and do not reflect a function as excitatory neurotransmitters. Still, it is of interest that the same group (Watkins *et al.*, 1966) has tested the *Lathyrus sativus* toxin and has observed that it is a more potent neuroexcitator of spinal interneurons and Betz cells in the cat than is glutamate.

Recent interest in a possible neurotransmitter role for glutamate and aspartate in animals has been stimulated by the demonstration that a highly specific high affinity uptake mechanism for glutamate and aspartate exists in synaptosomes (pinched off nerve terminals) from rat brain and spinal cord (Logan and Snyder, 1971; Wofsey *et al.*, 1971). Snyder and coworkers suggest that the existence of this uptake system is neurochemical evidence of a possible transmitter role for glutamate and aspartate in mammalian brain and spinal cord. The presumed function of the high affinity uptake system in nerve endings is to remove glutamate and aspartate from the synapatic cleft and thus terminate their synaptic activity.

Does the *Lathyrus sativus* toxin act in animal tissue as it does in yeast cells to block a glutamate or aspartate uptake mechanism? In particular, does it interfere with a glutamate or aspartate uptake mechanism necessary for normal nerve functions? Experiments to explore these possibilities are in progress.

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Molecular Composition and Sedimentation Characteristics of Soluble Antigen–Antibody Complexes†

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ABSTRACT: Soluble antigen(Ag)–antibody(Ab) complexes are formed in the presence of excess antigen. A limiting small immune complex is formed in high degrees of antigen excess. The molecular compositions of human serum albumin–anti-human serum albumin (rabbit) and bovine serum albumin–anti-bovine serum albumin (rhesus) complexes were investigated by ultracentrifugation. The two major species of limiting complexes were 11.0 S and 8.5 S, as determined by zonal ultracentrifugation, and 11.7 S and 9.0 S by analytical ultracentrifugation, with the smaller complexes predominating in moderate degrees of antigen excess. The molar composition of these complexes was determined by zonal ultracentrifugation using ^{131}I- and ^{125}I-labeled antigens and antibodies, respectively. The molar composition of the 11.0S complexes was Ag_2Ab_2 and the smaller complexes consisted of primarily Ag_1Ab_1. A computer model of the smaller complexes was constructed using current hydrodynamic and electron microscopic data for γG and bovine serum albumin molecules. Calculations of theoretical sedimentation coefficients disclosed that Ag_2Ab_1 and Ag_1Ab_1 complexes cannot be separated adequately by analytical ultracentrifugation, and that the Ag_1Ab_1 complexes observed experimentally did not result from dissociation of larger complexes. Thus the smallest, or limiting complex, in low to moderate degrees of antigen excess is predominantly Ag_1Ab_1.

Molecules of the γG[1] class of antibodies are bivalent. Antigens, however, may be monovalent to multivalent. Multivalent antigens form precipitates with their specific antibodies in the zone of antibody excess as well as at antigen–antibody equivalence due to lattice formation. Further increase in the amount of antigen results in a decrease of the precipitate and the formation of soluble antigen–antibody complexes. Complexes formed at low degrees of antigen excess are larger, with more lattice work, and possess more

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[1] Abbreviations used are: Ag, antigen; Ab, antibody; HSA, human serum albumin; BSA, bovine serum albumin; γG, γG-globulin; anti-HSA or anti-BSA, antibodies directed against HSA or BSA.