is formed by the heat treatment of ovalbumin.

The rapid increase in β-sheet structure was quantitatively observed during heat denaturation of ovalbumin. The formation of β-sheet structure of heat-denatured ovalbumin increased in higher protein concentration and salt concentration in which the intermolecular interaction was enhanced. It seems likely that protein interactions would be primarily of a hydrophobic nature under high-salt concentration. β-Sheet is generally thought to be stabilized by free energy gain from increased hydrogen-bond formation. It seems possible that the increased β-sheet structure was stabilized by hydrophobic interaction.

In addition, the content of β-sheet structure was increased in proportion to the molecular weight of the heat-denatured ovalbumin aggregates. Therefore, it is apparent that the β-sheet is intermolecularly formed between unfolded molecules. It is probable that the partially unfolded form with a significant amount of secondary structure is stabilized by the intermolecular interaction. Thus, the irreversibility of the heat denaturation of ovalbumin is derived from the intermolecular interaction formed by β-sheet structure. Taking into account the result that the exposed hydrophobic residues on the molecular surface remarkably increased with the heat denaturation of ovalbumin (Kato et al., 1983), the β-sheet structure may be liable to form in the hydrophobic environment. That is, the intermolecular β-structure may be strengthened by the exposed hydrophobic residues that exclude the water in the space between unfolded molecules. Thus, it seems reasonable to conclude that ovalbumin aggregates or gels are built from the partially unfolded molecules with a significant amount of secondary structure and cross-linked with the network of the intermolecular β-sheet structure strengthening by the exposed hydrophobic residues.

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Ability of L-Canavanine To Support Nitrogen Metabolism in the Jack Bean, *Canavalia ensiformis* (L.) DC.

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The ability of L-canavanine, a nonprotein amino acid of certain leguminous plants, to support the nitrogen metabolism of jack bean, *Canavalia ensiformis* [Leguminosae], was assessed by administration of L-[*guanidino*-N^3-^{15}N]arginine, L-[*guanidinooxy*-N^3-^{15}N]canavanine, or L-[*guanidinooxy*-N^1-^{15}N]canavanine into the cotyledons of 9-day-old plants. A strikingly similar pattern of ^{15}N assimilation into de novo synthesized amino and imino acids resulted from feeding L-[*guanidino*-N^3-^{15}N]arginine and L-[*guanidinooxy*-N^3-^{15}N]canavanine. Glutamic acid plus glutamine and alanine were the most heavily labeled of the detected compounds. Some transfer of ^{15}N from L-[*guanidino*-N^3-^{15}N]arginine to canavanine was noted. This may occur by a transamidination reaction between L-canaline and L-arginine. L-[*guanidinooxy*-N^1-^{15}N]Canavanine also supported amino and imino acid biosynthesis in this plant, but much more alanine and less glutamic acid and glutamine were labeled. These experiments provide substantive experimental evidence for the long-reputed hypothesis that canavanine functions as a nitrogen-storing metabolite.

L-Canavanine, the 2-amino-4-(guanidinooxy)butyric acid structural analogue of L-arginine, is distributed throughout the Lotoideae (Fabaceae), a major group of leguminous plants, and is found in such agronomically important plants as clover, alfalfa, trefoil, and Lespedezas (Bell et al., 1978; Rosenthal, 1979). Canavanine is frequently the principal nonprotein amino acid of the seed and often accounts for more than 3% of the dry matter (Van Etten et al., 1961, 1967). There is considerable evidence that this potentially toxic secondary metabolite functions in higher plant chemical defense against herbivores, particularly insects (Rosenthal, 1979; Rosenthal and Bell, 1979). In contrast, its putative role as a nitrogen-storing metabolite is far less firmly established experimentally. The storage of appreciable canavanine in the seed, which is precipitously lost during germination; its facile translocation from the cotyledons to the growing parts of the plant; its high

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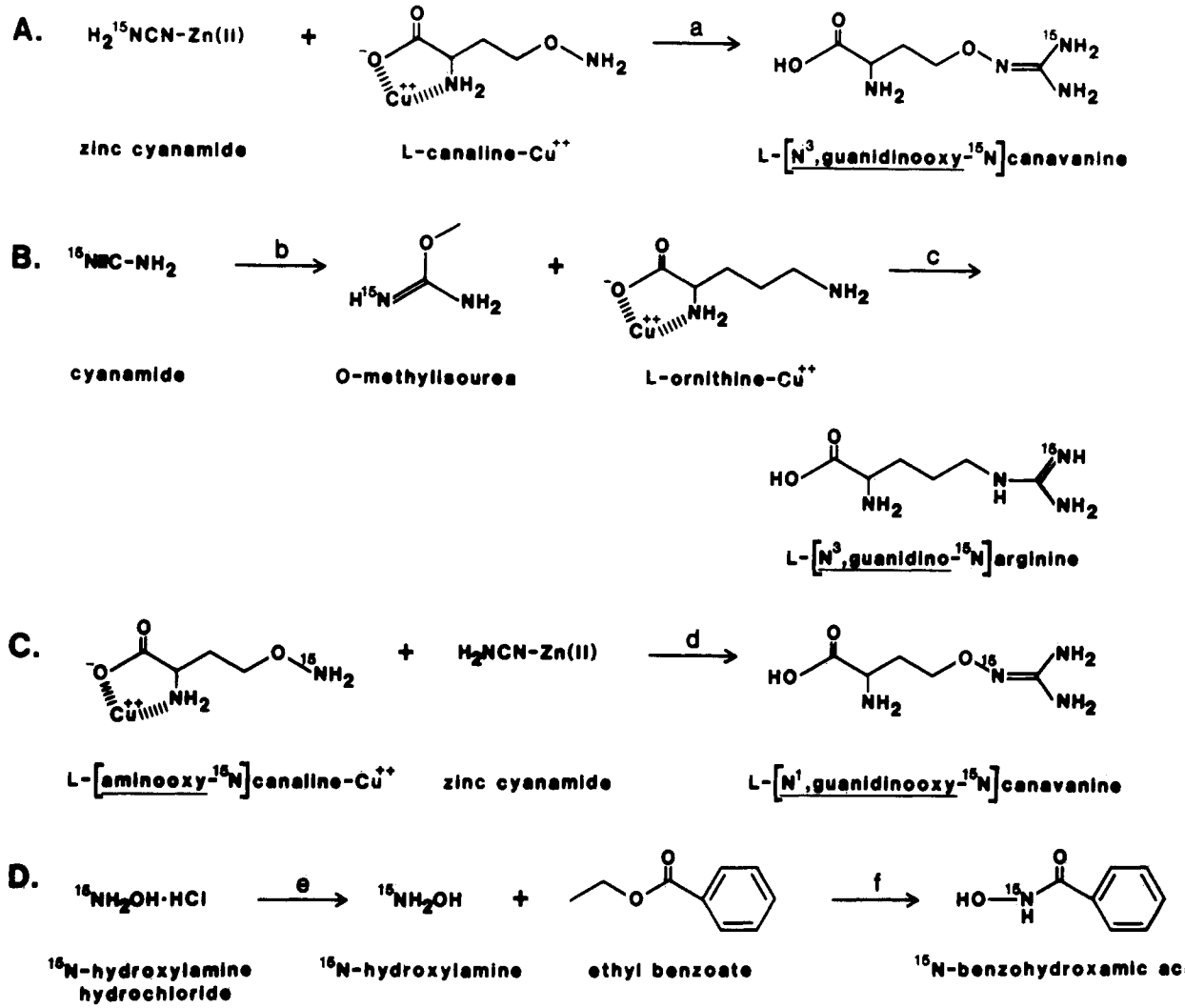
Scheme I. Isotopic Synthesis of L-[*guanidinooxy*-N^3-^{15}N]Canavanine, L-[*guanidinooxy*-N^1-^{15}N]Canavanine, L-[*guanidino*-N^3-^{15}N]Arginine, and [^{15}N]Benzohydroxamic Acid[a]



[a] Reaction conditions: (a) pH 6.4/45 °C/48 h, H_2S; (b) MeOH/HCl/45 °C/2 h; (c) pH 10.5/22 °C/48 h, H_2S; (d) pH 6.4/45 °C/48 h/H_2S; (e) MeOH/KOH, filter, dry; (f) DMF.

nitrogen to carbon ratio; and discovery of the mechanism for releasing the nitrogens of the guanidinooxy moiety as ammonia have supported the view that its raison dêtre is that of a nitrogen-storing metabolite. For example, studies of the jack bean, *Canavalia ensiformis* (L.) DC., reveal that L-[*guanidinooxy*-^{14}C]canavanine administered to the green cotyledons of 9-day-old plants is rapidly transported to the roots and above-ground parts of the plant (Rosenthal and Rhodes, 1984). While the above reasoning is compelling, direct experimental evidence supporting canavanine's role as a nitrogen-storing metabolite is lacking. As a result, we synthesized L-[*guanidinooxy*-N^3-^{15}N]canavanine (Scheme IA) and L-[*guanidino*-N^3-^{15}N]arginine (Scheme IB) and compared their effectiveness in supporting de novo synthesis of ^{15}N-containing amino and imino acids. L-Arginine was selected for this comparative study since its ability to function as a nitrogen-storing metabolite has been thoroughly established (Thompson, 1980).

L-Canavanine or L-arginine is catabolized by the jack bean plant via the action of arginase (EC 3.5.3.1), which mediates a hydrolytic cleavage to yield L-canaline or L-ornithine, respectively, and urea. Urease (EC 3.5.1.5) subsequently cleaves urea to carbon dioxide and ammonia. Thus, by combining the action of these two enzymes, the jack bean plant releases the nitrogen of the guanidinooxy or guanidino moiety of these amino acids as ammonia. In contrast, when *C. ensiformis* is provided L-[*guanidinooxy*-N^1-^{15}N]canavanine (Scheme IC), these enzymes yield urea and L-[*aminooxy*-^{15}N]canaline. In order for this heavy nitrogen atom to be utilized, catabolic reactions other than those described above must operate.

The availability of L-[*guanidinooxy*-N^3-^{15}N]canavanine and L-[*guanidinooxy*-N^1-^{15}N]canavanine therefore permits a comparative assessment of the effectiveness of arginase plus urease in mobilizing the external nitrogens (N^2, N^3) of the canavanine guanidinooxy group as compared to the reactions pathways(s) that foster utilization in the internal nitrogen (N^1) of the guanidinooxy group of canavanine.

EXPERIMENTAL SECTION

Preparation of L-[*guanidino*-N^3-^{15}N]Arginine. A mixture of L-ornithine (10 mmol) and 1.5 g of CuO was swirled in 10 mL of deionized water at 100 °C for 3 min and then stirred at 30 °C overnight. Unreacted CuO was removed by filtration, and the filtrate was adjusted to pH 10.5 with 4 N NaOH. ^{15}N-Labeled free cyanamide (99.5% ^{15}N, Kohler Isotopes; 3 mmol) was dissolved in 10 mL of absolute methanol, cooled in an ice bath for 10–15 min, and then reacted with a 10-fold molar excess of acetyl chloride. Care was taken to provide the acetyl chloride *very slowly* in small aliquots to avoid a violent reaction. After the final acetyl chloride addition, the reaction mix-

ture was stirred at 45 °C for 2 h; the reaction was terminated by evaporation of the methanol in vacuo. The representative yield of ^{15}N-labeled *O*-methylisourea was 96 ± 1%. [See Rosenthal et al. (1983) for additional experimental details.]

The ^{15}N-labeled *O*-methylisourea was dissolved in 3 mL of deionized water and combined with 1.75 equiv of L-ornithine–Cu^{2+} salt. After readjusting to pH 10.5 as above, the reaction mixture was stirred at 22 °C for 48 h. The reaction mixture was treated with H_2S and CuS removed by filtration. The filtrate was brought to pH 3.0 with 4 N HCl and the resulting precipitate removed by centrifugation at 12000*g* for 12 min. The supernatant solution was concentrated by rotary evaporation in vacuo and placed on a 20 × 150 mm column of Dowex-50 (H^+). Unreacted ^{15}N-labeled *O*-methylisourea was eluted with deionized water, concentrated by rotary evaporation in vacuo, and saved. Unreacted ornithine, an unknown compound, and L-[*guanidino*-N^3-^{15}N]arginine were eluted with 0.5 L of 400 mM NH_3. After the column effluent was dried by rotary evaporation in vacuo, the reaction products were stored at −20 °C.

The unknown basic substance, possibly 2-guanidino-5-aminopentanoic acid, never exceeded 5% of the final reaction product; but its concentration increased significantly at elevated reaction temperature, or if incomplete formation of the copper salt of L-ornithine occurred.

Recovered ^{15}N-labeled *O*-methylisourea was treated with the copper salt of L-ornithine as described above, and the isolated reaction products were combined with those of the initial preparation. The combined reaction products were acidified to pH 3.0 and purified on a 20 × 150 mm column of Dowex-50 (NH_4^+). Unreacted ornithine and the unknown were eluted with 50 mM NH_3; L-[*guanidino*-N^3-^{15}N]arginine was then obtained by elution with 400 mM NH_3. The column effluent was concentrated by rotary evaporation in vacuo, treated with decolorizing charcoal, concentrated in vacuo, and precipitated from aqueous ethanol. the representative yield for the tandem preparations was 65% (Scheme IB).

The purity of the L-[*guanidino*-N^3-^{15}N]arginine was established by automated amino acid analysis in which the column was massively loaded. Successful production of [^{15}N]arginine was established by the Sakaguchi colorimetric method as modified by Rosenthal and Naylor (1969) and mass spectroscopic analysis (Figure 1B).

Synthesis of L-[*guanidinooxy*-N^3-^{15}N]Canavanine. Reaction of the copper salt of L-canaline with zinc(II) ^{15}N-labeled cyanamide to prepare L-[*guanidinooxy*-N^3-^{15}N]canavanine was achieved after the method of Ozinskas and Rosenthal (1986a) (Scheme IA). Successful production of [^{15}N]canavanine was established by the pentacyanoammonioferrate colorimetric assay (Rosenthal, 1977) and mass spectroscopic analysis (Figure 1A).

Synthesis of L-[*guanidinooxy*-N^1-^{15}N]Canavanine. Zinc(II) cyanamide was reacted with L-[*aminooxy*-^{15}N]-canaline to form L-[*guanidinooxy*-N^1-^{15}N]canavanine (Ozinskas and Rosenthal, 1986a) (Scheme IC). L-[*aminooxy*-^{15}N]Canaline was obtained by reacting [^{15}N]-benzohydroxamic acid with benzyl L-2-[(carbobenzyloxy)amino]-4-[(*p*-tolylsulfonyl)oxy]butyrate; the latter was prepared by the method of Ozinskas and Rosenthal (1986b). The reaction product, benzyl L-2-[(carbobenzyloxy)amino]-4-(benzamidooxy)butyrate, was deprotected and L-[*aminooxy*-^{15}N]canaline isolated as previously described (Ozinskas and Rosenthal, 1986b).

[^{15}N]Benzohydroxamic acid was made by dissolving 3.55 mmol of ^{15}N-labeled NH_2OH·HCl (99% ^{15}N; ICN) in 2 mL of anhydrous methanol and adding a 4-fold excess of ethanolic KOH. KCl was removed by filtration (Scheme ID). [^{15}N]Hydroxylamine was reacted with 2 equiv of ethyl benzoate at 30 °C for 24 h. After the ethanol was removed by rotary evaporation in vacuo, the residue was dissolved in deionized water and taken to pH 2.0 with 6 N HCl. The aqueous solution was extracted four times with an equal volume of ethyl acetate. The extract was dried over Na_2SO_4, filtered, and evaporated. The residue was purified by flash chromatography on silica gel with acetone/hexane (2:3, v/v).

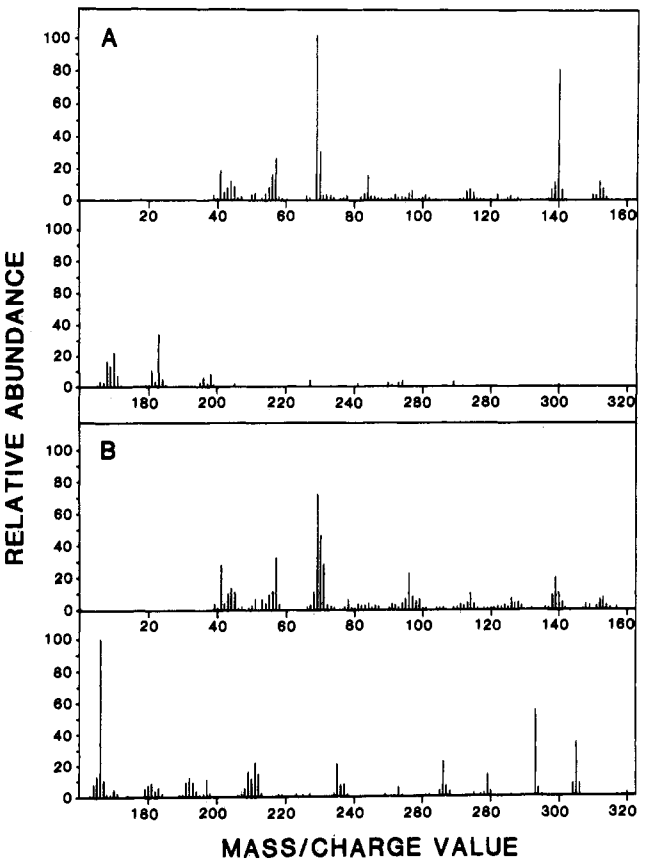


Figure 1. Mass spectrometry fragmentation pattern for (A) L-[*guanidinooxy*-N^3-^{15}N]canavanine and (B) L-[*guanidino*-N^3-^{15}N]arginine.

The appropriate fractions were pooled, and the solvent was removed by rotary evaporation in vacuo. After the residue was dissolved in deionized water, it was decolorized with charcoal, filtered, and concentrated by rotary evaporation in vacuo. [^{15}N]Benzohydroxamic acid was obtained by crystallization from the mother liquor with absolute ethanol. Final yield was 70%.

Growth of the Plants and ^{15}N-Labeled Compound Administration. The ^{15}N-labeled compounds were administered to 9-day-old jack bean plants by direct injection into the fleshy, green cotyledons (the storage tissues of the seed). Each of the two segments constituting the cotyledons received 12.5 μmol of labeled amino acid in 20 μL of sterile deionized water.

The plants were fed at 0930 on a sunny summer day in which the temperature reached a maximum of 32 °C. All treatments were replicated three times, and the aboveground parts minus the cotyledons were collected after 12 h and stored at −60 °C. Conditions for the growth of the plants prior to heavy-nitrogen administration were described previously (Rosenthal, 1982).

Preparation of the Plant Extract. Frozen plant materials were ground with a large excess of acetone at full power with a Sorvall Omni mixer. The resulting homo-

Table I. Distribution of ^{15}N in [^{15}N]Canavanine- and [^{15}N]Arginine-Fed Jack Bean Plants

amino acid	% ^{15}N incorporation		
	L-[*guanidinooxy*-N^1-^{15}N]-canavanine	L-[*guanidinooxy*-N^3-^{15}N]-canavanine	L-[*guanidino*-N^3-^{15}N]-arginine
alanine	14.2	5.0	5.5
aspartic acid + asparagine (amino N)	2.0	3.7	4.2
glutamic acid + glutamine (amino N)	4.8	11.1	12.1
homoserine	1.2	0.8	1.2
histidine	3.0	2.2	3.0
isoleucine	4.6	3.1	3.3
leucine	ND	T	T
lysine	0.6	0.6	0.5
methionine	T	T	T
proline	1.3	3.2	1.3
serine	0.5	1.1	1.2
valine	0.5	0.9	0.9
arginine	ND	T	53.8
canavanine	37.0	46.3	15.6

[a] Each plant (n = 3) received L-[*guanidinooxy*-N^1-^{15}N]canavanine, L-[*guanidinooxy*-N^3-^{15}N]canavanine, or L-[*guanidino*-N^3-^{15}N]arginine The three plants were pooled into a single sample, processed, analyzed as outlined in the text. Only those amino or imino acids that incorporated detectable ^{15}N are listed. Key: ND = not detected; T = trace. Each value is the mean of two determinations.

genate was filtered and the acetone-defatted plant material allowed to dry at 22 °C.

The plant materials were extracted with 150 mL of 50% aqueous ethanol containing 0.2 N sulfuric acid by mechanically stirring overnight at 4 °C. After centrifugation at 12000g for 15 min, the supernatant solution was concentrated by rotary evaporation in vacuo and adjusted to approximately pH 3 with 2 N NH_4OH. The resulting $(NH_4)_2SO_4$ was removed by treatment with $Ba(OH)_2$; $BaSO_4$ was eliminated by centrifugation as above.

The supernatant solution was rotary evaporated in vacuo to dryness, dissolved in deionized water, and evaporated as above. After the residue was taken up in a minimum volume of deionized water, it was filtered, adjusted to pH 3.5 with HCl, treated with charcoal, filtered, and purified on a 20 × 120 mm column of Dowex 50 (H^+). After the column was washed with 1 L of deionized water, the amino acids were eluted with 0.5 L of 400 mM NH_3. The effluent was concentrated in vacuo, treated with decolorizing charcoal, and concentrated by lyophilization. The samples were stored at −60 °C.

Mass Spectroscopy. The lyophilized amino acids were converted to their N-(trifluoroacetyl) n-butyl ester derivatives for analysis by GC–MS as described previously (Rosenthal et al., 1982). The small relative abundance of mass spectral fragments containing nitrogen prevented determination of the ^{15}N content of phenylalanine, tyrosine, and tryptophan.

RESULTS AND DISCUSSION

L-[*guanidino*-N^3-^{15}N]Arginine supported the de novo synthesis of a large number of protein amino and imino acids (Table I). The principle metabolic products derived from arginine utilization are glutamic acid and glutamine (our method of analysis does not distinguish between these amino acids). Previous study of [^{15}N]urea utilization by *C. ensiformis* also indicated glutamate and glutamine as the principal recipients of the heavy-nitrogen atom (Rosenthal and Rhodes, 1984). These reaction products would result if arginine is hydrolytically cleaved by arginase to generate L-ornithine and urea; urease would cleave the latter to generate ammonia. These enzymes are very active in newly developing jack bean plants and create the principal pathway for canavanine catabolism (Rosenthal, 1970). Once ammonia is produced it can react with 2-oxoglutarate in the presence of glutamic acid dehydrogenase (EC 1.4.1.2) to form glutamic acid; the latter, in turn, can react with ammonia to form glutamine via the action of glutamine synthetase (EC 6.3.1.2).

Jack bean plants administered L-[*guanidino*-N^3-^{15}N]-arginine can also produce [^{15}N]canavanine (Table I). This synthesis may be mediated by a transamidinase (EC 2.1.4) capable of transferring the ^{15}N-labeled amidino group of arginine to canaline to produce [^{15}N]canavanine. Transamidination between canavanine and ornithine to generate ^{15}N-labeled arginine is not observed under our conditions. This may result from the minute amount of free ornithine (<1 μmol) that is present in the young jack bean plant; in contrast, 15–20 μmol of canaline is produced daily (Rosenthal, 1972).

The ability of L-[*guanidinooxy*-N^3-^{15}N]canavanine to support the nitrogen metabolism of the developing jack bean is revealed strikingly in Table I. The ^{15}N-labeling pattern within the de novo synthesized amino and imino acids is virtually identical with that of L-[*guanidino*-N^3-^{15}N]arginine-treated plants. These data are the first experimental evidence substantiating the long-held belief that canavanine, like arginine, is an effective nitrogen-storing metabolite that can support higher plant nitrogen metabolism (Bell, 1971).

An interesting ^{15}N incorporation pattern results from administration of L-[*guanidinooxy*-N^1-^{15}N]canavanine to jack bean plants (Table I). In the presence of arginase, this secondary metabolite is converted to [^{15}N]canaline and urea; in comparison, L-[*guanidinooxy*-N^3-^{15}N]canavanine would generate canaline and [^{15}N]urea.

Plants provided L-[*guanidinooxy*-N^1-^{15}N]canavanine show far less incorporation of heavy nitrogen into glutamic acid and glutamine and an increase in the amount of [^{15}N]alanine. It is not clear presently how the aminooxy group of canaline supports L-alanine production, but it appears to do so at the expense of glutamate and/or glutamine. With the above exceptions, the pattern of ^{15}N incorporation for L-[*guanidinooxy*-N^1-^{15}N]canavanine was comparable to that of L-[*guanidinooxy*-N^3-^{15}N]canavanine. Thus, all of the nitrogen atoms of the guanidinooxy moiety of canavanine are able to support amino and imino acid production by the developing jack bean plant.

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Isolation and Identification of Trichothecenes from *Fusarium compactum* Suspected in the Aetiology of a Major Intoxication of Sandhill Cranes

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Isoneosolaniol (4,8-diacetoxy-12,13-epoxytrichothec-9-ene-3,15-diol) and other unidentified trichothecene mycotoxins were isolated from culture extracts of two highly toxigenic strains of *Fusarium compactum* cultured from waste peanuts involved in an acute intoxication of sandhill cranes (*Grus canadensis*). Neosolaniol and other unidentified trichothecenes were detected in waste peanuts collected from affected areas. The structure of isoneosolaniol was determined by ^{1}H and ^{13}C NMR analyses and by high-resolution mass spectrometry. Isoneosolaniol was highly toxic to 1-day-old chickens and to a HEp2 cell culture assay. It was concluded that the most logical cause of the sandhill crane intoxication was *Fusarium* spp. contaminated peanuts and various trichothecene mycotoxins acting alone or in conjunction with other *Fusarium* mycotoxins.

An estimated 9075 sandhill cranes (*Grus canadensis*) died at or near a major roost site at Cedar Lake in Gaines County, TX, from 1982 to 1987, with a major loss (5000) occurring during January and February 1985 (Windingstad et al., 1987). Concurrent studies have implicated waste peanuts contaminated with *Fusarium* species as the most probable cause (Windingstad et al., 1988; Nelson et al., 1988). Prominent clinical signs in sick cranes were the inability to hold their heads erect and difficulty in flying. Cranes that could fly did so with head, neck, and legs drooped perpendicularly to the body. Submandibular edema observed in sick and dead cranes may have resulted from this prolonged posture of the head.

Peanuts were the major component of the cranes' diet, with over 95% of the dead cranes having peanuts in their gizzards (Windingstad et al., 1988). Nelson et al. (1988) have shown that *Fusarium* was the major fungal genus on waste peanuts collected from the affected area. Although several of the species isolated (*Fusarium moniliforme*,

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