Ethylene production by slices was much higher in both green and red fruits treated with JA-Me than that of controls over a period of 1–10 days (figs 1 and 2). The effect of JA-Me on the ethylene production after 1 or 2 days is much more pronounced for red-ripe fruits (fig. 2) than for green ones (fig. 1). A rapid increase of ethylene production can be observed for both treated and control tissue on day 3, when the color of tissues turned pink (control) or yellow (treated with JA-Me) (fig. 1). On days 7–10 ethylene production decreased in control tissues while at the same time it remained almost at a steady level in treated ones (fig. 1).

Ethylene production by green fruits at different times after JA-Me treatment was about 1.6–7.9 times higher than by control tissues (fig. 1). Initially, treatment of red ripe fruits with methyl jasmonate accelerated ethylene production about 9 times compared to control tissues (fig. 2). However, on day 8 ethylene synthesis decreased in treated and control tissue about 3 times as compared to 1 day but differences between treatments remained at a similar level (fig. 2). The inhibitory effect of methyl jasmonate on lycopene accumulation[12] and the stimulatory effect on ethylene evolution in tomatoes indicates that ethylene production is not essential for lycopene synthesis. It is possible that methyl jasmonate blocks a physiological action of ethylene in processes connected with lycopene synthesis. Until now the presence of jasmonic acid or methyl jasmonate in tomato plants has not been reported.

Ethylene biosynthesis in tomatoes occurs via the normal pathway of methionine to S-adenosyl-methionine to 1-aminocyclopropane-1-carboxylic acid (ACC) to ethylene[15–18].

The mechanism of the strong stimulatory effect of methyl jasmonate on ethylene production in tomatoes remains unknown. Detailed study of the effect of JA-Me on ethylene production in intact fruits as well as the level of ACC and the effect of ethylene biosynthesis inhibitors on ethylene production after methyl jasmonate treatments will be undertaken.

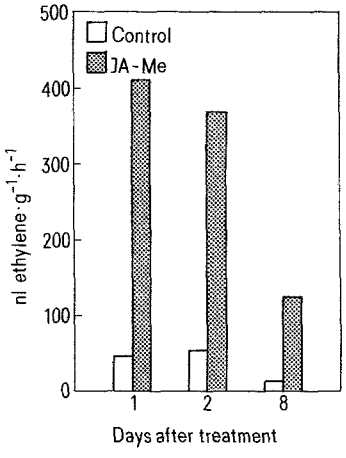

Figure 2. The effect of methyl jasmonate on ethylene production from slices from tomato fruits. Treatments were applied on red ripe fruits starting on day 0. Each bar represents means for that particular day of measurement. LSD 1% value are as follows; day 1, 149.1; day 2, 236.4; day 8, 97.6.

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Canavanine in alfalfa *(Medicago sativa)*

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Summary. Canavanine was extracted and characterized from various cultivars of alfalfa seed. Values obtained range from 8 g/kg for the Buffalo variety to 14–20 g/kg for the highly weevil resistant variety, Weevl-Chek.
Key words. Alfalfa; *Medicago sativa;* canavanine.

A color reaction between histidine and the pentacyanoferrate reagent, at neutral pH was reported in this journal, recently[1]. This reaction has been used in the past for the identification of canavanine[2–4]. In that paper, claim was made that the color reaction, with alfalfa extract, with the pentacyanoferrate reagent was due to its content of histidine and not canavanine. In reporting a fluorimetric method for canavanine assay, the method was applied to seeds, stems and roots of various cultivars of alfalfa *(Medicago sativa)*[5]. Contrary to the assertion of the author, in the recent paper cited[1], large amounts of canavanine were found in alfalfa seeds ranging from 8 to 16 g/kg. Much smaller amounts were found in the leaves and stems[6]. To explain these contradictions, canavanines were prepared from various cultivars of alfalfa seeds and their properties were compared to canavanine obtained from the jack bean *(Canavalia ensiformis)* and with histidine.

Materials and methods. Details of the method of extraction have been described in the papers cited[5,6]. Briefly, the canavanine was extracted from the ground seeds of the jack bean or the alfalfa variety with 0.25 M H_2SO_4. After centrifugation, the supernatant was freed of protein by shaking with chloroform and centrifuging. The protein collected at the interface. The H_2SO_4 was removed with $Ba(OH)_2$ and the canavanine was adsorbed on Dowex 50-X-2 column, 50–100 mesh, NH_4^+ form. It was eluted with 0.16 M NH_4OH and the eluate collected on a fraction collector. Only the fractions giving the magenta color with the pentacyanoferrate reagent were saved and lyophilized. The residue was recrystallized from 80% ethanol. If highly colored it was purified as the flavianate[3,4].

The pentacyanoferrate reagent was made by mixing equal volumes of 0.08 M sodium nitroprusside, 0.075 M potassium ferricyanide, and 0.6 M NaOH. The solution turns dark and then lightens to a pale yellow on standing in the refrigerator for 2 h. A dilution of the solution to be tested was made, depending upon the canavanine concentration. 0.2 ml of this solution was added to 2 ml of 0.5 M phosphate buffer, pH = 7.0, and 0.2 ml of the pentacyanoferrate reagent was added. Absorbance was measured at 520 nm, repetitively, until a maximum reading was obtained. The color then fades. The canavanine was also assayed by its reaction with phenanthrenequinone fluorometrically[6].

Hydrogen was bubbled into 25 ml of 4 mM solutions of histidine and the canavanine to be tested, containing 200 mg sodium borate (borax), and 100 mg 5% Pd on $BaSO_4$. After 1–2 h the test for canavanine with the pentacyanoferrate reagent became negative. Paper electrophoresis was then carried out on 20 µl of the solution, spotted at the origin. A 0.75 M lithium acetate buffer, pH 4.9, at 18 V/cm was applied for 45 min. The strips were sprayed with ninhydrin (Sigma) and pentacyanoferrate reagents.

Arginase (2000 U)[7] was incubated at 37 °C with 3 mmoles canavanine or histidine in 30 ml of pH 8.6 glycine buffer, 0.01 mM with respect to Mn^{2+}. Urea estimation[8] was carried out at 15-min intervals on 0.1-ml aliquots for 2 h, when the reaction seemed to be completed. After standing overnight, canaline was isolated as the picrate by adding 2 g of picric acid and shaking at 37 °C. The recrystallized from water picrate was freed of picric acid with H_2SO_4 and the canaline isolated[9]. No insoluble picrate was obtained from the histidine. With paper electrophoretic and thin layer chromatography studies it was shown that canavanine from alfalfa and jack bean had been hydrolyzed and histidine remained unchanged.

Results. It can be seen from figure 1 that the sensitivity for canavanine is about eight times that for histidine, at 520 nm. There are also significant differences in their spectra so that they can be readily distinguished.

Figure 2 shows the elution pattern obtained when 10 ml of 4 nM canavanine and 10 ml of 4 mM histidine were mixed and placed on a 1 × 60 cm, Dowex 50-X-8, 200–400 mesh column, NH_4^+ form, and eluted with 0.16 M NH_4OH as for the preparation of canavanine above. The canavanine was located with the pentacyanoferrate reagent, and the histidine with diazotized sulfanilic acid (Pauly reagent)[10]. When the column was lengthened to 100 cm, and the quantity of canavanine and his-

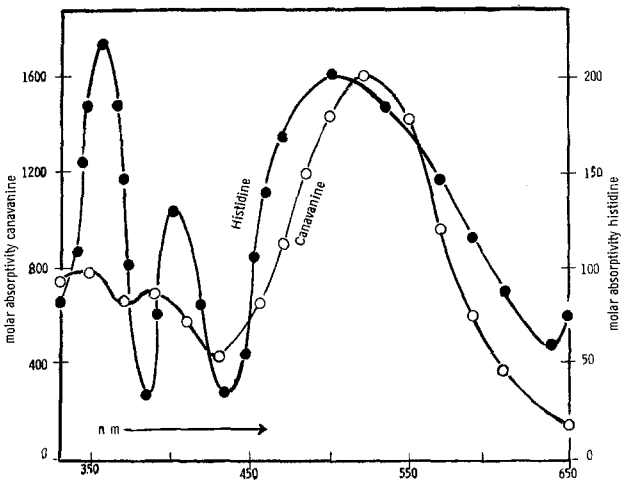

Figure 1. Comparison of the absorption spectrum of histidine and canavanine with the pentacyanoferrate reagent. The molar absorptivities (1 cm light path) are compared, with the scale for histidine 1/8 that of canavanine. The canavanine color is magenta. The color with histidine is more amber.

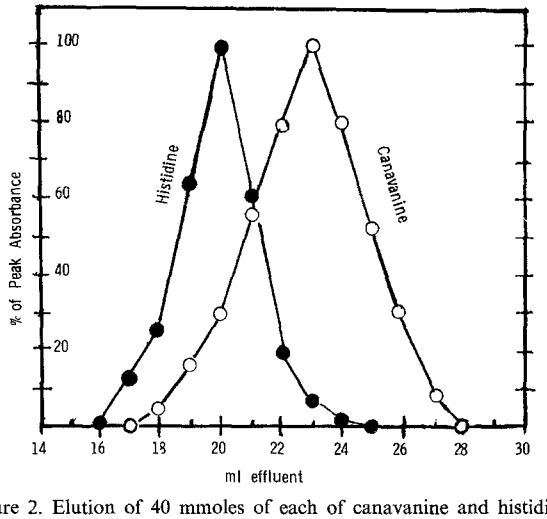

Figure 2. Elution of 40 mmoles of each of canavanine and histidine, simultaneously, from a 1 × 60 cm Dowex 50-X-8, 200–400 mesh column, with 0.16 M NH_4OH. The canavanine is located with the pentacyanoferrate reagent, and the histidine with diazotized sulfanilic acid.

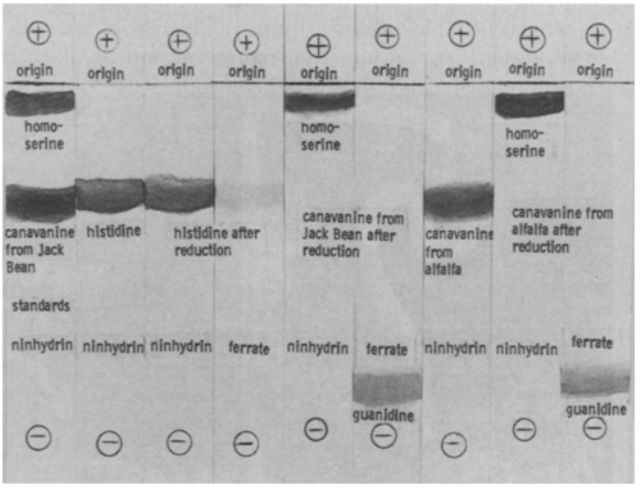

Figure 3. Demonstration, by paper electrophoresis, that canavanine from jack bean, and alfalfa both reduce to homoserine and guanidine. Histidine is unchanged. The spray, ninhydrin or the pentacyanoferrate reagent, is indicated on each strip. With ninhydrin, canavanine and homoserine are a royal blue. Histidine is lavender and turns to brown on aging. With the pentaferrate reagent, the canavanine and guanidine stains form promptly, and are a magenta. With histidine this stain produces a light pink on long standing. Note that all the canavanine has been destroyed after reduction. The pentacyanoferrate stain for canavanine was at the same location as with ninhydrin.

tidine reduced to half, the two peaks were of about equal width and well resolved.

Canavanine from jack bean and from alfalfa yielded guanidine and homoserine (fig. 3). All the canavanine was destroyed on reduction. On the other hand histidine was apparently unchanged.

From the table it can be seen that the canavanine obtained from alfalfa is not histidine and is similar to that obtained from the jack bean.

The amount of canavanine obtained from different samples of Weevl-Chek was variable and ranged from 14 to 20 g/kg. Other alfalfa varieties yielded as follows, Saranac, 10–13, Classic, 9–11, Arc, 9.5–12, Team, 8–11, Buffalo, 6–8.5 g/kg of canavanine.

The question arises as to how it was possible for the claim to be made that there was no canavanine in alfalfa[1]. Examining the procedure used to isolate canavanine from jack bean[11], substantial amounts of charcoal are used in the procedure. Activated charcoal will remove about 1 mg of canavanine per g of charcoal from solution[6]. When processing large quantities of jack beans or alfalfa seeds this is acceptable. For leaves and stems the canavanine would be removed. However, the author does not specify whether he extracted seeds, stems or leaves. If stems or leaves are extracted, the pentacyanoferrate reaction is not sensitive enough to detect the canavanine in alfalfa. A fluorometric procedure needs to be used[6]. In the fluorometric procedure, fluorescence intensity for canavanine is 3500 times that observed with histidine. Using 10 g/kg of alfalfa seeds as a base, to produce the fluorescence observed with alfalfa seed extracts, they would have to contain 35,000 g of histidine per kg. Using the pentacyanoferrate reaction (see fig. 1), they would have to contain at least 100 g of histidine per kg.

The histidine in alfalfa is mainly in the protein and occurs only in the expected small amounts[12,13]. In the reference referred to[11], protein is removed by neutralizing H_2SO_4 with NH_4OH. This brings the concentration of $(NH_4)_2SO_4$ to about 0.1 M in 50% ethanol. This will not precipitate all the protein. This may account for the presence of substantial amounts of histidine in the extracts.

Other statements in the paper are inexact[1]. The absorption spectrum of canavanine (fig. 1) seems to be that of a solution where the color has faded. It appears that the sensitivity with canavanine over histidine is four times. It is actually eight times (see fig. 1, this paper). The statement is also made that canavanine did not produce color with the Pauly reagent. This is true only with very low concentrations of canavanine. If 1 mg canavanine, serine, homoserine or aspartic acid is placed in a test tube and 2 ml of a 0.1% solution of diazotized sulfanilic acid, in molar NaOH is added, a deep red color will appear after about 1–2 h. This can be accelerated to minutes by warming in the flame. This color has absorption maxima at 520 and 405 nm. The color with histidine has an absorption maximum at 360 nm. In the visible (about 400 nm) this color is about 40 times as sensitive as with canavanine, and appears golden.

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Effects of methylene blue on electrical behavior of myenteric neurons

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Summary. Intracellular recording methods were used to investigate the action of methylene blue on electrical behavior of myenteric neurons in guinea pig small intestine. The neurophysiological studies were done in parallel with studies on contractile activity of the intestinal musculature. Methylene blue depolarized the membranes, increased the input resistance, augmented excitability and reduced postspike hyperpolarizing potentials in AH/Type 2 myenteric neurons. These effects, with the exception of suppression of postspike hyperpolarization, were reversed by exposure to elevated calcium. The mechanism of action of methylene blue appeared to be suppression of calcium-dependent potassium conductance in the neuronal membranes. The neuronal action of methylene blue was manifest as a release of excitatory neurontransmitter substances which evoked contraction of the small intestinal longitudinal muscle.

Key words. Guinea pig small intestine; myenteric neurons; electrical behavior; methylene blue; neuronal membranes; K^+-conductance, Ca^{++}-dependent.

Methylene blue (tetramethylthionine chloride) is an autoxidizable dye that has been used widely as a vital stain for nervous tissue in both vertebrate and invertebrate animals. Its pharmacological use as a therapeutic or diagnostic agent in humans is well known. Methylene blue was used as a stain to visualize the enteric neuronal plexuses in early extracellular studies of the electrophysiological behavior of enteric ganglion cells[1–3]. Reported results of these studies suggested that methylene blue did not alter ongoing action potential discharge of single neuronal units in the myenteric and submucosal plexuses of the intestine in a variety of species[1–3] and did not affect synaptic transmission in autonomic ganglia[4,5].

We now report the results of a study of effects of methylene blue on intracellularly-recorded electrical behavior of small intestinal myenteric ganglion cells. These results showed that methylene blue depolarized the membrane potential and aug-