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Canavanine: Detection and Occurrence in *Colutea arborescens*

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Canavanine, α -amino- δ -guanidinoxybutyric acid, is the only known example of a natural compound containing the guanidinoxy function,

Although canavanine occurs free to the extent of 2-3.5% of dry weight in the seeds of the jack bean, *Canavalia ensiformis*, and the closely-related *C. lineata*, its discoverer, M. Kitagawa, was unable to obtain it from any other leguminous plant (Kitagawa & Tomiyama, 1929; Kitagawa, 1937). It has, however, been found in the seeds of *C. obtusifolia* (Damodaran & Narayanan, 1939).

During a study of the colour reactions of pentacyanoferrate derivatives and substituted guanidines, it was observed that guanidinoxy compounds, including canavanine, reacted only within the range pH 5-7.5, whereas the alkyl-substituted guanidines, including arginine, reacted only in solutions more alkaline than pH 8 (Fearon, 1946). This is in agreement with the observations of Kitagawa (1937) and

Archibald (1946). As the result of an application of the pentacyanoferrate reactions to a survey of plant tissues, it has now been possible to show the presence of a guanidinoxy compound in some species of *Medicago* and *Ornithopus*, and canavanine has been isolated from the seeds of the bladder senna, *Colutea arborescens*.

EXPERIMENTAL

Reagents

A freshly prepared neutral aqueous solution of disodium pentacyanonitrosoferrate (sodium nitroprusside) does not react chromatically with a guanidoxine until the mixture has been exposed to daylight for about 5 min., when a colour, ranging from orange-red (*N*-methoxyguanidine) to magenta (canavanine) develops, and may persist for days. The pigment is stable to dilute acid, but is reversibly bleached by alkali. Alternatively, an aqueous solution of nitroprusside may be activated by irradiation before doing the test (Kitagawa & Yamada, 1932), though this seems to

offer no advantage over the direct method here described. In alkaline solution, photoactivated nitroprusside develops red colours with guanidine (Tiegs, 1924), urea (Ramsden, 1931) and other amidines, but these reactions are apt to be obscured by the chromatic versatility of the unchanged nitroprusside in presence of alkali.

Activation of the nitroprusside can be effected also by oxidizing agents in alkaline solution, including ferricyanide (Marston, 1925; Weber, 1928), persulphate (Pittarelli, 1928), Br_2 (Grote, 1931), I_2 (Fearon, 1937) and H_2O_2 (Archibald, 1946). The target of all these reagents is determined by the pH of the final mixture. In neutral or slightly acid solution, 'activated nitroprusside', however prepared, reacts characteristically with guanidinoxy compounds. The reagent chromogen in the mixture appears to be the ferric complex, pentacyanoaquoferrate (III), $[\text{Fe}(\text{CN})_5\text{OH}_2]^{3-}$, formed by oxidation of pentacyanoaquoferrate (II), $[\text{Fe}(\text{CN})_5\text{OH}_2]^{2-}$ a decomposition product of nitroprusside (Baudisch, 1948; van Sonsbeek, 1950; Fearon, 1946; Archibald, 1946). An objection to the use of 'activated nitroprusside' reagents is their instability and their liability to be contaminated by unchanged nitroprusside or by excess of activator. This can be overcome by employing a solution of trisodium pentacyanoammonioferrate, which can be prepared from sodium nitroprusside by Hofmann's method (Fearon, 1946; Herington, 1953) or obtained commercially.

Pentacyanoammonioferrate reagent (PCAF)

$\text{Na}_3[\text{Fe}(\text{CN})_5\text{NH}_2]$ (1%, w/v) in Cu-free distilled water. The freshly prepared reagent is in the ferrous state and is of low chromogenic activity, as noted by Archibald. After exposure to light and air for about 1 hr., it begins to undergo spontaneous oxidation. The resulting mixture of the ferric and ferrous complex is relatively stable and retains its potency for at least a month, if stored in a brown glass bottle at low temperature.

Phosphate buffer (pH 7)

0.2N-NaOH (29.54 ml.) and 0.2M- KH_2PO_4 (50 ml.) are made up to 500 ml. with water and stored at low temperature.

Canavanine standard. Aqueous solution of canavanine base (1.0 mg./ml.) or the equivalent of canavanine sulphate. The salt can be prepared from jack bean meal or obtained commercially (Bios Laboratories Inc., 17 West 60th Street, New York 23, N.Y., U.S.A.; California Foundation for Biochemical Research, 3408 Fowler Street, Los Angeles 63, California, U.S.A.; L. Light and Co., Colnbrook, Bucks.). The standard solution is preserved by addition of toluene and storage at low temperature.

Test

About 5 ml. of the test solution, buffered to pH 7, are mixed with 5 drops of PCAF. The presence of canavanine is shown by the gradual development of a purple colour. If the reagent has been only recently prepared, addition of a drop of 1% (w/v) aqueous potassium persulphate will hasten colour formation. Apart from canavanine, the test is negative with the natural amino acids, including arginine and arginine peptides (salmine, clupeine), citrulline, cysteine, cystine and methionine. On making the mixture strongly alkaline by addition of 5 drops of 5N-NaOH, the canavanine colour is discharged, but may be replaced by the

development of a secondary colour if other amidines are present in the solution: orange-red (acetamidine, cyanamide), red (guanidine, urea) or red-purple (methylguanidine, glycoylamine, creatine, dicyanodiamide, galegine, arginine, streptomycin). A blue colour, developing both in neutral and in alkaline solutions, is given by some sulphur compounds containing S-S and S-O groupings (thiosulphate, thiocyanate, thiourea, formamidine disulphide, thiouracil, 4-methylthiouracil).

Sensitivity. At pH values below 4 and above 9, no colour is given by *N*-methoxyguanidine or by canavanine. The characteristic reaction begins about pH 5, and increases in rate of development and in final intensity as the pH rises to 7. At pH 7-8 the colour comes up rapidly and soon fades. At pH 6.5-7 the pigment has maximum stability, and may persist for at least a week at room temperature. In this pH range, the test will detect canavanine in concentrations as low as 0.008%.

Application to plant tissue

For each test, about 500 mg. of the fresh or dried material were triturated with 5-10 ml. of the pH 7 buffer and 5 to 10 drops of PCAF. The appearance of a red-to-purple colour within 10 min. was recorded as a positive test.

Guanidinoxy reaction negative. No colour reactions were obtained with the seeds, leaves, or fruit of the following species:

Amaryllidaceae: *Alstroemeria chilensis*; Balsaminaceae: *Impatiens noli-me-tangere*; Boraginaceae: *Borago officinalis*, *Lithospermum arvense*, *Myosotis arvensis*; Celastraceae: *Euonymus latifolius*; Chenopodiaceae: *Chenopodium bonus henricus*; Compositae: *Carthamus lanatus*, *Dahlia variabilis*, *Lapsana communis*, *Tragopogon porrifolius*; Cruciferae: *Biscutella leiocarpa*, *Brassica oleracea*, *Bunias orientalis*, *Crambe maritima*; Gramineae: *Avena sativa*, *A. sterilis*, *Hordeum distichon*, *Triticum vulgare*; Labiatae: *Phlomis alpina*, *Melittis melissophyllum*, *Stachys germanica*; Leguminosae: *Desmodium tiliifolium*; Liliaceae: *Camassia esculenta*, *Scilla hispanica*; Onagraceae: *Eucharidium concinnum*; Papaveraceae: *Papaver rhoeas*; Primulaceae: *Cyclamen hederacifolium*; Ranunculaceae: *Clematis vitalba*; Resedaceae: *Reseda luteola*; Rosaceae: *Cotoneaster frigida*, *Geum rivale*; Saxifragaceae: *Tellima grandiflora*; Scrophulariaceae: *Scrophularia orientalis*; Solanaceae: *Browallia* sp., *Solanum dulcamara*; Umbelliferae: *Eryngium oliverarium*, *Perula communis*; Valerianaceae: *Valeriana* sp.

Guanidinoxy reaction positive:

Leguminosae: *Colutea arborescens*, *Medicago arborea*, *M. echinus*, *Ornithopus perpusillus*.

Colutea seed was found to give an intense colour with PCAF, and as the seeds could be obtained in sufficient quantity, they were submitted to the extraction procedure that had proved satisfactory in the preparation of canavanine from jack bean.

Isolation and characterization of canavanine from *Colutea arborescens*

The meal from 113 g. of finely ground seed was suspended in 400 ml. of distilled water and acidified to Congo red paper by dropwise addition of conc. H_2SO_4 . After remaining overnight at room temp., the pasty suspension was mixed with 1 vol. of water, stirred and filtered, to remove substances that interfered with the subsequent fractionation. The

residue was then extracted thoroughly with an equal volume of water and the filtrate, which gave a strongly positive PCAF test, was concentrated to about 200 ml. on a water bath. After filtration, the concentrate was mixed with 5 vol. ethanol and kept overnight at 5°. After decantation of the ethanol, the viscid residue adhering to the sides of the flask was rinsed out with a minimum amount (50 ml.) of cold water, and treated with an equal volume of cold 20% (w/v) aqueous flavianic acid. After keeping overnight at 5°, the crystalline flavianate which had formed was removed by filtration and recrystallized 4 times from hot water. It was then dissolved in 50 ml. hot water and treated drop wise with hot saturated aqueous $\text{Ba}(\text{OH})_2$, until no further separation of Ba salts could be observed and the mixture was alkaline (pH 9.5). After removal of the barium flavianate by filtration, the filtrate was acidified carefully to Congo red by addition of H_2SO_4 , to precipitate excess Ba^{2+} , and boiled with activated charcoal (British Drug Houses Ltd.), to remove traces of flavianic acid. The resulting colourless filtrate was concentrated to about 45 ml. on a water bath and, when cold, treated with 4 vol. of ethanol. A clear syrup separated on standing. After decantation of the supernatant layer, the residue was suspended in warm ethanol, to which water was added drop by drop, until, aided by warming and stirring, complete solution took place. The solution was allowed to cool slowly, and then transferred to a refrigerator. After keeping at 5° for 48 hr., a crop of colourless prismatic crystals had separated, and was transferred to a desiccator. Yield, 1.2 g. The crystals began to shrivel at 165° and melted with decomposition at 172°. Canavanine sulphate from jack bean (Kitagawa, 1937) had m.p. 172° (decomp.); (E.A.B.) 172° (decomp.). No depression of m.p. was observed when the *Colutea* compound was mixed with a specimen of canavanine sulphate from jack bean. (Found: C, 20.5; H, 5.4; N, 19.7. Calc. for $\text{C}_6\text{H}_{13}\text{O}_3\text{N}_4$, H_2SO_4 , H_2O : C, 20.5; H, 5.5; N, 19.2%.) Carboxyl-N (Van Slyke, MacFadyen & Hamilton, 1941), 4.56. Calc. for $\text{C}_6\text{H}_{13}\text{O}_3\text{N}_4$, H_2SO_4 , H_2O : 4.79%. The compound was very soluble in water, giving a solution acid to Congo red. $[\alpha]_D^{18.5}$, +18.62° in water (c, $\text{C}_6\text{H}_{13}\text{O}_3\text{N}_4$, H_2SO_4 , H_2O = 7.76% (w/v)). Calc. for $\text{C}_6\text{H}_{13}\text{O}_3\text{N}_4$, H_2SO_4 : +19.84°. Canavanine sulphate from jack bean (Kitagawa) $[\alpha]_D^{17}$, +19.41°).

Filter-paper chromatography (upward running on Whatman no. 4 paper at $18 \pm 1^\circ$) gave a single spot in phenol-water (R_F , 0.26) or in *n*-butanol-acetic acid-water (4:1:5, by vol.) (R_F , 0.13). The chromatograms were coloured by spraying with 0.4% ninhydrin in water-saturated *n*-butanol or with PCAF mixed with an equal vol. of pH 7 buffer or with 1% (w/v) aqueous sodium nitroprusside followed by exposure to daylight. Canavanine sulphate from jack bean behaved in the same way in all respects.

From these results it is concluded that the guanidinoxy compound present in *Colutea arborescens* is canavanine.

Colorimetric estimation of canavanine

To establish the working conditions, a curve showing the relationship between the optical density of the colour with PCAF and the concentration of canavanine was prepared by setting up a series of colorimeter tubes containing, respectively, 0.2, 0.4, 0.6, 0.8, 1.0, 1.4 and 2.0 ml. of canavanine standard. Each tube was treated with 0.5 ml. of PCAF and the volume made up to 10 ml. with pH 7 buffer. After mixing the contents, the tubes were exposed to diffused

daylight for 40 min. and then compared in an EEL photoelectric colorimeter with a blue-green '303' filter (Evans Electroselenium Ltd., Harlow, Essex). A solution containing 9.5 ml. of buffer and 0.5 ml. of reagent was used as the 'blank' control. The results are shown in Fig. 1.

Unlike the original nitroprusside reaction, the PCAF reaction is not highly photosensitive. With an active solution of the reagent, colour development can proceed in complete darkness and a difference of 10 min. either way during the period of exposure to daylight was found to have no appreciable effect on the colorimeter readings. The speed of colour development, however, does depend on the extent to which the reagent has undergone spontaneous oxidation during storage.

Procedure. To 1 ml. of the neutral solution for test, containing 0.1–1.0 mg. of canavanine, is added 0.5 ml. of PCAF; the solution is made up to 10 ml. with pH 7 buffer, shaking the mixture during the dilution. A working standard is prepared at the same time, using 1 ml. of the canavanine standard. After 40 min. exposure to daylight, the solutions are compared by means of a visual or photoelectric colorimeter with a blue or blue-green filter.

Results. Canavanine (g. free base/100 g. dry weight): bladder senna seed, 4.7; jack bean seed, 3.3; soya bean seed, 0.0.

Interference effects. (1) *Reducing agents.* The colours obtained with pure canavanine and PCAF or light-activated nitroprusside are relatively stable; the colours given by aqueous extracts of jack bean meal and PCAF tend to fade in a few hours, irrespective of whether the mixtures are kept in the light or the dark. The colour returns on addition of more of the reagent or an oxidizer, such as H_2O_2 or persulphate. The fading effect is attributed to the presence

Fig. 1. Relation between optical density of the pentacyanoammonioferrate complex and the canavanine concentration.

of agents capable of reducing the ferric component in the reagent. Ascorbic acid, hydroxymethylglyoxal (reductone) and cysteine can bring about fading of the canavanine colour, and if present in sufficient quantity can inhibit completely the colour test. These, and possibly other reducing factors, must be considered in testing biological material for guanidinoxy compounds; their effects can be overcome by pretreatment with aqueous iodine.

(2) *Competitors*. No guanidinoxy reaction could be observed with any animal secretion or tissue examined (milk, blood serum, cerebrospinal fluid, urine, liver, muscle, kidney), and low recovery values were obtained for canavanine added to these materials. Pretreatment with aqueous iodine had little if any effect on the low recovery, which suggested that it was not caused by destruction of the canavanine by arginase or inactivation of the reagent by reducing agents. Individual tests in presence of the chief urinary solutes showed that creatinine strongly inhibits the PCAF reactions with guanidinoxy compounds. This inhibition can be overcome by addition of an excess of the reagent, which indicates that the creatinine inhibition effect may arise from competition with the canavanine.

SUMMARY

1. Methods are described for the detection and estimation of guanidinoxy compounds, including canavanine, in biological material.

2. Canavanine has been isolated from the seeds of the bladder senna, *Colutea arborescens*.

3. A guanidinoxy compound, similar in its colour reactions to canavanine, has been detected in the seeds of *Medicago arborea*, *M. echinus*, and *Ornithopus perpusillus*.

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The Influence of Previous Diet on the Fasting Blood-Sugar Level and on Glucose Utilization in the Rat and Hamster

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Observations have been made from time to time on the effect of previous diet upon carbohydrate metabolism in animals. Samuels, Gilmore & Reinecke (1948) found that, after short periods of fasting, rats on a high-fat diet had lower blood-sugar levels than those on a high-carbohydrate diet. A reduced tolerance in man to orally or intravenously administered glucose (Sweeney, 1927; Himsworth, 1933) and depressed glucose utilization by isolated rat diaphragm (Lundbaek & Stevenson, 1948; Gilmore & Samuels, 1949; Hansen, Rutter & Samuels, 1951) has also been observed as a result of feeding a diet high in fat and low in carbohydrate. In these respects, fat-fed non-ruminant animals show a striking resemblance to the adult ruminant (Reid, 1950*a*, *b*; 1952).

The purpose of the present work was to confirm these findings in rats and hamsters with a view to reproducing the conditions obtaining in the adult

ruminant in non-ruminant animals by suitable modification of the diet. The effect of feeding various diets upon the fasting blood-sugar level, upon glucose tolerance, and upon glucose utilization and glycogen deposition in isolated diaphragm has therefore been examined. Studies on the hexokinase activity of various tissues were included in the hope that they would throw light upon the mechanisms involved.

While the work reported here was in progress, Long (1953) published his results on the hexokinase activity of tissues from rats fed from weaning on a variety of diets. Using homogenates of intestinal mucosa, highest activity was found in animals fed a high-carbohydrate, fat-free diet and lowest in those fed a high-fat, carbohydrate-free ration. As recorded below, similar results have been obtained using homogenates of rat and hamster kidney.