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In 1959 Kitagawa discovered and isolated the amino acid, canavanine. Since its discovery much interest has been centered on this amino acid because of its biological aspects; e. g. canavanine has been shown to inhibit the growth of bacteria, viruses, protozoa, and cancer cells.

The occurrence of canavanine is widespread in the leguminous plants and its identification and isolation for this reason has been restricted to these plants; of the leguminous plants, jack bean meal (Canavalia ensiformis) has been a readily available source.

The most notable of its properties is the characteristic ruby red color which canavanine forms with sodium nitroprusside. This color formation has been used for the identification of canavanine in natural products. An unfortunate reaction of canavanine is its deamination and cyclization to desaminocanavanine under neutral conditions. This conversion has been responsible for some of the loss

of canavanine during its isolation. A useful reaction which canavanine undergoes is the formation of an insoluble salt with flavianic acid. The formation of canavanine diflavianate is the usual method for the isolation of canavanine from crude extraction mixtures. However, various workers have experienced difficulty in the conversion of canavanine diflavianate to its free base and thus they have introduced various modifications in the procedure. The author in attempting to isolate canavanine from the crude extracts by the use of flavianic acid has also found the conversion of canavanine diflavianate to its free base (canavanine) difficult and tedious. It was found that in order to effect a complete conversion, a large excess of barium hydroxide must be added to an incipient boiling solution of canavanine diflavianate. Under these conditions a reliable isolation of canavanine was obtained.

Since the use of flavianic acid in the isolation of canavanine has proved to be difficult and tedious an ion exchange method which avoids the use of flavianic acid has been investigated. The results with this method indicated that a quantitative and practical isolation of canavanine directly from the crude extracts could be achieved. The use of a strongly acidic ion exchange resin in conjunction with a preferential eluting agent has been the key for the separation and isolation of canavanine as its free base from the impurities in the crude extract.

A METHOD FOR THE ISOLATION OF CANAVANINE BY ION EXCHANGE

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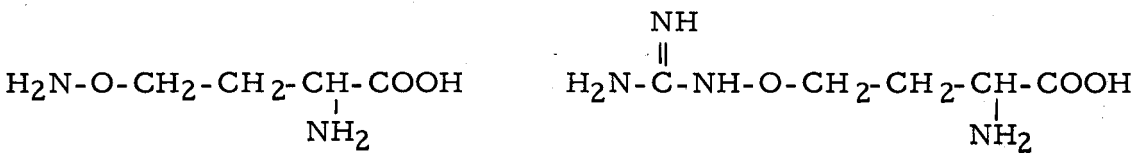
INTRODUCTION

In 1929 Kitagawa and co-workers (20) announced the discovery of a new amino acid to which they gave the name canavanine. This amino acid was isolated during the course of the preparation for the enzyme urease from the extracts of jack bean meal Canavalia ensiformis. From elemental analysis the empirical formula of canavanine was found to be $C_5H_{12}N_4O_3$. Treatment with the enzyme arginase (9, 21, 27, 28, 29) degraded canavanine into urea and another amino acid having a molecular formula $C_4H_{10}N_2O_3$. This latter amino acid was given the name canaline; its structure was determined in the following manner. Canaline was catalytically reduced whereupon a mole of homoserine (α-amino-γ-hydroxybutyric acid) and a mole of ammonia was formed (22). Canaline in turn was also reconverted into canavanine (25, p. 23-41). These results were surprising since this suggested that canaline was a α-amino-γ-aminooxybutyric acid; furthermore, that the structure of canavanine might be α-amino-γ-guanidinooxybutyric acid. That canavanine is a α-amino-γ-guanidinooxybutyric acid was established by its synthesis (25, p. 23-41) and also of canaline (24). Additional supports for this structure came from the direct degradation of canavanine to

α-amino-γ-bromobutyric acid with hydrobromic acid (14), and to homoserine and guanidine by bacterial cleavage (19), as well as hydrogenolysis (44, p. 233-245). Hence, the structure of canaline (I) and canavanine (II) can be written as shown in Figure 1.



(I) (II)

Figure 1

The rather remarkable feature of canavanine is that it appears to be the only amino acid occurring naturally that contains the guanidinooxy group.

The occurrence of canavanine is widespread in leguminous plants. Originally, its discoverer, Kitagawa was unable to find it in any other leguminous plants other than the jack bean (Canavalia ensiformis) where it occurs in the free state up to 2-3.5 percent of the dry weight of the plant and in Canavalia lineata (25, p. 23). However, subsequent work has shown that canavanine can be isolated in significant amounts from Canavalia obtusifolia (8) and from Colutea arborescens (11) seeds. To date canavanine has now been identified in the seeds of 27 other leguminous plants and isolated from two of them in significant quantities (11, 3, 4, 5, 36, 41). There has been a suggestion that the restriction of canavanine to leguminous plants

is due to the presence of nitrogen fixing bacteria in the plant roots which is responsible for its biosynthesis. This theory has been disproved (4).

Canavanine is very soluble in water, and insoluble in alcohol and ether. On crystallization from a water-ethanol mixture it forms colorless irregular prisms. Its optical rotation values are reported to be $[\alpha]_D^{17^\circ}$ + 8.1 and $[\alpha]_D^{20^\circ}$ + 7.9 in water (concentration 3.2 percent) (14; 25, p. 25). Canavanine and its degradation product canaline possess the L-configuration. In hydrochloric acid solutions the optical rotation is between +30 and +40 which lends further support for its L-configuration (2, 6). The melting points recorded for the free amino acid are 182-183°C dec. (25, p. 25) and 183-184°C (12).

Canavanine does not reduce Fehling's solution or ammoniacal silver nitrate. The aqueous solution of canavanine is alkaline to phenolphthalein (25, p. 25). Its isoelectric point has been reported to be 7.93 (42) and 8.2 (13, p. 2627). The fact that canavanine is a weaker base than arginine whose isoelectric point is 10.8 (10, p. 1649) indicates that the free base does not form a carbonate such as the other basic amino acids on exposure to air. Furthermore, canavanine is a weaker base than ammonia. The most reasonable explanation for the weaker basicity of canavanine compared to arginine stems from the substitution of the N-O linkage in canavanine for the N-C linkage in arginine in the guanidino portion of the molecule.

Canavanine forms a variety of salts; for example, the diflavianate, the sulfate, and the picrate (25, p. 26). The formation of the diflavianate salt by canavanine is one of the principal methods by which it is currently isolated from crude extraction mixtures. Canavanine diflavianate is soluble in water to the extent of 1:200 parts water, and melts at 210-215°C (dec.) with sintering at 190°C (25, p. 26) and 212°C (8).

Canavanine gives the following color reactions. It forms the typical purplish color with ninhydrin, characteristic of amino acids. Although canavanine possesses a guanidinooxy group, it does not give a positive Sakaguchi reaction which is characteristic for the guanidine group which occurs in arginine and, therefore, it may be distinguished from arginine. However, recent evidence indicates that in the determination of arginine by the Sakaguchi test in the presence of canavanine, the latter will interfere (35). This limitation can be circumvented by substituting sodium hypobromite for hypochlorite in the test. Occasionally, some canavanine preparations give a positive Sakaguchi reaction, a condition attributed to the presence of desaminocanavanine, a deamination product easily derived from canavanine (26, p. 373-385).

An impressive and characteristic color reaction of canavanine is the formation of a ruby red or magenta color with sodium nitroprusside (disodium pentacyanonitrosoferrate) (11; 25, p. 25). This

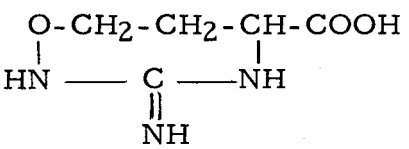
color reaction has been extensively used for the determination of canavanine in biological materials (3, 4, 5, 36, 41). The test is specific for the guanidinooxy group and is carried out in neutral aqueous solutions. Concentrations of canavanine as low as 1:10, 000 can be detected (25, p. 26). Under neutral conditions, the test is negative for the natural amino acids including arginine, cysteine, cystine, and methionine (11). A drawback to this test is the necessity of activating a fresh preparation of the nitroprusside reagent by sunlight for at least five minutes prior to its use. This objection may be overcome by employing an oxidizing agent such as peroxide in the activation process or by exposing the reagent to infrared light. The "activation" process appears to involve the formation of pentacyanoaquoferrate (III) $[Fe(CN)_5OH_2]^{=}$, the active chromogen, from nitroprusside (11).

Although canavanine resembles arginine in structure, its chemical stability toward acids and bases is not the same as those of arginine. Both arginine and canavanine are stable in acid solutions, but canavanine is less stable than arginine (25, p. 28).

In direct contrast, the opposite case is observed in basic solutions. Arginine is unstable in a basic solution, but canavanine is extremely stable (40). For example, when canavanine is boiled with five percent barium hydroxide for two hours, and even for seven hours, no alteration in canavanine due to basic hydrolysis occurs

(26, p. 378). The differences between arginine and canavanine again are a reflection of the difference in the guanidine portion of the molecule.

An interesting but usually undesired impurity which has been found to contaminate canavanine preparations is derived from canavanine in neutral aqueous solutions. Kitagawa who was able to remove this contaminant by boiling the canavanine preparations with ten percent hydrochloric acid described the impurity as desaminocanavanine (26, p. 373-385). Desaminocanavanine is formed from canavanine by the spontaneous loss of ammonia, and on the basis of this and further evidence, Kitagawa proposed the structure to be:



This structure has been confirmed by the catalytic hydrogenolysis of desaminocanavanine into N-amidinohomoserine (33).

Desaminocanavanine, as expected, does not give a positive ninhydrin reaction. Since it gives a positive Sakaguchi reaction (26, p. 380), this is the reason for some canavanine preparations to give the positive Sakaguchi test as mentioned previously. Desaminocanavanine, as well as canavanine, gives a positive nitroprusside reaction but at a slower rate and lesser sensitivity (1:500) (26, p. 379).

This is unfortunate, inasmuch as a mixture of canavanine and desaminocanavanine is, therefore, indistinguishable by this test.

An aqueous solution of desaminocanavanine is acidic (26, p. 380), and, obviously, from its structure the compound would be expected to be more acidic than canavanine. As a consequence, desaminocanavanine and canavanine are separable since the former is not precipitated by flavianic acid nor other basic amino acid precipitants (26, p. 380).

Since in the isolation of canavanine it is possible that desaminocanavanine can arise from the deamination of canavanine, a discussion of the conditions optimal for its formation certainly is warranted. Upon boiling canavanine with 90 percent alcohol for eight hours, 70 percent of the canavanine was converted to desaminocanavanine, while 76 percent was converted when boiled with water for seven hours (26, p. 375). The conversion of canavanine into desaminocanavanine occurs even at room temperature over a prolonged period. For example, at the end of 12 days at 28° 34 percent of the initial canavanine had become deaminated and cyclized to desaminocanavanine, while in 75 percent alcohol 16 percent of the canavanine had degraded in water. In general, the cyclization in alcohol is accelerated at low temperatures (26, p. 375).

The fact that canavanine is structurally analogous to arginine has led to some interesting speculations regarding the compound.

In most of the studies conducted, canavanine has been shown to be a growth inhibitor due to its competitive and antagonistic nature toward arginine. Thus, canavanine has been shown to inhibit the growth of some bacteria (18, 43), viruses (17, 38, 39), protozoa (44, p. 233-245), and cancer cells (32). Inhibition of growth in most cases stems from the interference with biosynthesis or utilization of arginine.

Specific interest in this compound stems from a study by K. S. Pilcher of the Department of Microbiology who observed the "Inhibition of Multiplication of Lee Influenza Virus by Canavanine" (39). Consequently, the need for canavanine for such studies has provided the impetus for this investigation.

At this point, a discussion of the methods of isolating canavanine is in order. All of the current methods utilize flavianic acid to separate canavanine from the crude extraction mixture.

Various modifications of the original Kitagawa procedure have been introduced to decompose the resulting canavanine diflavianate and to attain the purified free base.

According to the method of Kitagawa (25, p. 24), defatted jack bean meal is extracted three times with 50 percent ethyl alcohol, and the combined extracts are concentrated to a syrup. The syrup is poured into absolute ethanol to precipitate the crude canavanine. This precipitation from absolute ethanol is repeated twice. The precipitated crude canavanine is dissolved in water and acidified to congo

red end-point with sulfuric acid. Flavianic acid is then added to the acid solution, whereupon, canavanine diflavianate is formed. The canavanine diflavianate is recrystallized at least twice from water. Canavanine diflavianate is deflavianated by adding barium hydroxide and filtering the barium flavianate that is formed. Excess barium is removed with sulfuric acid. The small amount of flavianic acid that remains in solution is removed with charcoal. Excess barium or sulfate ions are quantitatively removed with dilute sulfuric or barium hydroxide solution. On concentration of the solution and addition of absolute alcohol crystallization of the free amino acid occurs slowly.

A criticism of Kitagawa's procedure is the lack of experimental details. His preparations were also occasionally contaminated with a substance which could be removed by boiling the crude canavanine solution with ten percent hydrochloric acid prior to the formation of the diflavianate.

Gulland and Morris (14) describe a procedure similar to Kitagawa's isolation of canavanine diflavianate. These workers also experienced difficulty in decomposing the diflavianate to obtain the free purified canavanine. They avoided this difficulty by converting the diflavianate to the ruffianate with ruffianic acid. Canavanine ruffianate in turn was decomposed to the free purified canavanine.

Gulland and Morris do not report a yield of the final isolated

product obtained by their method. From an inspection of the procedure and the fact that no yields are given, the practicality of their method in isolating canavanine is questionable.

Damodaran and Narayanan (8) describe a procedure by which canavanine is isolated from the seeds of Canavalia obtusifolia. Their method does not differ from that of Kitagawa's except in one respect. An impurity which had contaminated Kitagawa's preparations was removed in this procedure by the addition of basic lead acetate prior to the formation of canavanine diflavianate. This extra step led to a purer final product.

A method described by Cadden (6) should be mentioned despite the fact that his procedure is as vague as those of Kitagawa's.

Fearon and Bell (11), and Bell (3) were able to isolate canavanine from the seeds of Colutea arborescens and Anthyllis vulneraria, respectively. The former isolation is novel as to the method of extracting the crude extraction mixture. The initial extraction was carried out with only water which had been acidified with sulfuric acid. The aqueous acidic suspension of the seeds was allowed to stand overnight without any stirring! The procedure, otherwise, is the same as that of Kitagawa's.

The disadvantage of Fearon and Bell's procedure is the necessity for four or five recrystallizations of canavanine diflavianate. Of course since a different plant seed is used, this may require the

recrystallizations.

Konobu (31) recently was able to accomplish the isolation of canavanine from jack bean meal by the use of an ion exchange column. Basically, the procedure is the same as that of Kitagawa's. Briefly, the method involves the decomposition of canavanine diflavianate with barium hydroxide; removal of the barium flavianate that is formed; and adjustment of the pH to 4-5 with sulfuric acid. The solution of canavanine sulfate is then passed through a weakly basic anion exchange column (Amberlite IR-4B) which yields the free base in the effluent.

In the author's opinion, this procedure offers no distinct advantage over those methods previously discussed. It is the decomposition of canavanine diflavianate and separation of the freed canavanine from flavianic acid that is tedious and difficult. The method does avoid the necessity for the quantitative removal of barium and sulfate ions by titration with dilute sulfuric and barium hydroxide solutions when obtaining the free canavanine base.

Overall, Damodaran and Narayanan's method appeared to be the most feasible, but the author was unable to duplicate the results of these investigators.

Because of the difficulty of isolating canavanine from natural sources, attention has been given to the synthesis of this compound (12, 23, 24, 37). However, no practical synthesis has yet been

reported.

In view of the fact that a facile method for obtaining canavanine is not available, it was the purpose of this investigation to develop such a workable isolation of canavanine.

DISCUSSION PART I

The Isolation of Canavanine as the Diflavianate

The initial procedures used in this investigation were those of Damodaran and Narayanan (8). Since this laboratory had experienced much difficulty with this process and was unable to obtain the expected yields of canavanine, an investigation of the procedure was undertaken.

Except for the treatment with basic lead acetate in the original directions, the procedure of Damodaran and Narayanan adheres closely to the method of Kitagawa (25, p. 24). This procedure is as follows:

Defatted jack bean meal was extracted four times with 50 percent aqueous ethanol and the extracts were concentrated to a small volume. The concentrate was poured into absolute alcohol to precipitate the crude extract, and this precipitation was repeated. The precipitate was then dissolved in water and treated with a basic lead acetate solution until precipitation was complete. The precipitate was removed and the filtrate was acidified with sulfuric acid. After removal of the lead sulfate that had formed, the filtrate was acidified to pH 4 with sulfuric acid and then treated with excess flavianic acid. Canavanine diflavianate slowly crystallized and, after refrigeration overnight, the crystals were filtered. Canavanine diflavianate was

twice recrystallized from hot water.

Up to this point no difficulty was encountered in the duplication of Damodaran's work. For example, from extraction of one kilogram of jack bean meal a minimum of 40 grams of canavanine diflavianate which gave the correct analysis for nitrogen was obtained. On this basis nine to ten grams of canavanine, as the free base, should be isolated from one kilogram of bean meal if no losses were incurred in the subsequent conversion of canavanine diflavianate to free canavanine.

The melting points observed for canavanine diflavianate were perplexing. The experimental values that were observed generally ranged between 198-205°C compared to the reported vales of 210-215°C (25, p. 26). This discrepancy in melting points could not be readily explained since canavanine was the only basic amino acid that was present in the crude extract according to paper chromatographic analysis. It is known that flavianic acid precipitates only the basic amino acids such as arginine and lysine, neither of which was found to be present in the crude extract. Perhaps a small amount of canavanine monoflavianate is formed, a compound that is formed with a limited amount of flavianic acid.

From the foregoing discussion, the product that was isolated can be established as canavanine diflavianate, hence, no further elabration of Damodaran's procedure to this stage will be considered.

Proceeding with Damodaran's isolation of canavanine, the free base of canavanine was obtained by decomposing the diflavianate in the following manner. Canavanine diflavianate was dissolved in hot water and a saturated barium hydroxide solution was added until the mixture was strongly aklaline to litmus. After removal of the barium flavianate, excess barium ions were removed from the filtrate by the addition of sulfuric acid until the pH of the solution was 7. The gold-yellow solution containing only traces of flavianic acid was concentrated in vacuo and then treated with animal charcoal which removed the last traces of flavianic acid and left a colorless solution. The colorless solution was quantitatively freed of sulfate ions with dilute barium hydroxide and dilute sulfuric acid. Concentration of the solution was effected again in vacuo, and to the concentrate was added 95 percent ethanol. The mixture was kept cold, whereupon, canavanine crystallized slowly from solution.

It was in this stage of the procedure that the author was not able to reproduce Damodaran's work. Although a very small amount of canavanine was isolated, a second attempt yielded even less product. An inspection of the procedure discloses several sources for potential losses. If during the sequence of dissolving the canavanine diflavianate and adding the barium hydroxide the solution is not kept sufficiently hot, coprecipitation of canavanine diflavianate along with barium flavianate may occur. Furthermore, the decomposition of

canavanine diflavianate is an equilibrium process which requires that a sufficient amount of barium hydroxide be used. Thus, if enough barium hydroxide was not added not all of the canavanine diflavianate may have been decomposed.

For this reason, a number of runs using excess barium hydroxide was conducted by bringing the basicity of the solution (near incipient boiling) up to a pH of 11-12. Under these conditions, canavanine diflavianate yielded a minimum of 78 percent canavanine sulfate from canavanine diflavianate. Canavanine was isolated as the sulfate salt to facilitate the isolation procedure. However, in one run where canavanine was isolated as the free base, a 75 percent yield of canavanine was obtained from the diflavianate when the conditions mentioned above were followed. The possibility that some canavanine may precipitate as the insoluble barium canavanate was ruled out when the addition (to a pH 12) of excess barium hydroxide to a pure solution of canavanine gave no precipitate of barium canavanate.

Another problem that was encountered in the procedure was the removal of the last traces of flavianic acid that results from the decomposition of canavanine diflavianate. Even a trace of flavianic acid or barium flavianate intensely colored the solution and its removal was not accomplished with a small amount of charcoal. It was found, for instance, that as little as 10 mg. of flavianic acid in 200 ml. of water required at least two grams of charcoal at a pH of 2

to completely decolorize the solution. In a neutral solution, the same concentrations of flavianic acid was not removed even with four grams of charcoal. Furthermore, in two different experiments a solution of flavianic acid which underwent the same treatment as in the decomposition of canavanine diflavianate gave the same results, i. e. an acidic solution was decolorized and a neutral solution was not affected. Apparently, in strongly acidic solutions, the flavianic acid must be in the protonated form adsorbed by the charcoal, while in neutral solutions the acid exists as a non-adsorbable salt.

It was also observed that the use of a large amount of charcoal does not entail a significant loss in the amount of canavanine that is finally isolated. For example, when six grams of canavanine diflavianate was converted to canavanine during which two grams of animal charcoal was used to decolorize the solution, 1. 6 grams (75 percent) of canavanine sulfate was isolated.

In summarizing the investigation of Damodaran's procedure, the decomposition of canavanine diflavianate must be carried out with the addition of a large excess of barium hydroxide until the pH of the solution is at least 11-12, and this addition should be made to an incipient boiling solution of canavanine diflavianate.

DISCUSSION PART II

The Isolation of Canavanine with Ion Exchange

It is well established that many excellent separations and isolations of amino acids which otherwise would have been extremely difficult have been carried out with the aid of ion exchange resins (7, p. 255-298; 15; 34, p. 893-906). These works suggested that the isolation of canavanine could possibly be accomplished on a preparative scale directly from the crude extract with an ion exchange column. Since the conversion of canavanine diflavianate to free canavanine is tedious and difficult, the advantages of the ion exchange method are obvious. Furthermore, the ion exchange method may possibly provide a purer product due to isolation under milder conditions. For these reasons, an investigation was extended to a study of the direct isolation of canavanine from the crude extract with an ion exchange column and without any recourse to chemical treatments of precipitants.

Initially, an analysis of the crude extract was made. The analysis indicated that the crude extract was more complex than originally anticipated.

A Molisch and Anthrone test (30, p. 14; 16, p. 2) confirmed the presence of carbohydrates in the crude extract which was not unexpected in view of the aqueous ethanolic extractant used in the

procedure.

A Biuret test (30, p. 46) indicated the absence of any polypeptides or proteins which were expected to be present in the crude extract.

By paper chromatographic analysis in 80 percent phenol-water system, aspartic acid and glutamic acid in addition to canavanine were positively identified in the crude extract.

From estimates with paper chromatographic analysis, the relative amounts of the constituents in the crude extract were estimated to be 3 percent aspartic acid, 6 percent glutamic acid, 12 percent canavanine, and 79 percent neutrals (carbohydrates, organic acids, etc.). The validity of these estimates was supported by the data obtained in the isolation of canavanine as the diflavianate from the crude extract in earlier experiments. Later work on the separation of the amino acids from the crude extract with an ion exchange column also supported these estimates.

Knowing the constituents of the crude extract, preliminary experiments with both a weakly acidic (carboxylic acid) and weakly basic (polyamine) ion exchange resins were begun. The results indicated that impure canavanine was isolated. Due to these results attention was directed on the use of a strongly acidic (polystyrenesulfonic) ion exchange resin for the separation of the amino acids. The advantage of this resin is the complete separation of the amino acids

from the bulk of the neutrals in the crude extract. Then by preferential elution, the acidic amino acids may be separated from canavanine.

The problems of finding the proper conditions as well as an eluting agent which would preferentially elute the acidic amino acids but not the basic amino acids (canavanine) were investigated. For these studies a synthetic mixture consisting of aspartic acid, glutamic acid, and lysine (in place of canavanine) was used in substitution for the crude extract.

It was discovered that 0. 5N pyridine was sufficiently basic to preferentially elute the acidic amino acids and neutral amino acids such as valine but would not displace such basic amino acids as lysine. Furthermore, in a subsequent experiment with 0. 5N pyridine, it was found that pure canavanine was not eluted from the column. To elute the basic amino acids, including canavanine, 0. 5 N ammonia was found to be effective; canavanine obtained in this manner was isolated as the free base.

Having found the necessary conditions for achieving a separation and elution with synthetic mixtures, pilot isolations of canavanine from the crude extract were undertaken.

Accordingly, a solution of one gram of crude extract was added to a column containing the strongly acidic resin (Dowex 50W-X4). The column was washed thoroughly with water to remove the neutrals from the column. Half normal pyridine was next passed

through the column which eluted the acidic amino acids and then washed again with water to remove excess pyridine. Canavanine, isolated as the free base, was eluted from the column with 0.5N ammonia. The isolated canavanine was shown by paper chromatographic analysis to be homogeneous, and the product gave the correct carbon and hydrogen analysis. The yield obtained compared favorably with the estimates from paper chromatographic analysis and the estimate determined by isolating canavanine as the diflavianate. This result indicates that a quantitative isolation of canavanine was achieved.

In larger runs using 10 and 20 grams of crude extract, identical results were obtained; the yields of isolated canavanine were 1.25 grams and 2.57 grams, respectively, before recrystallization. A correct carbon, hydrogen, and nitrogen analysis was given by the canavanine. In order to remove a colored impurity, the isolated canavanine was recrystallized from aqueous alcohol after attempts to extract the impurity from the canavanine with absolute ethanol, 95 percent ethanol, and ether had failed. The yields of recrystallized canavanine were 0.8 gram and 1.5 grams respectively. It was observed that the yellow colored impurity was contained only in the canavanine fractions during the elution with ammonia. These results indicate a close similarity between the impurity and canavanine, or that the impurity is strongly adhered to canavanine.

In an attempt to recover more canavanine from the

recrystallization solution, a product that gave the correct analysis for desaminocanavanine was isolated instead. This behavior typifies the unstable nature of canavanine in aqueous or aqueous alcoholic solutions. In the author's opinion, canavanine is much more unstable than is implied in Kitagawa's work with desaminocanavanine (26, p. 373-385). It can not be overemphasized, that in working with a neutral solution of canavanine, there is always the possibility of some deamination and cyclization to desaminocanavanine.

Three reagents which were found to be very useful in the solution of these isolation problems were ninhydrin, the Sakaguchi reagent (30, p. 51), and the nitroprusside reagent (1, 3, 4, 11). These reagents reacted with canavanine, desaminocanavanine, and arginine in the following fashion: (see Table I)

TABLE I

	Ninhydrin	Sakaguchi	Nitroprusside
Canavanine	positive	negative	positive
Desaminocanavanine	negative	positive	positive
Arginine	positive	positive	negative

It can be seen from the table that the analysis for the presence or absence of arginine by the Sakaguchi test in the crude extract will lead to misleading results, if any desaminocanavanine is present.

However, the absence of arginine was shown by paper chromatographic analysis using a ninhydrin indicator.

EXPERIMENTAL PART I

Formation of Canavanine Diflavianate and its Conversion to Canavanine Sulfate and Canavanine

A. Extraction of Jack Bean Meal

One kilogram of jack bean meal (Canavalia ensiformis) that had been defatted with petroleum ether for several days was extracted with four liters of 50 percent aqueous ethanol (590 ml. of 95 percent ethanol to one liter with water) for two hours at a time. The extractions were carried out in a two gallon crock fitted with a stirrer. At the end of two hours of stirring the extract was decanted into a filtering bag. This extraction was carried out a total of four times. The extracts were concentrated separately in vacuo to approximately 400-500 ml. in a large scale evaporator. Each concentrated extract was kept cool until they were all combined and concentrated to a final volume of 350-450 ml. Generally, the evaporations were conducted at a temperature of 35-45°C and in no case should the temperature be allowed to rise above 50°C for any length of time.

The concentrated viscous gold-yellow liquid was dripped at a fast rate into five liters of ice-cold absolute ethanol that was vigorously stirred in a two gallon crock. The crude extract precipitated as a milky white material, and the extract was refrigerated overnight in the deep freeze. Next day, the alcohol was decanted, and the

precipitate filtered. The precipitate was dissolved in 300 ml. of water and again precipitated from alcohol as previously. After refrigeration overnight, the reprecipitated crude extract was filtered and dried in vacuo over P_2O_5. This drying process was repeated until the crude extract was bone dry. The yield of crude extract was approximately 65 grams. It is advisable to store the crude extract in the deep freeze until further use.

In order to determine the number of extractions necessary for this procedure, 500 grams of jack bean meal were extracted. The results of this experiment are as follows: The first three extracts contained a total of 26 grams of material; the fourth and fifth extracts contained ten and four grams, respectively; the total amount of crude extract was 40 grams.

B. Canavanine Diflavianate (8)

Forty grams of crude extract* from Part A was dissolved in 3 1/2 liters of water, and was treated with a saturated solution of basic lead acetate (lead subacetate) until precipitation was complete. The precipitate was centrifuged and washed with small quantities of warm water (save the filtrates).

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*In case the crude extract is not dried but is used immediately after the precipitation from alcohol, it is dissolved in 3 1/2 liters of water and treated with a saturated basic lead acetate (lead subacetate until precipitation is complete.

The combined filtrates were made acidic to congo red end point by the addition of dilute (0. 2N) sulfuric acid, filtered, and treated with excess of flavianic acid solution (200 grams/500 ml. of water) with vigorous stirring. A heavy orange-yellow precipitate consisting of microscopic needles appeared in a few minutes. The mixture was left in the refrigerator overnight, filtered, and the precipitate washed with ice-cold water. Canavanine diflavianate was recrystallized twice from 1 1/2 liters of hot water. M. p. 212°C after previous browning at 190°C. Kitagawa (25, p. 26) reports a m. p. of 210-215°C with semifluid at 190-192°C. The author found a m. p. of 198-201°C dec.

C. Canavanine Sulfate

To canavanine diflavianate (six grams) dissolved in 300 ml. of incipient boiling water was added with vigorous stirring a saturated solution of barium hydroxide. Enough base (approximately 65 ml.) was added to bring the pH of the solution to 12. After cooling the solution to room temperature (to decrease the solubility of barium flavianate), the barium flavianate was removed by filtration, then triturated three times with dilute barium hydroxide (30 ml. portions); the filtrates were all combined. Concentrated sulfuric acid was added to the combined solutions until the pH was 3. After removing the barium sulfate by centrifugation, the solution was decolorized

with about 2-2. 5 grams of animal charcoal, and then concentrated in vacuo to about 20-25 ml. ; 30-35 ml. of absolute ethanol was then added. The mixture was refrigerated overnight, whereupon, canavanine sulfate crystallized. If oiling out had occurred, the alcoholic solution was decanted and absolute ethanol added to the colorless oil to absorb the water. The yield of canavanine sulfate after drying over phosphorus pentoxide in vacuo was 1. 6 grams; m. p. 165-170°C. Note: canavanine sulfate is hygroscopic.

D. Canavanine

The colorless solution after treatment with animal charcoal (see Part C) was quantitatively freed of sulfate ions with 0. 1N barium hydroxide and 0. 1N sulfuric acid. After removal of barium sulfate, the solution was concentrated in vacuo and crystallized as previously described. The yield of free base of canavanine obtained from 4. 4 grams of canavanine diflavianate was 0. 72 gram; m. p. 175-178°C dec. (corr.).

EXPERIMENTAL PART II

The Isolation of Canvanine from the Crude Extract by Ion Exchange Methods

In order to determine the components of the crude extract, the material was subjected to a number of analytical tests.

A. Analytical Tests Applied to the Crude Extract

Molisch and Anthrone Tests (30, p. 14; 16, p. 2)

For the determination of carbohydrates in the crude extract, the Molisch and Anthrone tests were applied to a solution of the crude extract whose concentration was 0.25 percent w/v in water; both tests were positive for the presence of carbohydrates.

Biuret Test (30, p. 46)

The Biuret test was applied to a two percent and ten percent (w/v) solution of the crude extracts. The result was negative indicating the absence of both polypeptides and proteins.

Paper Chromatography

Preliminary studies using the solvent systems butanol:acetic acid:water 4:1:5 (v/v) and lutidine:collidine:water 1:1:1 (v/v) gave no separations of the amino acids. Resolution was achieved in 80 percent phenol-water (w/v) over one percent ammonia solvent system. This system was used in all of the paper chromatographic analyses.

A solution of the crude extract (40 mg. /ml.) and standards (5 mg. /ml.) of aspartic acid, glutamic acid, and arginine hydrochloride in ten percent isopropyl alcohol were spotted on Whatman #1 chromatography paper. The spots were applied twice for the standards and four times for the crude extract. The chromatogram was developed by the descending method at room temperature for a period of 24 hours in the phenol-water system. After drying overnight in the hood, the chromatogram was sprayed twice with 0. 2 percent ninhydrin (in water saturated butanol). The R_f's found were: aspartic acid 0. 16; glutamic acid 0. 26 canavanine 0. 64.

Experiments both by reinforcement of the crude extract with the standards and comparison with the standards indicated that the free amino acids in the crude extract were aspartic acid, glutamic acid, and canavanine. These tests proved the absence of arginine which had been indicated in earlier work.

Estimates by Paper Chromatography

Solutions of aspartic acid and glutamic acid (2. 5 mg. /ml.), and crude extract (40 mg. /ml.) in ten percent isopropyl alcohol were prepared. Twenty microliters of each solution was spotted on Whatman #1 chromatography paper. The chromatogram was developed in the same fashion as before in the phenol-water system. From calculations 50 micrograms of the acidic amino acids and 800 micrograms of the crude extract were applied. A visual examination of

the intensity of the ninhydrin spots indicated the composition of the crude extract to be 3 percent aspartic acid, 6 percent glutamic acid, 12 percent canavanine, and 79 percent neutrals (ninhydrin unreactive).

Estimation of Canavanine in the Crude Extract by Preparation of the Diflavianate

To two grams of crude extract dissolved in 100 ml. of water were added five grams of flavianic acid dissolved in 12.5 ml. of water with vigorous stirring. Crystals of canavanine diflavianate crystallized in five to ten minutes. The mixture was refrigerated overnight, and the diflavianate was filtered the next day. The diflavianate was recrystallized from approximately 25 ml. of hot water. The yield of canavanine diflavianate was 1.1 grams, m.p. 198-201°C dec. (corr.). Since canavanine diflavianate contains 22 percent free canavanine, 0.25 gram of canavanine was present in the crude extract. Therefore, canavanine is present to the extent of 12.5 percent of the dry weight of the crude extract. This compares quite favorably with the estimates made by the paper chromatographic method.

B. Determination of the Conditions for the Isolation of Canavanine from the Crude Extract with Synthetic Mixtures

In all of the experiments that follow, the strongly acidic ion exchange resin (Dowex 50W-X4; 50-100 mesh; hydrogen form) was always acid generated by washing first with four percent hydrochloric

acid and then with distilled water until the effluent was chloride free.

Elution of the Acidic Amino Acids

To a column containing Dowex 50 (four grams) was added a mixture consisting of 30 mg. each of aspartic acid and glutamic acid dissolved in ten ml. of water. The column was washed with 25 ml. of water, and the effluents checked with ninhydrin to insure that complete adsorption on the column had occurred. Elution of the amino acids was effected by adding 2N pyridine (redistilled, reagent) to the column. A quantitative elution was achieved after passing through 25 ml. of pyridine.

In later work with the actual isolation of canavanine from the crude extract, it was found that 0. 5N pyridine was sufficient for the elution of the acidic amino acids.

Elution of the Basic Amino Acids

To a column of Dowex 50 (four grams) was added a solution of 200 mg. of lysine hydrochloride dissolved in ten ml. of water. The column was washed with water until the effluent was free of chloride. To see if pyridine would elute the basic amino acids, 2N pyridine was added to the column in an attempt to elute lysine. After passing through 125 ml. of pyridine, no residue was found in the effluent upon its evaporation in vacuo.

After washing the column with water, 0. 5N ammonia was added which eluted the lysine. A quantitative elution of lysine was

obtained with 50 ml. of ammonia. To insure that canavanine was not eluted with 2N pyridine but was eluted with 0. 5N ammonia, an identical experiment was conducted with pure canavanine (100 mg. dissolved in ten ml. of water). Canavanine was not eluted on addition of 125 ml. of pyridine, but was eluted quantitatively with 50 ml. of 0. 5N ammonia.

Elution of the Neutral Amino Acids

A solution of valine (100 mg. in ten ml. of water) was added to a column of Dowex 50 (four grams). After washing the column with 50 ml. of water, 2N pyridine was added. A quantitative elution of valine was obtained with 25 ml. of pyridine.

C. Isolation of Canavanine from the Crude Extract

A solution of dry crude extract (one gram in ten ml. of water) was ultracentrifuged (10, 000 rpm for 20 minutes) to remove all suspended material. The clear, dark, yellow colored solution was added to a column of Dowex 50 (four grams) and passed through the column at a rate of approximately one ml. /min. As exchange on the column occurred a tan-brown band appeared (this band will turn to a darker brown color with time). The column was washed thoroughly with water (approximately 250 ml.) to remove the neutrals. The pH of the effluent wash drops to 3. 8 toward the end of the wash, and the effluent gives a weakly positive ninhydrin test due to some elution of

the acidic amino acids.

The acidic amino acids were eluted with 2N pyridine, the effluents being collected in five ml. fractions with the aid of a fraction collector. The movement of the colored bands can be observed during the progress of the elution with pyridine. The colored material was obtained in the fractions containing the acidic amino acids. The acidic amino acids were found in the first 20 ml. of effluent. After a total of 75 ml. of pyridine was collected, the column was washed with water (approximately 50 ml.) to remove excess pyridine. Canavanine was eluted next by using a total of 50 ml. of 0. 5N ammonia. Canavanine was concentrated in the first 25 ml. of the ammonia effluent.

To follow the progress of the elution, the acidic amino acids were determined in the pyridine fractions by spotting an aliquot on a strip of Whatman #1 chromatography paper and developing the spots with 0. 2 percent ninhydrin over a microburner. Canavanine was determined in the ammonia fractions by adding to a small aliquot (pH adjusted to near neutral with HCl) a drop of five percent sodium nitroprusside (disodium pentacyanonitrosoferrate) solution and then developing the color in sunlight for five to ten minutes. A ruby red color characteristic of canavanine results. In the absence of sunlight the color can be developed in infrared light but the coloration is much slower and less intense.

The fractions containing canavanine were combined and evaporated to dryness in vacuo at a water bath temperature of 35-40°C. The amount of canavanine isolated from one gram of crude dry extract was 0.128 gram, m.p. 178-180°C dec. (corr.).

A paper chromatographic analysis showed the isolated canavanine to be homogeneous. R_f 0.67 (80 percent phenol-water over one percent ammonia).

It is not necessary to collect fractions during the elution of the acidic amino acids with pyridine. Also, 0.5N pyridine was found to be sufficient for the elution of acidic amino acids in scaled up runs.

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