CV. SYNTHESIS OF THE *i*-HYDROXY- ASPARAGINES.

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A NOTE ON THEIR DISSOCIATION CONSTANTS.

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THE important part played by acid amides in the protein metabolism of the plant is too well known to require emphasis: these substances have long attracted the attention of plant chemists on account of the relative ease with which asparagine and glutamine—the only amides which have yet been found in natural products—can be isolated from plant extracts. It is perhaps less well known that the amount of these two amides which can be obtained in crystalline form from any given plant extract is always less than that which the “amide-nitrogen,” as determined by Sachsse’s method [1873], suggests should be present. Two explanations of this readily suggest themselves. In the first place the presence of other soluble plant products prevents the crystallisation of the total amount of asparagine or its copper salt. Where the amount of these other products is small, as in intensely etiolated lupin seedlings, Schulze [1880] obtained a yield of 90 % of crystalline asparagine; but in less intensely etiolated plants, or in the normal green plant wherein the other products predominate, the yield is always low and seldom exceeds 30 %. In the second place, it may well be that amides other than asparagine or glutamine are present in the plant, and that these have so far escaped detection because they possess different solubility properties from the other two amides. Schulze admitted this alternative, which appears the more probable from Vickery’s [1924] recent work on the water-soluble nitrogenous materials of green alfalfa plants. Vickery’s results suggest that the possible unknown amides were similar to asparagine and glutamine, *i.e.* they were monoamides of monoaminodicarboxylic acids. As on theoretical grounds it would seem possible that one of these might be a hydroxyasparagine, the synthesis of the *i*-hydroxyasparagines was undertaken so that future search for one of the possible new amides in plant products could be guided by a knowledge of the chemical and physical properties of these substances.

The synthesis presented in some ways the same difficulties which confronted

Piutti [1888] when he worked out the constitution and synthesis of the α - and β -asparagines. These arose chiefly from the fact that the action of hot alcoholic ammonia on the diethyl ester of aspartic acid did not give the expected diamide, but a ring product, "aspartimide," which was afterwards shown by Fischer to be the diamide of 2:5-diketopiperazine-3:6-diacetic acid. In the present instance it was found that the action of hot alcoholic ammonia on the diethyl ester of the *anti*-acid led to considerable decomposition, and no definite substance could be isolated from the resulting dark brown oil. The synthesis of the diamide was, however, readily accomplished by the method of Fischer and Königs [1904], who showed that the diethyl ester of *l*-aspartic acid and fluid ammonia at room temperature reacted to produce the diamide of *l*-aspartic acid (yield about 50 %) and "aspartimide." This diamide on standing with cold aqueous baryta gave a good yield of *l*- β -asparagine.

i-*para*- and *i*-*anti*-Hydroxyaspartic acids were synthesised from maleic acid without difficulty by Dakin's [1921] method and reacted with fluid ammonia to give the diamide in good yield, together with other products which probably correspond to "aspartimides." When the diamide was allowed to stand with cold aqueous baryta partial hydrolysis occurred, with the formation of both the α - and β -hydroxyasparagines.

The α - and β -hydroxyasparagines are readily differentiated by means of their copper salts. The β -amide (1) gives a normal copper salt containing one equivalent of the metal, whereas the α -amide (2), having the hydroxyl group contiguous to a carboxyl group, takes up two equivalents, indicating that the hydrogen of the hydroxyl has been replaced by metal. Similar abnormal salts are given by hydroxyaspartic acid itself, by *isoserine*, and, as is well known, by malic and tartaric acids. Confirmatory evidence of the position of the amide group in these two amides is given by the biuret reaction. Schiff [1900] showed that the colour developed depends on the number of carbon atoms separating the NH_2 -groups. The amide which the copper salt suggests is α -hydroxyasparagine gives a red-violet (oxamide type) colour, showing that the NH_2 -groups are attached to adjacent carbon atoms, while the β -hydroxyasparagine gives a blue-violet (malonamide type) colour, showing that the NH_2 -groups are connected through a chain of three carbon atoms. Similar colour reactions are given by α - and β -asparagine respectively.

The α -hydroxyasparagines crystallise with 1 H_2O in well-defined stars and long tapering needles, somewhat resembling glutamine. They are readily distinguishable from *l*- β -asparagine. The crystals of the β -hydroxyasparagines

however are anhydrous, and under the microscope exhibit rhombic hemihedral forms which are indistinguishable from those of *l*- β -asparagine. Unlike the latter they exhibit no tendency to form large crystals on slow evaporation of an aqueous extract, a difference which is still apparent when a mixture of the two is allowed slowly to crystallise.

With regard to the general properties of the hydroxyasparagines, the α - and β -forms, as mentioned above, are readily distinguishable by their differences in water of crystallisation, crystalline form, and copper content of the copper salts. They also exhibit different dissociation constants. No differences, however, have been noted—except perhaps slight ones in solubility—between the corresponding *anti*- and *para*-forms.

Both the α - and β -hydroxyasparagines reduce permanganate fairly readily in neutral solution, an observation which can be used to distinguish them readily from *l*- β -asparagine. They give no reaction with Fenton's reagent for tartaric acid, and their colour reactions with various phenols and sulphuric acid do not differ in any marked way from those given by *l*- β -asparagine. Like the latter, also, they have no sharp melting or decomposition point: when heated they slowly darken above 175° and decompose between 230° and 250°.

As to the possible presence of a hydroxyasparagine in plant products the following observations may be of use.

(1) With mercuric nitrate they give heavy white precipitates similar to that given by *l*- β -asparagine.

(2) The copper salts are practically insoluble in cold water, but are fairly readily soluble in boiling water in contradistinction to the copper salt of *l*- β -asparagine, which is soluble only with difficulty in boiling water. The copper salts of the hydroxyasparagines are soluble in excess of copper acetate. Boiling with excess of freshly precipitated cupric hydroxide or with copper carbonate is therefore recommended in any attempt to isolate these salts from plant extracts. Differences in the colours of these various salts which are noted below may prove, in practice, to be somewhat deceptive, as the exact shade of blue varies somewhat with the degree of fineness of the crystals.

(3) If one equivalent of aqueous baryta be added to a solution of any hydroxyasparagine and carbon dioxide passed, no precipitate of barium carbonate occurs, even on boiling, indicating the presence of either an abnormal barium salt or carbamate. Sufficient material has not been available further to investigate this interesting observation.

(4) The α -hydroxyasparagines crystallise so readily and have such a small solubility in cold water that it is improbable that they would have escaped detection had they been present to any extent in plant extracts. The β -hydroxyasparagines, however, are much more soluble in water, and have been found in certain cases to crystallise only with great difficulty in the presence of much foreign material. They might well be present in the syrupy mother liquors which are known to contain so much of the unaccounted "amide-nitrogen" of plant extracts.

(5) Plant chemists occasionally identify asparagine by its crystalline form and nitrogen content, and also glutamine by the copper content of its copper salt. It may not be out of place to call attention to the fact that for all practical purposes β -hydroxyasparagine ($C_4H_8O_4N_2$) and β -asparagine ($C_4H_8O_3N_2, H_2O$) have the same crystalline form and nitrogen content, and that the copper content of the copper salt of β -hydroxyasparagine is the same as that of the copper salt of glutamine.

EXPERIMENTAL.

i-Hydroxyaspartic acids. Dakin's method [1921] was followed in all essential details. Maleic acid was used as the starting material for the synthesis of bromomalic and chloromalic acids, which were then heated with strong ammonia at 100° for 10 hours to give a mixture of the *i*-hydroxyaspartic acids. These were separated by fractional crystallisation.

i-para-*Hydroxyaspartic acid diethyl ester.* 6 g. of finely powdered acid were suspended in 50 cc. of absolute alcohol and esterified in the usual way with hydrogen chloride. The ester hydrochlorides were decomposed with K_2CO_3 , the ethereal solution of the esters dried with anhydrous sodium sulphate and the ether removed in high vacuum at 30° . The ester was a pale yellow viscous oil. Yield 5.5 g. or 70 % of theory. No attempt was made to purify the product by distillation *in vacuo*, as Dakin states that this leads to considerable decomposition. (Found: C, 46.1; H, 7.2; N, 6.8. $C_8H_{15}O_5N$ requires C, 46.8; H, 7.3; N, 6.8 %.)

i-para-*Hydroxyaspartic acid diamide.* 6.3 g. of the diethyl ester were placed at the bottom of a thick-walled Carius tube, which was then cooled by solid carbon dioxide and acetone. Three to four volumes of fluid ammonia were then run in and the tube sealed. The tube was removed from the bath, allowed to attain room temperature and gently shaken to mix the ester and fluid ammonia. At the end of the first day small white crystals appeared in the liquid, and at the end of the fourth day, when the tube was opened, the walls of the tube in contact with the fluid were covered with a crystalline mat. The fluid ammonia was allowed to evaporate slowly. The resulting yellow crystalline mass was scraped out of the tube, powdered, extracted twice with 40 cc. of warm ethyl alcohol to remove unchanged ester and dried *in vacuo* over solid sodium hydroxide. Yield 4.2 g. Found: N, 27.1 %; $C_4H_9O_3N_3$ requires N, 28.6 %, so that products other than the diamide had been formed. These could not be separated by extraction with warm ethyl alcohol as in the case of the corresponding *anti*-preparations, for the diamide is itself somewhat soluble in this reagent. The mixture was very soluble in cold water, but on standing for some days a small amount of crystalline diamide separated. Recrystallised from a little hot water it was obtained in well-formed cubes and rectangular plates. (Found: C, 32.5; H, 6.1; N, 28.7. $C_4H_9O_3N_3$ requires C, 32.6; H, 6.1; N, 28.6 %.) Heated in an open capillary tube it darkens slowly above 170° , shrinks at 173° and melts with decomposition and evolution

of gas at 174° (corr.). The diamide reacts alkaline to litmus, and, as one would expect, gives an intense red-violet biuret reaction. It is freely soluble in warm water, and when pure is much less soluble in cold water. It is somewhat soluble in warm methyl and ethyl alcohols, only slightly soluble in the cold solvents or in acetone, and is insoluble in ether.

It gives a heavy white precipitate with mercuric nitrate, soluble in excess of nitric acid and a precipitate with mercuric chloride which dissolves on warming and reappears on cooling. It gives a small precipitate with phosphotungstic acid on standing, but none with chloroplatinic acid or with concentrated sulphuric acid. The aqueous solution on boiling slowly gives off ammonia. When 0.35 g. dissolved in 350 cc. of water was boiled gently in a flask about one-quarter of the total nitrogen was removed in about 16 hours. The solution slowly darkened and on allowing the residual liquid to evaporate slowly to a syrup in a desiccator a dark brown gum, similar to that given by the action of hot alcoholic ammonia on the ester, was obtained. The action of a mild alkali, such as baryta, results in the splitting off of one amide group, with the liberation of one-third of the total nitrogen as ammonia and the formation of a mixture of the two monoamides.

i-a-para-Hydroxyasparagine. 3.5 g. of the crude *para*-diamide were dissolved in a little water and placed in a Van Slyke apparatus for determining amide-N *in vacuo*. Water containing 6 g. of crystalline baryta was then added, and the total volume made up to 70 cc. At the end of 16 hours the ammonia set free was distilled off *in vacuo*, the residual liquor filtered to remove a small white deposit, which probably contained barium *para*-hydroxyaspartate, and barium removed exactly with sulphuric acid. The resulting solution was concentrated to 40 cc. *in vacuo* and allowed to stand overnight. 0.85 g. of crystalline material, consisting chiefly of tapering needles and complicated stars, was removed. On standing for a further 36 hours another 0.29 g. of similar material was obtained. The combined crystalline fractions were dissolved in 45 cc. of hot water, filtered from a small amount of insoluble residue and allowed to crystallise slowly. The resulting product, when viewed under the microscope, was seen to consist almost entirely of tapering prisms and stars, with only occasional plates of the β -amide. After two further crystallisations from water a product (0.8 g.) free from the β -amide was obtained. It crystallises from water in fine tapering prisms and stars of complicated form, and contains 1 H_2O which is only removed in high vacuum at 100° . (Found: C, 28.7; H, 5.9; N, 17.1; H_2O , 10.8. $\text{C}_4\text{H}_8\text{O}_4\text{N}_2$, H_2O requires C, 28.9; H, 6.0; N, 16.9; H_2O , 10.8 %.) It is freely soluble in boiling water, and crystallises readily from solution on cooling. At 18° it is soluble in about 125 parts of water. If a solution of the amide is first neutralised by the addition of one equivalent of sodium hydroxide and excess of a strong solution of copper acetate added, the solution turns a deep blue and becomes strongly acid to litmus, owing to the liberation of acetic acid and the formation of an abnormal salt containing two equivalents of copper. It crystallises readily in microscopic

aggregates of stars and needles containing 1 H_2O , which is not readily removed at 105° . (Found: H_2O , 8.0. $\text{Cu}(\text{C}_4\text{H}_6\text{O}_4\text{N}_2)$, H_2O requires H_2O , 7.9 %.) For analysis the material was dried *in vacuo* over P_2O_5 at 100° . (Found: C, 23.0; H, 3.1; N, 13.1; Cu, 29.6. $\text{Cu}(\text{C}_4\text{H}_6\text{O}_4\text{N}_2)$ requires C, 22.9; H, 2.8; N, 13.4; Cu, 30.4 %.)

The copper salt, which can be prepared equally well by boiling with freshly precipitated and well-washed cupric hydroxide, or by the addition of copper acetate without the previous addition of sodium hydroxide, is almost insoluble in cold water, but is moderately soluble in boiling water, from which it can be readily recrystallised. The blue colour of the salt is a little paler than that of crystalline copper sulphate and is easily distinguished from that of the corresponding β -hydroxyasparagine and also of *l*- β -asparagine.

i- β -para-Hydroxyasparagine. The mother liquor from the isolation of the corresponding α -monoamide mentioned above was allowed to evaporate slowly in a desiccator over sulphuric acid. When the volume had been reduced to less than 5 cc. crystals appeared, and after several days the syrupy residue had set nearly solid. It was filtered at the pump and washed with a little cold water. The white crystalline mass (0.9 g.) was dissolved in 20 cc. of warm water and allowed to evaporate slowly in a desiccator. The first two crops of crystals consisted chiefly of hexagonal plates, and on repeated recrystallisation from water there was finally obtained a fraction (0.4 g.) which appeared under the microscope to be uniform, consisting of simple and complicated rhombic plates and prisms, which are indistinguishable from the corresponding forms of *l*- β -asparagine. The β -monoamide contains no water of crystallisation. (Found: C, 32.2; H, 5.5; N, 18.6; amide-N [Sachsse, 1873], 9.3. $\text{C}_4\text{H}_8\text{O}_4\text{N}_2$ requires C, 32.4; H, 5.4; N, 18.9; amide-N, 9.4 %.) The β -monoamide dissolves in about 55 parts of cold water and is freely soluble in boiling water. In the presence of each other the α - and β -monoamides crystallise out readily in their respective forms, but in the presence of other substances their solubility, especially that of the β -monoamide, is greatly increased, and crystallisation is slow. The β -monoamide and *l*- β -asparagine crystallise readily in the presence of each other in crystals that appear uniform and may be mixed. The α -monoamide also crystallises readily in the presence of *l*- β -asparagine, but the two crystalline forms are distinct, and a tendency to form mixed crystals was not observed.

The copper salt is prepared in a similar way to that of the α -amide. It separates in small amorphous spheres, which, under high magnification, are seen to consist of star-shaped aggregates of stumpy needles. It contains one equivalent of copper, and 1 H_2O which is not completely removed in an oven at 105° . (Found: H_2O , 4.8. $\text{Cu}(\text{C}_4\text{H}_7\text{O}_4\text{N}_2)_2$, H_2O requires H_2O , 4.8 %.) For analysis the material was dried *in vacuo* at 100° over P_2O_5 . (Found: C, 26.8; H, 4.1; Cu, 17.8; N, 15.8. $\text{Cu}(\text{C}_4\text{H}_7\text{O}_4\text{N}_2)_2$ requires C, 26.8; H, 3.9; Cu, 17.8; N, 15.7 %.)

The synthesis of the amides of the *i*-anti-hydroxyaspartic acid was similar

to that described for the *para*-acid above, consequently only points of difference in the two series and necessary analyses are recorded.

i-anti-*Hydroxyaspartic acid diethyl ester*. This acid resembles glutamic acid in that a relatively large amount of alcohol is necessary to effect its initial solution. 6 g. of the finely pulverised acid were suspended in 60 cc. of absolute alcohol, and a rapid stream of dry hydrogen chloride was passed in until the alcohol boiled. The boiling was continued for 1 hour on the water-bath, but much acid still remained undissolved. The mixture was therefore cooled, and the dry gas again passed until the alcohol boiled. The boiling was continued for another hour, when complete solution of the acid was obtained. The ester hydrochloride, though fairly soluble in alcohol, is very insoluble in cold alcohol containing hydrogen chloride. Yield of ester, a pale yellow oil, 4.7 g. or 60 % of theory. It was not distilled. (Found: C, 46.4; H, 7.5; N, 6.6. $C_8H_{15}O_5N$ requires C, 46.8; H, 7.3; N, 6.8 %.)

i-anti-*Hydroxyaspartic acid diamide*. 4.5 g. of the ester were treated with fluid ammonia. The yield of crude diamide was 3.2 g. This was purified by extracting twice with 150 cc. of boiling absolute alcohol. The material (2.5 g.) contained 28.2 % N, and was therefore practically pure *anti*-acid diamide. A small amount of this was dissolved in hot water. On standing overnight it crystallised in small cubes and irregular oblong prisms. (Found: C, 32.7; H, 6.2; N, 28.6. $C_4H_5O_3N_3$ requires C, 32.6; H, 6.1; N, 28.6 %.) Heated in an open tube it commences to darken above 170°, shrinks at 176°, and melts with decomposition at 176.5° (corr.). It gave similar reactions to the *para*-diamide.

i- α -anti-*Hydroxyasparagine*. 2.1 g. of the diamide were dissolved in 70 cc. of water containing 4 g. of crystalline baryta and treated as in the case of the *para*-diamide. Barium was removed, the filtrate concentrated to 20 cc. and on standing overnight 0.8 g. of crystalline material separated. Recrystallised once from warm water it was obtained in blunted needles and prisms, free from the β -monoamide. It contains 1 H_2O which is not removed at 105°. (Found: H_2O , 10.8; amino-N, 8.4. $C_4H_8O_4N_2, H_2O$ requires H_2O , 10.8; amino-N, 8.4 %.) It dissolves in about 95 parts of cold water and is freely soluble in boiling water. The copper salt is prepared in a similar way to that of the *para*-amide, and has a similar appearance and solubilities. (Found: C, 20.8; H, 3.6; Cu, 28.3; H_2O , 8.0. $Cu(C_4H_6O_4N_2), H_2O$ requires C, 21.0; H, 3.5; Cu, 28.0; H_2O , 7.9 %.)

i- β -anti-*Hydroxyasparagine*. The mother liquor from the isolation of the α -monoamide described above was allowed to evaporate slowly in a desiccator. Crystallisation was slow, but after some days 0.47 g. had separated and was removed. Redissolved in 10 cc. of warm water it crystallised slowly in needles and stars of the α -monoamide and a few plates of the β -monoamide. After a tedious fractionation there was finally obtained 0.1 g. of material which consisted entirely of the rhombic plates characteristic of the β -monoamide. It contained no water of crystallisation. (Found: C, 32.8; H, 5.6; N, 18.0. $C_4H_8O_4N_2$ requires C, 32.4; H, 5.4; N, 18.9 %.) It is readily soluble in hot water,

and has about the same solubility in cold water as the corresponding α -mono-amide. The copper salt separates in nodular aggregates like those of the *para*- β -monoamide, containing one equivalent of copper and 1 H_2O . (Found: H_2O , 5.2. $\text{Cu}(\text{C}_4\text{H}_7\text{O}_4\text{N}_2)_2$, H_2O requires H_2O , 4.9 %.) For further analysis the material was dried in high vacuum over P_2O_5 . (Found: C, 27.0; H, 4.2; Cu, 18.0. $\text{Cu}(\text{C}_4\text{H}_7\text{O}_4\text{N}_2)_2$ requires C, 26.8; H, 3.9; Cu, 17.8 %.)

DISSOCIATION CONSTANTS.

Complete hydrogen electrode titrations of aspartic acid, hydroxyaspartic acid, *i*- β -asparagine, *i*- β -*para*-hydroxyasparagine and *i*- α -*anti*-hydroxyasparagine have been carried out. From the data the "titration constants" of each substance have been calculated. These values must be close approximations to the dissociation constants uncorrected for activity (p_K) and will be assumed to be identical with them. The mean values are assembled in Table I. Following Brønsted [1923], all constants have been formulated as hydrion dissociation constants. Consequently, the subscript numeral indicates only the order in which the several constants of a substance appear on the p_H scale. If we adopt the zwitterion hypothesis of Bjerrum [1923], assignment of the various constants may be made as follows:

- p_{K_1} , the first carboxyl of the dibasic acids and the carboxyl of the amides;
- p_{K_2} , the second carboxyl of the dibasic acids and the amino-group of the amides;
- p_{K_3} , the amino-group of the dibasic acids.

A number of determinations of the dissociation constants of aspartic acid have appeared in the literature. Some of these, recalculated to conform to the above treatment, are included in the table. Agreement is fair. We have found only one previous determination for β -asparagine. The method employed was that of measurement of the rates of ester hydrolysis. The values so obtained agree well with our results by the more precise electrometric method. The constants of the remaining substances have been determined for the first time. The assembled results form an interesting contribution to the problem of the effect of substitution upon ionisation. They do not, however, cover a sufficient number of cases to make profitable a detailed analysis from this point of view. Moreover, simple effects of substitution are undoubtedly complicated, in substances such as these, by powerful electrostatic forces between ionising groups. It is clear, however, that both the introduction of hydroxyl and the substitution of the hydroxyl group in carboxyl by amide increases the hydrion dissociation of both carboxyl and amino-groups. The magnitude of these effects is reduced to about one-third as the substituent is removed from the α - to the β -position. It is interesting to compare the influence of α -amide formation with that of peptide formation. Comparison with the constants of a typical amino-acid and its dipeptide indicates that these effects upon the amino-group are similar in sign and in magnitude.

Table I.

	COOH	COOH	NH ₂	Mol. conc.	Temp. ° C.	Author
	<i>pK₁'</i>	<i>pK₂'</i>	<i>pK₃'</i>			
Aspartic acid	1.90	—	9.47	—	25	Levene and Simms [1925]
	2.08	3.94	9.85	—	—	Hopfield <i>et al.</i> [1920]
	2.08	3.66	9.85	—	—	Harris [1924]
	2.05	3.76	9.85	0.02	25	Chibnall and Cannan
Hydroxyaspartic acid	1.95	3.47	9.03	0.02	25	Chibnall and Cannan
	<i>pK₁'</i>		<i>pK₂'</i>			
β -Asparagine	2.18	—	8.87	—	—	Lunden [1906]
	2.02	—	8.80	0.02	25	Chibnall and Cannan
β -Hydroxyasparagine	2.12	—	8.26	0.01	25	Chibnall and Cannan
α -Hydroxyasparagine	2.31	—	7.17	0.01	25	Chibnall and Cannan
Glycine	2.42	—	9.74	—	25]	Quoted by Kirk and Schmidt [1929]
Glycylglycine	3.15	—	8.10	—	25]	

EXPERIMENTAL.

The technique of titration followed that of Cannan and Knight [1927]. The temperature was accurately maintained at 25°. The reference electrode was a saturated calomel half-cell in electrical contact with the hydrogen electrode vessel through a saturated potassium chloride bridge. The standard of reference was 0.1 *M* hydrochloric acid which was assumed to have a hydrogen electrode potential of 0.0643 v. at 25°. The potentials of the liquid junctions, which were established in a reproducible fashion, were ignored. A pair of palladinised gold-plated platinum wires served as hydrogen electrodes. In no observation did they differ from one another by more than 0.2 mv. The calculation of "titration constants" followed the method of Simms [1926].

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