XV. THE ESTIMATION OF GLUTAMINE IN THE PRESENCE OF ASPARAGINE.

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IN connection with a research into the nitrogenous constituents of forage grasses which we are undertaking in this laboratory we have investigated the different methods used by plant chemists for determining the various groups of nitrogen present in the aqueous extract. It was necessary to find out whether the determination of amino-N by the Van Slyke method should be carried out (1) direct, (2) after removing ammonia and volatile bases by distillation with magnesia, or (3) after mild hydrolysis [Sachsse, 1873] to hydrolyse acid amides and subsequent removal of ammonia with magnesia. In most grass extracts very little difference was found between (1) and (2), but with other green leaves, as for instance, those of young sunflower or castor-oil plants there was a fall in amino-N after removal of ammonia which was difficult to account for. Ammonia itself gives only about $\frac{1}{3}$ of its N after ten minutes in the Van Slyke apparatus, and the fall observed was many times the total ammonia-N value. It seemed to us that the anomaly might be due to glutamine, and we accordingly investigated the behaviour of this substance under similar conditions.

We were surprised to find that glutamine gave nearly the whole of its N in the Van Slyke apparatus, that is, not only did the amino group react normally with nitrous acid, but the amide group also reacted. A search of the literature showed that this abnormal behaviour was noticed by Thierfelder and von Cramm [1919] and also by Schulze and Bosshard [1883], who showed that whereas asparagine gave half its N as "amido-N" by Sachsse and Kormann's [1875] method (based on the reaction between the modern "amino-groups" and nitrous acid), glutamine gave the whole of it. As far as we are aware these two observations have remained unnoticed by those plant chemists who have employed the Van Slyke method for determining amino-N.

It was shown by Bosshard [1883] that glutamine was much less stable than asparagine, and that considerable hydrolysis of the amide group occurred on boiling with magnesia. For this reason Schulze [1906] recommended that the

free ammonia-N of plant extracts should be determined by distillation with magnesia *in vacuo* at 40°. As the removal of ammonia in steam in the ordinary micro-Kjeldahl apparatus takes under 10 minutes we investigated the percentage breakdown of glutamine when distilled with magnesia at 100° in this apparatus. Ammonia representing about 27 % of the amide groups was obtained in 10 minutes. The residue from the distillation flask was withdrawn, made just acid with acetic acid, concentrated, and the amino-N determined by Van Slyke's method. To our surprise this had fallen to 28 % of the total N. It was clear that further hydrolysis of the amide group had occurred during the concentration in weak acid solution, but even so the conversion of the resulting glutamic acid into pyrrolidonecarboxylic acid—if that were the explanation of the large decrease in amino-N—had taken place much more rapidly than Foreman's [1914] researches on this transformation would suggest.

It seemed to us that the relative instability of glutamine compared with asparagine might be made the basis of a method for the separate determination of these two substances in plant extracts; we accordingly undertook a systematic investigation of the stability of glutamine and certain other substances at various p_H values, measuring both the amount of ammonia formed and the fall in amino-N under controlled conditions.

EXPERIMENTAL.

Estimation of ammonia- and amino-N.

The ordinary micro-Kjeldahl apparatus was used to determine ammonia in steam at 100°. For the determination *in vacuo* at 40° the micro-apparatus of Parnas and Heller [1924] was employed. The flask was made detachable to permit the withdrawal of the residual liquid after distillation with as little dilution as possible.

The procedure was as follows. 10 cc. of the solution or plant extract were washed into the flask, which was then connected to the apparatus. 3 cc. of a 10 % suspension of magnesia were then run in through the special burette provided for this purpose. The ammonia was removed by distilling for 15 minutes at a pressure of about 3 cm., the flask being immersed in a water-bath at 40°. A fine stream of air was drawn through the apparatus instead of the steam employed by Parnas and Heller, as this allowed a higher vacuum to be maintained without continuous watching and also concentrated the liquid in the distilling flask to a volume of about 5 cc. 8–10 cc. of distillate were collected. Control experiments with ammonium sulphate showed that 98 % of the ammonia passed over under these conditions. At the end of the operation the flask was detached from the apparatus and sufficient acetic acid was added to the residual liquid to dissolve the excess of magnesia. The solution was then carefully washed into a graduated 10 cc. flask and amino-N determined on 2 cc. aliquots in the Van Slyke micro-apparatus.

The relative stability of glutamine and asparagine.

The results of a series of experiments with 30 mg. samples of glutamine and asparagine are given in Table I. It will be seen that a reaction time of 10 minutes in the Van Slyke apparatus gives only 49.5 % of the total N of asparagine, but 92 % of that of glutamine.

Table I. *Showing the relative stability of asparagine and glutamine.*

(Values given are percentages of total N.)							
Substance	Whether magnesia used	Tempera- ture	Amide-N		Time of shaking in Van Slyke apparatus (mins.)	Amino-N	
			Time of distilla- tion (mins.)	Amide-N found		Found	Theory
Glutamine	—	—	—	Not determined	10	92	50
Glutamine	—	—	—	”	30*	94	50
Glutamine	—	—	—	”	150*	97.5	50
Asparagine	—	—	—	”	10	49.5	50
Glutamine	No	100°	5	8.9	10	78	50
Glutamine	”	40°	15	1	10	92	50
Glutamine	Yes	100°	10	13.5	10	74	50
Glutamine	”	40°	15	1	10	86	50
Asparagine	”	100°	10	1.4	10	50	50
Asparagine	”	40°	15	0.3	10	50	50
Urea	”	40°	15	0	—	10	No amino-N
Allantoin	”	40°	15	0	—	1.2	”

* Intermittent shaking.

It was shown by Plimmer [1925] that the amide groups of asparagine and of other amides such as acetamide and propionamide were stable to nitrous acid in acetic acid solution, but that they reacted completely in 24 hours in strong HCl solution. He suggested that in aqueous or acetic acid solution the amides had the formula $R.C(OH):NH$, which was converted into the tautomeric $R.CO.NH_2$ by mineral acid. They could then react with nitrous acid in virtue of the $-NH_2$ group. If this is so then glutamine appears to be the only amide possessing the $R.CO.NH_2$ structure in aqueous or acetic acid solution. When an aqueous solution of glutamine was boiled at 100° for 5 minutes 17.8 % of the amide-N was split off. This value was increased to 27 % in the presence of magnesia. In each case there was a corresponding fall in amino-N. Boiling an aqueous solution *in vacuo* at 40° for 15 minutes caused no decomposition and in the presence of magnesia there was only a small liberation of ammonia, equal to 2 % of the amide-N, and a fall in amino-N of 6 %, suggesting a small amount of anhydride formation. These results for amide-N confirm those of Schulze [1906]. In all subsequent experiments described in this paper amide-N was determined by distillation *in vacuo* with magnesia for 15 minutes, and in the case of glutamine the control value for amide-N was taken as 1 % and of the amino-N as 86 % of the total N.

We next proceeded to a more comprehensive series of experiments to

determine the stability at various p_H values. The procedure was as follows. 30 mg. samples of glutamine, asparagine and glutamic acid were placed in boiling-tubes and 10 cc. of the requisite buffer solution added. The tubes were then heated in a boiling water-bath for the required time, cooled and the contents transferred to the flask of the amide-N apparatus. Amide-N and amino-N were determined. The results are given in Table II.

It will be seen that asparagine is quite stable over the range p_H 4–8, and that the amide-N is only liberated to the extent of 7 % at p_H 2. Glutamine, however, is rapidly decomposed, and relatively large amounts of amide-N are given at p_H 3, 4, 5, 8, 9 and 10. Glutamic acid undergoes transformation to pyrrolidonecarboxylic acid at about the same rate as Foreman's [1914] results would suggest. The point of interest, however, is that the breakdown of glutamine cannot be due entirely to a simple hydrolysis to ammonia and glutamic acid, for the disappearance of the amino-N is greater than can be attributed to the transformation of this acid. It seems that two reactions are going on simultaneously. (1) The hydrolysis of the amide group to give ammonia and glutamic acid, the latter being transformed at once to pyrrolidonecarboxylic acid. (2) The glutamine, which is a γ -amide, is partially transformed into the anhydride, 2: 5-diketopiperazine-3: 6-dipropiondiamide, which is not so readily hydrolysed to give ammonia. This latter supposition is necessary to account for the extreme fall in the amino-N at p_H 6 and p_H 7, in which cases only small amounts of amide-N are found. The small amount of glutamine available was insufficient to permit further investigation into the chemistry of the reaction.

It was clear that the great difference in the stabilities of glutamine and asparagine at p_H 4 would enable us to estimate separately these two substances in aqueous solution. At a later stage of the research, when many data at this p_H had been accumulated, we found that urea, a possible constituent of many plant extracts, was also unstable at this p_H but very much less so at p_H 8 and the latter p_H was the one finally adopted for the estimation of glutamine in plant extracts. The small amount of glutamine available did not, however, permit us to repeat all our experiments at this p_H , so it is necessary to quote certain analyses carried out at p_H 4.

The rate of decomposition of glutamine when heated in aqueous solution at 100° (at both p_H 4 and 8) for periods of from 1 to 5 hours inclusive was next investigated; 3 hours seemed the most satisfactory period, and the data are given in Table III. It will be seen that at p_H 4 the value for amide-N is 44 % and for amino-N 5 % of the total N. The respective control values are 1 % and 86 %. The amide-N has therefore increased by 43 % and the amino-N has fallen by 81 % of the total N. Within the limits to which we shall use these figures there is no change for asparagine. If therefore a solution containing asparagine and glutamine is heated for 3 hours at p_H 4 there should be an increase of amide-N equivalent to 43 % and a decrease in amino-N equivalent to 81 % of the total glutamine-N present. The ratio decrease in amino-N/increase

in amide-N will be $\frac{81}{43} = 1.9$. The corresponding ratio at p_H 8 will be $\frac{86-8}{35.5-1} = 2.3$.

We redetermined the amount of ammonia yielded by asparagine and glutamine when hydrolysed by Sachsse's [1873] method using the strength of acid and time of hydrolysis recommended by Vickery and Pucher [1931] as best for plant extracts. 30 mg. samples were boiled with 2N H_2SO_4 for 4 hours, and amide-N and amino-N determined *in vacuo* as before. The results are given in Table III. The amide-N in each case is about 49 % of the total N, confirming the results of previous workers.

It will be readily seen that the results given by partial hydrolysis at p_H 4 and by complete hydrolysis with 2N H_2SO_4 enable the amounts of asparagine and glutamine present in any given extract to be calculated in the following way.

- (1) Asparagine amide-N + glutamine amide-N = increase of amide-N with 2N $H_2SO_4 \times \frac{50}{49}$.
- (2) Glutamine amide-N = increase of amide-N at p_H 4 $\times \frac{50}{43}$.
- (3) Asparagine amide-N = (1) - (2).

Analysis of a mixture of asparagine and glutamine.

Two solutions containing 120 mg. of glutamine and asparagine ($C_4H_8O_3N_2 \cdot H_2O$) respectively in 50 cc. of water were prepared. From them four other solutions were made, containing the following proportions by volume (1) 1 : 4, (2) 2 : 3, (3) 3 : 2, (4) 4 : 1. Amide-N and amino-N were determined on 6 cc. samples (a) under control conditions, (b) after heating with an equal volume of p_H 4 buffer at 100° for 3 hours, (c) after boiling with 2N H_2SO_4 for 4 hours.

The amounts of asparagine and glutamine amide-N were then calculated by means of the above equations. The agreement between the observed and actual values was excellent, as shown in Table IV.

The estimation of glutamine in plant extracts.

Before applying the above method to the estimation of asparagine and glutamine in plant extracts it was necessary to investigate certain other substances which under the appropriate conditions might give rise to amide-N or to loss of amino-N and, therefore, introduce errors.

Urea is known to be unstable in acid or alkaline solution, being converted to ammonium carbonate. Furthermore Plimmer [1925] showed that it yielded 5 % of its N as amino-N in half an hour by Van Slyke's method. Fosse [1928] and more recently Klein and Tauböck [1931] have produced evidence that this substance occurs frequently in plant extracts. We have therefore determined the stability of urea in aqueous solution when heated at 100° for three hours at various p_H values. The results are given in Tables II and III. It will be seen

Table II. *The decomposition of glutamine and other substances in aqueous solution at 100° at various p_H values.*

Substance	No. of hours hydro- lysis	p _H 2		p _H 3		p _H 4		p _H 5		p _H 6		p _H 7		p _H 8		p _H 9	
		Amide- N		Amide- N		Amide- N		Amide- N		Amide- N		Amide- N		Amide- N		Amide- N	
		Amino- N	Amide- N	Amino- N	Amide- N	Amino- N	Amide- N	Amino- N	Amide- N	Amino- N	Amide- N	Amino- N	Amide- N	Amino- N	Amide- N	Amino- N	Amide- N
Glutamine	1	—	—	—	—	33.5	28.1	—	—	—	—	—	—	—	—	—	—
Glutamine	2*	47.7	5.5	46.3	4.6	43.0	10.0	38.8	18.8	2.8	21.5	7.8	11.8	38.8	10.1	—	—
Glutamine	3	—	—	—	—	43.7	4.1	—	—	2.6	11.0	7.3	4.1	35.5	8.3	—	—
Glutamine	4	—	—	—	—	48.2	2.0	—	—	—	—	—	—	—	—	—	—
Glutamine	5	—	—	48.6	1.9	48.3	1.0	—	—	—	—	—	—	—	—	—	—
Asparagine	2	3.8	50.2	2.8	49.0	0.4	50.0	0.3	50.8	0.8	49.3	0.7	49.3	0.6	49.1	1.4	50.2
Asparagine	5	—	—	4.8	—	1.4	48.1	—	—	—	—	—	—	—	—	—	—
Glutamic acid	3	—	—	—	—	75.8	—	—	84.1	—	78.1	—	79.0	—	72.0	—	69.1
Urea	3	—	—	—	—	—	—	20.9	12.0	1.0	19.3	2.5	14.7	5.1	4.8	6.7	4.8
i-Hydroxyglutamic acid	3	—	—	—	—	34.4	5.8	—	63.5	—	82.0	—	80.9	—	57.4	—	—

* These values for glutamine were obtained by boiling the solution over a free flame instead of heating on a water-bath.

Table III. *Decomposition of glutamine and other substances under various conditions.*

Substance	MgO distillation	Control		After 3 hrs. at 100° at p _H 4		After 3 hrs. at 100° at p _H 8		Sachse hydrolysis			
		Amide-N		Amide-N		Amide-N		4 hrs. with 2N H ₂ SO ₄		2 hrs. with N H ₂ SO ₄	
		Amino-N	Amide-N	Amino-N	Amide-N	Amino-N	Amide-N	Amide-N	Amino-N	Amide-N	Amino-N
Glutamine	1.1	86.3	44.0	5.0	35.5	8.5	48.5	48.5	44.0	48.9	39.1
Asparagine	0.2	50.0	0.5	49.0	1.6	48.0	48.7	48.7	47.6	48.1	49.1
Glutamic acid	—	100.0	—	84.0	—	89.8	—	—	—	—	—
i-Hydroxyglutamic acid	—	—	0	63.0	0	57.4	—	—	—	—	—
i-Leucylglutamine	—	—	1.4	27.7	0.4	32.6	—	—	—	—	—
i-Hydroxyaspartic acid	—	—	0	99.0	0	100.0	—	—	—	—	—
i-α-Hydroxyasparagine	—	—	2.0	44.0	1.9	47.5	—	—	—	—	—
Urea	0	10.0	34.4	5.8	5.1	4.8	37.3	37.0	41.0	33.3	38.9
Allantoin	0	1.2	2.1	0.5	3.9	2.2	22.4	22.4	5.2	3.9	4.2

Table IV. Analysis of a mixture of asparagine and glutamine.

Mixture number	Glutamine amide-N taken mg.	Asparagine amide-N taken mg.	Control				After 3 hrs. at 100° at pH 4				Ratio		Sachse hydrolysis with NH_4SO_4 for 1 hr. amide-N mg.	Glutamine amide-N found mg.	Asparagine amide-N found mg.
			Amide-N mg.	Amino-N mg.	MgO distillation Amide-N mg.	Amino-N mg.	Inc. in amide-N mg.	Amide-N mg.	Amino-N mg.	Dec. in amide-N mg.	Dec. in amide-N	Inc. in amide-N			
1	0.28	1.07	0.01	1.53	0.01	1.77	0.23	0.24	1.12	0.41	1.8	1.8	1.30	0.28	1.05
2	0.55	0.80	0.01	1.77	0.01	1.77	0.46	0.47	0.90	0.87	1.9	1.9	1.30	0.54	0.79
3	0.83	0.53	0.02	1.97	0.02	1.97	0.70	0.72	0.65	1.32	1.9	1.9	1.30	0.81	0.52
4	1.10	0.27	0.02	2.14	0.02	2.14	0.93	0.95	0.35	1.79	1.9	1.9	1.31	1.08	0.25

Table V. Glutamine and asparagine contents of various plants.

Plant	Fresh wt. g.	Aqueous extract total N g.	Control				At pH 8				With NH_4SO_4				Dec. in amino-N				Glutamine amide-N				% of total water-soluble N			
			Amide-N g.	Amino-N g.	Amide-N g.	Amino-N g.	Amide-N g.	Amino-N g.	Amide-N g.	Amino-N g.	Amide-N g.	Amino-N g.	Amide-N g.	Amino-N g.	Dec. in amide-N at pH 8	Inc. in amide-N	Dec. in amide-N with NH_4SO_4	Glutamine amide-N	Amide-N	Glutamine amide-N	Asparagine amide-N	Amide-N	Glutamine amide-N	Asparagine amide-N	Amide-N	Glutamine amide-N
Seedlings of <i>Ricinus communis</i> (grown in light)	185	0.413	0.007	0.105	0.022	0.066	0.396	0.092	0.031	0.009	0.009	0.009	0.009	0.009	2.0	1.8	1.8	1.8	1.8	7.5	2.1	1.8	7.5	2.1	1.8	7.5
Seedlings of <i>Ricinus communis</i> (etiolated)	372	0.914	0.055	0.399	0.107	0.128	0.199	0.302	0.157	0.041	0.041	0.041	0.041	0.041	2.5	1.6	1.6	1.6	6.0	17.2	4.5	6.0	17.2	4.5	6.0	17.2
Seedlings of <i>Brassica oleracea</i> (grown in light)	500	0.859	0.041	0.149	0.007	0.134	0.055	0.167	0.009	0.046	0.046	0.046	0.046	0.046	2.0	*	*	*	5.0	(1.0)*	5.4	5.0	(1.0)*	5.4	5.0	(1.0)*
Seedlings of <i>Helianthus annuus</i> (etiolated)	56	0.148	0.001	0.049	0.005	0.033	0.016	0.040	0.007	0.009	0.009	0.009	0.009	0.009	3.0	0.8	0.8	0.8	0.7	6.1	7.5	0.7	6.1	7.5	0.7	6.1
Roots of <i>Apium graveolens</i>	1200	0.696	0.032	0.243	0.060	0.130	0.130	0.188	0.084	0.046	0.046	0.046	0.046	0.046	1.9	1.5	1.5	1.5	4.6	12.1	6.6	4.6	12.1	6.6	4.6	12.1
Seedlings of <i>Vicia faba</i> (grown in light)	635	2.461	0.010	1.324	0.042†	1.240†	0.653	1.305	0.048	0.605	0.605	0.605	0.605	0.605	2.0	2.4	2.4	2.4	0.4	(2.0)†	24.6	0.4	(2.0)†	24.6	0.4	(2.0)†

* There is an increase in amino-N with NH_4SO_4 , the small glutamine value is therefore rejected.

† Glutamine determined at pH 4 instead of pH 8 (see text).

that at p_H 4 34 % of the urea is converted into ammonia and at p_H 8 only 5 %; for this reason we recommend the latter p_H when dealing with plant extracts.

Urea gives no appreciable "decrease in amino-N" at p_H 4 or 8. Consequently if it is present to any extent in plant extracts the ratio "decrease in amino-N/increase in amide-N" will be much less than 2.3.

Glutamic acid and hydroxyglutamic acid both show a decrease in amino-N at p_H 8. Since they yield no amide-N they will, if present to any extent in plant extracts, give an enhanced value for the above ratio.

Schulze [1885] showed that allantoin gave about 5 % of its N as ammonia-N on hydrolysis with N H_2SO_4 for two hours and pointed out that it was a possible source of error in the determination of the total amide-N of plant extracts. We find that it gives 4 % of ammonia-N at p_H 8 and that it gives 22 % after hydrolysis with $2N$ H_2SO_4 for 4 hours. The possible error in the determination of total amide-N in this way is considerable and we therefore recommend the milder hydrolysis employed by Schulze when dealing with plant extracts. Data are given in Table III. We have not had an opportunity of investigating the behaviour of other ureides of the type which Klein and Tauböck [1931] suggest are present in plant extracts, but they will not be less stable at p_H 8 than urea itself.

Thierfelder and von Cramm [1919] showed that *l*-leucylglutamine and other synthetic peptides containing glutamine were much more stable towards acid hydrolysis than glutamine itself. As shown in Table III we find that at p_H 8 only 0.8 % of the amide-N is liberated, so that errors due to peptides containing glutamine will be small. Hydroxyaspartic acid and α -hydroxyasparagine [Chibnall and Cannan, 1930] are also stable at p_H 8.

It will be seen that urea and ureides are the only substances we can suggest which will give an increase of amide-N at p_H 8 and glutamic and hydroxyglutamic acids the only substances which will give a decrease in amino-N at the same p_H . It is possible for these substances to be present in the same plant extract in such proportion that the ratio "decrease in amino-N/increase of amide-N" at p_H 8 is about 2.3. The error in the determination of glutamine would then be considerable.

None of these substances gives a decrease in amino-N after hydrolysis with N H_2SO_4 , whereas glutamine, as shown in Table III, gives a decrease of about 50 % of the total N. We believe this large decrease in amino-N on acid hydrolysis to be a property unique to glutamine, but we have not made use of it in calculating the amount of the substance present in a plant extract because of possible errors introduced by the hydrolysis of peptides. The ratio "glutamine amide-N/decrease in amino-N with N H_2SO_4 " is about 1, and if determined should effectively expose any significant error due to the combination of the four substances mentioned above.

Having thus explored the field for possible sources of error, and bearing in mind the limit of accuracy with which the amino-N of a plant extract can be

determined by Van Slyke's method, we recommend that the glutamine and asparagine content of a plant extract be determined in the following way.

(1) Glutamine amide-N + asparagine amide-N = increase in amide-N with $N H_2SO_4$.

(2) Glutamine amide-N = increase in amide-N at $p_H 8 \times 1.4$.

(3) Asparagine amide-N = (1) - (2).

(4) The ratio "decrease in amino-N/increase in amide-N" at $p_H 8$ should be about 2.3.

(5) The ratio "glutamine amide-N/decrease in amino-N with $N H_2SO_4$ " should be about 1. It will be raised if peptide splitting occurs during the hydrolysis.

(6) The presence of urea can be detected by an increase in the value of (2) if determined at $p_H 4$.

(7) The small error due to allantoin falls almost entirely on the value for asparagine amide-N.

The amounts of glutamine and asparagine in various plants.

We have determined the amide content of several plants by the above method and the results are given in Table V. The aqueous extract of the fresh plant material was prepared by the method of Chibnall [1922], except that a small mincing machine was used instead of an end-runner mill, and the final residue of cell-wall material was not extracted with boiling water. The protein in the extract was coagulated by heating as quickly as possible with steam, and removed by filtration. The clear filtrate was then rapidly cooled to 40° and concentrated to the required volume *in vacuo*. It is necessary to carry out the analyses without delay, as glutamine is unstable in aqueous solution; a test sample after standing 14 days at $15-20^\circ$ gave only 64 % of the total N as amino-N. On account of the high buffering capacity of plant extracts it was found advantageous to titrate a sample with 0.1 *N* sodium hydroxide either electrometrically to $p_H 8$ or to a faint pink with phenolphthalein.

Ricinus communis (*castor-oil plant*). The 21-day etiolated seedlings contained a high proportion of amide-N, the major part of which was due to glutamine. Schulze [1897] obtained considerable amounts of glutamine, but was unable to isolate any asparagine.

As was expected, the seedlings grown in the light contained less total amide-N, but the major part was again due to glutamine. It is interesting to note that Green [1890] isolated a small amount of asparagine from young embryos germinating in the light.

Brassica oleracea var. bullata (*Brussels sprout*). The young seedlings grown in light did not contain much amide-N, and the major part of this was due to asparagine. Although the ratio "decrease in amino-N/increase in amide-N" at $p_H 8$ is 2, the small value (1.0 %) for glutamine amide-N is rejected because there is an increase in amino-N with $N H_2SO_4$ instead of the decrease which

should prove the presence of glutamine. As was pointed out in the earlier discussion, if the glutamine content is small the "decrease in amino-N with $N H_2SO_4$ " may be masked by peptide splitting. It is probable that this has occurred in the present case, because Schulze [1897] was able to isolate asparagine, and with great difficulty a very small amount of glutamine from *B. oleracea* var. *gongylodes*.

Helianthus annuus (*sunflower*). Schulze's pupil Frankfurt [1893] was able to isolate asparagine and glutamine from etiolated seedlings. Our results confirm the presence of both amides.

Apium graveolens (*celery*). Schulze [1897] isolated asparagine and glutamine from the roots. Again our results confirm the presence of both amides.

Vicia Faba (*broad bean*). Schulze, in a series of papers, showed that seedlings, especially etiolated seedlings, of the Leguminosae accumulate large quantities of asparagine, but he was never able to isolate glutamine. Our results for the seedlings of the broad bean grown in daylight show the presence of large amounts of asparagine. The increase in amide-N at p_H 4 (this experiment was made before the procedure was changed to p_H 8) is very small and is of the same order as that which would be given by the asparagine itself. The observed value for glutamine amide-N is therefore considered to be within the experimental error of the method and is rejected.

It will be seen from the above results that the micro-method described in this paper, which requires only 1-3 g. of fresh plant material, estimates to within an error of about 1 in 10 the amount of glutamine in the aqueous extract. Furthermore instability of this substance in aqueous solution, which has been clearly demonstrated, offers a ready explanation of the low yields, compared with that suggested by Sachsse-hydrolysis, of crystalline glutamine which Schulze always obtained from plant extracts.

SUMMARY.

Attention is called to the fact, previously discovered by Schulze and Thierfelder, that glutamine reacts abnormally with nitrous acid. It gives 92 % of its total N as amino-N in the Van Slyke apparatus.

The stability of glutamine in solution at 100° at various p_H values has been investigated by determining both amide-N and amino-N.

After heating at 100° at p_H 8 for 3 hours glutamine gives 35 % of the total N as amide-N, while the amino-N falls to 8.5 %. Asparagine is stable under these conditions.

This difference in behaviour has been made the basis of a method for the separate determination of asparagine and glutamine in plant extracts.

The method has been applied with success to a mixture of asparagine and glutamine, and also to 6 plant extracts, the amide contents of which were known from the previous work of Schulze.

In conclusion we should like to thank Prof. Winterstein for the gift of 2 g. of glutamine, Prof. Knoop for a sample of *l*-leucylglutamine synthesised by the late Prof. Thierfelder and Prof. Harington for a sample of synthetic *i*-hydroxyglutamic acid.

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