VIII. INVESTIGATIONS ON THE NITROGENOUS METABOLISM OF THE HIGHER PLANTS.

PART I.

THE ISOLATION OF PROTEINS FROM LEAVES

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IN spite of the importance of this subject, very little is known up to the present about the nitrogenous metabolism of the higher plants. This is due, to a great extent, to the extraordinary difficulty in the chemical technique. A large number of metabolic processes are continually taking place within the leaves, and many different substances must be present at the same time, for the separation of which no well-defined chemical methods have been evolved. The first object of these investigations was the elaboration of methods by means of which the chief nitrogenous constituents of the leaves could be isolated and identified.

The work was commenced in 1916 by one of the present authors (S. B. S.) in collaboration with Miss Laura M. Saunders, but had to be abandoned after a few months work owing to the pressure of other duties. Certain work of a preliminary nature was however brought to a conclusion, and the results obtained by Miss Saunders were utilised when the work was resumed by the present authors at the beginning of 1920.

The first subjects chosen for investigation were cabbages, not because they are ideal for the purpose, but because they could be readily obtained in quantity in London almost throughout the year. The experiments were directed, in the first instance, towards the discovery of a method by means of which the maximum amount of nitrogenous material could be removed from the leaves in the form either of true or colloidal solution. They were treated with water containing various cytolytic agents, and the effect of each was quantitatively investigated. An account of the results obtained is given in the following [see Chibnall and Schryver, 1920]. The most satisfactory agent was ether, and water saturated with ether was found to remove not far short of twice the amount of nitrogen which was dissolved out by water alone.

By treating minced leaves for a short period with water saturated with ether (five parts of leaves to four parts of the ether-water) and then subjecting the mixture enveloped in filter cloth to pressure in a tincture press, a green opalescent fluid was obtained, from which no particles separated on pouring through a folded filter-paper. This fluid however deposited a flocculent precipitate on keeping, very slowly in cold weather, but more readily in summer. On warming gently (to about 40°) a dense flocculent precipitate was formed almost immediately, which was found to consist chiefly of complex nitrogenous compounds. A method was hereby revealed for preparing in mass the proteins of the leaves, which has not been hitherto described. Only when a relatively large quantity of material was in hand appeared a paper [Osborne and Wakeman, 1920], in which a method of preparing proteins from spinach was described, and this must therefore be regarded as the first record of the preparation in mass of the proteins of the leaves. The method employed differs in certain important particulars from that described by the authors of the present paper, who, by applying their process to spinach, have obtained results not altogether in agreement with those of the American authors—the most notable difference being that of the nitrogen content of the products isolated.

The results obtained with cabbage leaves pointed to the possibility of obtaining a general method for isolating in mass a part, at any rate, of the proteins of leaves, and the more detailed examination of the simpler nitrogenous substances in the cabbage was relinquished for the moment, in order to ascertain whether the method for preparing proteins, as outlined above, was of general application to all leaves. In the meantime the investigation of the simpler nitrogenous products of the leaves has been undertaken by Mr H. W. Buston and Mr A. E. D. Burdett, some of whose results are utilised in the present paper.

The method of extracting the proteins by means of ether-water has been applied, in addition to the cabbage, to the leaves of Virginia creeper, grass, scarlet runners and spinach.

In the case of Virginia creeper, the leaves of which were collected just as they were about to change colour, and when active metabolism was apparently finished, no appreciable amount of protein was obtained. From the grass, proteins were separated, but they were obtained only in small quantities, and considerably contaminated with starch. From the leaves of the scarlet runner and spinach, on the other hand, proteins were obtained in fairly considerable quantities. Samples were prepared from two pickings of the former leaves, the earlier when the pods were in active process of formation, and the latter when they were fully formed, and when the general metabolism of the plant was on the down grade. The leaves of the earlier picking yielded a fair quantity of protein, the ether-water extract of the plant being converted into a semi-solid mass on warming, whereas those of the later picking gave a very much smaller yield. These results indicate that the proteins,

which can be isolated by means of ether-water, are obtained in the largest quantity when the plant is in an active state of metabolism, and the amount is likely to have some considerable physiological significance, and to represent the proteins which are in process of active translocation.

By means of ether-water a little less than two-thirds of the total nitrogen in the leaves has been removed. An examination of the residual nitrogen has not yet been made, but it forms part of the programme of subsequent research.

As already indicated, the main object of the present communication has been to give a general account of the method for isolating the proteins of plants, and none of the products obtained has as yet been subjected to exhaustive examination. It is not possible therefore at the present moment to give anything more than a very superficial account of their properties. Work is, however, proceeding on the 150 g. of the crude cabbage product prepared earlier in the year.

The proteins isolated appear to have a nitrogen content in the neighbourhood of 13 %, and that isolated from the cabbage has distinct acidic properties, dissolving in weak alkalis, and being precipitable from this solution by acids; this precipitate does not redissolve on addition of excess of acid.

When the general character of the proteins is considered, it will be seen that theoretically there can exist either distinctly alkaline or distinctly acid products, in addition to those that have the iso-electric point only slightly on either side of neutrality. The alkaline proteins should yield on hydrolysis preponderating amounts of di-amino-acids. The protamines, isolated from fish sperm, belong to this class. Acid proteins on the other hand should yield on hydrolysis large amounts of dicarboxylic acids (glutamic and aspartic acids). Proteins of this class have, so far, not been described. The plant globulins, however, on hydrolysis yield large amounts of dicarboxylic acids, but they are not distinctly acidic in character in that they also yield large amounts of ammonia, corresponding roughly to the excess of the carboxyl groups over the amino groups in the molecule. This indicates that these proteins have been isolated in the form of amides. Now it is worthy of note that the protein of cabbage which has been isolated is acidic in properties, and yields on hydrolysis an amount of ammonia (amino-nitrogen) corresponding to 5 % of the total nitrogen, which is distinctly below that of the other plant proteins. These results indicate that acidic proteins exist in the leaves, which, when they pass into the resting stage in the seed, undergo amidisation. Such proteins should contain a relatively small amount of nitrogen. Whether the low percentage found in the samples described in this paper is due to this cause or whether they are conjugated with some nitrogen-poor prosthetic group can only be determined by subsequent research.

There exists in the leaves an appreciable amount of ammonium salts, and the study of the equilibrium of these, the amino-acids, and the proteins, should throw considerable light on the nitrogenous metabolism of the plants.

The proteins from scarlet runner leaves yield an appreciably higher number

for the amide nitrogen than the cabbage protein. It is conceivable that the leaf proteins can exist more or less completely amidised in different stages of the development of the plants, and it is hoped that at an appropriate season opportunity will be offered for investigating this point. It may account for the difference in the properties of the proteins already isolated by the ether-water method. In the meanwhile, material obtained during the summer months is under examination, and the results will, it is hoped, form the subject of a later communication.

The rather full analytical details given for the cabbage are quoted as an illustration of the methods employed rather than as accurate totals, which must of necessity be incomplete until the nitrogen in the residue has been more fully investigated.

EXPERIMENTAL.

Preliminary experiments on cabbage leaves.

A preliminary series of experiments was made in 1916 by Miss Laura M. Saunders under the direction of one of the present authors (S. B. S.). The soft parts of the cabbage were minced, and treated with various solvents, as enumerated below, and the total nitrogen thereby passing into solution was estimated by Kjeldahl's method. Table I gives the results obtained. In each case the solvent was allowed to act for several hours, and the liquid afterwards expressed through muslin in a tincture press.

Table I. *Showing the effect of various cytolysing agents.*

Weight of cabbage used, 200 g.				Weight of N extracted in g.
Solvent				
Water saturated with ether	...	...	...	0.38
Water saturated with chloroform	...	...	...	0.335
<i>M</i> /2 benzoate of sodium	...	...	...	0.259
Water containing 20 g. chloral hydrate per litre	...	...	...	0.304
Distilled water	...	...	...	0.220

This indicated that ether-water was the most efficient agent, whilst further experiment showed that the proportion of five parts by weight of minced cabbage to four of the ether-water was ample, also that 20 minutes was sufficient time to leave the mixture before pressing.

The investigation of the liquid extract was taken up by the present authors in January 1920. A dozen cabbages, purchased in the ordinary market, were taken, the hard ribs and heart removed, and the remainder minced in a mechanical mincer, using a fairly coarse cutting plate. Very little, if any, liquid was expressed during this operation, but the leaves were well torn up and lacerated so as to expose a large surface to the cytolysing agent. They were then mixed with ether-water in the proportions stated above (five by weight of cabbage to four of the water), and after standing for 20 minutes the fluid was expressed through thick muslin in a tincture press. It was

green in colour, opalescent, and filtered slowly, but without leaving a residue, through filter paper at the pump. Attempts were next made to concentrate it under reduced pressure at 40°. It was found however that a green solid separated with rise of temperature, causing such violent frothing that the operation had to be suspended. This indicated that the liquid as it came from the press contained some substance in colloidal solution, and attempts were made to cause this to flocculate before concentration. Standing for several days, either alone, or with the usual flocculating salts, did not bring about the desired effect (the room temperature was about 13°; this will probably account for the difference observed in June, when the room temperature was 25°), whereas warming on a water-bath at the ordinary pressure to 40° caused the immediate formation of a thick precipitate, which settled at once on cooling, leaving a clear solution that could be concentrated quietly without trouble. This gave a method of obtaining the products of the extraction in a concentrated form suitable for storing, and 135 cabbages were forthwith so treated. Each batch of about 12 was extracted twice with the ether-water, the second extract from one batch being used as the first in the next, and so on. In all 70 kilos of minced material were treated, and the resultant clear liquid concentrated to about 10 litres after separation of the colloid.

The green colloidal precipitate. This was washed twice with two litres of 95 % alcohol, then with two litres of 0.730 ether and finally allowed to dry in the open air. (The alcoholic washings on evaporating to dryness and purifying by extraction with warm water gave a dark coloured oil consisting almost entirely of chlorophyll. The ether washings likewise consisted chiefly of pigment, giving a solid on evaporation.) On rubbing lightly through a 0.1 mm. sieve, 290 g. of a fine olive-green powder were obtained. The fine powder was then exhaustively extracted with ether in a Soxhlet, and the resultant product, of a dull green colour, weighed 165 g. It contained 11 % of moisture, and the dry material 10.6 % N and 0.255 % P. The extract from the Soxhlet consisted of fats and lipoids with a small amount of green colouring matter.

The high nitrogen content of this precipitate showed that a large proportion of it was apparently protein. Water extracted a small amount of non-nitrogenous matter, leaving a residue which now contained 11 % of nitrogen. The only solvent found for it was aqueous caustic soda, which first gelatinised, and then slowly dissolved a little more than half of it. The strength of the soda used was 0.3 %, and three extractions were made. The residue was centrifuged off, washed with graded strengths of alcohol, then ether, and dried in an oven at 108°. It contained 8.85 % of nitrogen. The alkaline solution remained clear on exact neutralisation, but gave an immediate precipitate on being made slightly acid. This was centrifuged off, washed with graded strengths of alcohol, then ether, and dried in the air. It contained 11 % of nitrogen. On redissolving in soda an appreciable amount was found to be insoluble. After this had been centrifuged off the clear liquid

was again made acid, and the resultant precipitate was found to contain 12.8 % N and a trace only of phosphorus. It was now completely soluble in soda, and could be reprecipitated unchanged. It gave a strong biuret reaction and was new in character in that it was insoluble in excess of acid. Table II shows the distribution of the nitrogen in this compound, as determined by Osborne's modification of Haussmann's method.

Table II. *Distribution of nitrogen in the cabbage leaf protein prepared in the winter.*

	% N.
Amide nitrogen	... 5.89
Humin „	... 5.23
Basic „	... 21.95
Mono-amino nitrogen (by diff.)	... 66.93

The clear mother liquid. The 10 litres that had been prepared were poured into an equal volume of 95 % alcohol, when a copious white precipitate was formed, which settled on standing. The supernatant liquid was siphoned off and the residue sucked dry at the filter-pump, washed with alcohol, then ether, and dried in the air. The clear alcoholic liquid was again concentrated under reduced pressure, and has been stored since under toluene and a little chloroform.

Mr H. W. Buston is at present at work on the isolation of the individual amino-acids in this liquid. He has shown that 9.49 % of the total nitrogen is in the form of ammonium salt, and after the removal of this, 25.1 % in the form of amino-nitrogen. Also that 20 % of the total nitrogen is precipitated by means of lead salts. After the removal of this the major part of the residual nitrogen is precipitable by phosphotungstic acid.

The alcoholic precipitate weighed 300 g., and was white when dry. On analysis it gave 6.5 % of moisture, and the dry substance 1.90 % of nitrogen and 1.64 % of phosphorus.

Part of it was extremely soluble in water, and could be purified by repeated reprecipitation in 80 % alcohol. Nothing further has been done to identify this substance. Several grams of it have been prepared, which give a colourless solution in water. A similar sample prepared by Miss Saunders in 1916 gave the following analysis: Ca, 24 %; SO₄, 18 %; P, 1 %. It probably contains free calcium sulphate.

Of the residue insoluble in water, part was soluble in 5 % acetic acid. It could be precipitated by alcohol, and was purified by repeated solution and reprecipitation. It gave an ash weight of 65 %, and the following analysis (Saunders): Ca, 22.5 %; P, 11.7 %; C, 12.2 %; H, 2.35 %. No nitrogen and a trace of halogen. Molisch's test was positive on standing. It is probably some conjugated calcium or calcium-magnesium phosphate, but does not appear to be phytin in that it is not precipitated from acetic acid solution by boiling. The material will be investigated later.

ANALYSIS OF THE PROTEIN OF THE CABBAGE EXTRACT.

Having made this brief survey of the various products that had thus been prepared, it was decided that, for the present, attention should be given to the substance that obviously contained much protein. To obtain accurate data as to its percentage weight in the cabbage, and the proportion of the total nitrogen that it contained, a quantitative analysis was made of another sample of six cabbages, purchased early in June. These were a little different from those used earlier in the year. They were smaller; both the inner and outer leaves appeared to contain more green colouring matter; and the area of small yellow leaves, growing around the heart, was much less.

As before, the heart and thick fibrous stems were removed, and the remainder minced. The weight of the minced material was 1877 g., and of this 297 g. (leaving 1580 g.) were placed in an oven heated to 108° for the purpose of obtaining the dry weight and the total nitrogen. From these data it was estimated that the 1580 g. used for the experiment contained 157.7 g. of solid matter; 6.18 g. N; and 25.4 g. of ash.

This 1580 g. was then thoroughly mixed with 1200 cc. of ether-water, allowed to stand for 20 minutes, and then pressed out through thick muslin in the tincture press. The total volume of the extract was 2440 cc. The residue, removed from the filter-cloth, was again passed through the mincer, and then mixed with another 1000 cc. of the ether-water, allowed to stand for 20 minutes, and pressed out. The volume of this extract was 965 cc. The residue was then again removed from the filter-cloth, crumbled up by rubbing between the hands, and treated successively with another 1000 cc. of the ether-water three times, the volumes of the extracts being 935, 940 and 940 cc. respectively.

The final residue, after the fifth pressing, was dried in an air oven at 108°. It weighed 67.5 g. and contained 2.32 g. N.

The nitrogen in the several extracts was also determined, the results being given in Table III. It will be observed that the first extract contains 80.9 %, and the first with second combined, 90.2 % of the extractable nitrogen.

To test if any of the nitrogen was held back in the pores of the filter-cloth, the latter was washed first with water and then three times with 80 % alcohol. A quantity of green colouring matter was removed, but the total nitrogen in all the washings was less than 0.05 g.

The green colloidal precipitate. It was not found necessary to heat the extracts so obtained to coagulate the protein material, as the temperature at which the experiment was being conducted, 22–25°, was sufficient to cause a heavy precipitate to come down during the course of the night, leaving an almost clear solution.

The precipitate from the first extract, called "A" henceforth, was separated by decanting and centrifuging, as was also that from the other four solutions combined, called henceforth "B."

Table III. *Showing the distribution of N after extracting five times with ether-water.*

				Total volume or weight	Weight of N in g.
1st ether-water extract	...	...	...	2440 cc.	3.066
2nd "	"	"	...	965 "	0.352
3rd "	"	"	...	935 "	0.191
4th "	"	"	...	940 "	0.096
5th "	"	"	...	940 "	0.088
Total extract	...	...	...	6180 "	3.793
Dry residue	...	...	...	67.5 g.	2.320
<i>Total nitrogen</i>	...	...	...	—	6.113
Taken for experiment (estimated)				—	6.18

Table IV. *Showing total solids precipitated by standing 24 hours (after washing with alcohol and ether).*

	Weight in g. moisture free	Weight of N in g.	% of N
Precipitate A	3.70	0.452	12.1
" B	0.448	0.052	11.9

Both A and B were washed with 80 % alcohol until all the chlorophyll was removed, then three times with 0.730 methylated ether, and left to dry at the room temperature.

Table IV summarises these weights in terms of the total cabbage taken.

The alcoholic washings of A and B consisted almost entirely of chlorophyll, with a certain small amount of the soluble nitrogenous substances carried down during the precipitation. The dry weight was 1.05 g., and the nitrogen content 0.056 g.

The ether washings of A and B on evaporation gave an orange-yellow grease, weighing 0.18 g.

The original extracts, after the precipitation of A and B, were still slightly cloudy. It was found that raising the temperature to boiling point caused another slight coagulation to occur, and a deposit, called henceforth C, to settle during the course of the night. After this had been removed by centrifuging, the nitrogen in each solution was again determined. All five were then mixed, and an aliquot part evaporated to dryness and heated to 108° in an oven. From this it was calculated that the weight of the water-soluble material was 73.1 g.

The coagulate C, the major portion of which was thrown down from the first extract, was washed with water, which removed 0.392 g. of material containing 0.021 g. N, then with alcohol, which removed a further 0.159 g. of material, containing less than 0.01 g. N and finally with ether, being allowed to dry off at room temperature. The weight, moisture free, and calculated for the total cabbage taken, was 0.608 g., and it contained 0.074 g. N.

The results of the preceding analyses are summarised in Table V.

Table V. *Showing distribution of nitrogen.*

					Weight in g. moisture free	Weight of N in g.
1st clear solution	}				73.1 of solid matter	2.370
2nd " "						0.312
3rd " "		...	...			0.165
4th " "						0.076
5th " "						0.078
Precipitate A	...	...	...		3.74	0.452
" B	...	...	...		0.45	0.054
Coagulate C	...	...	...		0.68	0.074
Alcoholic washing of A and B	...				1.05	0.056
Ether	"	"	...		0.18	?
Water washing of C	...	...	...		0.39	0.021
Alcoholic washing of C	...	...	...		0.16	?
Residue	...	...	...		67.50	2.32
<i>Total</i>	...	...	...		147.25	5.992
Taken for experiment (estimated)					157.7	6.18

Examination of precipitates A and B.

These were mixed and treated with 0.4 % caustic soda. Considerably more than half was found to go into solution this time, imparting to it as before a deep brown colour. The residue was centrifuged off and treated twice again in the same way. The third solution on being made acid gave very little precipitate, so it was resolved to treat with hot alcoholic alkali according to the method described by Osborne and Wakeman [1920] in their paper on spinach leaves, which had come into our hands about this time. The first application of 0.3 % potash in 60 % alcohol dissolved about half the residue, and gave quite a heavy precipitate on being made acid. A second application dissolved practically nothing. The residue, after being thus treated, was washed with graded strengths of alcohol, then ether, and dried at 108°. It weighed 0.136 g. and contained only 0.003 g. N.

The aqueous soda extract was made slightly acid, and the heavy precipitate allowed to settle. The supernatant liquid was poured off, and the remainder centrifuged. The solid was redissolved in the same strength soda. It was again precipitated with acid, centrifuged, washed with graded strengths of alcohol, then ether, and allowed to dry at the room temperature. The dried product weighed, moisture free, 2.247 g. and it contained 12.81 % N, and only a trace of phosphorus.

The residual acid solution, together with the alcoholic washings, was found to contain 0.129 g. of nitrogen.

The alcoholic soda extract was treated in a similar way. The final product weighed, moisture free, 0.162 g., and it contained 12.7 % N. The weight of nitrogen remaining in this solution and the washings was 0.005 g. Table VI shows the complete analysis of the precipitates A and B, calculated for the

total weight of cabbage taken. It will be seen that 63·3 % of the total weight of A and B is recovered from the aqueous soda solution, and 4·55 % from the alcoholic; in all 67·85 %. Of the total weight going into solution, 70·6 % is reprecipitated.

Table VI. *Showing the extraction of precipitates A and B by aqueous and alcoholic soda.*

	Weight in g.	Weight of N in g.
Protein, precipitated from aqueous soda	2·651	0·339
" " " alcoholic soda	0·191	0·024
Non-protein substances, soluble in aqueous soda and acid } " " " alcoholic soda and acid } (by diff.)	1·185	0·139
Insoluble residue	0·161	0·003
<i>Total</i> ...	4·188	0·516

Table VII. *Showing the distribution of nitrogen in the cabbage leaf protein prepared in summer.*

	% N
Amide nitrogen	5·62
Humin nitrogen	5·15
Basic nitrogen	21·31
Mono-amino nitrogen	67·92

The figure 12·8 for the % N in the final product is in agreement with that obtained for the similarly prepared compound from the cabbages purchased several months earlier. The internal distribution is also very similar, as will be seen from Table VII.

Table VIII shows the results of the complete analysis.

Table VIII. *Final results of the analysis.*

	Weight in g. moisture free	% weight, moisture free	Weight of N in g.	% N
In aqueous solution	73·49	46·59	3·02	48·87
In colloidal suspension in water:				
Protein	2·84	1·80	0·36	5·83
Non-protein substances	1·19	0·76	0·13	2·13
Albumin (? coagulated on boiling)	0·68	0·43	0·07	1·13
Soluble in alcohol and ether ...	1·39	0·88	0·07	1·13
Residue	67·66	42·80	2·32	37·64
<i>Total</i> ...	147·35	93·26	5·97	96·73
Taken for analysis (estimated) ...	157·70	—	6·18	—

CUT GRASS.

Short lengths of grass, as cut by a lawn-mower, were obtained from the Botanical Gardens, Chelsea. After being washed and air dried they were minced, with a fine cutter, and treated with ether-water as before. 1420 g. of minced material were used, and from experiments on an aliquot part it

was estimated that this amount contained 857 g. of solid matter, or 60.4 %, and 32.8 g. of nitrogen.

The amounts of nitrogen expressed in the five ether-water dressings are given in Table IX.

Table IX. *Showing the weights of N in the ether-water extracts from grass.*

	Weight of N in g.
1st extract	4.79
2nd „	2.35
3rd „	1.36
4th „	0.79
5th „	0.66
Total of 5 extracts	9.95

The solution from the press was olive-green as in the cabbage, and gave no deposit on prolonged centrifuging. Experiments to bring about flocculation were then tried. Standing for two days, either unchanged or after warming to 60°, failed to bring this about. The only reagents successful were alcohol in the proportion of 1 in 4 and mineral acid.

As the cabbage had given a final product that was precipitated from solution by acids, it was resolved to use this method in the present case. It was found that the addition of enough sulphuric acid to make the whole $N/12$ was sufficient to give a definite precipitate and leave a clear solution. Acid of this strength also would be insufficient to bring about any hydrolysis in the cold. The material so obtained was washed with alcohol and ether as before and air dried. It contained 6 % N only, but was soluble in 0.3 % aqueous soda from which it could be reprecipitated by acid. The product so obtained contained 8.25 % N. It failed to give any of the colour reactions for a protein, and as the amount obtained was small and we were desirous of investigating other leaves before the season became too late, nothing further was done with it.

SCARLET RUNNER LEAVES.

These were picked at the end of the first week in September, when the beans were still in process of formation. They were cut so as to leave no stem, and after being cleansed were minced, using a coarse cutting plate. They appeared to undergo very considerable disintegration, the minced product being a dark green pulp, containing a large quantity of expressed juice.

The total weight minced was 1331 g. and of this 110 g. were taken for dry-weight analysis. From the result of this it was estimated that the 1221 g. used in the experiment contained 185.8 g. of solids, or 15.2 %, and 8.21 g. N.

It was then treated five times successively with ether-water, being re-minced before the second application. 1000 cc. were used in each case, and the results are given in Table X.

Table X. *Showing distribution of nitrogen in bean leaves after extraction in the ether-water.*

	Weight of N in g.
1st extract	4.07
2nd „	0.75
3rd „	0.37
4th „	0.16
5th „	0.16
Total in 5 extracts	5.51
In the residue, weight 79 g. ...	2.60
<i>Total</i> ...	8.11
Taken for experiments (estimated)	8.21

In comparison with the cabbage it will be seen that the total nitrogen expressed in the first extract is about the same, *i.e.* nearly one-half; the residue similar, being 42 %, and the total expressed nitrogen greater, being 68.4 % as compared with 62.4 %.

All the extracts, especially the first, were deep green and opaque. A sample of the latter, on warming to 40°, flocculated into a solid mass, and the main bulk was diluted with an equal volume of water before being heated. The precipitate, called henceforth D, after washing with alcohol and ether as described above had a moisture-free weight of 15.5 g., and contained 10.3 % N.

The similar product from the second and third extracts, called henceforth E, weighed, moisture free, 2.2 g. and contained 8.5 % N.

Extracts 4 and 5 failed to flocculate on warming to 40°, but warming to 60° brought this about. This temperature also caused a second flocculation in the first three extracts, and the combined products, called henceforth F, after washing as before, weighed, moisture free, 4.7 g. and contained 10.8 % N.

All the extract solutions were now a clear light brown. The first on boiling gave a third small coagulate, but the others remained clear. This coagulate weighed 0.52 g., contained 9.1 % N and is probably the impure product from some water-soluble albumin. It appeared to be insoluble in all common solvents.

Table XI shows the results of the foregoing precipitation after allowance has been made for the quantities withdrawn for experiment.

Table XI. *Showing the products obtained by warming the ether-water extract of bean leaves.*

	Weight in g.	% N	g. N
Precipitate D ...	16.5	10.3	1.70
„ E ...	2.3	8.5	0.19
„ F ...	5.0	10.9	0.55
Coagulate on boiling	0.5	9.1	0.05
Total weight ...	24.3	—	2.49

It will be seen from this that 13.1 % of the total dry weight is precipitated in the form of a highly nitrogenous body containing 38.4 % of the total nitrogen.

Examination of Precipitates D, E and F.

Precipitate D was found to contain starch, and was freed from this by repeated washing with water at 60° until the washings gave no reaction with iodine solution. The resultant product was found to be almost insoluble in cold aqueous soda (0.3 %), but went up readily in alcoholic potash (0.3 % in 60 % alcohol) warmed up just to the boil on a water-bath. After centrifuging, it was just made acid with dilute sulphuric acid, when a copious precipitate came out, which, unlike the cabbage product, was soluble in excess. It was now partially soluble in cold aqueous soda. The remainder was taken up again in hot alcoholic potash, and on reprecipitation was found to be completely soluble in cold soda. The combined solutions in cold soda were centrifuged, yielding a clear liquid, which threw down a heavy white precipitate on being made just acid. This was centrifuged off, washed with alcohol and ether as before, and dried in the air. The product weighed, moisture free, 5.88 g., and contained 13.33 % N, and very small traces of phosphorus. It would appear (from its mode of preparation and properties) to be of the nature of an alkali albumin.

Precipitate E was worked up in the same way, starch being first removed. As before hot alcoholic potash was required, the product after two washings becoming soluble in cold soda. Whilst drying it was accidentally lost by the cracking of the beaker, but the yield appeared to be the same as in the other two preparations, *i.e.* about 40 %.

Precipitate F contained no starch, which probably accounts for its higher percentage of nitrogen. As before hot alcoholic potash was required to dissolve it, the product obtained after acidification becoming soluble in cold soda after a second washing. Washed and dried the final preparation weighed, moisture free, 2.15 g., and contained 13.4 % N.

The resultant products from D and F are probably the same, for they behave in exactly the same way with acids and alkalies, give a strong biuret reaction, and contain about 13.5 % N.

Table XII gives the distribution of the nitrogen in the product from D.

Table XII. *Showing the distribution of nitrogen in the bean leaf protein.*

	% N
Amide nitrogen	9.38
Humin nitrogen	7.95
Basic nitrogen	28.66
Mono-amino nitrogen	54.01

It will be observed that the amide nitrogen is considerably higher than in the case of the cabbage product, possibly accounting for its less acidic character.

The working up of this bean material was not done with quite the same care as that bestowed on the cabbage, so that small losses may have been entailed. Allowing for this, and calculating the product to be obtained from

E as 40 % of its weight, we estimate the total yield of this protein to be 9 g., containing 1.12 g. N. Expressed as percentages, 5 % of the dry weight and 13.8 % of the nitrogen are isolated in the form of a protein.

Considering that the combined weight of D, E and F was nearly 24 g., this yield is very low. A comparison of it with that of the cabbage and spinach (Osborne's figures) is given in Table XIII.

Table XIII. *Showing the different effects of aqueous and hot alcoholic alkali on the proteins of the cabbage, bean and spinach leaves.*

	% weight recovered		
	Cabbage	Bean (mean)	Spinach (Osborne)
From cold aqueous soda	63.3	—	58.6
From hot alcoholic potash	4.6	38	13.4
<i>Total</i> ...	67.9	38	72.0

These figures are instructive in that they show that a high yield is obtained only when the reaction takes place in cold aqueous soda. It seems probable therefore that warming with alcoholic potash to the boil brings about a greater degree of hydrolysis than we at first suspected, the solution containing degradation products that are not precipitated in slight excess of acid. With regard to this question of the degree of solubility in cold soda, we have been struck by the way in which this property seems to vary in the different preparations from the same plant. From the experience gained in working up the various products described in this paper we have come to the conclusion that the previous treatment the first precipitate undergoes in its washing with alcohol has something to do with it. In one case in particular, in which some of the precipitate from spinach was left over the week-end in absolute alcohol, we found the resultant product, dried by ether, to be quite insoluble in the cold. Possibly strong alcohol has some denaturing effect on the protein present.

SPINACH.

About a kilo. of spinach was worked up at the beginning of October by our method in order to compare the final product obtained with that isolated by Osborne and Wakeman [1920]. Only 57 % of the total nitrogen passed into solution, which was divided into two parts. One was warmed to 40° to flocculate the colloid by the method described above, the other was flocculated by adding a quarter volume of alcohol, as described by Osborne. In both cases the washed and dried product gave about 10.4 % N, showing that the two methods had apparently the same result. On extracting with cold soda and precipitating with acid a protein-like substance was isolated which gave only 12.6 % of nitrogen and was insoluble in excess of acid. It appeared therefore to be a substance with an acidic character similar to that isolated from the cabbage.

This result does not correspond at all with that of Osborne and Wakeman. Their method is first to grind the fresh leaves three times in a Nixtamal mill, then add water in the proportion of 1 litre to 1 kilo. of spinach, and regrind the mixture twice. They then centrifuge to remove the disintegrated cellular matter and obtain a green opaque solution containing a large quantity of protein in colloidal suspension. They flocculate as described above by adding alcohol, and after washing and drying obtain a substance averaging 14 % N. This substance they then treat in the same way as we do, *i.e.* with cold soda, and on making the solution slightly acid bring down a substance giving 15.3 % of nitrogen. This differed from all our preparations in that it was soluble in slight excess of both acid and alkali.

A chemical explanation of this difference we are at present unable to give, but it may be a purely physiological one. From the very large proportion of the total nitrogen that they actually isolate as protein (43 %), it would appear that they took their leaves at a period of great physiological activity. They do not give the time of year when their leaves were picked, nor the period between picking and working up. In our case the leaves were purchased in a shop, and were probably picked two days previously. This period may have allowed some enzyme action to have taken place, but if that were the case we should have expected a much higher nitrogen percentage in the protein of the bean leaves, which were treated the same day that they were picked. The explanation seems to us, in view of the small amount of evidence yet available, to be due to different chemical compounds being actually present in the leaves taken.

For comparison the distribution of the nitrogen found by them in their compound is given in Table XIV.

Table XIV. *Showing the distribution of nitrogen in the spinach leaf protein (Osborne and Wakeman).*

	% N
Amide nitrogen	7.50
Humin nitrogen	5.97
Basic nitrogen	22.49
Mono-amino nitrogen	64.04

SUMMARY.

- (1) A method has been described for obtaining the proteins of green leaves.
- (2) This consists in treating the disintegrated tissue with water containing a cytolytic agent, for which purpose ether was found to be the most suitable.
- (3) By means of water saturated with ether, opalescent colloidal solutions are obtained from which flocculation of the colloid takes place, either on standing, if the room temperature is sufficiently high (above 20°), or more rapidly on warming to 40°.

(4) The colloidal precipitates obtained in this manner from the leaves of cabbage, scarlet runners and spinach have been examined and found to consist for the most part of proteins, and to contain about 10 % N.

(5) Methods have been described for obtaining the proteins in a purer condition, and products containing about 13 % N have been prepared.

(6) The cabbage and spinach proteins are, when freshly prepared, soluble in weak alkaline solutions from which they can be precipitated by acids, in excess of which they are not speedily soluble. The protein of scarlet runner was insoluble in weak aqueous alkalies.

REFERENCES.

- Chibnall and Schryver (1920). *J. Physiol.* 54, Proc. July.
Osborne and Wakeman (1920). *J. Biol. Chem.* 13, 1.