LXXVII. A METHOD FOR THE SEPARATION OF AMINO-ACIDS FROM THE PRODUCTS OF HYDROLYSIS OF PROTEINS AND OTHER SOURCES.

PRELIMINARY COMMUNICATION.

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IN the course of investigations on the nitrogenous metabolism in green leaves, experiments were undertaken with the object of isolating the various nitrogenous substances contained in cabbages. It has already been shown by Chibnall and Schryver [1921] that nearly all the simpler nitrogenous products can be removed from the leaves when they are treated with water saturated with ether, and the liquid is then expressed in a tincture press. An opalescent solution is thereby obtained, which on warming to 40° deposits a flocculent precipitate, consisting for the most part of proteins. After filtration and warming further to 100° another fraction of proteins is precipitated. On adding an equal bulk of 95 % alcohol to the filtrate from this precipitate, a voluminous pale buff-coloured precipitate is produced, containing in addition to inorganic salts and other substances which account for only a relatively small percentage of the total nitrogen, an organic phosphate derivative which has not yet been further investigated. The filtrate from this phosphate fraction was concentrated under diminished pressure, acidified with sulphuric acid, and the requisite amount of phosphotungstic acid was added. 32.8 % of the nitrogen in the extract after removal of the protein and phosphate fractions was thereby precipitated; this extract contained also 11.4 % of its nitrogen in the form of ammonium salts. The filtrate and the washings from the phosphotungstate precipitate were treated with barium hydroxide (Congo red) to remove the greater part of the sulphuric acid and excess of phosphotungstic acid, and then evaporated *in vacuo*. The liquid thus obtained contained 53.55 % of its nitrogen in the amino form (Van Slyke). Experiments were then made to remove the dicarboxylic acids by barium hydroxide and alcohol. The first addition of the alkali produced a precipitate of phosphotungstate containing no nitrogen; this was filtered off, and a further addition of barium hydroxide in saturated solution was made, and then $1\frac{1}{4}$ the bulk of 95 % alcohol was

added. A precipitate of the dicarboxylates was thereby produced. It is not advisable to add a larger bulk of alcohol, otherwise the precipitate tends to become slimy. 13.9 % of the nitrogen in the extract (after removal of proteins and phosphates) is thereby precipitated.

After the separation of the barium dicarboxylates, carbon dioxide was passed into the liquid, *without previous removal of the alcohol*. A bulky precipitate was thereby produced, which contained appreciable quantities of nitrogen (1.66 % of the dry weight). This was filtered off, and the filtrate was saturated with barium hydroxide, and treated with carbon dioxide. Another precipitate was obtained, which was also relatively rich in nitrogen. These experiments were repeated with a larger bulk of the liquid. An extract freed from the dicarboxylic acids and alcohol was diluted with an equal volume of 95 % alcohol, and saturated with solid barium hydroxide; somewhat more was required than would saturate the amount of water present. Carbon dioxide was then passed through the solution. The precipitate was filtered off, the filtrate was re-saturated with barium hydroxide, and again treated with carbon dioxide. This treatment was repeated altogether seven times, and the precipitates contained the following percentages of nitrogen: (i) 1.70, (ii) 1.67, (iii) 0.92, (iv) 0.81, (v) 0.55, (vi) 0.29, (vii) 0.09. Altogether, 68.83 % of the nitrogen contained in the liquid from which the bases and dicarboxylic acids had been separated by the methods given above were thus removed. Furthermore, the residual liquid contained in the amino form only 2.6 % of the total nitrogen present in the liquid after removal of the dicarboxylates.

Determination of the optimal conditions of precipitation.

Many years ago Siegfried prepared the barium salts of carboxylates of amino-acids, possessing the general formula

by treating these acids with carbon dioxide and barium hydroxide. These barium salts readily decompose on warming, yielding barium carbonate and the free amino-acids. There is little doubt that the amino-acids have been precipitated in the form of the barium salts of their carboxylates by the method described above. Experiments were undertaken with the object of determining the optimal conditions of precipitation, a few of which are given in detail below.

After addition to the base-free extract of cabbage of the amount of barium hydroxide necessary to precipitate the barium salts of the dicarboxylic acids after addition of alcohol, the filtered liquid was concentrated *in vacuo*. To the liquid thus obtained varying quantities of alcohol and barium hydroxide were added before treatment with carbon dioxide. The gas was then passed

in and the amount of precipitate produced, and its percentage of nitrogen were determined. The results are given in the following table:

(a)	1000 cc. alcohol; 1000 cc. extract:				
	15 g. Ba(OH) ₂	...	...	Weight of precipitate	12.5 g.; N 1.61 %.
(b)	500 cc. alcohol; 500 cc. extract:				
	Baryta to saturation	...	...	" "	24.5 g.; N 1.70 %.
(c)	200 cc. extract; 400 cc. water;				
	and 600 cc. alcohol				
	(i) 35 g. Ba(OH) ₂	...	...	" "	31.5 g.; N 1.60 %.
	(ii) 30 g. Ba(OH) ₂	...	...	" "	— N 0.77 %.
(d)	200 cc. extract; 400 cc. alcohol:				
	10 g. Ba(OH) ₂	...	...	" "	9.5 g.; N 1.60 %.
(e)	200 cc. extract; 0 cc. alcohol:				
	30 g. Ba(OH) ₂	...	...	" "	21.5 g.; N 0.35 %.

As a result of the above experiments it will be seen that very little nitrogenous matter is precipitated by successive treatments with barium hydroxide and carbon dioxide in the absence of alcohol; on the other hand no advantage is obtained by diluting the solution from which the amino-acids are to be precipitated with more than an equal bulk of 95 % alcohol. By using therefore an equal bulk of alcohol and adding the baryta to saturation before treatment of the liquid with carbon dioxide, the best results are to be obtained. Under these conditions the results recorded in the preceding section were obtained from the cabbage extract.

The main disadvantage of the method lies in the fact that the alternate treatment of the liquid with barium hydroxide and carbon dioxide must be repeated several times before a nitrogen-free precipitate is obtained. To obviate the manipulations necessary for this process, an attempt was made to convert the amino-acids into their carboxylates by treatment with an excess of ethyl chloroformate in the presence of sufficient barium hydroxide to combine with the hydrochloric acid set free in the reaction, and the subsequent addition of a large excess of baryta. Several experiments of this character were carried out, but in no case did the precipitate obtained contain more than small amounts of nitrogen, and, compared with the first methods tried, this alternative method showed no advantages. Furthermore, it was found that after a little practice, when the amounts of reagents necessary were known, the successive operations of the earlier methods could be carried out without undue expenditure of time.

Decomposition of the nitrogenous barium precipitate.

150 g. of the precipitate containing 1.66 % of N (or 2.49 g. in all) were decomposed by boiling for 5–10 minutes with water; a Kjeldahl estimation on the filtrate showed that over 98 % of the nitrogen present in the precipitate was dissolved by this method. The brown-coloured filtrate was found to contain considerable amounts of barium in solution; this was accordingly removed *exactly* with sulphuric acid. The filtrate was decolorised by means of a little animal charcoal, and evaporated *in vacuo* to syrup, of amber colour. On

shaking this syrup with absolute alcohol it became changed to a cream-coloured, granular solid, while the alcohol became light orange-brown in colour. The solid was removed, washed two or three times with absolute alcohol, and finally with ether, and dried by leaving for two or three hours in a vacuum desiccator. By this method, it dried to a light buff powder, easily soluble in water, and containing 5.25 % of nitrogen. (Unless the washing with alcohol was thorough, and the drying rapid, the substance tended to change back to an amber-coloured, sticky mass.) Evaporation of the alcoholic extract gave a brown, non-crystalline mass. Judging from the percentage of nitrogen in the solid (*i.e.* 5.25 %), it appears that it contains about 40–50 % of amino-acid; the remainder consists probably of sugars and possibly oxy-acids. Further experiments to decide this point are to be made.

From the results of the above experiments it was found that a relatively large fraction of the simpler substances from the cabbage leaves could be isolated in a crystalline and but slightly pigmented form, and almost completely free from the resinous matters which hitherto have made the investigation of the crystalline substances contained in leaves a problem of great difficulty. The investigation of these substances of which a large proportion are nitrogenous is reserved for the future. It is worthy of note that leaves of plants contain relatively large amounts of nitrogenous substances which are neither bases nor amino-acids. The discovery of a method which removes nearly all these acids should considerably simplify the investigation of these unknown constituents.

In the meantime, it was thought advisable to ascertain whether the baryta-carbon dioxide method could be applied to the isolation in crystalline form of the amino-acids obtained by the hydrolysis of the proteins.

The discovery by Dakin of the butyl alcohol method forms a great advance in the technique of the isolation of the amino-acids, but this is not always convenient, as Dakin's recently published paper on the hydrolysis products of gelatin shows [Dakin, 1921]. It seemed that the method described in this communication might serve as an alternative one in such cases; it has the further advantage that it can be carried out much more rapidly than the butyl alcohol extraction method. The experiments described below on the isolation of the hydrolysis products of caseinogen and gelatin indicate that the method described in this paper is one of considerable promise.

Experiments with hydrolysed caseinogen.

The caseinogen was hydrolysed with sulphuric acid in the usual way; sulphuric acid was removed by means of baryta, and the liquid evaporated to small bulk to remove tyrosine. After removal of tyrosine, the amount of nitrogen present was 8.870 g. The concentrated liquid was diluted and precipitated with phosphotungstic acid in the ordinary way; 2.50 g. of nitrogen were thus removed, leaving 6.37 g. in solution. Dicarboxylic acids were now removed by treating the solution (concentrated to 500 cc., after removal of

excess of phosphotungstic and sulphuric acids) with 35 g. baryta, adding 1000 cc. alcohol, and allowing to stand for 20 hours. The product which crystallised out was removed, washed with 70 % alcohol and dried; its weight was 27 g., and it contained 1.6 g. of nitrogen.

The filtrate was concentrated to 250 cc. and contained 4.65 g. of nitrogen, of which 68.27 % (or 3.168 g.) was in the form of amino nitrogen. Of this solution, 125 cc., containing 2.325 g. of nitrogen and 1.584 g. amino nitrogen were used; baryta was added to saturation, then an equal bulk of 95 % alcohol, and CO₂ passed till precipitation was complete. The precipitate produced was of a gelatinous nature and gave some difficulty in washing and filtering; the gelatinous nature is less obvious in the fractions containing a lower percentage of nitrogen. Four such precipitations were made, and the amount of nitrogen brought down in each case was determined; the residual liquid was evaporated to a small bulk *in vacuo*, and the nitrogen remaining determined. The results are summarised in the Table below. A van Slyke determination on the residual solution gave 0.1175 g. amino nitrogen present, or 5.06 % of the total original nitrogen.

Each of the above four fractions of the barium precipitate was decomposed separately by boiling up twice with three times its weight of water for 5 minutes; after filtering and removing barium from the extract by means of the exact amount of sulphuric acid, the liquid was in each case examined for amino nitrogen. The results obtained are shown in the following table:

Caseinogen: solution after removal of dicarboxylic acids.

	Total N (Kjeldahl) g.	%	Amino N (van Slyke) g.	%	Non-amino N (by difference) g.
Original solution	2.325	100.00	1.584	68.27	0.741
Fraction I	0.906	39.00	0.6786	29.19	0.2274
" II	0.545	23.47	0.3995	17.18	0.1455
" III	0.298	12.79	0.2095	9.01	0.0885
" IV	0.275	11.85	0.1702	7.31	0.1048
Residue	0.280	12.04	0.1175	5.06	0.1625
Totals	2.304	99.15	1.5753	67.75	0.7267
(compare with top line, "Original solution")					

(N.B. In the above table, all percentages given are calculated as percentage of the total nitrogen present at the commencement of the series of experiments, 2.325 g.)

The solutions from decomposition of the barium precipitates, after the above determinations had been carried out, were evaporated to small bulk *in vacuo* till quite syrupy. The syrups were then shaken with a large bulk of absolute alcohol, when they were converted to pinkish-white granular masses; complete solidification is difficult to obtain unless the syrups be practically anhydrous. (A small portion of the solid was removed from alcohol, dried at 110°, and the nitrogen estimated—N = 10.84 %. The bulk of the

product was not dried down.) The nitrogen present in the solid product was entirely amino nitrogen; a portion of the alcohol was evaporated *in vacuo* and gave a semi-crystalline yellow mass.

Experiments with hydrolysed gelatin.

Precisely similar experiments to those carried out on caseinogen were also performed in the case of gelatin; the main object was to test the completeness of removal of amino nitrogen from solution, as the method of H. D. Dakin, using continuous extraction with butyl alcohol, breaks down when glycine is present in large quantity. Exact details of the method adopted need not be given here, as they were precisely the same as those described above. The main results were as follows:

Weight of nitrogen in hydrolysed liquid	= 21.25 g.
" " after phosphotungstate treatment	= 13.65 g.
" " " removal of Ba dicarboxylates	= 12.424 g.
" " as amino nitrogen	= 9.792 g.
= 78.81 % of total N present after removal of Ba dicarboxylates	

Only one-fourth of the solution at this stage (*i.e.* 100 cc. containing 3.106 g. of nitrogen) was worked up. The percentage of nitrogen contained in the first fractions of the barium precipitate was much higher than in the case of cabbage and caseinogen precipitates; the end point of the precipitation was, however, of the same order—87.73 % of the total nitrogen being removed (compared with 87.96 % in the caseinogen experiment).

The percentages of nitrogen in the four successive fractions of the barium precipitate were: 5.61 %, 2.30 %, 0.66 %, 0.29 %. Fraction I was decomposed and the extract examined separately; fractions II and III were combined and decomposed, etc.; fraction IV was decomposed and examined separately as before, the residual liquid was evaporated to small bulk and examined for total and amino nitrogen. The results are summarised below.

Gelatin: $\frac{1}{4}$ of solution after removal of dicarboxylates.

	Total N g.	%	Amino N g.	%	Non-amino N g.
Original solution	3.106	100.00	2.448	78.81	0.658
Fraction I	1.814	58.40	1.702	54.79	0.102
" II	0.907	28.13	0.644	19.93	0.263
" III					
" IV	0.015	0.48	—	—	—
Residual liquid	0.363	12.27	0.073	2.42	0.290
Totals	3.099	99.28	2.419	77.14	0.655
(cf. top line "Original solution")					

It will thus be seen that the removal of amino nitrogen by this method is almost complete in the case of gelatin.

The amino-acids themselves were obtained as before by evaporation of the solution from the decomposed barium precipitate *in vacuo* and treatment with absolute alcohol. The first fraction easily set to a white crystalline product, containing 12.96 % N, all in the form of amino nitrogen. The second fraction took much longer to granulate, being, after a week, still in a somewhat pasty form; this was due probably to incomplete drying in the vacuum desiccator. A small portion, dried at 105°, gave N = 11.56 %.

No experiments were made on the nature of the substances extracted by alcohol from the amino-acids; probably they were proline substances, which Dakin has shown to be present in large amount among the hydrolysis products of gelatin.

From the results given above, there can be little doubt that the baryta-carbon dioxide method can be applied to the isolation in crystalline form of the hydrolysis products of the proteins. In the above described experiments the separation of the amino-acids is not quite complete, but it is probable that it could be rendered more so by the addition of baryta and carbon dioxide to the filtrate from the four precipitations after this has been concentrated. It will be noticed that this residual fluid contains a relatively large amount of the nitrogen in the non-amino form.

It is proposed to investigate the method in greater detail, and to determine, amongst other things, whether it can be applied to a partial separation of the amino-acids from one another. It is also possible that it may be used for separating the diamino-acids from other bases. In view of the amount of time the various subsidiary investigations mentioned in this paper will take to complete, it has been thought advisable to publish a short account of some of the results obtained up to the present date.

REFERENCES.

- Chibnall and Schryver (1921). *Biochem. J.* 15, 60.
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