CCXXVIII. THE DICARBOXYLIC ACID-NITROGEN OF PROTEINS.
WITH A NOTE ON THE ALCOHOL-SOLUBLE PROTEIN FROM RAGI (*ELEUSINE CORACANA*).

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THE usual procedure for the Van Slyke determination of the nitrogen distribution of proteins [Plimmer, 1917] makes no provision for the dicarboxylic acids. A modification was described by Andersen and Roed-Muller [1915] in which the dicarboxylic acids were determined indirectly by the base bound at neutrality to phenolphthalein. With the only two proteins investigated by these authors the method gave satisfactory results but, involving as it does the complete absence of calcium salts and the previous removal of the diamino-acids, it calls for considerable modification of the ordinary Van Slyke procedure by which all the known proteins have been analysed. The presence of ammonia, of glucosamine or of organic acids was also shown to give rise to error. Probably for these reasons the method of Andersen and Roed-Muller has found no general application.

In the course of many protein analyses carried out in this laboratory it has been found that the lime-alcohol method of Foreman [1914] for precipitating the dicarboxylic acids gave very satisfactory results and that it could be easily incorporated into the ordinary procedure for Van Slyke analyses, so that the total dicarboxylic acids could be rapidly determined when working with only small quantities of material and without actual isolation of the amino-acids themselves.

The addition of this new group to the usual nitrogen distribution scheme is considered all the more desirable in view of the many errors to which the determination of the bases has been shown to be liable. The separation of the bases by means of phosphotungstic acid is neither quantitative nor specific [Thimann, 1930; Plimmer and Rosedale, 1925, 1; Gortner and Sandstrøm, 1925]. According to Plimmer [1916], the decomposition of histidine, amounting to 3–4 %, tends to give a higher value for arginine. To this must be added the possible presence in protein hydrolysates of the newly discovered amino-acid, citrulline [Wada, 1930], which also decomposes, giving ammonia on treatment with alkali. Finally it has to be remembered that peptides and, under certain

conditions, amino-acids such as phenylalanine and proline, are precipitated by phosphotungstic acid.

Apart from widening the scope of the Van Slyke method of analysis and providing an additional criterion for the characterisation of proteins in general, a knowledge of the dicarboxylic acid content of plant proteins is of importance on account of the possibly predominant rôle played by asparagine and glutamine in plant metabolism [Onslow, 1930].

The technique previously adopted in this laboratory was the modification of the method of Foreman [1914] described by Kingston and Schryver [1924] wherein barium hydroxide was substituted for calcium hydroxide. This modification is convenient in dealing with large amounts of protein as the quantity of alcohol required is much smaller, but it has the disadvantage that the amount of extraneous matter precipitated is appreciable [Jones and Moeller, 1928; Damodaran, 1931]. It was therefore decided to attempt to apply Foreman's original procedure to the Van Slyke method of small scale analyses.

Experiments were carried out on the following lines. (i) The completeness of the precipitation by alcohol of the calcium salts of glutamic and aspartic acids when these were present in small amount was tested. (ii) As the dicarboxylic acid precipitate is known to be contaminated with a small amount of material precipitable by phosphotungstic acid it was necessary to see if due allowance could easily be made for such material and a reasonably correct figure thus obtained for the basic N. (iii) When protein hydrolysates are treated by the Foreman method it is found that, after apparently complete precipitation of the dicarboxylic acids (as tested by adding small amounts of alcohol), a further flocculent precipitate always forms slowly either on standing for a long time or on the addition of large volumes of alcohol. With caseinogen Foreman showed that this precipitate contained no dicarboxylic acids. It was considered desirable to verify this with at least one other protein of a different class and to this end experiments were carried out on gliadin. (iv) Experiments were made on gliadin to see if reprecipitation were necessary when using calcium hydroxide. (v) The new modification was finally tested on five proteins whose dicarboxylic acid contents have been definitely determined by large scale analyses and was then applied to a number of proteins of different classes, the dicarboxylic acid contents of which are either unknown or not accurately known.

EXPERIMENTAL.

Completeness of the precipitation of glutamic and aspartic acids by calcium hydroxide and alcohol. 5 cc. of a solution of glutamic acid hydrochloride containing 0·1 g. of glutamic acid were made alkaline with calcium hydroxide and treated with 95 % alcohol till precipitation was complete, approximately 50 cc. being used. The precipitate was filtered at the pump and washed three times with 20 cc. portions of 95 % alcohol; 98·8 % of the glutamic acid was precipitated. A similar experiment with 5 cc. of a 0·2 % solution of aspartic acid showed that 99·1 % of the acid was precipitated.

Analysis of gliadin. It will be convenient here to describe in full the modified Foreman method as applied to gliadin.

Hydrolysis. 6 g. of gliadin (for duplicate analyses) were hydrolysed by boiling with 20 % hydrochloric acid for 18 hours. On an aliquot total N and amide-N were determined.

Amide-N. It was found convenient to determine the amide-N by distilling in steam a small aliquot of the hydrolysate with sodium hydroxide in a Parnas-Wagner micro-distillation apparatus for 4½ minutes. In most cases slightly higher results were obtained than those previously recorded, but this can hardly be ascribed to decomposition of arginine or other amino-acids as the period of distillation involved is so short.

The main fraction of the hydrolysate was next divided into two portions each of which was treated in the following way.

Removal of humin and ammonia. The solution was made strongly alkaline with calcium hydroxide (10 % suspension), the precipitated humin with the excess of calcium oxide centrifuged off and washed free from chlorides (three washings each with 50 cc. of warm water were usually required). The solution with the washings was evaporated *in vacuo* at 40° to a volume of 10–15 cc. The air admitted during distillation was freed from carbon dioxide by means of a soda-lime tube.

Precipitation of the dicarboxylic acids. To the syrup in the flask about 10 vols. of 95 % alcohol were added very slowly in small quantities at a time, with vigorous stirring, till precipitation was complete. This point was determined by transferring the mixture to a 250 cc. centrifuge-tube, centrifuging and testing by the addition of 10 cc. portions of alcohol. When no further precipitate formed the clear liquid was poured off and the precipitate, some of which adhered to the sides of the flask, was washed twice with 50 cc. portions of 95 % alcohol.

Treatment of the dicarboxylic precipitate. The precipitate was dissolved in a little acidulated water and the solution after making up to 25 cc. was analysed for nitrogen. The value obtained was 28·6 % of the total N. The remainder of the solution was transferred to a centrifuge-tube, and treated with 5 cc. of concentrated hydrochloric acid and a saturated aqueous solution of 5 g. of phosphotungstic acid. The precipitate formed was redissolved by warming in a water-bath and allowed to crystallise at room temperature for 2 days. It was then separated by centrifuging, washed with a solution of 2·5 g. phosphotungstic acid and 3·5 g. hydrochloric acid in 100 cc. water, and finally dissolved in a strong solution of sodium hydroxide. Nitrogen was determined on an aliquot. The basic material thus separated from the dicarboxylic acid precipitate contained 0·6 % of the total N. Allowing for this the value for the dicarboxylic acid-N was 28·0 %.

Precipitation of the bases. The alcoholic mother-liquor and washings from the calcium dicarboxylates were made neutral with acetic acid, evaporated *in vacuo* to remove all alcohol and made up to a volume of 50 cc. The precipitation

with phosphotungstic acid was now carried out according to the Van Slyke procedure, except that the precipitate was separated by centrifuging instead of by filtration. It was dissolved in a strong solution of sodium hydroxide and nitrogen was determined on an aliquot.

The mother-liquor and washings were treated as in the usual Van Slyke analysis and both total N and amino-N determined.

Effect of the precipitation of the dicarboxylic acids on the "basic" N. Parallel with the analysis of gliadin already described another determination was made in which the normal Van Slyke procedure was followed, the bases being precipitated directly after the removal of ammonia. The value thus obtained was 10·7 %. No solubility correction was applied in view of the recent work of Thimann [1930] on the bases. In the first analysis, the basic material precipitated from the dicarboxylic acid fraction was 0·6 % (in terms of total N) and 10·2 % from the remaining solution, thus giving a total value of 10·8 %. The agreement is considered sufficiently good for present purposes.

Similar experiments were made with certain other proteins, the basic N being determined in one case directly and in the other after precipitation of the dicarboxylic acids. It was found that varying amounts of basic material were removed with the dicarboxylic acids, a maximum of 2·2 % of the total N being found with caseinogen. Experience suggests that the governing factor is not the percentage of the bases present in the protein, but the physical condition of the precipitate and that by adding the alcohol in small quantities with vigorous shaking the amount of basic impurities could be reduced to a minimum. In all cases the bases were quantitatively recovered by precipitating the dicarboxylic acid solution itself with phosphotungstic acid.

Examination of the small precipitate obtained on adding large volumes of alcohol to the solution from which the calcium dicarboxylates had been precipitated as above. 10 g. of gliadin were hydrolysed and dicarboxylic acids separated as before. To the mother-liquid thus obtained an equal volume of alcohol was added and the solution kept overnight. The precipitate was filtered off and dissolved in the minimal amount of concentrated hydrochloric acid; the solution was saturated with hydrogen chloride gas and kept in the ice-chest. No separation of glutamic acid hydrochloride was observed. A similar small precipitate obtained from another 10 g. of gliadin hydrolysate was treated with copper oxide. No insoluble copper salt was obtained. These results confirm those of Foreman with caseinogen.

Effect of dissolving the dicarboxylic acid fraction and reprecipitating with alcohol. The precipitate obtained from gliadin with calcium hydroxide and alcohol, as already described, which contained 28·0 % of the total nitrogen was dissolved in about 15 cc. of water and reprecipitated by the addition of alcohol as before. The nitrogen content of the second precipitate thus obtained was 27·7 % of the total protein-N, showing that the amount of impurities removed by a second precipitation was inconsiderable.

Analysis of various proteins.

Of the proteins analysed the following were prepared in the laboratory according to the usual methods: gliadin, zein, edestin (from hemp-seed), haemoglobin and serum-globulin (from horse-blood). B.D.H. preparations of the following proteins were used: caseinogen, egg-albumin, pepsin. The leaf proteins analysed were the original preparations of these proteins made by Prof. Chibnall to whom I am indebted for their gift.

The nitrogen contents of the preparations used (calculated on an ash- and moisture-free basis) are given in column 3 of Table I. Though complete analyses were carried out on most of the proteins examined only the figures relevant to the present purpose are quoted. The amide-N figures are included in every case on account of their significance with regard to the relationship that is usually assumed to exist between the ammonia and the dicarboxylic acids resulting from the acid hydrolysis of proteins.

Table I. *Analysis of certain proteins.*

All figures given are in percentages of total protein nitrogen.
(A, present analyses. B, other workers*.)

							Basic N				
							A				
								Indirectly precipitated			
			Amide-N		Dicarboxyl c acid-N						
	Protein	Total N	A	B	A	B	Directly pre-cipitated	In dicarb-oxylic acid fraction	In main solution	Total	B
Proteins recently analysed for their dicarboxylic acid content:											
1	Gliadin	17·3	25·7	25·52 24·61 26·13 25·9	28·0	26·79[1]	10·7	0·61	10·2	10·8	7·26 5·61 12·91 10·97
2	Edestin	18·5	10·2	9·99	15·0	15·6[1]	35·1	1·84	32·63	34·5	38·15 31·7
3	Caseinogen	14·6	10·3	10·31 10·43	15·4	14·8[2]	23·1	2·20	20·45	22·7	24·12
4	Egg-albumin	14·9	8·8	8·65	14·7	12·3[1]	24·2	0·84	23·09	23·9	21·27
5	Zein	16·0	19·6	20·75	17·5	18·56[3]	—	0·56	2·90	3·5	3·1
Other proteins:											
6	Haemoglobin	16·9	5·6	5·24 6·37	9·4	—	—	0·44	28·80	29·2	31·3
7	Serum-globulin	15·9	8·1	—	13·1	—	—	1·21	22·77	24·0	25·25
8	Pepsin	13·4	3·6	—	12·3	—	—	0·35	21·2	21·6	—
Leaf proteins:											
9	Spinacin	16·3	—	6·93[4]	17·7						
10	*Zea Mays*	14·4	—	7·44[4]	21·9						
11	Runner bean	15·9	—	5·69[4]	20·8						
12	Alfalfa	15·7	—	5·5[4]	20·1						

(1) Jones and Moeller [1928]. (2) Foreman [1914]. (3) Dakin [1923]. (4) Chibnall and Grover [1926].

* Except where specific reference numbers are given all data in columns B are taken from Plimmer [1917], or Hoffmann and Gortner [1925].

Discussion.

The values for the dicarboxylic acids for the proteins 1–5 given by the method now described are in close agreement with those of previous workers who have used modern methods for the direct isolation of the acids themselves, and show that a good insight into the dicarboxylic acid content of proteins can be obtained by this simple addition to the usual procedure of Van Slyke. The values for the basic N determined directly and indirectly also show that the modification introduces no appreciable error in the determination of this group.

It is interesting to re-examine in the light of the analytical results now recorded the close agreement found by Osborne, Leavenworth and Brautlecht [1908] between the amide-N of proteins and their content of dicarboxylic acids. Assuming that one carboxyl of the acids was present as an amide group they calculated the proportion of ammonia which should be liberated by acid hydrolysis and compared this with the amount actually found. In Table II their results, recalculated to conform with the data given in Table I, are compared with those now available. Whereas Osborne found the agreement was fair in most cases, in others, especially the prolamins, the divergence was wide. The discovery of hydroxyglutamic acid and improvements in the methods for estimating glutamic and aspartic acids have so increased the total dicarboxylic acid content that the prolamins now constitute the only group which shows anything approaching agreement; the others all have an excess of dicarboxylic acids. This is very marked in the case of the leaf proteins which exhibit, as a class, the same regularity in dicarboxylic acid content as Chibnall and Grover [1926] found for the bases. Such a close agreement in amino-acid composition is not found in any class of seed proteins.

Table II.

Protein	Dicarboxylic N (Osborne)	Dicarboxylic N (from Table I)	Amide-N (Osborne)
Edestin	9·68	15·6	9·99
Glutenin	13·33	16·4[1]	18·87
Caseinogen... ...	7·28	15·4	8·49
Ovalbumin... ...	7·12	14·7	8·66
Gliadin	20·47	28·0	24·61
Zein...	11·69	18·56	18·44
Hordein	20·1	23·6[2]	23·31
Prolamin from Ragi	—	22·3[3]	21·4[3]

[1] Damodaran [1931]. [2] Kleinschmidt [1907]. [3] Damodaran, this paper.

In view of the large margin of error in calculating the values for the different amino-acids in the basic precipitate it is tentatively suggested that the determination of the following groups would give a reliable distribution of nitrogen for the characterisation of proteins:

(i) Amide-N.
(ii) Humin-N.
(iii) Dicarboxylic acid-N.
(iv) Basic N.

(v) Arginine-N, determined directly according to Plimmer and Rosedale [1925, 2].

(vi) Non-amino-N.

(vii) Monoaminomonocarboxylic acid-N.

NOTE ON THE ALCOHOL-SOLUBLE PROTEIN FROM RAGI (*ELEUSINE CORACANA*).

Ragi (*Eleusine coracana*) forms the staple cereal food of the people in some of the drier parts of India. It was hoped to make a study of all the proteins in this seed, but unfortunately only one, a prolamin, could be prepared in a state of purity.

The average protein content of the seed was 6·8 % (N × 6·35) and it consisted of a globulin, a prolamin, and a glutenin. From the finely ground ether-extracted seed a 9 % solution of sodium chloride (found by trial to be the optimal strength) extracted 29·2 % of the total N. The residue after being washed free from sodium chloride and air-dried was exhaustively extracted with warm 73 % alcohol to dissolve out the prolamin. 40·2 % of the total N was removed by this treatment. The residual material was finally treated with 0·4 % sodium hydroxide which dissolved a further 12·6 % of the N. 82·8 % of the seed-N was thus accounted for. Attempts to isolate the globulin by dialysis of the sodium chloride extract and of the glutenin by precipitation from the sodium hydroxide solution gave products which were impure and contained only about 12 % N. Variations in the method, such as treatment with takadiastase to remove carbohydrate, and extraction with alcoholic potash as recommended by Jones and Csonka [1927] were tried without success.

EXPERIMENTAL.

It was found that the quickest and most satisfactory method for preparing the prolamin was to extract the finely ground seed with boiling 73 % alcohol for 1 hour, filter and evaporate *in vacuo* to small volume. By preventing the alcoholic strength from going much below 70 %, either by the addition of further portions of the extract or of alcohol, the extract could be concentrated to a thick syrup without causing any denaturation of the protein. The syrupy solution so obtained was poured with vigorous stirring, while still warm, into about 20 volumes of acetone, when the protein was precipitated as a fine powder.

The substance, after filtering at the pump and drying in air, was purified by redissolving in a small volume of warm 73 % alcohol and reprecipitating with acetone as before. The air-dried powder contained 6·1 % moisture, 0·9 % ash, and moisture- and ash-free, 16·4 % N. A portion was redissolved in alcohol and again precipitated with acetone; but the nitrogen content was not

thereby increased. Tests for sulphur were negative. The isoelectric point of the protein, determined approximately by dialysing a suspension in distilled water in a cellophane bag for 48 hours, and measuring electrometrically the minimal p_H attained, gave a value between p_H 4·8 and p_H 5·0.

The prolamin was analysed by the method outlined in this paper.

Nitrogen distribution.

	% total N
Humin-N	0·8
Amide-N	21·4
Dicarboxylic acid-N...	22·5
Basic N	5·8
Arginine-N	2·2
Monoamino-monocarboxylic acid-N	44·1
Non-amino-N	5·1

It will be seen that the protein has an amino-acid composition characteristic of the prolamins. The amide-N and dicarboxylic acid-N are high, and in close agreement, while the basic N is relatively low [Damodaran, 1931].

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