CIX. THE NON-PROTEIN NITROGEN OF PULSES.

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The saline extract obtained in the preparation of globulins and albumins from pulses contains a non-protein nitrogenous fraction, which is dialysable, not coagulated by heat and not precipitated by saturation with salts or even by reagents such as trichloroacetic acid. Work on foodstuffs relates chiefly to the predominant protein characterising the seed and in the case of pulses the well defined and easily characterisable globulins have received the greatest amount of attention. The methods usually employed for their isolation render recovery of the non-protein nitrogenous bodies extremely difficult and cumbersome. Further, at the moment, there are no systematic methods for investigating a mixture of the complexity represented by this fraction. It is not surprising therefore that this valuable fraction has not hitherto received adequate attention.

It has been suggested that this non-protein fraction arises during the course of the preparation of proteins and that it is not originally present as such in the seed; but experiments have shown that it persists under carefully controlled conditions which preclude its enzymic formation. Whatever be the mechanism of its origin, it has not been generally taken into account in any of the investigations carried out on pulses.

Blish [1918] has investigated the non-protein nitrogen of wheat flour and indicated the probable presence of a non-protein nitrogenous substance not precipitated by copper hydroxide, non-amino and non-peptide in character. Ranga Rao and Sreenivasaya [1933, 1] have shown that the non-protein fractions of the body fluids of the lac insect obtained by (1) salt saturation and (2) coagulation by acetic acid, contain simple polypeptides which have an average complexity (ratio of total to amino-nitrogen) of 4·7. They [1933, 2] have further demonstrated the presence of free tyrosine which occurs to the extent of about 2·5 % of the non-protein fraction. Sreenivasan and Subrahmanyan [1934] have drawn attention to the presence of non-coagulable forms of nitrogen in the saline extract of leguminous seeds which may have great nutritive value.

In view of their simpler structure these nitrogenous compounds should be among the most easily digestible and assimilable; they may help the peptisation of the associated proteins and influence their enzymic digestion by providing the necessary activators and further they may supplement the nutritional deficiencies of the associated proteins and enhance their biological value. It is therefore obvious that this non-protein fraction merits a detailed investigation from all points of view and the present study relates to a determination of the percentage and average complexity of the non-protein nitrogen lost during the preparation of proteins by various methods.

Experimental.

Nine of the well-known Indian pulses, after air-drying, were powdered to pass through a 40-mesh sieve; with the exception of *Cajanus indicus*, *Lens esculenta*, *Cicer arietinum* and *Phaseolus mungo* which could be easily freed from husks, the pulses were powdered along with the husk. The meal (100 g.) was extracted for 24 hours at 0° with 100 ml. of 5 % NaCl by shaking the mixture at frequent intervals. On filtering the mixture a clear solution was obtained. The entire operation was carried out in a refrigerator maintained at 0° and in presence of toluene.

Total nitrogen was determined by the Kjeldahl method and amino-nitrogen by the method of Van Slyke in all the solutions examined. Ultrafiltration was carried out through collodion membranes under a pressure of 50 kg. per cm.^{2} employing 50 ml. of the saline extract, which was kept electromagnetically stirred during the filtration. This facilitated rapid filtration and prevented clogging of the pores of the membrane.

The trichloroacetic acid filtrate was obtained by treating 20 ml. of the saline extract with 5 ml. of 20 % trichloroacetic acid. The precipitate was filtered off, washed with 2 % trichloroacetic acid and the filtrate made up to 50 ml. Aliquots of 20 and 10 ml. were used for the total and amino-nitrogen respectively.

20 ml. of the saline extract were kept in a steam steriliser for 1 hour, the coagulated protein was filtered off, washed with 5 % NaCl and the filtrate made up to 50 ml. 20 and 10 ml. were used for the total and amino-nitrogen respectively.

Dialysis was carried out in collodion bags (prepared from a 6 % solution of pyroxylin (B.D.H.) in ether-alcohol mixture) against distilled water until the dialysate was free from chloride. The contents of the bag along with the precipitated protein were transferred to a beaker and treated with a sufficient quantity of sodium chloride to redissolve the precipitate and the volume was made up to 150 ml. with 5 % NaCl. An aliquot of 20 ml. was used for total nitrogen. The difference between this value and the total nitrogen of the crude extract gave the value for the diffusible non-protein nitrogen.

Ammonium sulphate was employed for the salt saturation experiments, since it is commonly used in all preparative work. The precipitate thus obtained was centrifuged, washed with a saturated solution of ammonium sulphate, redissolved in water and reprecipitated with 10 ml. of 20 % trichloroacetic acid. The precipitate was filtered off, washed with 2 % trichloroacetic acid until free from ammonia and used for determination of the total nitrogen. The difference

Table I.

	Percentage of nitrogen lost during the isolation of proteins by				
	Ultra-filtration	Trichloro-acetic acid pptn.	Heat coagulation	Dialysis	Saturation with $(NH_4)_2SO_4$
Cicer arietinum	12·1	19·1	—	19·6	23·4
P. mungo	9·3	24·7	—	—	24·2
P. radiatus	8·5	7·1	12·7	—	—
Vigna catiang	11·9	14·5	23·6	25·4	39·8
Dolichos Lablab	16·0	14·9	36·0	4·6	26·1
Cajanus indicus	14·3	23·5	45·6	25·7	23·6
Lens esculenta	26·1	29·3	46·2	52·1	43·6
P. aconitifolius	27·6	29·5	40·5	55·3	43·3
Dolichos biflorus	20·7	29·2	55·1	55·2	40·7

between this value and the total nitrogen of the saline extract represents the non-protein fraction not usually investigated.

Table I gives the percentage of nitrogen lost during the isolation of proteins by several methods adopted, whilst Table II gives the average complexity of the nitrogenous bodies in the crude saline extract and in the several non-protein fractions.

Table II. *Average complexity of the nitrogenous bodies in the crude saline extract and in the non-protein fraction.*

	Crude extract	Ultrafiltrate	$CCl_3.COOH$ pptn.	Heat coagulation
Cicer arietinum	10·6	3·8	3·5	6·3
P. mungo	13·6	2·5	3·7	7·0
P. radiatus	15·9	1·8	2·8	4·5
Vigna catiang	10·4	2·3	2·1	3·1
Dolichos Lablab	11·2	2·9	2·6	6·8
Cajanus indicus	6·4	2·0	2·0	4·0
Lens esculenta	7·1	2·5	2·9	4·8
P. aconitifolius	7·0	2·0	2·7	2·8
Dolichos biflorus	7·4	2·6	2·6	4·4

Discussion.

The non-protein nitrogen has been determined by five different methods each of them representing a well-defined process conventionally adopted in the study of proteins. The quantity thus lost varies from 10 to 55 % of the nitrogen extractable by saline solution, depending upon (1) the nature of the pulse under investigation and (2) the method adopted for the estimation of the non-protein fraction. Trichloroacetic acid is usually employed as a precipitant for proteins in various physiological fluids. Dialysis is a recognised method for the isolation of the globulins which are thrown out of solution as the dialysis proceeds, while saturation with ammonium sulphate offers a convenient method for the precipitation and purification of globulins and albumins. Ultrafiltration was adopted as a method of isolating the non-protein nitrogen of the saline extracts, since it offers possibilities of fractionating the nitrogenous bodies without contaminating extracts with acids and salts difficult to eliminate at a later stage. In the present study however only one grade of membrane has been used for all the pulses investigated.

Heat brings about coagulation not only of the globulins but also of the albumins and practically the same result is achieved by saturation with ammonium sulphate but with this difference, that the proteins are not denatured. There should therefore be fair agreement between the two sets of values; such agreement does indeed exist in the cases of the last three pulses in Table II. The higher the average complexity of the nitrogenous bodies in the crude extract, the less will be the nitrogen lost in the filtrate after heat coagulation. *P. radiatus*, for example, whose crude saline extract has the highest average complexity in the series (15·9), suffers the least loss of nitrogen not only during heat coagulation but also during ultrafiltration and precipitation with trichloroacetic acid. Similarly it will be observed that the last four pulses in Tables I and II, whose nitrogenous bodies in the saline extract possess lower complexities (6·4–7·4), yield high percentages of the non-protein fraction on heat coagulation and also on ultrafiltration and precipitation with trichloroacetic acid.

It will be seen that there is general agreement between ultrafiltration and trichloroacetic acid precipitation except in the case of *P. mungo*, which indicates

that the membrane used for ultrafiltration is impermeable to all that the trichloroacetic acid is able to precipitate. This is born out by the fact that none of the ultrafiltrates yields any precipitate with trichloroacetic acid.

The average complexities of the "non-protein" fractions obtained by ultrafiltration and precipitation with trichloroacetic acid, in the case of the pulses examined, are always lower than the corresponding figure for the fraction obtained by heat coagulation, whose uniformly higher complexity indicates the presence or peptones and higher polypeptides in the filtrate. Further confirmation of this fact is obtained by the observation that the filtrates after heat coagulation give a further precipitate with trichloroacetic acid. Particular interest attaches to the heat-coagulated fraction since heat coagulation represents the nearest approach to the conditions of cooking and corresponds to the portion generally administered to invalids and children. It is interesting to observe that the average complexities of the fractions in general run parallel with the recognised ease of their digestibilities. The non-protein fraction of *P. aconitifolius*, for instance, has the lowest ratio of 2·8 in the heat coagulation series, a fact which is in harmony with the reputation which this pulse enjoys as a very easily digestible and assimilable source of nitrogen during convalescence. This is apparently contradictory to the findings of Niyogi *et al.* [1932] that the protein of *P. aconitifolius* is poor both as regards its digestibility and biological value. It only suggests the supplemental value of the non-protein fraction and emphasises its importance in influencing the digestibility and biological value of the protein.

The saline extracts, the ultrafiltrates and the heat-coagulated filtrates were examined to see if they contained any of the essential amino-acids in a free condition. An active preparation of tyrosinase from *Dolichos Lablab*, was employed to test for tyrosine and related compounds like 3:4-dihydroxyphenylalanine. In view of the high concentration of chlorides and carbohydrates, tryptophan could not be tested for with glyoxylic acid. Results of the tyrosine test, given in Table III, indicate that only two of the pulses examined, *P. aconitifolius* and *P. radiatus*, show the presence of tyrosine definitely, the former containing a much higher concentration of the amino-acid, in all the three different filtrates tested. In the case of *Dolichos*, tyrosine could not be detected in the heat-coagulated filtrate, while its presence was unmistakable in the crude extract and in the ultrafiltrate. This observation together with the fact that the *Dolichos* extract gradually darkens on keeping, points to the conclusion that tyrosine is gradually liberated from the proteins through the action of the associated protease which becomes inactivated during the process of heat coagulation.

Table III.

	Saline extract	Ultrafiltrate	Heat-coagulated filtrate
P. aconitifolius	+ + +	+ + +	+ + +
P. radiatus	+	+	+
D. Lablab	+	+	−

Summary.

The saline extracts obtained during the preparation of globulins and albumins from pulses, contain a non-protein nitrogenous fraction, which occurs to the extent of 10–55 % of the total nitrogen depending upon the nature of the pulse and the method of determination.

This non-protein fraction has been shown to contain the simpler, easily digestible and assimilable peptides which have an average complexity varying from 1·8 to 3·8. In some cases the existence of free tyrosine or simple derivatives thereof has been demonstrated.

Attention has been drawn to the fact that heat coagulation constitutes the nearest approach to the conditions of cooking and the fraction thus obtained represents the portion usually administered to children and invalids.

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REFERENCES.

Blish (1918). *J. Biol. Chem.* **33**, 551.
Niyogi, Narayana and Desai (1932). *Ind. J. Med. Res.* **19**, 859.
Ranga Rao and Sreenivasaya (1933, 1). *J. Ind. Inst. Sci.* **16** A, 76.
—— —— (1933, 2). *Current Sci.* **1**, 378.
Sreenivasan and Subrahmanyan (1934). *J. Ind. Inst. Sci.* **17** A, 49.