STUDIES IN THE PROTEINS OF INDIAN FOODSTUFFS.

PART VIII. ON THE HEAT COAGULATION OF GLOBULINS FROM *VIGNA CATIANG*, WALP., & *P. ACONITIFOLIUS* JACQ.

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One of the striking properties of globulins in general, is its coagulability by heat, which is accompanied by certain changes—physical, chemical and physiological.

The more important physical changes induced in proteins may be summarised as follows:—(1) The viscosity *e.g.*, of the egg albumin is increased by denaturation (Loughlin and Lewis, *Biochem. J.*, 1932, **26**,476), and the extent of increase depends upon protein concentration, P_H and other factors; (2) there is definite increase in refractivity, as observed by Barker (*J. Biol. Chem.*, 1934, **104**, 667.) in the case of egg albumin; and (3) the specific optical rotation is increased (in a negative sense) by denaturation (Holden and Freeman, *Australian J. Exp. Biol. and Med. Sci.*, 1930, 7, 13).

CHEMICAL CHANGES.

It is known that during the denaturation of proteins by heat, nitrogen is not split off (Sorensen and Sorensen, *Compt. Rend. Lab. Carlsberg*, 1925, **9**, 15) and that sulphydryl groups, not detectable in native protein, are detectable in the denatured proteins (Arnold, *Z. Physiol. Chem.*, 1910, **70**, 300). Denaturation is catalysed by an increase in acidity or alkalinity, by alcohol, acetone, urea, thiocynate, iodide and salicylate. Heavy metal ions catalyse denaturation in acid solution (Thomas and Norris, *J. Amer. Chem. Soc.*, 1925, **47**, 501).

PHYSIOLOGICAL CHANGES.

Wu, Broeck and Li (*Proc. Soc. Exp. Biol. & Med.*, 1927, **24**, 472) have investigated the antigenic properties of egg albumin and have concluded that both the precipitin and anaphylactic reactions are altered by denaturation. Bizano (*J. Physiol.*, 1913, **46**, 267) and Mastin and Schryver (*Biochem. J.*, 1926, **20**, 1177) have demonstrated that heating increases the ease of digestibility by pepsin and trypsin.

The present investigation relates to a study of (1) the physical changes accompanying coagulation, and (2) to determine if the method of heat coagulation could be developed, by careful experimental control, into a method of fractionating a mixture of proteins. Johns and Waterman have, by the method of heat coagulation, shown the presence of an α and a β fraction in the total globulins of the velvet bean (*J. Biol. Chem.*, 1920, **43**, 59) and mung bean (*J. Biol. Chem.*, 1920, **44**, 303) but an analysis of these fractions have not been carried out. We have extended the study to a fractionation of the globulins of the cow pea and the aconite bean and have carried out an analysis of all the fractions.

EXPERIMENTAL.

Both the globulins from cow pea as well as from aconite bean, were subjected to a fractionation by the method of heat coagulation and at the separation of each successive fraction, a physico-chemical study of the resulting filtrate was made. This involved (1) a determination of the hydrogen ion concentration, (2) optical activity, and (3) total nitrogen. Cow pea globulins for these studies were obtained by dialysis, while the globulins of the aconite bean were prepared by the method of dilution and carbon dioxide saturation. The protein preparations in their "wet" condition, were redispersed in saline solution, filtered and the contents of their total nitrogen (consequently their protein content) were determined by Kjeldahl.

With a view to determine the temperatures of coagulation of the various protein fractions, a preliminary experiment was carried out in an apparatus specially constructed for the purpose. The arrangement consists of a water-bath whose temperature is gradually raised by a micro-burner at the slow rate of 0.5° per minute. A thin-walled test-tube containing the clear protein solution under experiment is immersed in the bath. A thermometer in the test-tube and another one in the bath respectively, recorded the temperatures of the solution and the bath. The development of the slightest turbidity or opalescence can be easily detected by the dark background and a diagonally oriented illumination which are provided in the arrangement. The temperature at which the opalescence is developed, is recorded and this temperature is maintained for 30 minutes during which a complete flocculation of the fraction coagulable at that temperature, takes place. The solution is centrifuged or filtered off, and the clear liquid heated gradually to a higher temperature. This operation is continued in stages until the boiling point of the water-bath. The temperatures at which definite fractions

come down with the globulins of cow pea and aconite bean, are given in Table I.

TABLE I.

(*Degrees Centigrade*)

	1	2	3	4	5
Cow pea	52–53°	72–73°	78–79°	89–90°	93–94°
Aconite bean	68–69°	80–81°	84–85°	90–91°	94–95°

These preliminary experiments were also helpful in obtaining an idea of the approximate yield of the fractions at each of the coagulation points. Another set of experiments was carried out with the globulins of cow pea, where a slight modification in the experimental procedure was introduced. 25 c.c. of the saline extract measured into 25 c.c. volumetric flasks, I, II, III, IV and V, were respectively immersed in baths maintained at 52°, 72°, 78°, 89°, and 93° for one hour. The flasks were cooled, the contents filtered and aliquots of the filtrate were utilised for the determination of total nitrogen, hydrogen ion concentration and optical activity. The results are given in Table II.

TABLE II.

(*Cow pea Globulins*)

Temperature of coagulation (Degrees Centigrade)	P_H	Mgms. of nitrogen removed	Fall in rotation per 100 mgms. of N removed
52	4·95	0·00	0·00
72	5·10	0·60	1·17
78	5·15	35·4	0·31
89	..	195·8	0·22

It will be seen from Table II that the highest yield of protein is obtained at 89°C. and that the fraction removed at 72°C. has the highest specific rotation. Basing our experience on these results, the experiments were repeated on a larger scale with 400 c.c. of the globulin extract, with a view to obtain useful amounts of the several fractions

for subsequent analysis. Table III gives the results of these experiments. The differences observed in the two sets of values given in Tables II and III, are attributable to a change in procedure adopted in the large-scale experiment. While in the previous experiments, separate batches of the globulin extract, were subjected to heating for a definite interval of time, in the next experiment, the same batch of extract was employed for obtaining the several fractions at various temperatures and the extract was, therefore, subjected to heating for a very much longer time.

TABLE III.

(Cow pea Globulins).

Temperature of coagulation (Degrees Centigrade)	Fall in optical rotation	Mgms. of Nitrogen removed	Fall in optical rotation per 100 mgms. of N removed
52—53	0·00	0·0	0·0
71—72	0·14	19·0	0·74
78—79	0·10	13·2	0·76
88—89	0·25	152·9	0·16
95—96	0·28	191·9	0·14

The above set of experiments was repeated with the globulins of aconite bean and Table IV incorporates the results.

TABLE IV.

(Aconite Bean Globulins)

Temperature of coagulation	Fall in optical rotation	Mgms. of Nitrogen removed	Fall in optical rotation per 100 mgms. of N removed
Total globulins.	0 00	0·00	0·00
68—69	0·64	33·6	1·91
78—79	0·14	32·0	0·42
84—85	0·23	101·8	0·23
90—91	0·58	169·8	0·34
94—95	0·11	65·0	0·17

The filtrates, after removal of the last fraction, were tested with (1) trichloracetic acid, and (2) dilution with water and CO_2 saturation. It was found that cow pea filtrate did not give any precipitate with either of the treatments and the residual nitrogen amounted to about 9 per cent. of the total nitrogen present at the start. The filtrate from the aconite bean globulins, gave precipitates with both the treatments and 50 per cent. of the total was accounted for as the residual nitrogen in the final filtrate, thereby showing the presence of a thermostable globulin in the aconite bean. Further confirmation of this is obtained by the high rotation obtaining in the filtrate (0.33° in 100 mm. tube) in the case of the aconite bean. The corresponding value for cow pea filtrate is 0.03°.

It will be observed from the two Tables III and IV that a definite fractionation has been accomplished by the method of heat coagulation and this is supported by two facts: (1) The fractions are obtained at definite and reproducible temperatures of coagulation, and (2) the fall in optical rotation per 100 mgms. of nitrogen (given in column four of the Tables) is different, at each removal of the fraction. If the same type of protein were being removed, there should not have been any variation in the fall of the optical rotation per 100 mgms. of nitrogen. Further confirmation of the fractionation is obtained by the analysis of the essential amino acids of these fractions (according to the method of Folin and Merenzi, *J. Biol. Chem.*, 1929, **83**, 89, 103).

TABLE V.

(*Percentages on the Weight of the Protein*)

	Cow pea			Aconite Bean		
	Moisture	Ash	Nitrogen	Moisture	Ash	Nitrogen
			(Moisture and Ash free)			(Moisture and Ash free)
I.	9·55	0·60	15·92	11·00	..	13·39
II.	9·03	0·40	17·81	11·54	0·22	16·86
III.	9·33	0·52	15·73	10·09	..	16·65
IV.	10·30	0·20	15·77	11·41	1·20	17·00

It will be seen from the above Table that purer preparations are obtained at higher temperatures of coagulation. The first fraction represents the least pure preparation, since the coagulation carries down all the associated impurities by adsorption.

TABLE VI.

(*Percentages on Total Nitrogen*)

		Cystine	Tyrosine	Tryptophane
Cow pea	I.	0·53	4·37	0·82
	II.	0·33	4·03	0·54
	III.	0·14	2·94	0·39
	IV.	0·33	3·13	0·69
Aconite bean	I.	0·57	1·94	0·51
	II.	0·56	1·57	0·37
	III.	0·41	1·47	0·41
	IV.	0·34	1·42	0·43

A study of the Table VI will reveal distinct differences in amino acid make-up of the fractions, establishing thereby that a definite fractionation of the total globulins has been achieved by the method of heat coagulation.

SUMMARY AND CONCLUSIONS.

1. The mechanism of the nature of heat denaturation, physical, chemical and physiological, is discussed and the whole phenomena require further elucidation and clarification.

2. The globulins of cow pea and aconite bean can be definitely fractionated by the method of heat coagulation; this is supported by (1) the sharp temperatures of coagulation for each of the fractions, (2) the variations in the fall of optical activity per 100 milligrams of nitrogen removed (Tables IV & V), and (3) striking and significant differences in the essential amino acid make-up of the fractions (Table VI).

3. The existence of a thermostable globulin to the extent of 50 per cent. in aconite bean is noteworthy.

My best thanks are due to Mr. M. Sreenivasaya for his many useful suggestions and to Dr. V. Subrahmanyan for his kind interest in the investigations.

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[Received, 9–12–1935.]

2114-35—Printed at The Bangalore Press, Mysore Road, Bangalore City, by G. Sreenivasa Rao, Superintendent.