II.—STUDIES IN ENZYME ACTION—PART VI.
HEAT INACTIVATION OF PANCREATIC AMYLASE.

By K. Venkata Giri.

The rate of inactivation of enzymes under varying conditions has been studied by several observers. It has been found in general that the course of the reaction is unimolecular. Tammann (*Z. Physikal. Chem.*, 1895, 18, 426) showed this to be the case for emulsin, and Pace (*Biochem. J.*, 1930, 24, 606) obtained similar results with purified trypsin. Pancreatic amylase is an enzyme which loses its activity very rapidly, inactivation occurring at an appreciable rate at room temperatures. It thus forms a very suitable material for examination, in addition to which its importance in physiological processes and industry renders its study very desirable with a view to finding means of retarding as far as possible its spontaneous inactivation. The following experiments were consequently undertaken.

Experimental.

A preparation of pancreatin supplied by Messrs. The British Drug Houses, Ltd., was used in the present investigation. In view of its hygroscopic character, the specimen was dried in a desiccator over anhydrous calcium chloride for at least 24 hours before use. The solutions of pancreatin were made up with conductivity water particularly for experiments in which the kinetics of inactivation had to be followed without the addition of buffers. The starch solution used for the experiments was prepared from the B.D.H., A.R. soluble starch which was washed several times with water, then with alcohol and finally with ether and dried. A 2 per cent. solution of starch was generally used for determining the activity of the enzyme. The reaction was adjusted to P_H 7·1 by adding Sörensen's phosphate buffer. Pure sodium chloride was added to make up a 0·3 per cent. solution of that salt so as to secure the maximum activity of the enzyme under the experimental conditions. Activity was determined according to Sherman and Schlesinger (*J. Amer. Chem. Soc.*, 1915, 37, 1305).

Effect of concentration on the rate of inactivation of pancreatic amylase.—Preliminary observations having shown that in the initial stage of the reaction the rate of inactivation of pancreatic amylase at 40° conforms to the law for a unimolecular reaction, the effect of varying the concentration was examined. Solutions containing 0·05, 0·10, and 0·16 per cent., respectively, of the solid enzyme were maintained at 40° in a thermostat and the activities determined at frequent intervals. The results are shown in Table I. Curves showing the relation between the logarithm of the activity and time are given in Fig. I.

It will be observed that in the initial stages of the reaction these are approximately straight lines and parallel to each other, showing that the

Fig. I

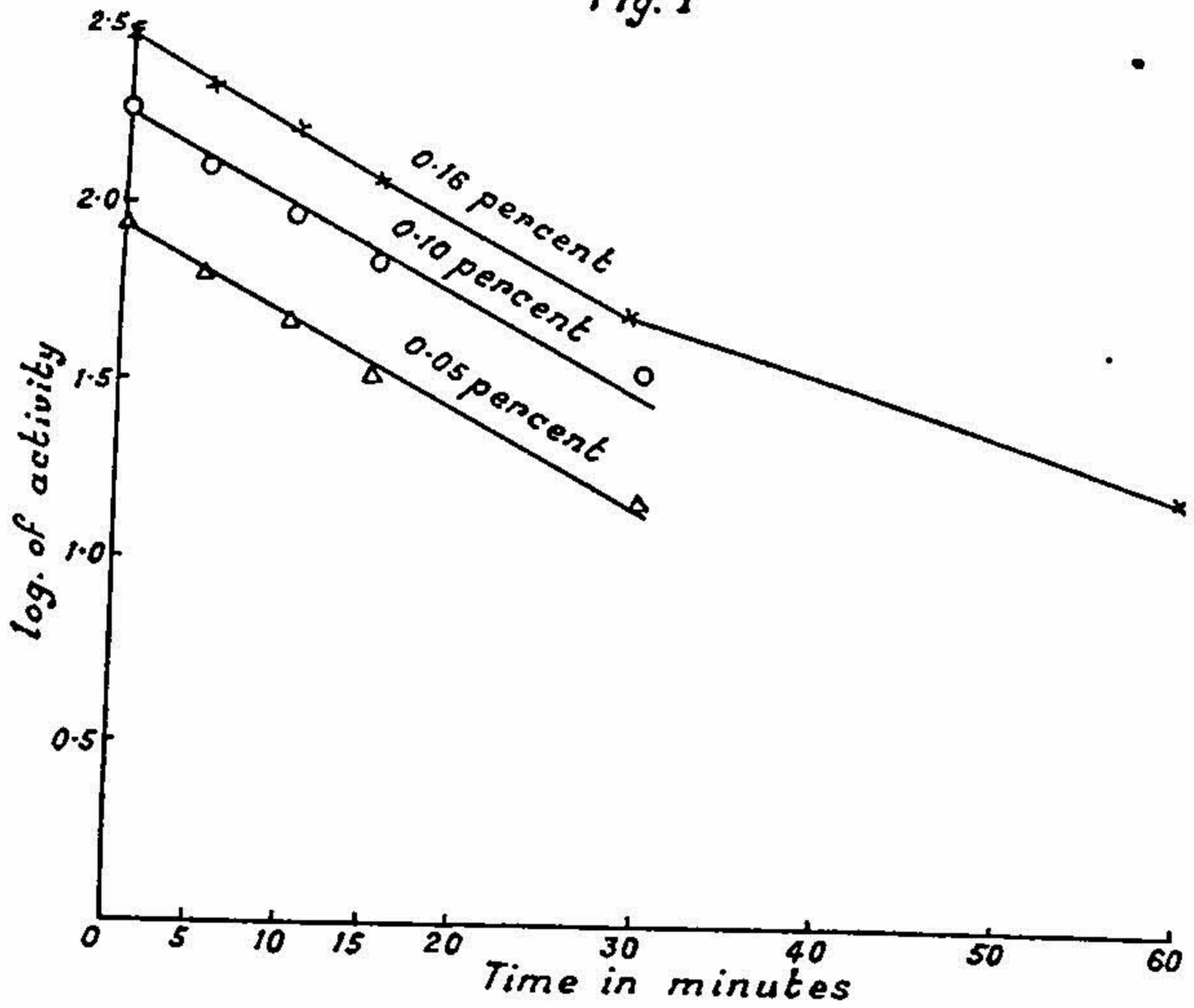
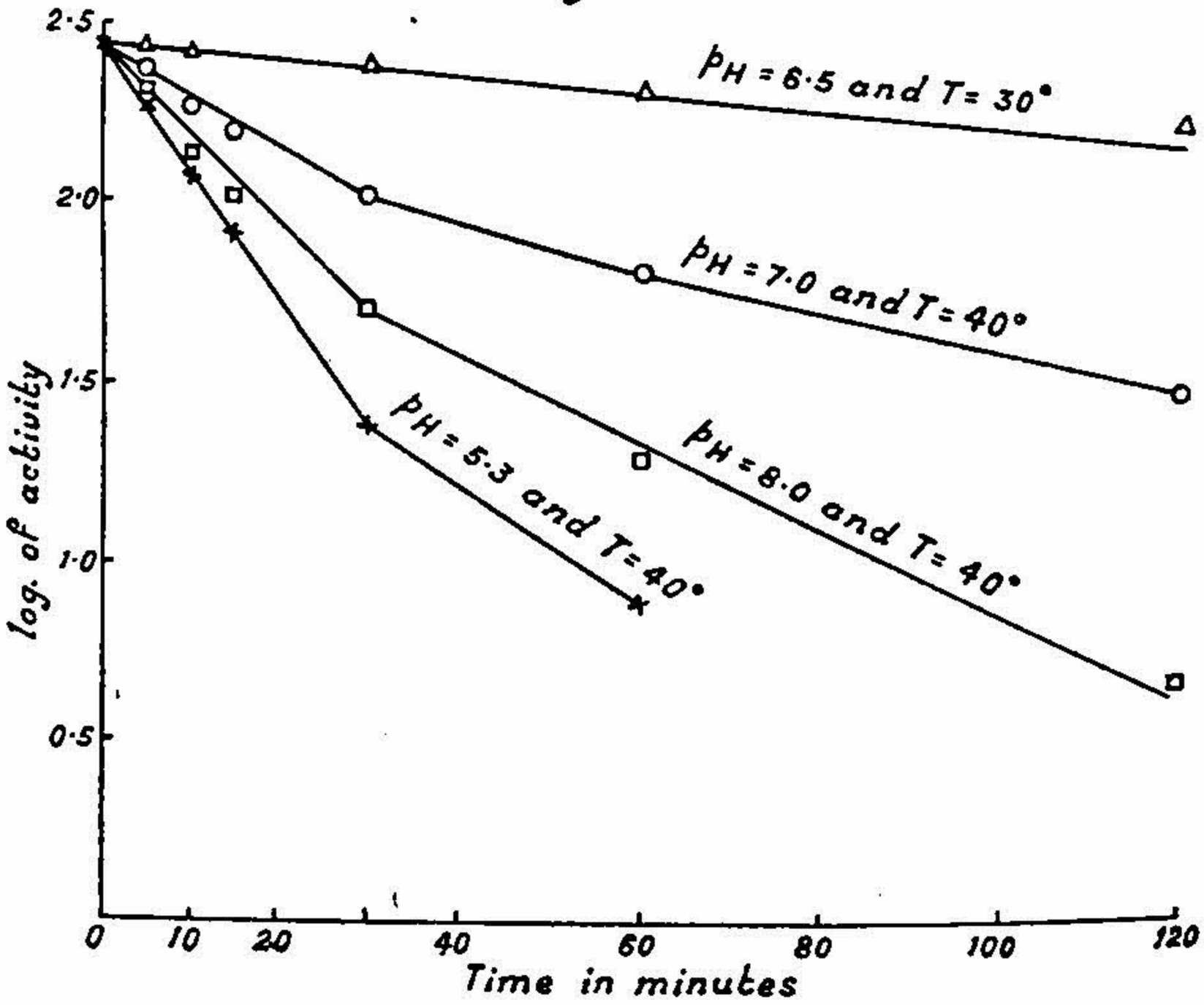

2·5
2·0
1·5
1·0
0·5
log. of activity
0·16 percent
0·10 percent
0·05 percent
0
5
10
15
20
30
40
50
60
Time in minutes

Fig. II


2·5
2·0
1·5
1·0
0·5
log. of activity
pH = 6·5 and T = 30°
pH = 7·0 and T = 40°
pH = 8·0 and T = 40°
pH = 5·3 and T = 40°
0
10
20
40
60
80
100
120
Time in minutes

reaction velocity is independent of the concentration and that the reaction itself is unimolecular. As the reaction progresses, the rate diminishes, indicating the presence of disturbing factors, or a complexity of the enzyme. As in the case of all enzymes the rate of decomposition varies with P_H, and more definite results were likely to be obtained by using buffered solutions to maintain P_H constant.

TABLE I.

Time in minutes	Activity in mg. of Cu_2O		
	Concentration per cent.		
	0·16	0·10	0·05
0	292·0	183·4	87·8
5	220·4	133·0	63·9
10	158·6	95·4	46·8
15	118·0	70·0	33·4
30	51·0	35·0	15·4
60	17·8	..	..
120	6·0	..	..

Effect of P_H *upon the heat inactivation of pancreatic amylase.*—Flasks each containing 80 c.c. of phosphate buffer in varying hydrogen-ion concentration were taken. Pancreatin (0·16 g.) was added to each and sufficient distilled water to make up the volume in each case to 100 c.c. The mixtures were maintained at 40° and the activities determined, the results being shown in Table II.

TABLE II.

Time in minutes	Activity in mg. of Cu_2O					
	P_H					
	5·3	5·9	6·5	7·0	7·2	8·0
0	284·1	284·6	283·4	282·0	279·8	285·4
5	192·0	220·8	236·0	238·2	228·4	208·0
10	121·3	163·0	193·0	190·0	182·0	153·0
15	83·9	130·0	160·0	160·0	147·0	112·1
30	25·0	77·4	104·0	108·1	88·2	50·4
60	8·3	41·2	70·2	67·3	56·8	21·0
120	..	11·8	30·2	31·0	25·3	5·0
K	0·0805	0·0504	0·0376	0·0353	0·0411	0·0613

The observations show that the rate of inactivation of the enzyme is the minimum at P_H 6·5–7·0, and increases rapidly as the reaction becomes either more acid or more alkaline. Thus, at P_H 5·3 the activity is only 3 per cent. of its original value at the end of one hour as compared with 25 per cent. at P_H 7·0. This behaviour resembles that of pepsin and catalase studied by Michaelis and Rothstein (*Biochem. Z.*, 1922, **133**, 487) and Morgulis and Befer (*J. Biol. Chem.*, 1928, **77**, 115), respectively.

Fig. II shows the relation between the logarithm of the activity and the time for some of these solutions. The curves resemble those of the unbuffered solutions but appear to exhibit a fairly definite break after about 30 minutes, the initial and final portions being nearly straight. The physical significance is not obvious although the presence of two enzymes is indicated. The simultaneous unimolecular inactivation of two enzymes at different rates would follow an equation of the type $C=ae^{-kt}+be^{-k't}$, but it has not been found possible to fit such an equation to the experimental results. It must be remembered, however, that the expression for the activity is more or less empirical being proportional to the amount of sugar formed under standard conditions in 30 minutes. Since the reaction with starch probably occurs in two stages, liquefaction followed by saccharification, the quantity of sugar is not necessarily proportional to the concentration of the enzymes; but assuming two enzymes one of which acts on the product of the other, the activity is given by the expression

$$la\left\{ t\,\frac{l}{s}\,(e^{-st}-1)\right\},$$

where l is the concentration of the liquefying and s, that of the saccharifying enzyme at any time and t, the time the mixture is allowed to react on the starch. This assumes that l and s remain constant during the reaction which is not the case, and in reality a more complicated expression for the activity is required. The above suffices to show, however, that strictly quantitative results for the decrease in activity can only be obtained when the ratio between the activities of the two enzymes is known and the course of the reaction with starch is followed in detail. Sherman and Schlesinger (*loc.cit.*) have shown that in the case of prolonged action, pancreatin destroys a quantity of starch which is nearly twice the weight of the sugar formed, but this result does not necessarily apply to the initial stages and more work is necessary to elucidate the matter. Unfortunately the experimental difficulties are considerable.

It follows that the present results must be regarded as being only qualitative as far as the kinetics of the reaction are concerned owing to the uncertainty of the method of measuring the activity. At the same time it is probable that for changes in activity which are not large the method gives results which are relatively correct and thus the main conclusion, *viz.*, the inactivation according to a unimolecular equation in the initial stages, the reduction in rate as the reaction proceeds and the effect of hydrogen-ion concentration, may be considered as established.

Influence of temperature on the velocity coefficient.—The experiments described in the foregoing section were repeated at P_H 6·5 and 7·0, which represent the region for optimum stability at 30°. The results are given in table III, and the corresponding graph for P_H 6·5 in Fig. II.

TABLE III.

Time in minutes	Activity in mg. Cu_2O	
	P_H	
	6.5	7.0
0	282·8	290·0
10	269·2	279·0
15	261·8	272·8
30	244·8	257·0
60	213·8	227·6
120	180·0	204·8
K	0·0048	0·0040

At 40° the rates of decomposition at P_H 6·5 and 7·0 were substantially the same; but at 30° the velocity at P_H 7·0 is distinctly less, and as may be seen from the graph, the reaction followed the unimolecular law throughout the period during which observations were taken.

The energy of activation calculated by means of the formula

$$E=R\ (\log K_1-\log K_2)\ \frac{T_1T_2}{(T_2-T_1)}$$

is 39,100 cals. for P_H 6·5 and 41,000 for P_H 7·0.

These results are of the same order as those obtained by Lewis (*Biochem. J.*, 1926, **20**, 965) for the heat denaturation of proteins and by Chick and Martin (*J. Physiol.*, 1910, **40**, 404) for the heat denaturation of oxyhæmoglobin. This suggests that the inactivation of pancreatic amylase may be due to the denaturation of proteins present in the preparation; on the other hand, the marked variation of the rate with the hydrogen-ion concentration of the solution might be ascribed to hydrolysis and the data available afford no indication as to which of these hypotheses is more likely to be correct.

Summary.

A study of conditions relating to heat inactivation of pancreatic amylase shows that solutions of the enzyme are readily inactivated even at the laboratory temperature. The rate of inactivation is at a minimum at P_H 6·5 to 7.0, in the neighbourhood of the optimum reaction for the enzyme.

The course of inactivation follows that of a monomolecular reaction particularly in the early stages. The calculated value of the energy of activation approaches that for protein denaturation. There is evidence to suggest that the heat inactivation of pancreatic amylase is either due to the denaturation of proteins associated with it, or to the hydrolysis of the enzyme itself.

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III.—STUDIES IN THE PROTEINS OF INDIAN FOODSTUFFS—PART IV.

THE PROTEINS OF FENUGREEK.

(*TRIGONELLA FOENUM GRAECUM*).

By Yelandur Venkanna Sreenivasa Rau and Motnahalli Sreenivasaya.

Fenugreek (*Trigonella fœnum Græcum*) is a leguminous annual, widely cultivated throughout India. Leaves of the young plants are used as green vegetable while the seeds are valued as a condiment and form an essential ingredient of many Indian preparations; they are valued also in Indian medicine for their tonic, astringent and emollient properties. Recently, its medicinal applications have been more widely recognised (Indian Science Congress Abstracts, 1932). On incineration the seeds leave about 7 per cent. of ash which is rich in phosphoric acid (25 per cent. on the weight of the ash). Reutter has noted the presence of several bases in fenugreek such as choline, trigonelline, methylamine, neurine and betain which are derived from the splitting of the lecithin. It is stated that fenugreek can be employed as a substitute for cod-liver oil wherever the latter is prescribed, as in rickets, anæmia and debility. A comprehensive study of the constituents of this seed has been opened by us and this paper relates to the isolation and analysis of two among its proteins.

Experimental.

The sun-dried seeds were ground to flour and passed through a 40-mesh sieve. The soft, snow-white cotyledons constituting the mucilaginous portion separate, and cannot be ground. The flour is light yellow and on analysis gave the following percentages calculated on moisture-free material.

TABLE I.

Ash	Ether extractives	Crude protein N × 6.25	Starch	Cellulose	Lignin	Other Carbohydrates
3·55	8·10	29·90	2·11	21·00	2·23	33·02

Preparation and analysis of the proteins.—Preliminary extraction trials established that a 6 per cent. salt solution removed the maximum quantity of nitrogen representing about 40 per cent. of the total. Distilled water, 6 per cent. saline solution and cold 70 per cent. alcohol, when used consecutively, extract 15, 25, and 5 per cent. respectively, accounting for 45 per cent. of the total nitrogen. The presence of mucilage in the seed renders filtration extremely slow and difficult. Simple gravity filtration yielded clear extracts and was found preferable to centrifuging or pressure filtration.

The flour (500 g.) was mechanically stirred for 2 hours with a 6 per cent. sodium chloride solution (2·5 l.): after filtering through cheese-cloth the residue was again extracted with saline solution (2 l.). Gravity filtration of the extract gave 3 l. of a clear, pale yellow liquid which was dialysed against a

stream of cold distilled water for 5 days until the dialysate was free from chlorides. The precipitated protein (Fraction A) was purified by dissolving in 4 per cent. salt solution followed by a second dialysis; on acidifying the filtrate from this fraction, fraction B was precipitated, and recovered on the centrifuge.

Both preparations were washed several times with distilled water, dehydrated with alcohol and ether, dried, powdered and passed through a 100-mesh sieve. Preparation A (6 g.) was a light, cream-white powder while preparation B (2 g.) was slightly brownish. On analysis, they gave the following percentages:—

TABLE II.

Fraction	Moisture	Ash	Ash and moisture free		
			Nitrogen	Phosphorus[1]	Sulphur[2]
A	4·74	0·50	17·2	0·00	0·14
B	7·96	0·90	15·91	0·31	0·285

[1] Micro-method of Pregl.

[2] Micro-combustion method of Carius.

The two preparations were analysed by the method of Van Slyke (*J. Biol. Chem.*, 1911, **10**, 15) as modified by Plimmer and Rosedale (*Biochem. J.*, 1925, **19**, 1004), also Plimmer and Lowndes (*Biochem. J.*, 1927, **21**, 247). Arginine was estimated not only in the basic fraction but also separately in the original hydrolysate (Plimmer and Rosedale, *loc. cit.*, 1020). Tyrosine and tryptophan were estimated separately in the original proteins according to the method of Folin and Marenzie (*J. Biol. Chem.*, 1929, **83**, 89). Cystine was estimated by the method of Rimington (*Biochem. J.*, 1930, **24**, 1114). The results are given in tables III and IV (columns 1 and 2), and are compared with globulins of some of the local leguminous seeds (Niyogi, Narayana and Desai, *Ind. J. Med. Res.*, 1931–32, **19**, 1041) expressed as per cent. of total nitrogen.

TABLE III.

Distribution of Nitrogen (per cent. of total).

Form of Nitrogen	Fenugreek		Average of 10 globulins
	A	B	
Melanin	0·6	1·8	1·7
Amide	6·8	7·2	9·7
Basic:			
Arginine	21·2	18·7	16·2
Histidine	18·5	4·7	4·1
Cystine	0·4	0·9	0·6
Lysine	1·9	6·1	8·8
Non-basic:			
Amino	48·9	58·9	55·6
Non-amino	1·9	1·8	3·1
Total ..	100·2	100·1	99·8

The essential amino-acids in the proteins A and B of fenugreek, compared with other globulins, and expressed as percentages of protein (ash and moisture-free), are as follows:—

TABLE IV.

Amino-acid	Fenugreek		Average of 10 globulins
	A	B	
Lysine	1·7	4·9	7·1
Histidine	11·6	2·8	2·3
Arginine (Van Slyke) ..	11·2	9·3	8·0
,, (Direct) ..	12·4	10·5	8·9
Cystine	0·6	1·2	1·9
Tyrosine	5·7	2·1	4·0
Tryptophan	0·5	Present	0·5

Summary.

Two proteins A and B from fenugreek have been isolated and analysed. The globulin (fraction A) is characterised by a surprisingly high content of histidine which is about five times the average amount (2·27 per cent. of protein) contained in the average of ten typical globulins. The excellent and reputed tonic properties of the seed may be partly attributable to this circumstance, histidine being an essential constituent of hæmoglobin. Fraction B is albuminous and is an interesting protein since it appears to contain both phosphorus and sulphur.

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