CCXLII. ENZYMIC PROTEOLYSIS

I. LIBERATION OF AMMONIA FROM PROTEINS¹

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THE isolation of asparagine [Damodaran, 1932] and of glutamine [Damodaran *et al.* 1932] from enzymic digests of proteins has given direct demonstration of the existence of amide groups in the protein molecule. Before, however, any attempt can be made to decide if the ammonia, which is an invariable product of protein hydrolysis, arises solely from dicarboxylic acid amides, it becomes essential to have more exact information than is at present available on the deamidizing activity of the proteolytic enzymes. Such information is in the first place necessary to enable a proper choice of enzymes and conditions of experiment to be made, so that digestion of protein could be effected with the least amount of decomposition of the ammonia-yielding complexes. Secondly, comparative study of the liberation of ammonia from proteins and from synthetic substrates is likely to provide analogies from which the nature of the precursors of ammonia might be inferred.

The rate of liberation of ammonia in tryptic digests of casein, gliadin and Witte's peptone was studied by Hunter & Smith [1925], who found that the

Fig. 1. Digestion of casein. ○ Amino. × Amide. — Digest. ---- Control.

formation of ammonia proceeded much more slowly than the liberation of amino groups. According to these authors "the differences were so great as to suggest

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that the two processes are really catalysed by different enzymes and that pure trypsin might produce from proteins no ammonia at all".

As a preliminary to the work already cited on the isolation of asparagine and glutamine, a number of experiments were carried out on the splitting of

Fig. 2. Digestion of gliadin. $\odot$ Amino. $\times$ Amide. — Digest. ---- Control.

Fig. 3. Digestion of edestin. $\odot$ Amino. $\times$ Amide. — Digest. ---- Control.

amide and peptide bonds during the action of proteolytic enzymes on gliadin and edestin. Attention was, however, mainly directed to securing the conditions under which a far-reaching hydrolysis of protein could be effected with minimal

liberation of ammonia and no conclusions were drawn as to any relation between proteolytic and deamidizing actions of the enzymes used.

The present paper describes a systematic study of the formation of ammonia and the hydrolysis of peptide linkages during the action of pepsin, trypsin, erepsin and papain on three typical proteins, casein, edestin and gliadin. Figs. 1-3 represent graphically the whole course of the digestion of each of these proteins by the three animal proteases acting in succession. The numerical data and details of procedure are given in Tables I, II, III. It will be seen from the curves, which show the progressive liberation of amino groups and of free ammonia during the course of digestion, that the rates of splitting in the two cases do not by any means run parallel. With each enzyme, in the earlier stages of action

Table I. *Pepsin, trypsin and erepsin on casein*

A. 22 g. casein (N 15.68 %) dissolved in about 500 ml. *N*/10 HCl+475 ml. water + 1 g. B.D.H. pepsin dissolved in 25 ml. water.

B. 60 ml. of A (at the end of 8 days) boiled at pH 6 to inactivate pepsin, brought back to pH 1.6 and made up to 100 ml.

C. 450 ml. of A (at the end of 7 days) boiled at pH 6, brought to pH 8.6 with NaOH, 25 ml. of a solution of 0.5 g. of B.D.H. trypsin and sufficient water to make up to 500 ml.

D. 30 ml. of C (at the end of 15 days on trypsin) boiled at pH 6, adjusted to pH 8.6 and made up to 50 ml.

E. 140 ml. of C (after 22 days on trypsin) inactivated at pH 6, brought to pH 7.6 and made up to 250 ml. after the addition of 15 ml. of erepsin solution. After inactivation and before addition of erepsin the digest had to be left in the ice-chest for about 3 weeks.

F. 30 ml. of E boiled at pH 6 and made up to 50 ml. after adjusting to pH 7.6.

Time hr. days	Hydrolysis %											
	Pepsin				Trypsin				Erepsin			
	A		B (after inactivation)		C		D (after inactivation)		E		F (after inactivation)	
	Peptide	Amide	Peptide	Amide	Peptide	Amide	Peptide	Amide	Peptide	Amide	Peptide	Amide
0	—	—	—	—	27.30	8.50	—	—	79.70	35.00	—	—
1	33.29	—	—	—	—	—	—	—	—	—	—	—
2	3.43	—	—	—	39.10	8.50	—	—	80.80	35.00	—	—
4	6.57	—	—	—	46.10	—	—	—	—	—	—	—
6	9.43	—	—	—	—	—	—	—	81.30	35.00	—	—
8	10.42	—	—	—	54.05	—	—	—	—	—	—	—
12	11.00	0.80	—	—	55.60	—	—	—	—	—	—	—
1	15.29	1.60	—	—	62.10	10.10	—	—	84.60	37.20	—	—
2	18.43	3.06	—	—	63.60	11.70	—	—	85.80	39.70	—	—
3	—	—	—	—	—	—	—	—	86.40	43.60	—	—
4	21.00	4.95	—	—	67.10	14.30	—	—	87.30	44.20	—	—
5	—	—	—	—	70.30	16.90	—	—	88.90	49.30	—	—
6	21.86	6.82	—	—	—	—	—	—	—	—	—	—
7	23.30	8.29	—	—	—	—	—	—	90.70	53.60	90.80	53.10
8	24.10	9.49	24.25	8.62	—	—	—	—	—	—	—	—
10	25.90	10.56	—	—	—	—	—	—	—	—	—	—
11	26.40	11.23	—	—	73.10	24.10	—	—	—	—	—	—
15	—	—	—	—	76.30	29.30	76.70	29.70	—	—	—	—
16	27.57	16.31	24.25	12.82	—	—	—	—	—	—	—	—
22	—	—	—	—	77.90	33.10	—	—	92.90	57.40	90.80	57.60
31	—	—	—	—	—	—	76.70	37.60	—	—	—	—
47	30.13	25.00	24.25	18.84	—	—	—	—	—	—	—	—
49	—	—	—	—	—	—	76.70	45.30	—	—	—	—
66	—	—	—	—	—	—	76.70	52.20	—	—	—	—

Table II. *Pepsin, trypsin and erepsin on gliadin*

A. 11 g. gliadin (N 17.25 %) suspended in about 400 ml. of *N*/20 HCl and treated with 0.5 g. of pepsin dissolved in a few ml. of *N*/20 HCl; clear solution obtained after incubation for 3 hr.

B. 30 ml. of A boiled at pH 6 on the 7th day, pH brought again to 1.6 and made up to 50 ml.

C. 350 ml. of A on the 10th day boiled at pH 6, brought to pH 8.6, 0.4 g. B.D.H. trypsin added and made up to 500 ml.

D. 30 ml. of C (after 20 days on trypsin) boiled at pH 6, reaction adjusted to pH 8.6 and made up to 50 ml.

E. 175 ml. of C (after 16 days on trypsin) boiled at pH 6, adjusted to pH 7.8 and 25 ml. of erepsin solution added.

F. 30 ml. of E boiled at pH 6 after 3 days on erepsin, pH readjusted to 7.8 and made up to 50 ml.

Time hr.	Hydrolysis %											
	Pepsin				Trypsin				Erepsin			
	A		B (after inacti- vation)		C		D (after inacti- vation)		E		F (after inacti- vation)	
	Peptide	Amide	Peptide	Amide	Peptide	Amide	Peptide	Amide	Peptide	Amide	Peptide	Amide
	—	—	—	—	—	—	—	—	—	—	—	—
0	—	—	—	—	16.40	15.12	—	—	63.50	29.00	—	—
1	—	—	—	—	23.70	15.12	—	—	—	—	—	—
2	—	—	—	—	30.16	15.12	—	—	65.50	29.00	—	—
4	—	—	—	—	35.60	15.12	—	—	66.10	29.00	—	—
6	—	—	—	—	38.30	15.12	—	—	67.30	29.00	—	—
7	0.81	0.04	—	—	—	—	—	—	—	—	—	—
10	2.24	0.90	—	—	—	—	—	—	—	—	—	—
12	—	—	—	—	43.10	15.29	—	—	67.50	29.90	—	—
days	—	—	—	—	—	—	—	—	—	—	—	—
1	6.52	4.05	—	—	44.50	15.70	—	—	69.20	30.50	—	—
2	8.56	7.70	—	—	49.00	16.29	—	—	72.00	33.20	—	—
3	10.52	10.68	—	—	54.30	17.64	—	—	—	—	73.30	39.20
4	12.16	12.69	—	—	—	—	—	—	74.40	36.50	—	—
5	—	—	—	—	56.70	23.18	—	—	75.50	39.20	—	—
6	13.72	16.38	—	—	—	—	—	—	—	—	—	—
7	—	—	14.10	16.55	—	—	—	—	—	—	—	—
8	—	—	—	—	—	—	—	—	76.10	43.10	—	—
9	—	—	—	—	57.70	25.20	—	—	—	—	—	—
10	16.54	21.60	—	—	—	—	—	—	—	—	—	—
11	—	—	—	—	—	—	—	—	—	—	73.30	48.00
13	—	—	—	—	63.60	28.40	—	—	76.50	45.60	—	—
17	—	—	14.10	20.30	64.00	30.41	—	—	—	—	—	—
20	—	—	—	—	—	—	64.60	28.34	—	—	—	—
27	—	—	—	—	—	—	—	—	78.80	60.80	—	—
32	—	—	—	—	—	—	64.60	38.59	—	—	73.30	60.70
49	—	—	—	—	—	—	64.60	54.04	—	—	—	—

when the rate of peptide hydrolysis is at its highest, ammonia liberation is hardly noticeable and becomes significant only after the products of digestion have accumulated to some extent. On the other hand when the most active stage of proteolysis is past and formaldehyde titrations are increasing only very slowly, as indicated by the flattening of the amino-N curves, the liberation of ammonia continues at a steady rate and shows no tendency to slow down. These results are clearly brought out in Table IV and Figs. 4 and 5, in which the extents of amide and peptide hydrolysis at intervals at the beginning and end of the digestion are compared. The observations are readily explicable on the assumption that the ammonia formed is, at least in great part, not directly connected with

Table III. *Pepsin, trypsin and erepsin on edestin*

A. 17.5 g. of crystalline edestin stirred up with 375 ml. of *N*/10 HCl and kept at 37° for 1 hr., 350 ml. of water and 0.75 g. of pepsin in 25 ml. added to the clear solution.

B. 30 ml. of A boiled at pH 6 on the 18th day brought to pH 1.6 and made up to 50 ml.

C. 425 ml. of A on the 9th day boiled at pH 6 brought to pH 8.6, 0.5 g. B.D.H. trypsin added and made up to 500 ml.

D. 30 ml. of C boiled at pH 6 (after 15 days on trypsin) adjusted to pH 8.6 and made up to 50 ml.

E. 175 ml. of C (after 13 days on trypsin) boiled at pH 6 adjusted to pH 7.8 and 25 ml. of erepsin solution added.

F. 40 ml. of E boiled at pH 6 (after 17 days on erepsin), readjusted to pH 7.8 and made up to 50 ml.

		Hydrolysis %											
		Pepsin				Trypsin				Erepsin			
		A		B (after inactivation)		C		D (after inactivation)		E		F (after inactivation)	
Time	Peptide	Amide	Peptide	Amide	Peptide	Amide	Peptide	Amide	Peptide	Amide	Peptide	Amide	
hr.	0	—	—	—	—	20.10	11.50	—	—	62.10	34.80	—	—
1	—	—	—	—	—	27.10	—	—	—	—	—	—	—
2	2.44	—	—	—	—	—	—	—	—	65.10	34.80	—	—
3	5.49	—	—	—	—	31.80	11.50	—	—	—	—	—	—
4	—	—	—	—	—	—	—	—	—	65.50	—	—	—
5	—	—	—	—	—	33.40	—	—	—	—	—	—	—
6	7.93	—	—	—	—	—	—	—	—	66.40	—	—	—
8	—	—	—	—	—	37.80	11.80	—	—	—	—	—	—
12	10.54	0.34	—	—	—	—	—	—	—	—	—	—	—
days													
1	11.10	1.02	—	—	42.30	13.40	—	—	73.20	37.10	—	—	—
2	13.29	2.38	—	—	44.60	15.40	—	—	76.00	40.70	—	—	—
3	14.76	4.14	—	—	49.70	17.30	—	—	76.20	42.60	—	—	—
4	16.83	5.21	—	—	—	—	—	—	76.80	—	—	—	—
5	—	—	—	—	53.40	21.40	—	—	—	—	—	—	—
6	17.68	6.18	—	—	—	—	—	—	—	—	—	—	—
7	18.29	7.35	—	—	—	—	—	—	—	—	—	—	—
8	—	—	—	—	57.80	27.70	—	—	79.20	46.30	79.80	43.60	—
9	—	—	—	—	59.90	29.00	—	—	—	—	—	—	—
10	—	8.22	—	—	62.00	29.90	—	—	—	—	—	—	—
12	—	—	—	—	62.30	31.00	—	—	—	—	—	—	—
14	—	—	—	—	65.30	35.40	—	—	—	—	—	—	—
15	—	—	—	—	—	—	63.20	35.90	—	—	—	—	—
17	—	—	—	—	—	—	—	—	—	—	80.50	56.60	—
18	—	—	17.48	7.19	—	—	—	—	—	—	—	—	—
22	21.96	14.08	—	—	—	—	—	—	—	—	—	—	—
24	—	—	—	—	—	—	63.20	48.10	—	—	—	—	—
25	—	—	—	—	—	—	—	—	88.90	59.20	—	—	—
32	—	—	18.90	13.98	—	—	—	—	—	—	—	—	—
38	—	—	—	—	74.00	54.70	—	—	—	—	—	—	—
42	—	—	—	—	—	—	—	—	89.10	67.50	—	—	—
43	—	—	—	—	—	—	—	—	—	—	81.20	61.60	—
56	—	—	—	—	—	—	63.20	63.90	—	—	—	—	—
74	—	—	18.91	22.77	—	—	—	—	—	—	—	—	—

proteolytic action but arises from the decomposition of the primary products of protein cleavage similar to the γ -peptides of glutamine studied by Melville [1935]. It also seems safe to conclude that this secondary decomposition is not enzymic in nature but is brought about by the acidic or alkaline reactions involved. Thus when peptic, tryptic and ereptic digests, in which the increase in amino groups have progressed considerably, are boiled to inactivate the enzyme

and incubated the formation of ammonia goes on at approximately the same rate as in unboiled digests containing the active enzyme, although the former

Fig. 4. Digestion of casein, initial stages. — Amino. --- Amide. $\odot$ Pepsin. $\times$ Trypsin. Δ Erepsin.

Fig. 5. Digestion of casein, final stages. $\odot$ Pepsin. $\times$ Trypsin. Δ Erepsin. ---- Amide. — Amino.

show no further increase in amino groups. Similar results were obtained with purified trypsin and erepsin (Fig. 6 and Table V).

Table IV

Protein	Enzyme	Interval in days		% hydrolysis in the interval	
		From	To	Peptide	Amide
Casein	(a) Pepsin	0	2	18.43	3.06
		8	47	6.03	15.51
	(b) Control	8	47	0.00	10.22
	(a) Trypsin	0	1	34.80	1.60
		15	22	1.60	3.80
	(b) Control	15	66	0.00	22.50
	(a) Erepsin	0	1	4.90	2.20
		7	22	2.20	3.80
	(b) Control	7	22	0.00	4.50
Gliadin	(a) Pepsin	0	2	8.56	7.70
		6	10	2.82	5.22
	(b) Control	6	36	0.00	10.44
	(a) Trypsin	0	2	32.60	1.17
		17	35	0.80	17.59
	(b) Control	17	46	0.00	25.74
	(a) Erepsin	0	2	8.50	4.20
		2	27	6.80	27.60
	(b) Control	3	31	0.00	21.50
Edestin	(a) Pepsin	0	2	13.29	2.38
		22	64	7.29	10.73
	(b) Control	18	74	1.33	15.58
	(a) Trypsin	0	2	24.50	1.90
		14	38	8.70	19.30
	(b) Control	15	56	0.00	28.00
	(a) Erepsin	0	2	13.90	5.90
		8	42	9.90	21.20
	(b) Control	8	43	1.40	17.50

Table VI gives the results of an interesting experiment in which the deamidizing action of pepsin was tested on casein previously digested with trypsin and erepsin. It is seen that pepsin is unable to bring about any further hydrolysis of peptide groups but that ammonia production takes place as in a peptic digest of native casein. Further, the amount of ammonia produced is not higher than in a portion of the trypsin-erepsin digest kept at the *pH* necessary for the action of pepsin, but without the addition of the latter. This experiment brings out another interesting result not, however, connected with deamidation. When casein is acted upon by pepsin, trypsin and erepsin in that order the extent of hydrolysis is nearly 90 % of complete hydrolysis (about 20 % due to pepsin and the remaining 70 % due to the other two enzymes). When trypsin and erepsin are used first the splitting produced by them is still 70 %, but pepsin is now unable to act further on this digest and contribute its quota to bring up the hydrolysis to the usual 90 %. This result would suggest that the deciding factor for the action of pepsin is the position of the bonds attacked rather than their nature.

Table VII and Fig. 8 show the action of papain on casein, and Table VIII the action of papain on edestin. The results summarized in Table IX for the action of papain on casein are entirely different from those obtained with the animal proteases. It must be stated at the outset that, notwithstanding the

Table V. *Purified trypsin and erepsin on casein*

A. 15 g. casein dissolved in very dilute alkali, boiled for a minute and made up to 700 ml. at pH 8.6, 100 ml. of erepsin-free trypsin solution, also at pH 8.6, added.

B. 200 ml. of A on the 26th day boiled at pH 6 and after adjusting the reaction to pH 7.8, 25 ml. of trypsin-free erepsin added and the volume made up to 250 ml.

Time hr. days	Hydrolysis %			
	A Erepsin-free trypsin		B Trypsin-free erepsin	
	Peptide	Amide	Peptide	Amide
0	—	—	48.70	27.31
1	3.50	—	—	—
2	6.10	—	50.44	—
6	—	—	55.60	27.52
1	32.90	1.76	63.50	31.30
2	35.10	5.18	69.40	34.66
3	—	—	74.80	36.97
4	40.20	8.47	75.20	39.50
5	—	—	76.60	40.55
6	45.10	9.24	—	—
7	—	—	76.80	44.75
8	45.40	11.96	—	—
11	—	—	76.90	50.21
12	45.40	16.59	—	—
24	—	—	79.30	55.88
25	48.80	26.57	—	—

Fig. 6. Purified trypsin and erepsin on casein. ○ Amino. × Amide.

statements in the literature that activated papain is able to act on natural proteins, we have never succeeded in getting any of the proteins used in these experiments into solution by the action of papain-HCN either with commercial samples or with the enzyme prepared from the fresh latex. In all experiments

Table VI. *Pepsin after trypsin and erepsin on casein*

A. 22 g. casein dissolved in dilute NaOH, pH adjusted to 8.6, 1 g. B.D.H. trypsin added and made up to 1 l.

B. 800 ml. of A on the 8th day boiled at pH 6, made up to 1 l. at pH 7.8, with 100 ml. of erepsin solution and water.

C. 400 ml. of B on the 9th day inactivated by boiling at pH 6 brought to pH 1.8 with *N* HCl and made up to 500 ml. 250 ml. pipetted out and 0.25 g. of B.D.H. pepsin powder stirred in.

D. 250 ml. of C without the addition of pepsin.

Time hr.	Hydrolysis %							
	Trypsin		Erepsin		Pepsin			
	A		B		C		D	
	Peptide	Amide	Peptide	Amide	Peptide	Amide	Peptide	Amide
0	—	—	46.38	14.25	72.90	30.08	—	—
1	14.10	—	—	—	72.90	30.31	—	—
2	—	—	49.19	—	72.90	30.31	—	—
4	—	—	50.66	14.25	72.90	31.09	—	—
6	—	—	—	—	72.90	31.20	—	—
7	25.10	0.27	—	—	—	—	—	—
days								
1	35.60	0.95	59.57	16.65	72.90	35.75	72.90	35.88
2	39.20	4.89	62.91	19.27	72.90	41.40	72.90	40.56
3	—	—	—	—	72.90	44.35	72.90	42.04
4	43.70	8.98	70.52	23.27	—	—	—	—
5	45.70	9.93	71.25	—	—	—	—	—
6	—	—	72.56	29.25	—	—	—	—
7	48.20	13.56	—	—	72.90	48.37	72.90	47.46

Fig. 7. Pepsin after trypsin and erepsin. ○ Amino. × Amide.
— Digest. ---- Control.

Table VII. *Papain and papain-HCN on casein*

Casein digested with pepsin-HCl for 24 hr. and inactivated by boiling at pH 5.
 A. 200 ml. of casein peptone and 0.225 g. papain powder in 50 ml. of water.
 B. 40 ml. of A boiled on the 34th day and made up to 50 ml.
 C. 200 ml. of casein peptone and 50 ml. of papain-HCN (obtained by adding 10 ml. of 3 % KCN solution neutralized to methyl red with HCl and incubating at 37° for 1 hr).
 D. 80 ml. of C boiled after 15 days and made up to 100 ml.

Time hr. days	Hydrolysis %							
	Papain				Papain-HCN			
	A		B		C		D	
	Peptide	Amide	Peptide	Amide	Peptide	Amide	Peptide	Amide
0	13.55	8.81	—	—	13.55	8.81	—	—
5	—	—	—	—	31.25	18.84	—	—
1	21.43	18.62	—	—	43.78	29.64	—	—
2	23.82	19.15	—	—	47.06	32.66	—	—
4	24.33	21.24	—	—	53.21	36.85	—	—
6	25.00	22.56	—	—	56.36	38.56	—	—
9	—	—	—	—	60.39	41.71	—	—
10	—	—	—	—	—	—	63.81	41.30
15	—	—	—	—	62.63	44.27	—	—
16	31.23	26.89	—	—	—	—	—	—
17	—	—	—	—	—	—	63.81	41.62
34	33.36	28.60	33.40	28.62	68.92	48.93	—	—
35	—	—	—	—	—	—	63.81	44.25
41	—	—	33.40	28.77	—	—	—	—
59	—	—	33.40	30.16	—	—	—	—

Fig. 8. Casein peptone + papain. ○ Amino. × Amide.
 — Digest. Boiled control.

Table VIII. *Papain and papain-HCN on edestin*

Edestin digested with pepsin-HCl for 24 hr.

A, C and D prepared from edestin peptone as in Table VIII.

Time hr. days	Hydrolysis %					
	Papain A		Papain-HCN			
	Peptide	Amide	Peptide	Amide	Peptide	Amide
0	12.96	0.64	12.96	0.64	—	—
2	—	—	18.68	4.67	—	—
5	—	—	23.40	7.51	—	—
1	19.36	6.05	34.51	13.90	—	—
2	21.80	6.87	41.08	18.05	—	—
5	—	—	47.57	22.22	—	—
9	22.22	8.27	52.36	26.83	52.40	26.90
13	—	—	55.55	28.03	—	—
27	—	—	—	—	52.40	29.00

Table IX

Enzyme, etc.	Interval in days		% hydrolysis in the interval	
	From	To	Peptide	Amide
Casein peptone and papain-HCN	0	1	30.23	20.83
	1	15	18.85	14.63
	15	34	6.29	4.66
Control	9	34	0.00	2.95
Casein peptone and papain	0	1	7.88	9.81
	1	16	9.78	8.27
	16	34	2.15	1.71
Control	9	34	0.00	1.54

with papain, therefore, substrates used were proteins which were brought into solution by a 24 hr. digestion with pepsin. There is no doubt that in papain digests ammonia formation is connected with enzyme action. There is a certain parallelism between amide and peptide hydrolyses and further, activation by HCN accelerates the liberation of both amino groups and of free ammonia. The ammonia formed by secondary effects of the reaction medium in boiled digests is only a small fraction of the total ammonia produced during enzyme action. Further investigations are, however, necessary before it can be decided whether the power to hydrolyse peptide and amide bonds resides in the same enzyme or whether papain contains two components with those specific functions.

EXPERIMENTAL

Materials. The proteins used were prepared in the laboratory shortly before use. We have invariably found that commercial preparations of proteins are entirely unsuitable for quantitative digestion experiments on account of their physical properties which cause difficulty in getting them into solution or uniform suspension. The preparations were obtained by the usual methods, dehydrated with acetone without allowing prolonged contact, and the acetone removed rapidly by a current of air.

Enzymes. In most of the experiments B.D.H. preparations of pepsin and trypsin were used. In the series with purified enzymes erepsin-free trypsin (proteinase + carboxypolypeptidase) was prepared from defatted pig's pancreas by the method described by Waldschmidt-Leitz & Harteneck [1925]. Erepsin solution was obtained for ordinary experiments from an 87% glycerol extract of pig's intestinal mucosa by the following procedure, all operations involved being carried out at 0°. One volume of the glycerol extract was diluted with three volumes of ice-cold water and centrifuged. The supernatant was mixed with one-tenth its volume of *N*/10 acetic acid and again centrifuged to remove extraneous protein. The clear centrifugate was brought to pH 7.8 with alkali and immediately used. Trypsin-free erepsin (aminopolypeptidase + dipeptidase) was obtained from the crude solution obtained above by adsorption at pH 4.8 on aluminium hydroxide, *C*_γ, and elution with phosphate buffer at pH 8.3 [Waldschmidt-Leitz & Schaffner, 1926].

Papain used in these investigations was prepared in the laboratory from fresh papaya latex. The latex liberated by scarifying the skin of the fresh green fruits with a bone spatula was collected in a porcelain dish, the thick white fluid squeezed through muslin and precipitated by adding several volumes of ice-cold acetone. The precipitate was washed twice with acetone and dried over sulphuric acid *in vacuo*. A snow-white powder was thus obtained which dispersed in water to give a slightly opalescent solution. The preparation gave tests for peroxidase. For activation by HCN the method of Willstätter & Grassmann [1924] was used.

Preparation of digests. The digests were generally made up to contain 2 g. of protein and 0.1 g. of enzyme per 100 ml.

The starting of the digestion with pepsin was slightly different with the three proteins. With casein a solution in *N*/10 HCl could be readily obtained and after the addition of water and pepsin solution readings could be commenced immediately after mixing. Edestin suspended in *N*/10 HCl went into solution on being kept at 37° for 1 hr. with repeated shaking. Gliadin on the other hand formed a sticky mass on being suspended in *N*/10 HCl and went into solution only after digestion with pepsin for 3 hr. In all cases quantitative measurements were commenced only after homogeneous solutions were obtained. For digestion with papain the proteins were first brought into solution by means of pepsin which was then inactivated by boiling.

At the end of the action of pepsin, after leaving a small quantity for studying the effect of prolonged digestion, the main portion of the digest was brought to pH 6 and boiled to inactivate the enzyme. At this reaction it was found that boiling for 15 min. caused no appreciable increase in amino-N or ammonia. The solution was now cooled to 37° and, after the reaction was adjusted to pH 8.6 with NaOH, transferred to a measuring flask, mixed with a 5% trypsin solution containing one-twentieth the weight of the protein now present, and the volume made up at 37°. Readings were commenced immediately. A similar procedure was adopted in transferring the digest from trypsin to erepsin, 100 ml. of the latter being added to each 900 ml. of the inactivated tryptic digest. After the action of each enzyme had proceeded to a considerable extent a portion of the digest was inactivated and incubated to study the secondary formation of ammonia from the cleavage products under the influence of the reaction of the medium. The inactivation was brought about by boiling at pH 6, the reaction being then adjusted to the original pH of the particular digest.

Methods of analysis. On account of the difficulty of quantitatively dissolving a weighed amount of protein in a definite volume of liquid, calculation of percentage hydrolysis was made not on the basis of the weight of protein but on

the results of analysis of an aliquot of the digest subjected to complete hydrolysis by HCl and in which amino-N and ammonia-N were determined in the same way as in the enzyme digest. Such analyses were carried out after the addition of each enzyme and also in digests inactivated by boiling.

Nitrogen was determined by micro-Kjeldahl, amino nitrogen by Sørensen's formol titration (from pH 6.8 in aqueous solution to distinct pink with phenolphthalein in formalin). Free ammonia was determined by distillation *in vacuo* at 40° in the apparatus of Parnas & Heller [1924] but without the use of steam; a 10 % solution of sodium carbonate was used for making the solution alkaline; it was found that distillation was always complete in 10 min. during which time 9–10 ml. of the distillate were collected.

Certain unavoidable errors in the methods of analysis have to be noted, viz. the interference in the formaldehyde titration by the ammonia present in the digest, and in the case of purified ereptic digests by phosphate in the buffer solution used for elution, as also the possible formation of ammonia from cyanide during vacuum distillation with sodium carbonate of papain-HCN digests. But experimentally and by calculation it could be shown that these errors are not of sufficient magnitude to affect the significance of the results obtained.

SUMMARY

A study has been made of the progressive liberation of ammonia and of amino-groups during the action of pepsin, trypsin, erepsin and of papain on three typical proteins, casein, edestin and gliadin.

It is shown that amide and peptide hydrolyses do not by any means run parallel and that the ammonia formed is, at least in great part, not directly connected with proteolytic action but arises from the decomposition of the primary products of protein cleavage. This secondary decomposition is not enzymic in nature but is brought about by the acidic or alkaline reactions of the digestion media.

In papain digestion, on the other hand, ammonia formation appears to be an enzymic function. Papain either possesses deamidase activity itself or contains a component which has such activity.

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