CCXLVII. ENZYMIC PROTEOLYSIS

II. THE FORMATION OF FREE TYROSINE IN PEPTIC DIGESTION

BY M. DAMODARAN AND P. S. KRISHNAN

From the University Biochemical Laboratory, Cheparuk, Madras

(Received 21 July 1938)

THE liberation of free amino-acids during the digestion of proteins by pepsin has recently become the subject of controversy [cf. Abderhalden, 1937]. The view generally held has been that the action of pepsin ends with the fission of proteins into high-molecular complexes from which amino-acids are set free only by the further action of enzymes with specific peptidase function. In considering the evidence for this view it is necessary to bear in mind that most of the experiments on which it is based were carried out more than 30 years ago [Abderhalden, Baumann, Kautzsch, Körösy & London, 1905-7] and were designed mainly to throw light on the physiological question of the fate of protein in the alimentary tract. The procedure adopted in the well-known experiments of Abderhalden, London and co-workers cited above was to analyse the intestinal contents of dogs collected soon after a protein meal either by killing the animals or by means of fistulae established at different points of the alimentary tract. The experiments led to valuable results on which much of our present knowledge of digestion and absorption of proteins is based, but the chemical technique available was hardly adequate to elucidate the finer chemical differences in the mode of action of the proteolytic enzymes concerned. Thus in one paper by Abderhalden *et al.* [1906] we have the interesting statement, "Eine quantitative Methode zur Bestimmung von Aminosäuren aus Gemischen kennen wir nicht". Distillation after esterification was the method adopted for the detection of amino-acids in the mixtures they were dealing with. From stomach brei, in contrast to material obtained from the duodenum, jejunum etc., amino-acid esters were obtained either not at all, or only in traces. In view of the fact that esterification itself involved processes that might cause protein hydrolysis, it is not surprising that Abderhalden [1931] came to the conclusion that peptic digestion differs from that by trypsin and erepsin in not leading to the formation of free amino-acids.

More recently the same conclusion has been reached by Waldschmidt-Leitz & Künstner [1927] on less justifiable grounds. These authors used alcoholic titration for studying the extent of proteolysis of thymus histone and, on the assumption that peptides in contradistinction to amino-acids titrate quantitatively in 50% alcohol (for which we have found no theoretical or practical justification [cf. Harris, 1929]), claim to have proved the absence of amino-acids from peptic digests.

The evidence for the contrary view rests upon the findings of Northrop [1930] and Calvery & Schock [1936]. The earlier claim of Felix [1925] to have isolated lysine from peptic digests of thymus histone (as benzoyllysine) cannot be accepted without further confirmation, for, according to this author the liberation of free lysine was unaccompanied by any increase in amino or carboxyl groups and involves the assumption that the combination of lysine in histone is different

from that in other proteins. Northrop [1930] found that during the auto-digestion of crystalline pepsin "a large amount of tyrosine crystallised out". From 100 g. of egg albumin digested with 20 g. of pepsin for 26 days Calvery & Schock [1936] isolated 565 mg. of tyrosine; from the same amount of pepsin allowed to digest itself only 150 mg. of tyrosine were obtained.

Abderhalden [1937], on repeating these experiments with casein, was unable to obtain any tyrosine by direct crystallization and ascribes Calvery's findings to bacterial contamination, which according to the former is possible even in the strongly acid medium required for peptic digestion. Exception has been taken to these results also on the ground that the tyrosine present might have been formed through the agency of the acid present in the digest [Oppenheimer, 1936].

We have frequently noticed the crystallization of small quantities of tyrosine in concentrating large scale peptic digests of proteins prepared for various researches. But at the same time the validity of the objections raised by Abderhalden and Oppenheimer could not be denied as the digests were usually allowed to stand for varying periods and no special precautions against bacterial contamination had been taken. Calvery also allowed a digestion period of 26 days and the solution from which he obtained his tyrosine had been kept for 4 months in the ice chest. Digestion experiments were therefore carried out with casein, taking all precautions for excluding bacterial action, with proper controls to take into account the possible formation of tyrosine by the action of the acid medium or by the self-digestion of the enzyme employed, and limiting enzyme action to a short period. From a number of such digests, after preliminary treatment with phosphotungstic acid, we have been able to crystallize out appreciable quantities of tyrosine without any difficulty.

Quantitative determinations were also made during the course of digestion of the extent of peptide groups hydrolysed by formaldehyde titration and of the amount of tyrosine not precipitable by phosphotungstic acid by the colorimetric method of Folin & Ciocalteu [1927]. It is perhaps necessary to make it clear that the latter method does not determine free as distinct from combined tyrosine. Being based upon Millon's reaction which requires only the phenolic hydroxyl of tyrosine to be uncombined [Vaubel, 1900] the determination is valid for tyrosine in solution whether free or in peptide combination, though the fact that it is generally applied to hydrolysed protein has probably caused some misunderstanding. In a method due to Zuwerkalow [1927], of which the Folin-Ciocalteu method is a modification, the determination is made on the intact protein and we have found that satisfactory values of the tyrosine content of proteins can be obtained by this procedure. Emphasis has been laid on this point because in similar experiments on peptic digests analytical values meant to show that arginine was present in the form of low-molecular compounds not precipitable by salicylsulphonic acid [Lieben & Lieber, 1934] have been misconstrued [Calvery *et al.*, 1936] to mean the presence of free arginine.

In the present experiments the tyrosine values were determined after different periods of digestion on the filtrates after treatment of the digest with phosphotungstic acid in H_2SO_4 ; these values, therefore, represent the amount of tyrosine not combined in the form of large polypeptides (which can justifiably be assumed to be precipitable by phosphotungstic acid) and serve the purpose of setting the upper limit to the amount of free tyrosine present at any stage of digestion. The values given in Table I show the progress of peptide hydrolysis and the liberation of tyrosine, in so far as the latter is indicated by the amount present in the phosphotungstic acid filtrate, in a typical experiment. At the

end of 7 days' digestion with pepsin 6.13% of the tyrosine in casein was in a form not precipitable by phosphotungstic acid. Analysis of the digest at this stage also showed that the filtrate from phosphotungstic acid contained 6.02% of the total N of the digest, of which 45% was in peptide form (Table II).

The yield of pure tyrosine isolated from one such digest was 240 mg. from 203.3 g. of casein, equivalent to 1.8% of the total tyrosine present in the casein or 61% of the tyrosine not precipitable by phosphotungstic acid. From the mother liquor from the first crop of tyrosine a second solid fraction weighing 342 mg. could be obtained by further evaporation which, according to colorimetric estimation, contained 103 mg. of tyrosine; but the pure amino-acid could not be isolated from this fraction by recrystallization and it is not possible to say if this tyrosine was present in the free or combined state. In a control experiment without the addition of pepsin, in which an equal weight of casein was kept under otherwise identical conditions of temperature and acidity for a period of 14 days, not only could no tyrosine be isolated but the phosphotungstic acid filtrate did not show the slightest trace of tyrosine free or combined on being concentrated and treated with Millon's reagent. In an enzyme control in which an equal weight of pepsin was allowed to digest itself the phosphotungstic acid filtrate contained less than 17 mg. of tyrosine.

Abderhalden, in contradicting the findings of Calvery, has laid emphasis on the possibility of micro-organisms being active in peptic digests in spite of the acidity of the medium, as lumps of protein are usually present during the initial stages and the interiors of these would not be accessible to the bactericidal action of the acid. In one experiment, therefore, casein suspended in water was first sterilized by heat before the addition of H_2SO_4 and pepsin. From 202.5 g. of sterilized casein digested for 14 days 234 mg. of pure tyrosine and a further 145 mg. of the nearly pure amino-acid were isolated. From sterilized casein without the addition of pepsin, but kept under otherwise identical conditions, no tyrosine could be detected in solution.

There can be no doubt, therefore, that peptic digestion gives rise to free tyrosine. The amount of free amino-acids produced by the action of pepsin may be too small to be of physiological significance on account of the short time during which the chyme remains in the stomach, but in studying the chemical mode of action of the enzyme and its relation to protein structure, the ability of pepsin to liberate amino-acids will undoubtedly have to be taken into account.

EXPERIMENTAL

Rate of liberation of tyrosine

50 g. casein were digested with 2.5 g. pepsin under conditions described later in the section on isolation. Peptide hydrolysis was determined daily on 5 ml. aliquots by formaldehyde titration. Simultaneously, 100 ml. of the digest were pipetted out for determination of tyrosine not precipitable by phosphotungstic acid by the following procedure: the solution was made 5% acidic with 50% H_2SO_4 and treated with a 30% solution of phosphotungstic acid in 5% H_2SO_4 added in slight excess. The precipitate was separated by centrifuging and washed thoroughly with dilute phosphotungstic acid- H_2SO_4 . The centrifugate and washings were combined, freed from phosphotungstic acid and H_2SO_4 by baryta which was itself quantitatively eliminated by H_2SO_4 , concentrated *in vacuo* at 40° and finally made up to 25 ml. Tyrosine was estimated on 10 ml. aliquots according to Folin & Ciocalteu [1927]. Total tyrosine in the digest was determined by the method of Zuwerkalow [1927].

The extent of peptide hydrolysis at each stage was calculated from the increase in amino-N as determined by formaldehyde titration, by comparison with the amino-N on complete hydrolysis. The value of the latter was taken to be 68.14% of the total N as calculated from Van Slyke's data for the N distribution of casein [Plimmer, 1917] by adding together the whole of the amino-N, $\frac{1}{4}$ of the arginine-N, $\frac{1}{3}$ of the histidine-N and the whole of the lysine- and cystine-N; the total N in the solution was estimated in a 5 ml. aliquot by the micro-Kjeldahl method.

The results are summarized in Table I.

Table I

50 g. B.D.H. "Light White" casein + 2.5 g. B.D.H. pepsin in 1 litre approximately $N/20$ H_2SO_4 , pH 1.8.

Total N	= 30.07 mg./5 ml.			
Amino-N on complete hydrolysis (calc.)	= 20.48 mg./5 ml.			
	= 14.63 ml. $N/10$ NaOH			
Total tyrosine	= 261 mg./100 ml.			
Days	Formaldehyde titre; ml. $N/10$ NaOH	Peptide hydrolysis %	Tyrosine in phosphotungstic acid filtrate	
			mg./100 ml. of digest	% of total tyrosine
0	0.83	—	—	—
1	1.97	7.79	4.41	1.69
2	2.43	10.93	6.37	2.44
3	3.12	15.65	7.93	3.04
4	3.44	17.83	12.00	4.60
5	3.53	18.45	13.22	5.07
6	3.62	19.06	15.73	6.03
7	3.70	19.62	16.00	6.13

Composition of the phosphotungstic acid filtrate at the end of digestion

On the 7th day 200 ml. of the digest were pipetted out and treated with phosphotungstic acid as already described. After removal of reagents the solution was concentrated *in vacuo* and made up to 25 ml. Total N (micro-Kjeldahl) and amino-N (Van Slyke) were determined on aliquots. 10 ml. of the solution were hydrolysed with 30 ml. of conc. HCl for 10 hr.; the HCl was removed as completely as possible by distillation to dryness *in vacuo*, the solution made faintly alkaline by adding a 10% suspension of baryta and distilled again under reduced pressure to expel any ammonia present. The residual solution was made up to 25 ml. and amino-N estimated in aliquots. The results are given in Table II.

Table II

	mg./200 ml. digest	%
Total N in digest	1202.6	—
Total N in phosphotungstic acid filtrate	72.4	6.02
Amino-N in phosphotungstic acid filtrate before hydrolysis	37.68	52.05
Amino-N in phosphotungstic acid filtrate after hydrolysis	70.55	97.45
Peptide-N	—	45.40

Isolation of tyrosine

250 g. of B.D.H. light white casein were ground up to a uniform suspension with 2.5 l. of $N/10$ H_2SO_4 at 37° and mixed with an equal volume of sterile water in a sterilized pyrex flask. After adjusting to pH 1.8, 7.5 g. of B.D.H. pepsin dissolved in a small quantity of $N/20$ H_2SO_4 were added and the mixture incubated

at 37°. Care was taken to ensure that there were no particles of protein adhering to the sides of the flask above the liquid level and sufficient toluene was added to form a thick layer reaching to the neck of the flask. After a day's digestion the pH, which had changed to 2.2, was readjusted and a further 5 g. of pepsin dissolved in 25 ml. of $N/20$ H_2SO_4 added. The pH showed no appreciable change after the third day. After the first few days the "paranuclein" separating out of the solution settled to the bottom, giving a clear golden brown supernatant liquid.

After 7 days, when formaldehyde titration showed a hydrolysis of 18.01 %, 3800 ml. of the digest (containing on the basis of N determinations 203.3 g. of casein) were filtered, the clear solution cooled in ice and treated with sufficient 50% H_2SO_4 also previously cooled in ice, to make the whole solution 5% acidic with respect to H_2SO_4 . A concentrated solution of phosphotungstic acid in 5% H_2SO_4 was now added with vigorous mechanical stirring until precipitation was complete, the temperature being maintained throughout at 0°. About 600 g. phosphotungstic acid were required. The mixture was left in the ice chest for 2 days after which the bulky precipitate was removed by centrifuging and washed with ice-cold dilute phosphotungstic acid. To the combined solution and washings baryta was added in the form of a thin cream. The precipitate of barium phosphotungstate and sulphate was separated on the centrifuge and washed thoroughly with baryta water. The combined supernatant liquids were then freed from Ba quantitatively by means of H_2SO_4 . The final solution, which was about 5 l. in volume and quite colourless, was concentrated under reduced pressure at 45° to about 700 ml. The solution, which at this stage had a pale bluish tinge, was again made strongly alkaline with baryta to precipitate residual phosphotungstic acid, the small precipitate formed centrifuged off and the Ba again quantitatively removed from the solution by dilute H_2SO_4 . The solution was again concentrated *in vacuo* to about 125 ml. and then transferred to a crystallizing dish in a vacuum desiccator over H_2SO_4 . Characteristic crystals of tyrosine separated overnight. They were filtered with suction, washed with a little ice-cold water, then with alcohol and ether and finally dried in the vacuum desiccator over H_2SO_4 . The solid was perfectly white and weighed 243 mg.

A specimen dried *in vacuo* over P_2O_5 at 100° gave on analysis N, 7.597%, amino-N, 7.602%. $C_9H_{11}NO_3$ requires N, 7.735%. After a single recrystallization from hot water, without the use of charcoal, the values were N, 7.716%, amino-N, 7.732%.

The mother liquor on further concentration in the vacuum desiccator gave a thick deposit of solids which was filtered on a glass filter with suction. The material was inclined to be sticky but was obtained as a granular powder by repeated washing with 95% alcohol. It weighed 342 mg. and contained 9.605% N and 9.171% amino-N. Pure tyrosine could not be isolated from this fraction by recrystallization, but colorimetric estimation showed that it contained 103 mg. of the amino-acid. A trace of the solid gave a violet coloration with quinone in the cold in the presence of a drop of sodium carbonate.

The alcoholic washings from the second crop of solids on evaporation to dryness gave a thick syrup which contained 26.05 mg. total N and 13.02 mg. amino-N indicating the presence of peptides. Tests for tryptophan were negative; Millon's reagent gave a feeble colour.

Substrate control

250 g. of casein were suspended in 5 l. of $N/20$ H_2SO_4 and, after adjustment of the pH to 1.8, left in the incubator under toluene. After 14 days the clear liquid was filtered from the unaltered casein and treated with phosphotungstic acid as already described. The slight precipitate that was obtained was filtered off and the filtrate freed from reagents. The final solution, having a volume of about 5 l., gave no colour with Millon's reagent. It was concentrated under reduced pressure to about 500 ml. At this stage also Millon's reaction was negative. The last traces of H_2SO_4 were now quantitatively removed and the solution concentrated to about 10 ml. when a minute amount of crystalline material separated out, the Millon's reaction being, however, still negative. The separated crystals were inorganic.

Enzyme control

2.5 g. of pepsin were dissolved in 1 l. of $N/20$ H_2SO_4 (same concentration of enzyme as used in the digests) and incubated at 37° under toluene. After 7 days 200 ml. of the digest were pipetted out and precipitated with phosphotungstic acid in the usual way. The filtrate, after removal of phosphotungstic acid, etc., was concentrated *in vacuo* and made up to 25 ml. Aliquots of 10 ml. were taken for the duplicate estimation of tyrosine by Folin & Ciocalteu's method. Since the amount of tyrosine contained in the solution was small a known volume of standard tyrosine solution was added to the unknown before making colour comparison and the amount originally present obtained by difference. 200 ml. of the enzyme solution were found to contain 0.692 mg. of tyrosine not precipitable by phosphotungstic acid.

Isolation of tyrosine from sterilized casein digest

Casein prepared according to Cohn & Hendry [1930] was used directly after the final precipitation and washing without drying. Sufficient wet casein to give about 250 g. dry protein was suspended in 2.5 l. of water and sterilized by heating in a steamer for 1 hr. each day on three successive days. To the suspension cooled to 37° 2.5 l. of approximately $N/10$ H_2SO_4 were added slowly and the pH adjusted to 1.8. With vigorous mechanical stirring during the addition of acid formation of lumps could be completely avoided. 12.5 g. of pepsin in dilute H_2SO_4 were added and the mixture incubated under a thick layer of toluene. To avoid all chances of bacterial contamination no samples were withdrawn until the end of the digestion period of 14 days. The procedure after this stage was exactly as described before.

From 4.5 l. of the digest which, according to analysis, contained 1.32 g. of tyrosine on the whole and 517 mg. in a form not precipitable by phosphotungstic acid, two crops of crystals weighing 234 mg. and 145 mg. respectively were isolated. The first fraction gave N, 7.36% and amino-N, 7.49% on isolation and 7.67% and 7.65% respectively after one recrystallization. The second fraction which was not recrystallized gave N, 7.85% and amino-N, 7.97%. The result with sterilized casein suspended in acid without pepsin was exactly the same as with the substrate control already described.

SUMMARY

The liberation of tyrosine during peptic digestion of casein has been quantitatively followed. It is shown that after 7 days' digestion about 6% of the total tyrosine and roughly the same proportion of the total N of the protein are in a form not precipitable by phosphotungstic acid.

From peptic digests of casein, carried out under conditions where bacterial contamination was completely excluded, tyrosine has been isolated in yields amounting to 1.7–1.8% of the total tyrosine in the protein.

The tyrosine produced by self-digestion of the enzyme forms but a small fraction of that obtained from the casein digest.

Action of acid in the concentration necessary for peptic digestion does not lead to the liberation of the free amino-acid from casein.

REFERENCES

- Abderhalden (1905). *Hoppe-Seyl. Z.* **44**, 17.
— (1931). *Lehrbuch der physiologischen Chemie*, 6th ed., p. 329. Berlin.
— (1937). *Fermentforschung*, **15**, 315.
— Baumann & London (1907). *Hoppe-Seyl. Z.* **51**, 384.
— Kautzsch & London (1906). *Hoppe-Seyl. Z.* **48**, 549.
— Körösy & London (1907). *Hoppe-Seyl. Z.* **53**, 148.
Calvery & Schock (1936). *J. biol. Chem.* **113**, 15.
— Block & Schock (1936). *J. biol. Chem.* **113**, 21.
Cohn & Hendry (1930). *Organic Syntheses*, **10**, 16.
Felix (1925). *Hoppe-Seyl. Z.* **146**, 103.
Folin & Ciocalteu (1927). *J. biol. Chem.* **73**, 627.
Harris (1929). *J. biol. Chem.* **84**, 302.
Lieben & Lieber (1934). *Biochem. Z.* **275**, 38.
Northrop (1930). *J. gen. Physiol.* **13**, 739.
Oppenheimer (1936). *Die Fermente und ihre Wirkungen*, 5th ed. suppl. pp. 672, 845. The Hague.
Plimmer (1917). *The Chemical Constitution of the Proteins*, Part I, p. 132.
Vaubel (1900). *Z. angew. Chem.* 1126.
Waldschmidt-Leitz & Künstner (1927). *Hoppe-Seyl. Z.* **171**, 290.
Zuwerkalow (1927). *Hoppe-Seyl. Z.* **163**, 185.