- Levy, G. A. (1948). *Biochem. J.* **42**, 2.
 Levy, G. A. (1952). *Biochem. J.* **52**, 464.
 Levy, G. A. (1954). *Biochem. J.* **53**, 462.
 Levy, G. A. & Marsh, C. A. (1952). *Biochem. J.* **52**, 690.
 Lohmar, R., Dimler, R. J., Moore, S. & Link, K. P. (1942). *J. biol. Chem.* **143**, 551.
 Marsh, C. A. (1952). *J. chem. Soc.* p. 1578.
 Marsh, C. A. (1954). *Biochem. J.* **53**, 609.
 Marsh, C. A., Alexander, F. & Levy, G. A. (1952). *Nature, Lond.*, **170**, 163.
 Mills, G. T., Paul, J. & Smith, E. E. B. (1953). *Biochem. J.* **53**, 232.
 Miwa, T. (1932). *Acta phytochim.*, Tokyo, **6**, 154.
 Owen, L. N., Peat, S. & Jones, W. J. G. (1941). *J. chem. Soc.* p. 339.
 Pryde, J. & Williams, R. T. (1933). *Biochem. J.* **27**, 1210.
 Reichstein, T. & Grüssner, A. (1934). *Helv. chim. acta*, **17**, 311.
 Robinson, D., Smith, J. N. & Williams, R. T. (1953). *Biochem. J.* **53**, 125.
 Sammons, H. G. & Williams, R. T. (1946). *Biochem. J.* **40**, 223.
 Smith, F. (1944). *J. chem. Soc.* p. 633.
 Smith, F. (1951). *J. chem. Soc.* p. 2646.
 Spencer, B. & Williams, R. T. (1951). *Biochem. J.* **48**, 537.
 Treibs, W. & Franke, I. (1950). *Ann. Chim. (Phys.)*, **570**, 76.
 Tsou, K.-C. & Seligman, A. M. (1952). *J. Amer. chem. Soc.* **74**, 5605.
 Vischer, E. & Reichstein, T. (1944). *Helv. chim. acta*, **27**, 1332.
 Wong, E. & Rossiter, R. J. (1951). *Canad. J. med. Sci.* **29**, 195.
 Yackel, E. C. & Kenyon, W. O. (1942). *J. Amer. chem. Soc.* **64**, 121.

The N-Terminal Amino Acid Residues of Gelatin

3. ENZYMIC DEGRADATION

By A. COURTS

The British Gelatine and Glue Research Association, 2a Dalmeny Avenue, London, N. 7

(Received 13 September 1954)

The specific action of proteolytic enzymes on small peptides is well known (for reviews see Neurath & Schwert, 1950; Smith, 1951). The work reported in the present paper describes the hydrolytic effect of pepsin, trypsin, chymotrypsin and papain on gelatin using the *N*-terminal residue technique to follow the extent of degradation and to examine enzyme specificity. The *N*-terminal residue technique was employed in Part 2 of this series (Courts, 1954*b*) to examine thermal degradation of gelatin catalysed by hydrogen and hydroxyl ions. The technique does not permit the bonds broken to be specified, but indicates the amino acid residues carrying the amino groups which are released during hydrolysis.

MATERIALS AND METHODS

Gelatin. An alkali-processed ox-bone gelatin of isoionic point pH 5.1 was used, being identical with the material used in Part 2 of this series. The substrate was 2.5 g. in 50 ml. water adjusted to a suitable pH value.

Trypsin. Crystallized trypsin (Armour Laboratories, Chicago, U.S.A.) (0.02 g.) was used at pH 8.5. The reaction was allowed to proceed for 2.5 hr. at 21, 35, 44, 56 and 65°. The author is most grateful to Dr Watson of the Courtauld Laboratories for a gift of this enzyme.

Pepsin. Crystallized pepsin (Armour Laboratories) (0.02 g.) was used at pH 1.87 and 37°. The reaction was carried out for 6 and 19 hr. Controls were run without enzyme to allow for thermal degradation at this pH value.

Chymotrypsin. Crystallized chymotrypsin (Armour Laboratories) (0.02 g.) was used at pH 8.0 and 37°. Reaction times were 2, 4 and 6 hr. A second series was carried out to observe the effect of enzyme concentration and of variation of pH; thus 0.002 g. of chymotrypsin was incubated at 37° for 4 hr. with solutions of substrate at pH 5.1 (isoionic point of the gelatin) and at pH 8.0 (reputed optimum for this enzyme).

Papain. Commercial papain (Norman Evans and Rais, Manchester) (0.002 g.) was used at 37°. Hydrolysis was allowed to continue for 2 and 5 days at (a) pH 5.05, the pH optimum of papain for partially hydrolysed protein and (b) pH 6.95, the pH optimum for intact protein substrates. The extent of thermal degradation under these conditions was also examined.

N-Terminal residue determinations

The enzymically degraded gelatins were adjusted to pH 8.5 and allowed to react with 1:2:4-fluorodinitrobenzene (FDNB). The preparation of dinitrophenyl (DNP) derivatives, their subsequent hydrolysis and the evaluation of *N*-terminal residues were all carried out using the methods previously described by Courts (1954*a*, *b*).

Considerations of proteinase inactivation

No attempts were made to remove or inactivate the proteinase before allowing its digest to react with FDNB. Pepsin would be automatically inactivated at pH 8.5. It is likely that chymotrypsin would be inactivated by FDNB through substitution at its tyrosyl phenolic groups (cf. Sizer, 1945). Papain may likewise be inactivated by blocking of sulphhydryl groupings (Smith, 1951), although for both

these reactions there may be delay before they are complete. There is no evidence to show that the active centres of trypsin are affected by dinitrophenyl or similar substitution. Since the principal objective was to study the amino end-groups released, it would not materially affect any conclusions if some tryptic hydrolysis continued into the early stages of the FDNB reaction.

RESULTS

Papain. After digesting the gelatin with papain the *N*-terminal residues detected were qualitatively similar to those obtained in intact gelatin (Courts, 1954*a*) and in gelatin which had been thermally degraded. Quantitative measurements showed that the number of end groups had substantially increased (Table 1), and that the enzyme exhibited a marked specificity for peptide bonds involving the amino groups of alanine. Both the amount of degradation and the degree of specificity were considerably more pronounced for digestions at pH 5 than at pH 7.

Chymotrypsin. The change in end groups when chymotrypsin was used for digesting gelatin is summarized in Table 2. Observations reported in Table 1 suggest that thermal degradation under

these conditions was negligible. All the *N*-terminal residues corresponded to those originally found in undegraded gelatin and were present in similar proportions but substantially increased in amount. Certain other points are illustrated by Table 2: (i) contrary to the findings with papain, chymotrypsin was more vigorous at the normal pH optimum than at pH 5.1; (ii) the effect of increasing enzyme concentration by 1000 % only increased the number of peptide bonds broken by about 60 %; (iii) the rate of breakdown was initially very rapid, falling off gradually after 2 hr., suggesting exhaustion of some readily attacked bonds.

Pepsin. The rate of peptic hydrolysis of gelatin was comparatively slow in the conditions used. An increase occurred in all the *N*-terminal residues found in the non-degraded material. There was an 'above average' attack on peptide bonds involving alanine groups ($\times 8$ at 6 hr.; $\times 12$ at 19 hr.) and similarly for valine, the major constituent of others in the table ($\times 9$ at 6 and 19 hr.). There was, correspondingly, a 'below average' attack on bonds involving glycine groups. This is illustrated in Table 3.

Table 1. *Liberation of N-terminal residues during degradation of gelatin by papain*

For conditions, see text. End groups are expressed as moles/100 000 g. gelatin. M_n is number-average molecular weight calculated from end groups.

	Undegraded	Thermal degradation				Enzymic degradation			
		pH 5.05		pH 6.95		pH 5.05		pH 6.95	
		2 days	5 days	2 days	5 days	2 days	5 days	2 days	5 days
Glycine	0.95	0.97	1.23	1.00	1.21	7.10	14.00	2.44	6.32
Serine	0.29	0.31	0.30	0.27	0.33	2.28	2.40	0.48	1.05
Threonine	0.12	0.10	0.15	0.18	0.16	2.16	4.00	0.57	1.00
Alanine	0.14	0.16	0.16	0.14	0.19	9.03	12.24	1.18	4.61
Aspartic acid	0.08	0.11	0.16	0.16	0.16	1.28	3.42	0.41	0.77
Glutamic acid	0.14	0.15	0.15	0.16	0.20	0.49	1.78	0.20	0.57
Others	0.00	0.14	0.14	0.14	0.15	1.10	1.50	0.24	1.10
Total	1.73	1.94	2.29	2.05	2.40	23.44	39.34	5.52	15.42
M_n	58 000	52 000	44 000	50 000	42 000	4300	2500	17 000	6400
Approx. increase in glycine						$\times 7$	$\times 14$	$\times 2\frac{1}{2}$	$\times 6$
Approx. increase in alanine						$\times 80$	$\times 100$	$\times 10$	$\times 30$

Table 2. *Degradation of gelatin by chymotrypsin*

For conditions, see text. End groups are expressed as moles/100 000 g. gelatin. M_n is number-average molecular weight calculated from end groups.

	Undegraded	8 mg. enzyme/g. gelatin			0.8 mg. enzyme/g. gelatin	
		pH 8.0			pH 8.0	pH 5.1
		2 hr.	4 hr.	6 hr.	4 hr.	4 hr.
Glycine	0.95	5.08	7.56	8.34	4.53	2.4
Serine	0.29	0.90	0.92	1.25	0.92	0.59
Threonine	0.12	0.52	0.62	0.71	0.43	0.29
Alanine	0.14	1.00	1.24	1.40	1.00	0.57
Aspartic acid	0.08	0.55	0.61	0.75	0.36	0.33
Glutamic acid	0.14	0.37	0.58	0.85	0.33	0.16
Others	0.00	1.00	1.50	2.20	0.90	0.57
Total	1.73	9.42	13.03	15.50	8.48	4.92
M_n	58 000	10 000	7 200	6 000	11 000	19 200

Table 3. *Degradation of gelatin with pepsin*

For conditions, see text. End groups are expressed as moles/100 000 g. gelatin. M_n is number-average molecular weight calculated from end groups.

	Undegraded	Thermal degradation		Enzymic degradation	
		6 hr.	19 hr.	6 hr.	19 hr.
Glycine	0.95	1.09	1.20	1.89	2.81
Serine	0.29	0.28	0.27	0.58	0.68
Threonine	0.12	0.16	0.17	0.65	0.72
Alanine	0.14	0.17	0.17	1.41	2.14
Aspartic acid	0.08	0.15	0.19	0.41	0.69
Glutamic acid	0.14	0.15	0.11	0.19	0.45
Others	0.00	0.00	0.20	1.20	2.00
Total	1.73	2.00	2.31	6.33	9.49
M_n	58 000	50 000	43 300	15 700	10 500

Table 4. *Degradation of gelatin by trypsin*

For conditions, see text. End groups are expressed as moles/100 000 g. gelatin. M_n is number-average molecular weight calculated from end groups.

	21°	35°	44°	56°	65°
Glycine	27.26	36.92	40.44	28.51	22.20
Serine	0.80	0.81	1.17	1.05	1.18
Threonine	1.09	1.50	1.36	1.22	1.03
Alanine	0.71	0.46	0.85	0.82	0.83
Aspartic acid	0.89	1.07	1.25	0.74	0.62
Glutamic acid	1.36	0.43	0.80	0.51	0.80
Others	0.90	1.50	1.30	1.10	2.30
Total	33.01	42.69	47.17	33.15	28.96
M_n	3 000	2 300	2 100	3 000	3 450

Trypsin. While a small increase in all the expected *N*-terminal residues occurred during tryptic hydrolysis of gelatin, by far the greater number were derived from peptide bonds involving the amino group of glycine (see Table 4). This is prevalent throughout the temperature range examined (21–65°). The temperature optimum appears to be at about 44° for the 2.5 hr. digestion.

Artifact. It was observed during these experiments involving the mild degradation of gelatin at 37°, that the DNP-derivatives on hydrolysis gave rise to a slow moving artifact (*R*, 0.02) on a Celite column buffered at pH 4 using chloroform–ether (90:10 by vol.) as the organic phase eluent. This *R* value does not correspond to a DNP-derivative of any of the amino acids known to occur in gelatin hydrolysates, neither does it correspond to DNP-glucosamine (*R*, 0.12), DNP-chondrosamine (*R*, 0.12) or DNP- β -hydroxyglutamic acid (*R*, 0.33).

DISCUSSION

The general conclusion from results for the four enzymes used is that specificity is much less restricted than for peptide substrates, and, where it occurs, it does not correspond with the results for peptides. The classical investigations of Fruton & Bergmann (1939), using pepsin on synthetic substrates, suggested that tyrosine and phenylalanine would occur as the principal *N*-terminal residues of

peptic digests. This has not been found with gelatin. In this instance the enzyme has attacked more readily bonds involving the amino groups of alanine, and valine, but in addition many other bonds are attacked. Other protein–pepsin hydrolysates examined by the Sanger technique also afford some evidence of multiple specificity. These are compared in Table 5.

The modes of attack of the four proteinases on gelatin are compared in Table 6. The results suggest that a number of types of bonds are attacked by all four enzymes. Somewhat similar conclusions were reached by Röver, Desnuelle & Bonjour (1950) from observations on horse globin hydrolysed by pancreatic proteinases. The development of end-group methods of analysis is likely to provide further evidence of discrepancies between enzymic specificity towards small peptide units and large protein units. Thus, Gladner & Neurath (1952) have shown that the *C*-terminal residues resulting from the tryptic conversion of chymotrypsinogen into chymotrypsin are tyrosine and leucine, whereas tryptic hydrolysis of synthetic substrates has led to rupture at the carboxyl groupings of arginine and lysine (Neurath & Schwert, 1950).

It appears likely that the emphasis placed upon the importance of synthetic substrates for elucidating protein structure (Smith, 1951; Neurath & Schwert, 1950; also others) may have to be reviewed. The possibility arises that a proteinase reacts in two

Table 5. *N-Terminal residues released in proteins by pepsin*

Protein	Reference	Primary specificity	Secondary specificity
Gelatin	Present work	Alanine, valine	Glycine, serine, threonine, aspartic acid, glutamic acid
Ovalbumin	Desnuelle, Roverý & Bonjour (1950)	Complex mixture	—
Horse globin	Desnuelle <i>et al.</i> (1950)	Alanine, phenylalanine	Leucine, serine
Oxidized insulin A chain	Sanger & Thompson (1953)	Tyrosine, glutamic acid, aspartic acid	Leucine, cysteic acid
Oxidized insulin B chain	Sanger & Tuppy (1951)	Tyrosine, valine, leucine, phenylalanine	Alanine, histidine

Table 6. *N-Terminal residues released during the proteolysis of gelatin*

Proteinase	Primary specificity	Secondary specificity
Pepsin	Alanine, valine	Serine, threonine, aspartic acid, glutamic acid, glycine
Trypsin	Glycine	Serine, threonine, aspartic acid, glutamic acid, alanine
Chymotrypsin	—	Serine, threonine, aspartic acid, glutamic acid, alanine, glycine, valine
Papain	Alanine	Serine, threonine, aspartic acid, glutamic acid, glycine

distinctly different ways. First, it appears to be acting as a true catalyst, enabling aqueous hydrolysis to occur at greatly reduced temperatures. Under these circumstances it is probable that the peptide bonds, which are most sensitive to thermal hydrolysis, would be the most labile, and it may be significant that the *N*-terminal residues found in all the gelatin digests examined are the same as those released during aqueous hydrolysis at high temperatures in the presence of acid or alkali (Courts, 1954*b*). In this respect wool keratin is very similar to gelatin. Native wool shows a wide variety of *N*-terminal residues (Middlebrook, 1951) qualitatively identical with those of gelatin. Middlebrook (1951) showed that degradation of wool with hydrochloric acid provided polypeptide units involving the same end groups. Digestion of wool with papain, in the presence of urea and sodium bisulphite, again yields material with *N*-terminal residues which, except for leucine, are identical with those in the native protein, although quantitatively enhanced (Blackburn, 1950). The second mode of action appears to involve enzyme specificity. Although Desnuelle *et al.* (1950) consider that specific bond-breaking activity precedes random bond breaking, there is no evidence for this in the present work. Enzymic hydrolysis of oxidized insulin chains (Sanger & Tuppy, 1951; Sanger & Thompson, 1953) shows specificities similar in some instances to synthetic peptide substrates, together with secondary specificities. This indicates that the specificity experienced by the synthetic peptides may arise in protein

digests when the polypeptide chains are small enough.

SUMMARY

1. Gelatin has been degraded by pepsin, trypsin, chymotrypsin and papain. The *N*-terminal residues of the hydrolysates were determined by Sanger's fluorodinitrobenzene technique.
2. Attacks on similar peptide bonds are shown in all four cases, although to different degrees so far as observations from the amino end can discern. Papain, for example, attacks bonds involving alanine, and, to a lesser extent, glycine, serine, threonine, aspartic acid and glutamic acid. The results are related to those of certain other workers using different proteins.
3. It is suggested that proteolysis occurs in two ways; one involving a random breaking of peptide bonds, which closely follows the course of thermal degradation, the other involving a specific bond breaking action.

I am grateful to Mr A. G. Ward for the benefit of helpful criticism. This paper is published by permission of the Director and Council of The British Gelatine and Glue Research Association.

REFERENCES

Blackburn, S. (1950). *Biochem. J.* **47**, 443.
Courts, A. (1954*a*). *Biochem. J.* **58**, 70.
Courts, A. (1954*b*). *Biochem. J.* **58**, 74.
Desnuelle, P., Roverý, M. & Bonjour, G. (1950). *Biochim. biophys. Acta*, **5**, 116.

- Fruton, J. S. & Bergmann, M. (1939). *J. biol. Chem.* **127**, 627.
- Gladner, J. A. & Neurath, H. (1952). *Biochim. biophys. Acta*, **9**, 335.
- Middlebrook, W. R. (1951). *Biochim. biophys. Acta*, **7**, 547.
- Neurath, H. & Schwert, G. W. (1950). *Chem. Rev.* **46**, 69.
- Rover, M., Desnuelle, P. & Bonjour, G. (1950). *Biochim. biophys. Acta*, **6**, 166.
- Sanger, F. & Thompson, E. O. P. (1953). *Biochem. J.* **53**, 366.
- Sanger, F. & Tuppy, H. (1951). *Biochem. J.* **49**, 481.
- Sizer, I. W. (1945). *J. biol. Chem.* **160**, 547.
- Smith, E. L. (1951). *The Enzymes*, **1** (2), 793, eds. Sumner & Myrback. New York: Academic Press.

Fish Muscle Riboside Hydrolases

By H. L. A. TARR

Pacific Fisheries Experimental Station, Vancouver, Canada

(Received 14 June 1954)

During an investigation concerning the cause and control of Maillard browning in heated flesh materials it was found that free ribose, in concentrations which occasionally exceeded 0.1% of the wet weight, appeared when muscles of certain fishes were stored at 0° or at higher temperatures. This property was not exhibited by all fish to the same extent, but the muscles of those which were active formed ribose from added ribonucleic acid, adenosine triphosphate, ribonucleotides, ribosides and ribose 5-phosphate (Tarr, 1953, 1954*a*). The total pentose content of the muscles of certain marine fishes examined has ranged from 0.1 to 0.3%, but it is not as yet known whether this occurs as free nucleic acid, as nucleic acid loosely combined with protein as in nucleotopomyosin (Hamoir, 1951*a*, *b*) or in some other form. Since nucleosides have not been observed to be present in appreciable amounts in muscles of fish which have been frozen in liquid nitrogen while still alive, but are often present in relatively high concentrations in such tissue *post mortem*, it would seem probable that they arise by enzymic degradation of nucleic acid. This suggests that fish muscles contain a ribonuclease and other enzymes which hydrolyse ribonucleic acid to its constituent mononucleosides.

The formation of free ribose from these mononucleosides could occur by their direct hydrolysis by riboside hydrolase enzymes, or by nucleoside phosphorylation followed by hydrolysis of the ribose 1-phosphate formed. Riboside hydrolase enzymes have not often been described, phosphorylative mechanisms apparently occurring with greater frequency (Laskowski, 1951). However, Carter (1951) isolated a specific non-phosphorolytic uridine nucleosidase from yeast, and Lampen & Wang (1952) obtained both purine and pyrimidine hydrolytic nucleosidases from *Escherichia coli*. Ribose was not formed from either ribose 1-phos-

phate or ribose 5-phosphate by these two last-named preparations. The present experiments, which have already been described briefly (Tarr, 1954*a*), have shown that fish muscles contain non-phosphorolytic nucleosidases.

EXPERIMENTAL

Materials. The nucleosides, ribose, barium ribose 5-phosphate and yeast ribose nucleic acid were obtained from Nutritional Biochemicals Corporation, Cleveland, Ohio, or from Schwarz Laboratories, Mount Vernon, New York. Cation exchange resin XE64, a finely ground form of IRC50, was purchased from Rohm and Haas Co., Philadelphia, Pennsylvania. The resin was washed and suspended in 0.2M sodium phosphate buffer as described by Hirs, Moore & Stein (1953).

Paper electrophoresis. The technique used was practically identical with that described by Kunkel & Tiselius (1951). Enzyme preparations were freeze-dried before dissolving in 0.1M veronal buffer (pH 8.6) for application in amounts not exceeding 0.05 ml. to Munktel 20S paper. The conditions used were a 16 hr. development at about 3° and 6v/cm. The papers were stained with bromophenol blue and eluted into 0.01N-NaOH as outlined by the above investigators.

Paper chromatography. Ribose was determined quantitatively by paper chromatography (Tarr, 1954*b*). Five separate 10 μ l. amounts of reaction mixture were applied to Whatman no. 1 papers. The papers were developed 16 hr. at 20–22° with ethyl acetate-acetic acid-water solvent (3:1:3, by vol.) (Jermyn & Isherwood, 1949) and sprayed with aniline hydrogen phthalate reagent (Partridge, 1949). The above solvent separates D-ribose from other pentose sugars (Hochster & Watson, 1954), and it was found that the pentose formed from ribosides by the enzymes to be described had an R_f value identical with that of D-ribose. Nucleosides and their corresponding purine and pyrimidine bases were determined by quantitative paper chromatography (Paladini & Leloir, 1952). Five 5 μ l. amounts were applied to Whatman no. 1 papers, which were developed for 3 hr. at 37° with freshly prepared ethyl acetate-acetic acid-water (3:1:1.75, by vol.) solvent. The R_f values were not