The Effects of Proteolytic Enzymes on the Hyaluronic Acid Complex of Ox Synovial Fluid

By B. S. BLUMBERG* AND A. G. OGSTON

Department of Biochemistry, University of Oxford

(Received 11 December 1956)

Since the isolation of hyaluronic acid from vitreous humour by Meyer & Palmer (1934), there has been some uncertainty concerning the relationship between the polysaccharide and protein *in vivo*; Jeanloz (1956) has drawn attention to the need for more information on the relationship between proteins and mucopolysaccharides in general. Most methods of preparation have been designed to remove all protein from the polysaccharide. However, Ogston & Stanier (1950, 1952) claimed that hyaluronic acid isolated from ox synovial fluid by filtration is in the form of a firm complex with a definite proportion of protein, and that attempts to remove this affect the physicochemical properties of the material in solution, particularly its non-Newtonian viscosity. Curtin (1955) showed that this protein is mainly an α -globulin under physiological conditions.

In the present work, specific degradation of this protein has been used to obtain critical proof that it plays an essential part in the constitution and properties of the complex. The results suggest the possibility that systems containing hyaluronic acid may be susceptible to proteinase action *in vivo*, and that such action may play a part in the pathology of diseases in which hyaluronic acid is affected.

EXPERIMENTAL

Materials

Hyaluronic acid complex. (Complex.) Synovial fluid was collected from the astragalotibial joints of oxen immediately after death, as described by Ogston & Stanier (1950). The fluid was centrifuged in a Spinco Model L ultracentrifuge at 73 000 *g* for 30 min. The complex was separated by filtration, with 15 washings, at 4° on a 5-on-3 sintered-glass filter of 1 μ (or less) average pore diameter (Baird & Tatlock Ltd.); the buffer used for washing was 0.2 M-NaCl, 0.0077 M- Na_2HPO_4 , 0.0023 M- KH_2PO_4 (pH 7.3). The complex was finally dissolved in the required buffer, to about half the volume of the original fluid and centrifuged at 73 000 *g* for 30 min. Some samples showed an appreciable amount of 'fast component' (Johnston, 1955), which led to high blank values in the estimation of proteolysis by the Anson method; this was removed by centrifuging in a Spinco Model L

ultracentrifuge at 105 000 *g* for 180 min. The complex was finally dialysed against the required buffer.

Papain. This was twice crystallized, from crude papain B.P. Codex (British Drug Houses Ltd.), by the method of Kimmel & Smith (1954). The product was freeze-dried. Stock solutions were made by dissolving the dry material in buffer to 1 g./100 ml.

Chymotrypsin. Crystallized bovine chymotrypsin (Armour Laboratories) was used. Its activity was compared with that of trypsin, by the method of Kunitz (1947) as modified by Laskowski (1955), with casein (Light white, British Drug Houses Ltd.) as substrate, and was found to be 3130 Armour units/g. [One Armour unit, assayed by the method of Anson (1938), gives in 10 min. at 25° colour equivalent to 1 μg . of tyrosine.] Stock solutions contained 1 g./100 ml. of buffer.

Trypsin. Crystallized trypsin (Armour Laboratories, salt-free) was used, of stated activity 2740 Armour units/g. Stock solutions contained 1 g./100 ml. of buffer.

Ox-serum albumin. Crude albumin was prepared from ox serum. The globulins were removed by 50% saturation with ammonium sulphate. The albumin was then precipitated by 80% saturation with ammonium sulphate, separated by filtration, dialysed against water and freeze-dried.

Buffers. For the experiments with papain the buffer was 0.0453 M- Na_2HPO_4 , 0.0213 M- KH_2PO_4 (pH 7.0); for experiments with chymotrypsin and trypsin the buffer was 0.0533 M- Na_2HPO_4 , 0.0133 M- KH_2PO_4 (pH 7.4).

Methods

Total nitrogen. This was determined by the micro-Kjeldahl method. A selenium catalyst (Fawcett, 1954) with 6 hr. digestion and the Markham (1942) still were used.

Dry weight. Solutions of complex were dialysed against five lots of 2 l. of distilled water during 2 days. Weighed samples of solution were then dried to constant weight at 105°.

Viscosity. This was measured with the Couette viscometer of Ogston & Stanier (1953) at 25°, with velocity gradients between 0.25 and 40 sec^{-1} . Viscosity at high velocity gradient was measured with an Ostwald viscometer of 2 ml. capacity and water time 11 sec.

Activity of papain. This was determined by the Greenberg (1955) modification of the method of Anson (1938), with ox-serum albumin as substrate.

Sedimentation. This was measured in a Svedberg oil-turbine ultracentrifuge at 900 rev./sec. by the method of Cecil & Ogston (1948). The optical system was Baldwin's (1953) modification of the diagonal-schlieren method of Philpot (1938).

* Fellow of the Arthritis and Rheumatism Foundation, U.S.A.

Treatment of the complex with papain. Stock (1%) solution of papain was first activated by addition of KCN and ethylenediaminetetra-acetate (EDTA) in amounts to give concentrations of 5 mM in the reaction mixture. The reaction mixture was made by adding 1 ml. of the activated papain to 10 ml. of a solution containing approx. 0.2 g. of complex/100 ml. of buffer, pH 7.0. In preliminary experiments, in those on the effect of digestion on mucin formation, and in the digestion performed in the Couette viscometer, the temperature of incubation was 25°; in all other experiments incubation was for 24 hr. at 4°, to reduce the chance of bacterial contamination. Solutions were examined microscopically after incubation, and no micro-organisms could be seen.

Inhibition of papain. Papain was inhibited by treatment with HgCl_2 at a final concn. of 1 mM (Krebs, 1930, 1931; Kimmel & Smith, 1954) or with iodoacetamide at a final concn. of 0.25 mM (Kimmel & Smith, 1954) for 2 hr. at room temp. and 10 hr. at 4° before use. The inhibitions were shown to be complete by the milk-clotting test of Balls & Hoover (1937).

Treatment of complex with chymotrypsin and trypsin. This was performed as with papain, with the omission of KCN and EDTA, in buffer (pH 7.4) for 24 hr. at 4°.

Detection of proteolysis. The proteolytic actions of the enzymes on complex were tested by the method of Anson (1938).

Effect of proteolysis on the precipitation of mucin. Three ml. of an approx. 0.2% solution of complex was mixed with 0.5 ml. of stock enzyme and incubated for 2 hr. at 25°. It was then brought to pH 4.3 with 10% (v/v) acetic acid and 1.2 ml. of 1% ox-serum albumin was added. The appearance of the precipitate was compared with that obtained with undigested complex.

Recovery of hyaluronic acid after digestion of complex. After treatment of complex with papain, the digestion mixture was treated by filtration, as in the isolation of complex from synovial fluid, with five washings on the filter with buffer (pH 7.0).

RESULTS

Detection of proteolysis of the complex. Qualitative experiments by the method of Anson (1938) showed that activated papain digests the protein of the

Fig. 1. Variation of relative viscosity with velocity gradient, measured in the Couette viscometer in pH 7 buffer at 25°. ●, Hyaluronic acid complex treated with papain for 24 hr. at 4°; ○, untreated complex.

complex, whereas papain inhibited by mercuric chloride, or iodoacetamide, chymotrypsin and trypsin, have no detectable action.

Effect of proteolysis on viscosity. Treatment of the complex with activated papain, under the conditions described (24 hr. at 4°), abolishes the non-Newtonian viscosity, but has much less effect on the viscosity measured at higher velocity gradients (Fig. 1). The viscosity measured at high velocity

Fig. 2. Progressive effect of papain on the viscosity of hyaluronic acid complex (0.11 g./100 ml.). The reaction was carried out in the Couette viscometer at 25°. ●, η_0 , relative viscosity extrapolated to zero velocity gradient; ○, η_{50} , relative viscosity extrapolated to velocity gradient 50 sec.⁻¹; ●, η_0/η_{50} .

Fig. 3. Variation of relative viscosity with velocity gradient, measured in the Couette viscometer at 25° in the pH 7 buffer. ○, Hyaluronic acid complex treated with papain inhibited with iodoacetamide, for 24 hr. at 4°; ●, untreated complex. Similar results were obtained with papain inhibited with mercuric chloride, with chymotrypsin and with trypsin.

gradient (in the Ostwald viscometer) was little affected. Fig. 2 shows the time course of the effect on viscosity at 25°. Papain inhibited by mercuric chloride or by iodoacetamide (Fig. 3), chymotrypsin, trypsin and chymotrypsin and trypsin together, had no effect on the viscosity after 24 hr. digestion at 4°. Treatment of complex with 0.8 mM-cysteine (equivalent to approx. twice the -SH of the papain used) in the same buffer containing KCN and EDTA had no effect on the viscosity.

Effect of proteolysis on mucin formation. Untreated complex, or complex treated with chymotrypsin or trypsin, or with 0.8 mM-cysteine, gave a normal mucin precipitate, which rapidly contracted into a compact clot, leaving the remaining solution quite clear. Complex treated with papain gave a friable, fragmented precipitate, part of which remained finely dispersed in the solution.

Properties of hyaluronic acid recovered after treatment of complex with papain. The material isolated after five filtrations accounted for only 42% of the weight of the original complex, the remainder having passed through the filter; this lost material must have comprised nearly half of the polysaccharide of the complex, as well as most of its protein. The nitrogen content of the recovered material was 3.8%, compared with 5.9% in the untreated complex. Ogston & Stanier (1952) showed that, after thorough dialysis, about half the carboxylic groups are ionized; the expected nitrogen content for dialysed sodium hyaluronate, consisting of half-ionized glucuronic acid-acetyl glucosamine co-polymer, is 3.6%.

Fig. 4. Intrinsic viscosity, determined by extrapolating the plot of $(\eta_{rel} - 1)/c$ to zero value of c . Measurements were made with the Ostwald viscometer at 25° in the pH 7 buffer.

The intrinsic viscosity of the recovered material was measured in the Ostwald viscometer (Fig. 4) and its sedimentation in the ultracentrifuge was measured at three concentrations, and extrapolated to zero concentration by plotting $1/S_{20}$ against concentration (Fig. 5). The results are given in Table 1.

DISCUSSION

The observations that papain hydrolyses the protein component of the hyaluronic acid complex, whereas chymotrypsin and trypsin are without action, closely resemble those made by Muir (1956) on a complex between protein and chondroitin sulphate. The simultaneous effects on the viscosity (particularly the non-Newtonian viscosity) and on the sedimentation show that removal of protein has

Fig. 5. Sedimentation coefficients of hyaluronic acid complex which has been treated with papain. Measurements were made at 900 rev./sec. at approx. 26° in the pH 7 buffer. The values have been corrected to water at 20°.

Table 1. *Results of dynamic measurements on hyaluronic acid complex treated with papain, with deduced characteristics of its particles*

S_0 is the sedimentation coefficient at zero concentration; $[\eta]_c$ is the intrinsic viscosity, c being in g./100 ml.; M is the average particle weight, p the ellipticity, and V' the hydrodynamic specific volume, based on a spheroidal model of the particle (Ogston, 1953). The values for untreated complex are taken from Fessler, Ogston & Stanier (1954).

	Treated material	Untreated material
$10^{13} S_0$	6.9	14.7
$10^{-13} d(1/S)/dc$	3.1	3.3
$[\eta]_c$	22	55
$10^{-6} M$	2	10
p	7.7	8.3
V' (ml./g.)	230	510

an important effect on the physicochemical properties of the material. Calculation of the particle characteristics by the method of Fessler & Ogston (1951) and Ogston (1953) (Table 1) shows that a marked decrease of the average particle weight has occurred; the apparent ellipticity p is little affected; the reduction of the hydrodynamic specific volume V' would be expected to accompany a reduction of the particle weight of a random-coil type of material.

The possibility that this degradative action of papain may be due to an action other than proteolytic is excluded. Crude papain contains a lysozyme (Meyer, Hahnel & Steinberg, 1946; Smith, Kimmel, Brown & Thompson, 1955) which might attack the polysaccharide component. However, the activity of this in twice-crystallized material at pH 7.0 is very low and its action is unaffected by iodoacetamide and only partly inhibited by mercuric chloride (Smith *et al.* 1955). These completely inhibit the proteolytic activity of papain and abolish its actions on the hyaluronic acid complex. Mercuric chloride is not known to inhibit any hyaluronidase.

These findings suggest that hyaluronic acid occurs in synovial fluid, under physiological conditions, as a complex with protein which may serve to bind the polysaccharide units together. Removal of this protein leads to important changes in the physicochemical properties of the material. Claims have been made at various times (e.g. Roseman, Watson, Duff & Robinson, 1955) to have prepared hyaluronic acid complex from synovial fluid, in an undegraded state but containing a substantially lowered proportion of protein; but in no such case has it been established satisfactorily that its physicochemical properties are unchanged. It must be remembered that viscosity measured at high velocity gradient is rather insensitive to the type of change brought about by removal of protein.

The precipitation of hyaluronic acid as mucin has been widely used (for example, in the turbidimetric method of Kass & Seastone, 1944) to estimate hyaluronic acid; and also, from the type of the precipitate obtained, to assess whether hyaluronic acid is in a normal or altered state in patients suffering from rheumatic disease. The action of hyaluronidase is known to affect the amount and type of the precipitate, and changes in these have usually been ascribed to degradative changes, or to alterations of the degree of polymerization, of the polysaccharide. Our results show that caution is needed in accepting such interpretation, since the type of precipitate is shown to depend also on the presence or state of the protein which forms a complex with hyaluronic acid.

The action of hyaluronidase is often assumed as a

factor in the metabolism of tissue hyaluronic acid and as a cause of pathological changes in tissue ground substance, though the evidence for its occurrence or action is not strong (Mathews & Dorfman, 1955). The possibility that proteolytic enzymes may act in such systems should also be considered.

SUMMARY

1. Activated papain attacks the protein component of the hyaluronic acid complex of ox synovial fluid, leading to removal of protein from the polysaccharide. Papain inhibited with mercuric chloride or iodoacetamide, chymotrypsin and trypsin are without action.

2. Proteolysis causes a strong reduction of the non-Newtonian viscosity of solutions of the complex, while having relatively little effect on the viscosity measured at high velocity gradients. The sedimentation rate is lowered, and the average particle weight is reduced fivefold.

3. The character of the mucin clot formed by hyaluronic acid complex with protein at acid pH is altered by treatment with papain.

4. It is concluded that protein, in complex with hyaluronic acid, is essential to its maintenance in the state in which it occurs in synovial fluid *in vivo*.

REFERENCES

- Anson, M. (1938). *J. gen. Physiol.* **22**, 79.
 Baldwin, R. L. (1953). *Brit. J. exp. Path.* **34**, 217.
 Balls, A. K. & Hoover, S. R. (1937). *J. biol. Chem.* **121**, 737.
 Cecil, R. & Ogston, A. G. (1948). *Biochem. J.* **43**, 592.
 Curtain, C. C. (1955). *Biochem. J.* **61**, 688.
 Fawcett, J. K. (1954). *J. Med. Lab. Tech.* **12**, 1.
 Fessler, J. H. & Ogston, A. G. (1951). *Trans. Faraday Soc.* **47**, 667.
 Fessler, J. H., Ogston, A. G. & Stanier, J. E. (1954). *Biochem. J.* **58**, 656.
 Greenberg, D. M. (1955). In *Methods in Enzymology*, vol. 2, p. 54. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press.
 Jeanloz, R. W. (1956). *3rd Int. Congr. Biochem. Brussels*, 1955.
 Johnston, J. P. (1955). *Biochem. J.* **59**, 626.
 Kass, E. H. & Seastone, C. V. (1944). *J. exp. Med.* **79**, 319.
 Kimmel, J. R. & Smith, E. L. (1954). *J. biol. Chem.* **207**, 515.
 Krebs, H. A. (1930). *Biochem. Z.* **220**, 289.
 Krebs, H. A. (1931). *Naturwissenschaften*, **19**, 133.
 Kunitz, M. (1947). *J. gen. Physiol.* **30**, 291.
 Laskowski, M. (1955). In *Methods in Enzymology*, vol. 2, pp. 8, 26. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press.
 Markham, R. (1942). *Biochem. J.* **36**, 790.
 Mathews, M. B. & Dorfman, A. (1955). *Physiol. Rev.* **35**, 381.

- Meyer, K., Hahnel, E. & Steinberg, A. (1946). *J. biol. Chem.* **163**, 733.
- Meyer, K. & Palmer, J. W. (1934). *J. biol. Chem.* **107**, 629.
- Muir, H. (1956). *Biochem. J.* **62**, 26P.
- Ogston, A. G. (1953). *Trans. Faraday Soc.* **49**, 1481.
- Ogston, A. G. & Stanier, J. E. (1950). *Biochem. J.* **46**, 364.
- Ogston, A. G. & Stanier, J. E. (1952). *Biochem. J.* **52**, 149.
- Ogston, A. G. & Stanier, J. E. (1953). *Biochem. J.* **53**, 4.
- Philpot, J. St L. (1938). *Nature, Lond.*, **141**, 283.
- Roseman, S., Watson, D. R., Duff, I. F. & Robinson, W. D. (1955). *Ann. Rheum. Dis.* **15**, 67.
- Smith, E. L., Kimmel, J. R., Brown, D. M. & Thompson, E. O. (1955). *J. biol. Chem.* **215**, 67.

The Acid Phosphatases of Rat Liver

By G. A. J. GOODLAD AND G. T. MILLS

Biochemistry Department, The University of Glasgow, Scotland

(Received 14 November 1956)

It was shown by Norberg (1949) that the shape of the pH-activity curve of rat-liver acid phosphatase varied during the process of regeneration after partial hepatectomy. It has also been reported that rat-liver acid phosphatase activity, assayed at pH 5.5, showed a 50% increase in animals maintained on a low protein, low choline diet, although the activity assayed at pH 3.75 showed no change (Goodlad, Mills & Smith, 1951).

It thus appears possible that at least two non-specific acid phosphatases may be present in rat liver. The present work was undertaken to obtain more information on the nature of these enzymes. The effects of various factors on the pH-activity curve of unfractionated rat-liver homogenates have been examined. An attempt has been made to fractionate the enzymes by acetone precipitation in the presence of Zn^{2+} ions, and the intracellular distribution of rat-liver acid phosphatase activity has been studied by differential centrifuging. A preliminary account of this work has been published (Goodlad & Mills, 1956).

MATERIALS AND METHODS

p-Nitrophenyl phosphate was prepared by the method of Bessey & Love (1952). Glucose 6-phosphate was prepared enzymically from glucose 1-phosphate, a partially purified preparation of phosphoglucomutase (Najjar, 1948) being used, followed by purification by the method of McCready & Hassid (1944). The glucose 1-phosphate was prepared by the method of Hanes (1940) as modified by McCready & Hassid (1944). Phenyl phosphate was obtained from British Drug Houses Ltd. Adenosine 3'-phosphate and adenosine 5'-phosphate were supplied by L. Light and Co. Ltd. β -Glycerophosphate was obtained from The General Chemical and Pharmaceutical Co. Ltd.

Assay of phosphatase activity. A general method, applicable to all substrates, was based on the β -glycerophosphate method of Bodansky (1933) where the inorganic phosphate liberated was separated by the method of Delory (1938), and subsequently determined by the method of Allen

(1940). Buffer solution (0.5 ml.), 0.1 ml. of substrate solution, 0.1 ml. of water (or a solution of activator or inhibitor) and 0.3 ml. of enzyme solution, usually a 1/50 tissue homogenate, were incubated at 38° for a specified time, usually 1 hr., after which the reaction was stopped by the addition of 2 ml. of 5% (w/v) trichloroacetic acid. Precipitated protein was removed by centrifuging and 2 ml. samples were made alkaline to phenolphthalein with aq. NH_3 soln. (sp. gr. 0.880); 1 ml. of 2.5% (w/v) CaCl_2 was then added, followed by 1 ml. of 0.5% MgCO_3 (light) suspension. The tubes were shaken at intervals for 30 min., centrifuged and the precipitates washed once with 1 ml. of 5% (w/v) aq. NH_3 soln. The tubes were drained for 2–3 min. in an inverted position, the precipitate was dissolved in 1 ml. of 2.5 N- H_2SO_4 and colour developed by the addition of 0.4 ml. of 1% (w/v) amidol (2:4-diaminophenol hydrochloride) in 20% (w/v) sodium metabisulphite, 0.2 ml. of 8.3% (w/v) ammonium molybdate and 3.4 ml. of water. The intensities of the blue colour were estimated on the Hilger Spekker absorptiometer (Ilford 608, red filter) between 10 and 30 min. after addition of reagents. Phosphate was determined from a calibration curve for the range 5–40 μg . of P. Controls were employed to determine the inorganic phosphate in the tissue and that formed by autolysis during incubation. In these, the substrate was added after the addition of the trichloroacetic acid. One unit of activity = 1 μg . of P liberated/hr.

When phenyl phosphate was used as substrate, the method of assay was essentially that of King (1946). Enzyme preparation (0.1 ml.), usually a 1/100 tissue homogenate, was used in a 1 ml. incubation volume. Tissue blanks were determined by addition of substrate after the phenol reagent. No measurable breakdown of substrate occurred in the absence of the enzyme at any pH value used. One unit of activity = 1 μg . of phenol liberated/hr.

A modification of the procedure of Bessey, Lowry & Brock (1946) was employed when *p*-nitrophenyl phosphate was used as substrate. Enzyme preparation (0.3 ml.), usually a 1/400 tissue homogenate, was used in a 1 ml. incubation volume with a final substrate concentration of 0.0025 M. One unit of activity = 1 μg . of *p*-nitrophenol liberated/hr.

Preparation of tissue extracts. Male albino rats weighing 175–300 g. were killed by stunning and exsanguination. The livers were removed, washed with water to remove blood,