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Degradation of the Hyaluronic acid Complex of Synovial Fluid by Proteolytic Enzymes and by Ethylenediaminetetra-acetic acid

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Blumberg & Ogston (1957a, b) observed that papain in the presence of ethylenediaminetetra-acetic acid hydrolysed the protein present in the hyaluronic acid complex of ox-synovial fluid, strongly reduced the non-Newtonian viscosity of the complex and caused a considerable drop in its molecular weight. They concluded that the protein of this complex is essential to the integrity of its macromolecular structure and to its physicochemical properties in solution and, consequently, that the effect of papain on the viscosity of hyaluronic acid from other sources may provide evidence of its existence as a complex with protein.

Experiments done in collaboration with Dr B. I. Aldrich (Aldrich, 1958) in which ethylenediaminetetra-acetic acid was used in an attempt to remove calcium from the complex, showed that buffered solutions of complex containing ethylenediaminetetra-acetic acid suffered loss of viscosity during measurements in the stainless-steel Couette viscometer, but that this did not occur when an analogue of ethylenediaminetetra-acetic acid was used instead, nor when ethylenediaminetetra-acetic acid was used in a glass viscometer. These results suggested that ethylenediaminetetra-acetic acid might be concerned in catalytic degradation of the carbohydrate component of the complex; they thus also cast doubts upon the conclusions of Blumberg & Ogston, whose work with papain involved the use of ethylenediaminetetra-acetic acid as an activator and of the stainless-steel viscometer.

Further investigations on the effects of ethylenediaminetetra-acetic acid and of proteolytic enzymes on the complex were therefore undertaken.

EXPERIMENTAL

Materials

Hyaluronic acid complex. (Complex.) Synovial fluid was collected from the astragalotibial joints of oxen soon after death, as described by Ogston & Stanier (1950). The fluid was centrifuged in a Spinco model L ultracentrifuge at 73 000 g for 60 min. to remove particulate matter, and the complex was separated by filtration, with 9–11 washings, at 4° on a 5-on-3 sintered-glass filter of 1 μ (or less) average pore diameter (Baird and 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 used for experiments with trypsin, chymotrypsin and pepsin was finally dissolved in this same buffer to approximately the volume of the original fluid; that used for experiments with papain and cysteine was dissolved in 0.0453 M- Na_2HPO_4 -0.0213 M- KH_2PO_4 buffer (pH 7.0) to about half the volume of the original fluid. In all cases, the complex was then centrifuged at 73 000 g for 45 min. and dialysed against the buffer in which it was finally dissolved. Complex that was used for the determination of proteolysis by the method of Anson (1938) was further centrifuged at 105 000 g for 180 min. to remove any 'fast component' (Blumberg & Ogston, 1957a; Johnston, 1955).

Trypsin. This was Armour salt-free material of stated activity 1800 units/mg.

Chymotrypsin. This was Armour salt-free material of stated activity 975 units/mg.

Pepsin. This was Armour salt-free material.

Papain. This was twice-crystallized from crude papain (British Drug Houses Ltd.) by the method of Kimmel & Smith (1954). Stock solutions were made by dissolving freeze-dried material to 1 g./100 ml. in 0.0453M- Na_2HPO_4 -0.0213M- KH_2PO_4 buffer (pH 7.0). In some of the experiments a sample of papain obtained from Schering A. G., Berlin, and of about the same activity as that mentioned above, was used in the same manner. We are also indebted to Mr C. F. Phelps for a sample also prepared by the method of Kimmel & Smith.

Ox-serum albumin. This was from material isolated by Dr B. S. Blumberg (Blumberg & Ogston, 1957a).

Ethylenediaminetetra-acetic acid. (Hereafter denoted by EDTA.) The same sample was used as that used by Blumberg & Ogston (1957a).

Ethylene glycol bis-(β -aminoethyl ether)-NN-tetra-acetic acid. (Hereafter denoted by EGTA.) This was kindly supplied by Dr W. Bartley from a sample obtained from J. R. Geigy, S.A., Basle, Switzerland.

Cysteine. This was British Drug Houses Ltd. laboratory-grade reagent.

Metal salts. These were from British Drug Houses Ltd., analytical-grade reagents or equivalent.

Methods

Viscosity. This was measured with the Couette viscometer of Ogston & Stanier (1953) at 25°, with velocity gradients approximately between 0.4 and 40.0 sec^{-1} . Viscosity at high-velocity gradient was measured with an Ostwald viscometer of about 2.5 ml. capacity and water time 48 sec.

Electrodialysis. This was performed with an apparatus designed and constructed by Dr O. Smithies (Smithies, 1951) and previously described (Ogston & Sherman, 1958). Samples were placed in the central compartment, which was separated from the outer chambers by Millipore (Millipore Filter Corp., Watertown, Mass., U.S.A.) HA membranes of pore diameter 0.4 μ , and electro-dialysed for 9–11 hr. Buffer, 0.0153M- KH_2PO_4 -0.0097M- Na_2HPO_4 (pH 6.7), was circulated through the outer chambers. The entire apparatus was used in the cold room at about 2°.

Ultraviolet absorption. This was measured at 280 $m\mu$ (corrected for scatter at 350 $m\mu$) in a Hilger Uvispek spectrophotometer.

Mucin clot. (a) Quantitative: Mucin weights were determined by heating the clot formed by acidification of 1 ml. samples of hyaluronic acid solutions (Ogston & Stanier, 1952) to constant weight at 105° in an electric oven. Hyaluronic acid complex was taken as 45% of the mucin weight (Ogston & Stanier, 1952).

(b) Qualitative: The type of clot formed by acidification of samples of hyaluronic acid was noted, as the action of some agents upon hyaluronic acid causes a dispersed precipitate to be formed instead of the normal compact clot (Blumberg, 1957).

Treatment of the complex with trypsin and chymotrypsin. Weighed amounts of trypsin and chymotrypsin were added directly to buffered solutions (0.2M- NaCl -0.0077M- Na_2HPO_4 -0.0023M- KH_2PO_4 , pH 7.3) of the complex to the extent of 0.5 mg. of each per 1 ml. of solution. Incubations were at 4°.

Treatment of the complex with pepsin. The complex was first dialysed against 0.18M- NaCl -0.01M- HCl (pH 2.0).

Pepsin was then added directly to the resulting solution of the complex to the extent of 1 mg. per 1 ml. of solution. Incubations were at 4°.

Treatment of the complex with papain. Stock (1%) solution of papain was first activated by addition of KCN and EDTA in amounts to give concentrations of 5 mM in the reaction mixture, or for EGTA to give a concentration of 2.5 mM in the reaction mixture. (In one experiment EDTA was also used at 2.5 mM.) The reaction mixture was made by adding 1 ml. of the activated papain solution, adjusted to pH 7 by the addition of a very small quantity of 0.2M- HCl , to 9 ml. of a solution of the complex (pH 7.0). Incubations were at 4°.

Treatment of the complex with cysteine. This was carried out in the same manner as for papain, except that the stock cysteine solution was made to give a reaction mixture of 0.4 mM-cysteine (equivalent to about the same SH-group concentration as the papain reaction mixture). KCN and EDTA or EGTA were added as with the papain.

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

Detection of proteolysis of the complex. (a) With papain: This was accomplished by the Blumberg (1957) modification of the method of Anson (1938), except that ox-serum albumin was added to the digestion mixture immediately before the addition of trichloroacetic acid (but after the solution had been cooled to 4° and acidified to pH 5.0), to assist the precipitation of hyaluronic acid; the albumin [3 parts to 1 (w/w) of hyaluronic acid] was added in 2 ml. of water. Control experiments were done to measure any self-digestion of the papain.

(b) With trypsin and chymotrypsin, and with pepsin: Protein content (measured by ultraviolet absorption at 280 $m\mu$) of a sample of complex incubated with the enzyme and then isolated by electrodialysis was compared with that of an undigested control; allowance was made for a small loss of hyaluronic acid during electrodialysis, as determined by mucin clot weights. A lower protein content in this material indicated that proteolysis had occurred, as electrodialysis itself does not remove any protein from the complex (Ogston & Sherman, 1958).

RESULTS

Effects of trypsin and chymotrypsin and of pepsin on the complex. Treatment of the complex with trypsin and chymotrypsin together for 7 days had no detectable effect upon the viscosity, which was compared in the Couette viscometer with a control sample incubated without enzymes (Fig. 1). The mucin clots obtained from the digested and control samples were identical both in appearance and in weight/ml. of solution. The occurrence of proteolysis was demonstrated in a sample digested for 5 days and then electrodialysed, by comparing the absorption at 280 $m\mu$ with the weight of mucin clot in this and in a control sample; this showed that 65% of the protein originally present had been removed. In the 5-days' experiment, 14% of the polysaccharide was lost during electrodialysis, so that the viscosity after treatment was not

directly comparable with that of the control; however, the ratio of viscosities at velocity gradients 0 and 50 sec^{-1} (Ogston & Stanier, 1953) was normal, showing that its viscous properties were unaffected.

Preliminary experiments only were done with pepsin; these indicated that very little protein could be eliminated with this enzyme, and its use was therefore abandoned. The observation was made, however, that the complex could be kept at pH 2.0 for 2 days without any change of non-Newtonian viscosity.

Effects of ethylenediaminetetra-acetic acid and of EGTA on the complex. EDTA alone caused a complete disappearance of non-Newtonian viscosity and a lowering of viscosity at all velocity gradients within 1 hr. at 25° in the Couette viscometer. Material which had been so treated showed a marked reduction of viscosity as compared with a control sample kept in glass, when tested in the glass Ostwald viscometer. A solution of complex in EDTA showed no loss of viscosity in the Ostwald viscometer, even when sheared by repeated passage through the capillary. When EDTA was replaced by EGTA very little change of viscosity occurred, even in the Couette viscometer for 1 hr. at 25°.

The apparent implication of the material (stainless steel) of the Couette viscometer in this effect led to the testing in the glass Ostwald viscometer of the effects of a number of ions (Fe^{3+} , Cr^{3+} , Ni^{2+} , Mg^{2+} , Mn^{2+} , Co^{2+} , Zn^{2+} , Cu^{2+}) added in a final concentration of about 20 mM to synovial fluid and EDTA. None of these ions, all in their highest stable states of oxidation, had any significant effect upon the viscosity. However, Sn^{2+} , Cu^{+} and Fe^{2+} ions caused loss of viscosity; with Fe^{2+} ions the degradation was not dependent upon the presence of EDTA, and appeared to be inhibited at low concentrations of Fe^{2+} ions by

EGTA. When ascorbic acid (25 mM) was added to the complex, the viscosity (in glass) was reduced; this effect was increased by inclusion of EDTA but inhibited by EGTA.

Effects of papain and of cysteine on the complex. The activity of papain upon ox-serum albumin was found to be identical in the presence of EDTA and of EGTA. It was confirmed that papain causes proteolysis of the complex in the presence of 5 mM-EDTA (Blumberg & Ogston, 1957a); identical results were obtained in the presence of 2.5 mM-EGTA.

However, the effects of papain on the non-Newtonian viscosity were much greater with EDTA

Fig. 2. Variation of relative viscosity with velocity gradient, measured in the Couette viscometer in buffer, pH 7.0, at 25°. ●, Control: hyaluronic acid complex treated only with EGTA and KCN; ○, complex treated with papain, EGTA and KCN for 24 hr. at 4°; ×, complex treated with papain, EDTA and KCN for 24 hr. at 4°.

Fig. 1. Variation of relative viscosity with velocity gradient, measured in the Couette viscometer in buffer, pH 7.3, at 25°. ●, Hyaluronic acid complex treated with trypsin and chymotrypsin for 7 days at 4°; ○, untreated complex.

Fig. 3. Variation of relative viscosity with velocity gradient, measured in the Couette viscometer in buffer, pH 7.0, at 25°. ●, Control: hyaluronic acid complex treated only with EDTA and KCN; ○, complex treated with papain, EDTA and KCN for 25 hr. at 4°; ×, complex treated with cysteine, EDTA and KCN for 25 hr. at 4°.

than with EGTA (Fig. 2). The change of viscosity with papain and EDTA was markedly greater than with EDTA alone. In view of the apparent unimportance of proteolysis, a careful comparison was made of the effects of papain and cysteine (at the same concentration of SH groups) with EDTA and EGTA measured in the Couette viscometer. With EDTA (Fig. 3) and with EGTA (Fig. 4) the effects of papain and cysteine were found to be closely similar. The only difference that could be observed between the actions of cysteine and papain on viscosity was that addition of 0.1 mM- Cu^{2+} ions increased the effect of cysteine with EDTA, but decreased that of papain.

After incubation of the complex with cysteine and EDTA in glass, the mucin clot formed was abnormal, resembling that formed after similar treatment with papain.

DISCUSSION

Action of ethylenediaminetetra-acetic acid on the hyaluronic acid complex. Although we have not worked out the nature of the effect in detail, it seems clear that EDTA can assist in a non-proteolytic degradation of the complex, and it is likely that this is an oxidative process exerted upon the hyaluronic acid. The effect of EDTA on the viscosity, in the presence of ascorbic acid in glass, suggests that a complex between EDTA and a metal ion present in the preparation acts as an oxidation catalyst (Pirie & van Heyningen, 1954; Udenfriend, Clark, Axelrod & Brodie, 1954; Stadtman, 1955; Green, Mazur & Schorr, 1956; Mason, 1957). The effect of EDTA and cysteine (in

glass) upon the ability to form a mucin clot suggests that cysteine also can act as a reducing agent in the system (Hale, 1944). The effect of EDTA, when observed in the stainless-steel viscometer, together with the effects (in glass) of EDTA and Sn^{2+} or Cu^{+} ions, suggests that EDTA may also act by bringing such ions into solution from the steel. EGTA appears to be far less active in the catalytic role than is EDTA, and even to act as an inhibitor of the reaction with ascorbic acid.

In view of this, the use of EDTA in preparing hyaluronic acid from tissues appears to be most undesirable, since both metal ions capable of forming an oxidation catalyst with EDTA and reducing agents (apart from papain) are likely to be present, and the product is therefore likely to have undergone oxidative degradation. Stadtman (1955) has issued a strong warning against its use to protect thiols against oxidation in enzyme systems.

Effects of proteolytic enzymes on the hyaluronic acid complex. We have confirmed Blumberg & Ogston (1957a) in showing that papain does hydrolyse the protein of the complex, but we have shown that trypsin and chymotrypsin together have a proteolytic action also (which Blumberg & Ogston did not find).

Since the actions of the non-SH enzymes produce no change of viscosity or of the ability to form mucin, the action of papain on these properties, which is closely imitated by cysteine, cannot now be ascribed to proteolysis. It is still possible that papain, under the conditions used, may affect the viscosity by a more complete proteolysis than that produced by trypsin and chymotrypsin, but this would not be easy to prove in the presence of the oxidative degradation. Blumberg & Ogston's (1957b) finding that plasmin causes proteolysis and loss of viscosity (in the absence of EDTA) is inconclusive because of the ill-defined nature of the enzyme.

It follows that any change of viscosity brought about by papain cannot be taken as evidence for the existence of a complex, as Blumberg & Ogston (1957b) claimed; conversely, lack of change of viscosity on proteolysis is not evidence against the existence of a complex.

Hyaluronic acid-protein complex of ox-synovial fluid. These results do nothing to contradict the earlier evidence for the existence of a characteristic complex between hyaluronic acid and protein in synovial fluid (Ogston & Stanier, 1950, 1952; Curtain, 1955), which has been confirmed by the more recent evidence of electrodialysis (Ogston & Sherman, 1958) and of the composition of material isolated by precipitation with cetylpyridinium chloride (Blumberg & Ogston, 1957b). They do strongly suggest, however, that this protein, or a

Fig. 4. Variation of relative viscosity with velocity gradient, measured in the Couette viscometer in buffer, pH 7.0, at 25°. ●, Control: hyaluronic acid complex treated only with EGTA and KCN; ○, complex treated with papain, EGTA and KCN for 48 hr. at 4°; x, complex treated with cysteine, EGTA and KCN for 48 hr. at 4°.

considerable part of it, is not important in determining the physicochemical properties of hyaluronic acid in solution. Although it remains true that no sample free from protein appears to have been isolated with so high an intrinsic viscosity as that of the complex it seems likely that this is because methods powerful enough to remove all the protein can also cause depolymerization of the polysaccharide.

SUMMARY

1. Ethylenediaminetetra-acetic acid aids the degradation of hyaluronic acid complex under various conditions.

2. Papain has a proteolytic activity on the complex but appears, as a reducing agent, to cause more general degradation as well.

3. Trypsin and chymotrypsin together can remove 65% of the protein from the complex without any loss of viscosity by the complex.

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Factors Influencing the Incorporation of Amino Acid into the Protein of Microsome and Mitochondria Preparations of Rat Liver and Liver Tumour

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The requirement of the soluble cytoplasm (cell sap) for the incorporation of amino acids into the protein of isolated liver microsomes has been demonstrated by Zamecnik & Keller (1954). Both for microsomes derived from rat liver and mouse Ehrlich ascites tumour cells, the active components of the cell sap are precipitated when the pH is adjusted to 5.2 (pH 5 fraction) (Keller & Zamecnik, 1956; Littlefield & Keller, 1957). It has previously been observed that microsome preparations from rat-liver tumours are considerably less active for amino acid incorporation than are similar preparations from normal liver (Campbell & Greengard, 1959). Further, it appeared that not only

were the microsomes deficient in this respect, but that the cell sap contributed to this difference. The extent to which the pH 5 fraction from the two tissues reflects the differences found with the whole cell sap has now been investigated.

For amino acid incorporation into isolated mitochondria, the role of the cell sap seems to differ from that with microsomes. In the presence of a suitable source of energy, there is no absolute requirement for cell sap (McLean, Cohn, Brandt & Simpson, 1958; Greengard, 1959), although its inclusion in the reaction mixture enhances the incorporation of amino acids. Other factors influencing the extent of incorporation of amino