Hyaluronic Acid

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Formerly hyaluronic acid was determined by means of turbidimetric methods. However, the results depended on the degree of polymerization of the hyaluronic acid. Other methods are based on the hydrolysis of hyaluronic acid and the quantitative determination of its constituents[1]. All these methods are unspecific for hyaluronic acid.
In contrast, the enzymatic methods described here[2,3] are specific and virtually independent of the degree of polymerization.

1. Spectrophotometric Method

Principle

The degradation of hyaluronic acid by bacterial hyaluronidase[*] results in the almost quantitative yield of a disaccharide, which has been characterized as a 4,5-unsaturated uronide (D-Δ^4-glucosyl-pyranosyl-uronic acid-β[1→3]-2-acetamino-2-deoxy-D-glucose)[4]. Like the homologous oligosaccharides it has an absorption maximum around 230 mμ[2]. Hyaluronic acid has a low absorption at this wavelength (Fig. 1).

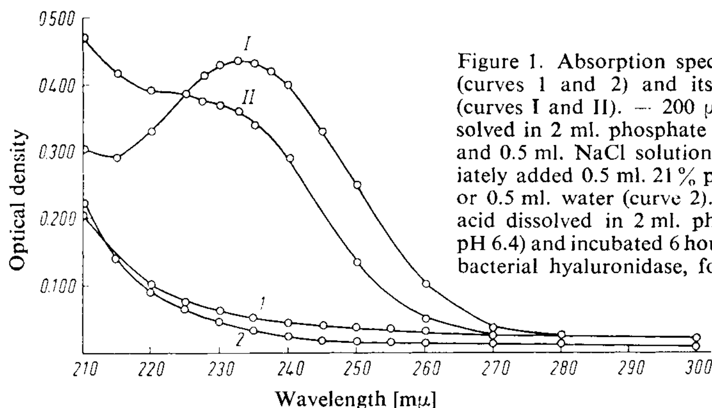

Figure 1. Absorption spectra of hyaluronic acid (curves 1 and 2) and its degradation products (curves I and II). — 200 μg. hyaluronic acid dissolved in 2 ml. phosphate buffer (M/15; pH 6.4) and 0.5 ml. NaCl solution (0.15 M), and immediately added 0.5 ml. 21% perchloric acid (curve 1) or 0.5 ml. water (curve 2). — 200 μg. hyaluronic acid dissolved in 2 ml. phosphate buffer (M/15; pH 6.4) and incubated 6 hours at 37°C with 100 μg. bacterial hyaluronidase, followed by addition of 0.5 ml. 21% perchloric acid (curve I) or 0.5 ml. water (curve II).

Reagents

1. Potassium dihydrogen phosphate, KH_2PO_4, A. R.
2. Disodium hydrogen phosphate, $Na_2HPO_4 \cdot 2H_2O$, A. R.
3. Sodium chloride, A. R.
4. Perchloric acid, A. R., sp. gr. 1.67 (*ca.* 70% w/w)
5. Bacterial hyaluronidase
   from *Staphylococcus aureus* or streptococci[2]. Isolation, see p. 92.
6. Potassium hyaluronate
   obtained from umbilical cord or vitreous humor and purified[2]. Commercial preparation, see p. 1021.

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[*] Hyaluronatelyase.

[1] *H. Gibian:* Mucopolysaccharide und Mucopolysaccharidasen. F. Deuticke, Vienna 1959, p. 90.

[2] *H. Greiling*, Hoppe-Seylers Z. physiol. Chem. *309*, 239 [1957].

[3] *H. Greiling*, *Th. Günther* and *T. Eberhard*, Hoppe-Seylers Z. physiol. Chem. *319*, 161 [1960].

[4] *A. Linker*, *K. Meyer* and *Ph. Hoffmann*, J. biol. Chemistry *219*, 13 [1956].

Purity of the enzyme preparation

The hyaluronidase preparation should have a specific activity of at least 20 units*). Contamination with the glycolytic enzymes does not interfere with the determination. Peptidase should not be detectable.

Preparation of Solutions (for *ca.* 20 determinations)

I. Phosphate buffer (M/15; pH 6.4):
In a 250 ml. volumetric flask, dilute 66 ml. of a solution of disodium hydrogen phosphate (11.876 g./litre) with a solution of potassium dihydrogen phosphate (9.078 g./litre).

II. Sodium chloride (0.15 M):
Dissolve 0.9 g. NaCl, A. R., in distilled water and make up to 100 ml.

III. Perchloric acid (*ca.* 20% w/v):
Dilute *ca.* 13 ml. $HClO_4$, A. R., (sp. gr. 1.67) to 75 ml. with distilled water.

IV. Bacterial hyaluronidase (*ca.* 1 mg. protein/ml.):
Dissolve 7 mg. dry preparation in 7 ml. 0.15 M NaCl.

V. Potassium hyaluronate (200 μg./ml.):
Dissolve 20 mg. potassium hyaluronate in 100 ml. phosphate buffer (solution I).

Stability of the solutions

The solutions are stored, stoppered, in a refrigerator at 0 – 4°C. Under these conditions the hyaluronidase solution shows no substantial loss of activity within 3 months. The other solutions are stable indefinitely so long as no bacterial growth occurs.

Procedure

Experimental material in solution may be used directly without further preparation, dissolve or suspend solid matter in NaCl solution (II).

Enzymatic reaction

The assay mixture, set up in conical centrifuge tubes, consists of:

Experimental and standard tubes

1. 2.0 ml. buffer (solution I)
   0.5 ml. sample
   0.5 ml. NaCl solution (II)

2. 1.5 ml. buffer (solution I)
   0.5 ml. potassium hyaluronate solution (V)
   0.5 ml. sample
   0.5 ml. NaCl solution (II)

3. 1.0 ml. buffer (solution I)
   1.0 ml. potassium hyaluronate solution (V)
   0.5 ml. sample
   0.5 ml. NaCl solution (II)

Control tube

4. 2.0 ml. buffer (solution I)
   0.5 ml. sample
   0.6 ml. NaCl solution (II)

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*) According to [2] a unit is the amount of enzyme contained in 1 ml. which increases the optical density of a solution containing 1 mg. hyaluronic acid/ml. by 0.100 in 100 sec. at 230 mμ and 30°C, and with a 1 cm. light path.

Heat all the solutions for 10 min. at 70° C. This preliminary treatment can be omitted if the sample contains no protein.

After cooling solutions 1—3 add 0.1 ml. bacterial hyaluronidase (solution IV). Then incubate all the tubes for 6 hours at 37° C (water bath).

Deproteinization and spectrophotometric measurements

After the 6 hour incubation add 0.5 ml. perchloric acid (solution III) to tubes 1—4, mix well, centrifuge for 20 min. at 5000 g. Decant the supernatant fluids into silica cuvettes and measure the optical density at 230 mμ against control tube 4. E_1, E_2 and E_3 are the optical densities of experimental tubes 1—3, E_2 and E_3 contain known amounts of hyaluronic acid.

Calculations

Beer's law is obeyed between 20 and 700 μg. potassium hyaluronate[3]. For the calculations E_1 is related to the optical density found for 100 μg. potassium hyaluronate. That is E_2-E_1 or E_3-E_2 or $(E_3-E_1)/2$. The average is:

$$E_{100\,\mu g.} = \frac{(E_2-E_1)+(E_3-E_2)}{2}$$

The amount of hyaluronic acid in 1 ml. of sample is given by:

$$\frac{E_1 \times 100 \times 2}{E_{100\,\mu g.}} = \mu g.\ \text{potassium hyaluronate/ml. sample}$$

Example

Sample: 0.5 ml. ascites serum. After incubation and deproteinization, measured at 230 mμ against control tube 4. $E_1 = 0.250$; $E_2 = 0.470$; $E_3 = 0.700$; $(E_2-E_1) = 0.220$; $(E_3-E_2) = 0.230$. $E_{100\,\mu g.} = (0.220 + 0.230)/2 = 0.225$

$$\frac{0.250 \times 100}{0.225} = 111\ \mu g.\ \text{potassium hyaluronate/0.5 ml. ascites serum.}$$

Sources of Error

Substances which absorb at 230 mμ and are not precipitated by perchloric acid, for instance high concentrations of nucleotides, interfere with the determination. α-Heparin and the chondroitin sulphates A and B competitively inhibit bacterial hyaluronidase (Fig. 2).

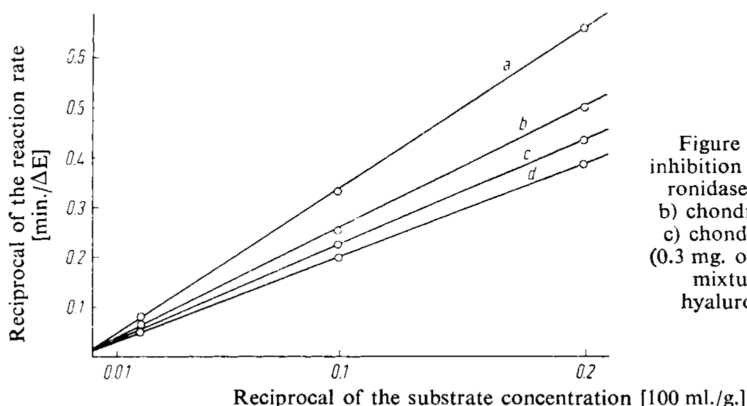

Figure 2. Competitive inhibition of bacterial hyaluronidase by a) α-heparin, b) chondroitin sulphate B, c) chondroitin sulphate A (0.3 mg. of each/ml. reaction mixture). Curve d: hyaluronic acid alone

In the presence of large amounts of these substances the enzymatic determination must be preceded by an isolation of the hyaluronic acid.

Specificity and Dependence of the Method on the Degree of Polymerization

α-Heparin, chondroitin sulphate A and B are not decomposed by bacterial hyaluronidase. Chondroitin is only hydrolysed to a slight extent. Moreover, the pH optimum of the chondroitin hydrolysis is in the acid range (pH 5.7 with the same ionic strength of 0.12). Contamination of the hyaluronic acid with up to 30% chondroitin does not interfere with the determination[5]. So far chrondroitin has only been found in the cornea[6,7].

Table 1 shows that the method is independent of the molecular weight up to a mean of about 20 for the degree of polymerization of the hyaluronic acid. With an average molecular weight of 1600 the optical density change after 6 hours incubation with bacterial hyaluronidase is about 12% too low, which can be explained by the decrease in the number of hydrolysable bonds with lower degrees of polymerization.

Table 1. Michaelis constants, maximum velocities and optical density changes on incubation of 150 µg. hyaluronic acid of different degrees of polymerization, with 100 µg. bacterial hyaluronidase for 6 hours. — The mean degree of polymerization was calculated according to:

$$\frac{2 \times \text{hexosamine} (\mu\text{moles N-acetylglucosamine/mg.})}{\text{reducing value} (\mu\text{moles /mg.})}$$

Degree of polymerization (mean)	Mol.wt. (mean)	K_M [g./100 ml.]	V_{max} [µg./min.]	E_{230}/150 µg. after 6 hrs. incubation
184	32973	1.1×10^{-1}	67	0.282
108	19354	1.0×10^{-1}	66	0.274
88	15770	1.1×10^{-1}	67	0.291
72	12902	1.0×10^{-1}	67	0.280
36	6451	1.0×10^{-1}	68	0.283
25	4480	0.95×10^{-1}	67	0.275
20	3584	1.0×10^{-1}	67	0.286
8	1434	1.0×10^{-1}	66	0.247

2. Colorimetric Method

Principle

Bacterial hyaluronidase degrades hyaluronic acid quantitatively to an unsaturated disaccharide, thus liberating *N*-acetylglucosamine end groups. These can be determined colorimetrically with the *Morgan-Elson* reaction: Thus on heating *N*-acetylglucosamine in alkali, furan derivatives are formed, which react with *p*-dimethylaminobenzaldehyde to form a red dye[8]. The condensation product of *p*-dimethylaminobenzaldehyde and the disaccharide from hyaluronic acid has two absorption maxima at 544 mµ and 585 mµ (Fig. 3) if the reaction is carried out according to *Leloir*[9]. In the method described here the optical density of the reaction mixture is measured at 585 mµ.[9a]

Reagents

1.—6. as for the spectrophotometric method (p. 87).

Additional:

7. Boric acid, A. R.
8. Potassium hydroxide, A. R.

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[5] *H. Greiling, Th. Günther* and *T. Eberhard*, unpublished.
[6] *E. A. Davidson* and *K. Meyer*, J. biol. Chemistry *211*, 605 [1954].
[7] *K. Meyer, E. A. Davidson, A. Linker* and *P. Hoffmann*, Biochim. biophysica Acta *21*, 506 [1956].
[8] *R. Kuhn*, Angew. Chem. *69*, 23 [1957].
[9] *J. L. Reissig, J. L. Strohminger* and *L. F. Leloir*, J. biol. Chemistry *217*, 959 [1955].
[9a] *H. Greiling*, Z. Rheumaforsch. *20*, 298 [1961].

9. Acetic acid, A. R.
10. Hydrochloric acid, A. R., 10 N
11. *p*-Dimethylaminobenzaldehyde, A. R.

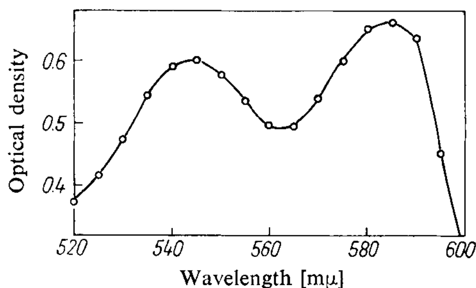

Fig. 3. Absorption spectrum of the *Morgan-Elson* reaction products of the disaccharide formed from hyaluronic acid by bacterial hyaluronidase[9]. Pure hyaluronic acid has no absorption between 520 and 600 mμ.

Preparation of Solutions

Solutions I—V as for the spectrophotometric method (p. 88).

Additional:

VI. Potassium tetraborate (0.8 M):
Dissolve 24.7 g. boric acid and 43.87 g. potassium hydroxide in distilled water and make up to 500 ml.

VII. *p*-Dimethylaminobenzaldehyde reagent:
Dissolve 10 g. *p*-dimethylaminobenzaldehyde in a mixture of 100 ml. acetic acid and 12.5 ml. 10 N HCl. Immediately before use dilute 1:10 with acetic acid.

Stability of the solutions

The *p*-dimethylaminobenzaldehyde solution must be stored in a dark bottle and should be prepared freshly each week.

Procedure

Enzymatic reaction

As for the spectrophotometric method (p. 88).

Deproteinization and colour reaction

After 6 hours incubation with the enzyme add to each tube

0.5 ml. perchloric acid (solution III),

mix thoroughly and centrifuge for 20 min. at 5000 g.

Pipette into test tubes:

2 ml. supernatant
0.6 ml. potassium tetraborate solution (VI).

Heat for 3 min. in a boiling water bath and then cool 15 min. in an ice bath. Potassium perchlorate precipitates out. Pipette into test tubes:

1 ml. $KClO_4$-free supernatant
3 ml. *p*-dimethylaminobenzaldehyde solution (VII).

Incubate for 20 min. at 37°C. Read the optical densities of solutions 1—3 against control solution 4 in 1 cm. cuvettes at 585 mμ. The optical densities E_2 and E_3 of the solutions 2 and 3 are standards.

Calculations

Calculate the hyaluronic acid content of the sample by means of the standards (see p. 89).

Example

The reaction mixture contained 0.1 ml. of synovial fluid which was diluted with 0.4 ml. sodium chloride solution. After the colour reaction the following values were measured at 585 mμ against the control cuvette 4:

$E_1 = 0.257$; $E_2 = 0.388$; $E_3 = 0.523$; $(E_2 - E_1) = 0.131$; $(E_3 - E_2) = 0.135$

$$E_{100\,\mu g.} = \frac{0.131 + 0.135}{2} = 0.133$$

$$\frac{0.257 \times 100}{0.133} = 193 \text{ μg. potassium hyaluronate/0.1 ml. synovial fluid}$$

Discussion

The colorimetric method has the advantage over the spectrophotometric method that the measurements can be made in the presence of nucleotides, amino acids and small amounts of protein. In this method also the mixture must be heated to 70°C before incubating with bacterial hyaluronidase in order to remove inhibitors.

The colorimetric method is more suitable for the determination of hyaluronic acid in body fluids than the spectrophotometric method, which is more suitable for hyaluronic acid solutions low in protein.

Appendix

Isolation of bacterial hyaluronidase (hyaluronate lyase)

Hyaluronidase is produced in the growth phase by strains of streptococci, staphylococci, pneumococci and *Clostridium perfringens*. The isolation of a bacterial hyaluronidase from a strain of group A streptococci is described below.

Grow[10] the organisms in casein hydrolysate with the addition of hyaluronic acid from umbilical cords (0.2%). Centrifuge the 24 hour culture at high speed at 0°C. Add ammonium sulphate to the supernatant and collect the protein fraction precipitating between $^1/_3$ and $^2/_3$ saturation by centrifuging at high speed. Dissolve the precipitate in distilled water and dialyse against 0.005 M phosphate buffer (pH 7). Adsorb the contents of the dialysis sac onto a column of DEAE-cellulose and elute with: (a) 0.02 M phosphate buffer (pH 6); (b) 0.05 M NaH_2PO_4 solution and (c) solutions of 0.05 M NaH_2PO_4 in 0.02 M to 0.1 M NaCl.

The preparation contains 4 isoenzymes of hyaluronidase. The main fraction with the highest specific activity is eluted with 0.05 M NaH_2PO_4 in 0.05 M NaCl. Dialyse this eluate and then freeze-dry[11]. Enzyme preparations obtained in this way have a specific activity of about 100 units[12]/mg.

[10] *H. J. Rogers*, Biochem. J. *40*, 583 [1946].
[11] *H. Greiling*, Österr. Chemiker-Ztg. *63*, 285 [1962].
[12] *H. Greiling*, Hoppe-Seylers Z. physiol. Chem. *309*, 239 [1957].