CHARACTERISTICS OF TESTICULAR HYALURONIDASE

by

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Hyaluronidase is a general term applied to a group of enzymes hydrolysing hyaluronic acid. In 1929 DURAN-REYNALS[5] observed a "spreading factor" in extracts of bovine testis and in 1936 MEYER and co-workers[17] described a bacteriolytic component —later called hyaluronidase—which hydrolyzed the polysaccharide of vitreous humor and umbilical cord. The chemical properties and biological importance of testicular and bacterial hyaluronidase have been the subject of intense studies by, among others, CHAIN AND DUTHIE[3], MADINAVEITIA[15] and MEYER AND RAPPORT[18].

It is, however, difficult to get a uniform picture of the properties of hyaluronidase from the existing literature. There are appreciable discrepancies between the data given by various investigators. It is obvious that such discrepancies can be due to difference of the preparation methods of the hyaluronidase. In the present paper physico-chemical and activity data are presented of various hyaluronidase preparations, *viz.* crude extract of bovine testis and hyaluronidase preparations purified according to HAHN[7], TINT AND BOGASH (Wyeth, Inc.)[23], and HÖGBERG (Leo, Inc.)[11]. The last-mentioned preparations have been analyzed as to activity and chemical characteristics.

MATERIALS AND METHODS

Enzyme. Preparation according to HAHN: Fractionation of testicular extracts with $(NH_4)_2SO_4$, followed by $CuSO_4$, NaCl and Pb-acetate precipitation of non-active material and repeated $(NH_4)_2SO_4$ fractionation. No use is made of organic solvents as precipitants. This preparation will be referred to as hyaluronidase I.

Preparation according to TINT AND BOGASH: Fractionation of testicular extracts with $(NH_4)_2SO_4$, alcohol fractionation in the cold and refractionation of the precipitate. This preparation will be referred to as hyaluronidase II.

Preparation according to HÖGBERG: Extracts of dried bull testis powder fractionated with $(NH_4)_2SO_4$, chloroform shaking at room temperature in order to denature proteins of high molecular weight, electrophoretic purification. This preparation will be referred to as hyaluronidase III.

Substrate. Sodium hyaluronate from human umbilical cords prepared according to BLIX AND SNELLMAN[2]. The crude hyaluronate was purified from ester sulphate containing polysaccharides according to SYLVÉN AND MALMGREN[27]. The particle weight of the substrate was about $6 \cdot 10^4$.

Measurements of activity. Viscosity reducing activity was determined in a capillary viscosimeter (Ostwald) at 25° C. 5 ml 0.15% substrate solution (veronal buffer, pH = 6.9; ionic strength 0.2 of which 0.1 NaCl) has been incubated with 1 ml enzyme solution; for details, see[12]. For calculation of enzyme activity of the breakdown of a charged macromolecule the following formula is valid (MALMGREN AND INGELMAN[12], HULTIN[9]).

$$A = J \cdot c_s^n \frac{\mathrm{d}\frac{1}{\eta_{sp}}}{\mathrm{d}t}$$

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A = the activity; J = an ionic factor, c_s = substrate concentration, η_{sp} = specific viscosity and t = time, n = 2 (theoretically) but the exponent can vary with the nature of the substrate. As the ionic factor cannot be determined directly it is suitable to use the quantity $d\frac{I}{\eta_{sp}}$ as a relative measure of the enzyme activity in comparison experiments using the same substrate concentration and the same buffer. The derivative $d\frac{I}{\eta_{sp}}/dt$ (expressed in min^{-1}) is a linear function for the first phase of the enzymatic breakdown of hyaluronic acid, and is directly proportional to the enzyme concentration (Fig. 1).

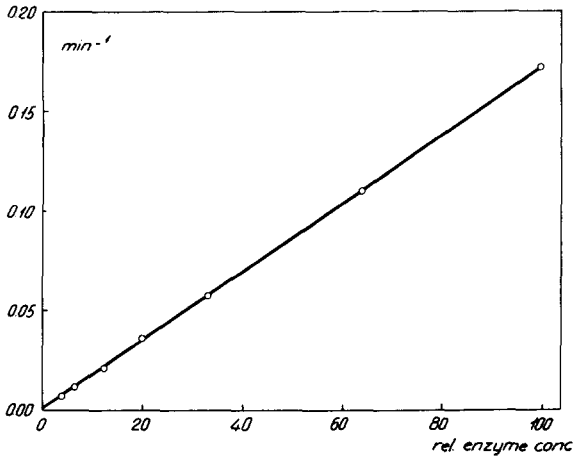

Fig. 1. The derivative $d\frac{I}{\eta_{sp}}/dt$ as a function of the enzyme concentration.

HULTIN[10] suggested the use of the above formula for calculation of the hyaluronidase activity, but he computed data from previous experiments performed without special regard to this method of calculation.

In this paper only the first viscosity reducing step of enzymatic breakdown of hyaluronic acid will be discussed. Other methods, such as the mucin-clot prevention test (ROBERTSON, ROPES AND BAUER[22]), determination of turbidity reducing units (KASS AND SEASTONE[13]) or reducing groups (MEYER[19], PARK AND JOHNSON[21]) are not considered here.

PHYSICO-CHEMICAL DATA

Electrophoresis experiments. The electrophoretic mobilities of the different preparations were determined according to the technique of TISELIUS AND SVENSSON[24, 26]. The determinations were made both by the usual optical recording and also by measurements of the activity of the original solution and of the activities in different parts of the cell after the run. Acetate, phosphate and barbiturate buffers of ionic strength 0.1 were used. The dependence of the mobility on the pH is presented in Fig. 2.

As seen from Fig. 2 the isoelectric point of the preparations investigated do not coincide. Hyaluronidase I has the I.P. at pH 5.7, hyaluronidase II at 5.9 and hyaluronidase III at 6.5. The latter preparation presents only one peak (in the electrophoretic pattern) moving with the same mobility as the activity (Fig. 3). The hyaluronidase II shows three components (Fig. 3). The activity is associated with the main constituent. The electrophoresis curves of preparation I do not indicate a quite uniform substance (HAHN[7] 1943).

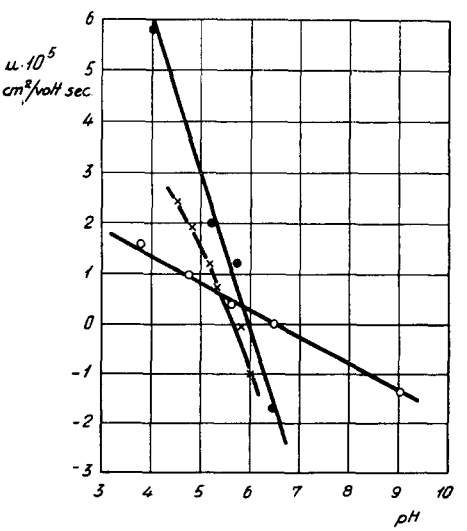

Fig. 2. Electrophoretic mobility as a function of the pH. × = hyaluronidase I (Values taken from HAHN[7]); ● = hyaluronidase II; ○ = hyaluronidase III.

Sedimentation. Sedimentation experiments were performed in the ultracentrifuge.

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The sedimentation was recorded both by the usual scale method (LAMM[14]) and by using a separation cell and determination of the hyaluronidase activity in the upper and lower part of the cell after the run. The centrifugations were carried out in phosphate buffers at pH = 6.8 and of ionic strength 0.1. The results have been drawn up in Table I.

As seen from Table I the sedimentation constant of preparation III is slightly dependent on the concentration. At zero concentration a value of 1.2 was extrapolated.

The centrifuge diagram of this preparation shows only one component that only slowly leaves the meniscus. The hyaluronidase preparation II exhibits two components, the active one ($s = 3.6$) being the main constituent.

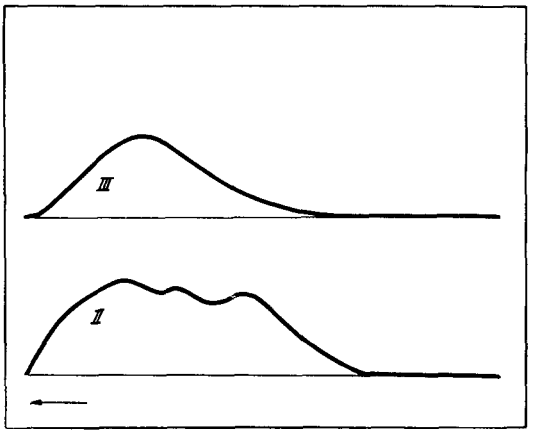

Fig. 3. Electrophoretic pattern of hyaluronidase II and III. Hyaluronidase II after 193 min at 6.0 volt/cm, pH = 4.0; hyaluronidase III after 180 min at 3.4 volt/cm, pH = 4.8. Direction of migration indicated by the arrow.

TABLE I

SEDIMENTATION CONSTANT OF SOME HYALURONIDASE PREPARATIONS

Preparation	*Conc.*	*Sedimentation constant*	
		By optical recording	*By activity measurements*
Crude testis extract	6.1 mg N/ml		4.3
Crude testis extract	2.7 mg N/ml		4.7
Hyaluronidase I		4.6*	4.3*
Hyaluronidase II	0.50%	3.6 and 5.2	
	0.25%	3.6 and 5.1	
	0.03%	—	3.2
Hyaluronidase III	0.86%	1.03	
	0.50%	1.10	
	0.20%	1.15	
	0.04%	—	1.6

* Value reported by HAHN[7].

Diffusion. Diffusion experiments were carried out according to LAMM[14]. Only the hyaluronidase III was investigated, the other preparations not being sufficiently homogeneous. The medium used was phosphate buffer at pH = 6.8 and ionic strength 0.1, temp. 25° C. The diffusion coefficient calculated from the area of the curve was $9.7 \cdot 10^{-7}$ c.g.s., calculated acc. to the moment method $10.0 \cdot 10^{-7}$ c.g.s. Accordingly, the enzyme is rather homogeneous.

Molecular weight. The partial specific volume of the hyaluronidase III has been determined 0.73. Following the insertion of ultracentrifugal and diffusion data in SVEDBERG's formula[25] the molecular weight of the enzyme was calculated to be about 11,000. This value is rather consistent with that obtained by ultracentrifugation ac-

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cording to ARCHIBALD[1] which method yields a particle size of about 14,000. Thus, the activity is bound to a medium sized polypeptide (*cf.* the analytical data below). It must be mentioned that already in 1938 MADINAVEITIA[15] inferred a particle size of his hyaluronidase preparation of the order of magnitude of 10,000. However, the analytical data of his hyaluronidase do not indicate a high degree of purity (*cf.* below).

ANALYTICAL DATA

The most homogeneous of the preparations investigated, *i.e.* hyaluronidase III has been analyzed for the chemical composition. The analytical data are found below.

ANALYTICAL DATA ON HYALURONIDASE III

Nitrogen content: Kjeldahl method		15.0	% dry weight
Amino nitrogen: VAN SLYKE		13.7	% dry weight
Protein (6.25 × N)		94	% dry weight
Lipid content, chloroform-ethanol extraction		0.1	% dry weight
Carbohydrates, orcinol reaction		2	% dry weight
Nucleic acid (U.V. absorption)		0	% dry weight
Sulphur		0.2	% dry weight
Phosphorus	less than	0.001	% dry weight
Ash	less than	0.1	% dry weight

These values differ very much from those obtained by other authors. MADINAVEITIA[15], for instance, found only 6.6% N but 9% ash; FAVILLI AND BERGAMINI[6] reported an active "nitrogen-free" hyaluronidase preparation.

Amino acids. The following amino acids were identified by means of two-dimensional paper chromatography according to CONSDEN, GORDON AND MARTIN[4] using butanole glacial acetic acid-water: aspartic acid, serine, glutamic acid, glycine, threonine, alanine, proline, tyrosine, valine, phenylalanine, leucine, *iso*leucine, lysine. In addition, histidine and arginine may be present but could not be demonstrated. The sulphur content indicates the presence of methionine and/or cystine.

The minute carbohydrate moiety present could not be analysed. During electrophoresis, however, the carbohydrate moves together with the enzymes, which suggests that the carbohydrate is associated with the enzyme. It may also be mentioned that preparation II contains 1.6% carbohydrate (orcinol reaction).

Presence of other enzymes. Hyaluronidase III has been tested for the presence of other enzymes. Proteolytic enzymes, alkaline and acid phosphatases, cholinesterase and pyrophosphatase could not be detected. Furthermore, after a long digestion of a highly purified hyaluronate with this enzyme the smallest splitting product probably was a disaccharide and not a monosaccharide—as revealed by adsorption analysis (MALMGREN[16]). Accordingly the presence of β-glucuronidase is excluded (*cf.* HAHN[8], MEYER AND LINKER[20]).

VISCOSITY REDUCING ACTIVITIES

The viscosity reducing activities per mg N of crude testis extracts and of the hyaluronidase II and III have been drawn up in Table II.

The purification of hyaluronidase III is $3 \cdot 10^3$. The corresponding value for hyaluronidase II cannot be given as the starting material was not available. In comparison it may be stated that HAHN[7] claimed a purification of 10^4 for his best preparation.

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However, a preparation method involving chloroform shaking probably causes partial denaturation and hence loss of activity.

TABLE II

VISCOSITY REDUCING ACTIVITIES

Preparation	*mg N/ml enzyme solution*	*Rel. acitvity*	*Rel. act./mg N*
Crude extracts of bovine testis	6.11	0.02 min^{-1}	0.003
Hyaluronidase II	0.151	0.598	3.96
Hyaluronidase III	0.094	0.842	8.96

DISCUSSION

The hyaluronidase preparations investigated differ from each other with respect to isoelectric point and sedimentation constant. In the crude extract of bovine testis the hyaluronidase activity sediments with a fairly high velocity. After purification the sedimentation constant of hyaluronidase activity decreases, the harder the purification method the lower the sedimentation constant. Thus, the original enzyme activity is associated with a rather large particle that must not necessarily be of pure protein nature (*cf.* the carbohydrate content). During purification this "carrier" is partly or completely removed which could explain why various preparations have different physico-chemical constants. However, the active polypeptide cannot be linked to the carrier with covalent bonds. Only a linkage of lower energy content, *e.g.* a hydrogen bond, can be expected to be broken up by fractionation with inorganic salts and/or chloroform shaking.

The hyaluronidase activity is associated with a medium sized polypeptide M $\sim$ 11,000. Perhaps it might be possible in some way to split this molecule in smaller parts some of which still exhibit activity.

ACKNOWLEDGEMENTS

Hyaluronidase preparations have been generously supplied by Leo Pharmaceutical Co, Inc., Hälsingborg, and Wyeth Research Laboratories, Philadelphia. The costs have been defrayed by institutional grant to the Cancer Research Division from the Eli Lilly and Co, Indianapolis, U.S.A.

SUMMARY

1. Testicular hyaluronidases prepared according to different methods have been investigated with respect to their physico-chemical properties.

2. The results indicate that native hyaluronidase in some way is associated with a "carrier" which can be removed during purification of the enzyme.

3. After purification there remains an active polypeptide with a molecular weight of about 11,000.

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RÉSUMÉ

1. Nous avons étudié les propriétés physico-chimiques de certaines hyaluronidases testiculaires préparées selon des méthodes différentes.

2. Les résultats indiquent que l'hyaluronidase native est associée d'une manière ou d'une autre à un "porteur" qui peut être supprimé pendant la purification de l'enzyme.

3. Après purification l'on obtient un polypeptide actif ayant un poids moléculaire d'environ 11,000.

ZUSAMMENFASSUNG

1. Nach verschiedenen Methoden aus Testikeln hergestellte Hyaluronidasen wurden hinsichtsichtlich ihrer physikalisch-chemischen Eigenschaften untersucht.

2. Die Ergebnisse zeigen, dass native Hyaluronidase in gewisser Hinsicht mit einem "Träger" verbunden ist, der während der Reinigung des Enzyms entfernt werden kann.

3. Nach der Reinigung bleibt ein aktives Polypeptid mit einem Molekulargewicht von ungefähr 11,000 zurück.

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Received February 26th, 1953