Biochem. J. (1968) **110**, 19
Printed in Great Britain

Canine Submandibular-Gland Hyaluronidase

PURIFICATION AND PROPERTIES

BY Y. H. TAN AND J. M. BOWNESS
Department of Biochemistry, Faculty of Medicine, University of Manitoba, Winnipeg 3, Man., Canada

(*Received 22 April* 1968)

1. Methods for the purification of dog submandibular-gland hyaluronidase from sedimentable and non-sedimentable portions of a homogenate and from the whole homogenate are presented. The method consists of three main steps: removal of mucin by acid precipitation or gel filtration on Sephadex G-200, ammonium sulphate precipitation and CM-cellulose chromatography. By this method specific activities of up to 1·28 and 0·78 μmoles of *N*-acetylglucosamine/min./mg. of protein were obtained for the purified freeze-dried non-sedimentable hyaluronidase and for the sedimentable hyaluronidase respectively. 2. A comparison of some of the properties of the non-sedimentable and the sedimentable hyaluronidase preparation indicated that there was little difference between the two and that they both resembled lysosomal hyaluronidase from rat liver.

In the preceding paper (Tan & Bowness, 1968) the occurrence, characterization and subcellular distribution of an acid-active hyaluronidase from canine submandibular gland was described. About 40% of the hyaluronidase was sedimentable and 60% was in the soluble supernatant fraction. The supernatant of submandibular-gland extracts was viscous; this is presumably due to the content of mucin (Hashimoto & Pigman, 1962). The purification procedure described was designed to remove the mucin, or the mucin plus the sucrose of the extraction medium, before fractionation of the remaining proteins to obtain an active hyaluronidase fraction.

EXPERIMENTAL

Materials. Enzyme-grade $(NH_4)_2SO_4$ was from Mann Research Laboratories (New York, N.Y., U.S.A.). Sephadex G-200 and G-100 were obtained from Pharmacia (Uppsala, Sweden) and CM-cellulose (new fibrous Whatman CM23) was from W. and R. Balston Ltd. (Maidstone, Kent). Sodium hyaluronate was prepared from human umbilical cord by method IVa of Jeanloz & Forchielli (1950). The dried hyaluronate preparation was redissolved in water and dialysed for 36hr. against four changes of water (41.) at 4° and then freeze-dried. Analysis of the preparation showed a glucuronic acid : hexosamine molar ratio 1:1·08, and the viscosity at 30° for a 1mg./ml. solution in 0·1M-sodium phosphate–citric acid buffer, pH4·0, was 1·13 centipoise. This preparation is referred to as hyaluronate A.

Sodium hyaluronate of higher mean molecular weight was prepared from commercial sodium hyaluronate (Koch–Light Laboratories Ltd., Colnbrook, Bucks.) by the method of Houck & Pearce (1957). Only the fraction precipitated at the ethanol concentration producing the first cloudiness was collected; 30mg. of reprecipitated hyaluronate was obtained from 150mg. of Koch–Light hyaluronate. The viscosity at 30° for a 1mg./ml. solution in 0·1M-sodium phosphate–citric acid buffer, pH4·0, was 1·58 centipoise. The glucuronic acid/hexosamine molar ratio was 1:1·10. This preparation is referred to as hyaluronate B.

Ox tracheal chondroitin sulphate was obtained from Koch–Light Laboratories Ltd. and shark cartilage chondroitin sulphate from Kaken Yaku Koko Co. Ltd. (Tokyo, Japan); i.r. spectra showed that the former was principally chondroitin 4-sulphate and the latter chondroitin 6-sulphate. Desulphated chondroitin 4-sulphate was prepared by the method of Kantor & Schubert (1957) and dermatan sulphate was obtained by the method of Jeanloz (1965). Glucuronic acid/hexosamine/sulphate molar proportions were 1·00:1·13:0·93 for chondroitin 4-sulphate, 1·00:1·18:0·97 for chondroitin 6-sulphate and 1·00:1·22:0·00 for desulphated chondroitin 4-sulphate.

Assays. The procedures used for the assay of hyaluronidase, β-*N*-acetylhexosaminidase, protein and uronic acid were as described in the preceding paper (Tan & Bowness, 1968), except where otherwise stated.

The reductimetric method of Park & Johnson (1949) was used to assay the activity of hyaluronidase preparations against different substrates (Table 2). The incubation procedure for the reductimetric method was similar to the one described for the *N*-acetylglucosamine assay (Tan & Bowness, 1968), except that the substrates were prepared in 0·1M-sodium acetate buffer, pH3·8; the acetate buffer was neutralized by 0·1N-NaOH after incubation at 37°. Hyaluronidase activity against chondroitin 4-sulphate or chondroitin 6-sulphate was also assayed by the spectrophotometric titration procedure of Bowness (1965). Total carbohydrate was assayed by the anthrone procedure

(Ashwell, 1957) with D-glucose as standard. Inorganic sulphate was assayed by the method of Dodgson & Price (1962). Total hexosamine content was assayed by the method of Boas (1953); slight modifications were made in the procedure for hydrolysis (5–15mg. of each material was hydrolysed in a sealed tube with 2ml. of 2N-HCl at 100–105° for 8hr.).

Viscosities were determined at $30 \pm 0{\cdot}1°$ with a Cannon 100 viscometer previously calibrated with sucrose solution and water.

Homogenization of glands. Freshly obtained canine submandibular glands (33g.) were chopped with a pair of scalpel blades into tiny pieces; portions of the chopped gland weighing about 2g. were suspended in 14ml. of 0·25M-sucrose and placed in the tube of a Potter–Elvehjem homogenizer with the Teflon pestle rotating at 1000rev./min.; three up-and-down strokes were made during the homogenization of the tissue. The homogenate was centrifuged at $400g_{av.}$ for 5min., and the supernatant was poured off and retained. The pellet was resuspended in 4vol. of 0·25M-sucrose, homogenized again and then centrifuged again at $400g_{av.}$ for 5min. Both supernatant fractions from all homogenizations were pooled (total volume about 300ml.) and then centrifuged at $101000g_{av.}$ for 30min. in a Spinco model L centrifuge (no. 40L rotor). The pellet then contained the sedimentable hyaluronidase (fractions M, L and P; Tan & Bowness, 1968) and the supernatant fraction S contained the non-sedimentable hyaluronidase. The pellet was resuspended in glass-distilled water (56ml.) at 4°, homogenized for three 15sec. periods at 20000rev./min. in a VirTis homogenizer to break up the subcellular particles and then centrifuged at $3500g_{av.}$ for 10min. All the operations described were performed at 4°. The supernatant (fraction MLP), containing the originally sedimentable hyaluronidase, and the originally non-sedimentable hyaluronidase (fraction S) were each purified separately by the procedure described in the following sections.

Gel filtration. About one-third of fraction S (88ml.) or the whole of fraction MLP (45ml.) was subjected to reversed-flow gel filtration on a Sephadex G-200 column (5cm. × 75cm.) prepared in 2M-NaCl–0·1M-sodium phosphate buffer, pH6·0. The flow rate of the applied sample of fraction S in a 0·1ml. pipette was about one-third that of water, whereas that of fraction MLP did not differ greatly from that of water. During application of the samples of fraction S or fraction MLP the flow rate of the column was adjusted to 20–30ml./hr. The column was developed with the buffer used in the preparation of the column at a flow rate of 48ml./hr., and effluent fractions (16ml.) were collected. The procedure was repeated twice more to dispose of the remainder of fraction S. This gel filtration is shown in Fig. 1.

Precipitation of mucin with acid. A sample (24g.) of the chopped canine submandibular gland was homogenized in 0·1M-sodium phosphate buffer, pH7·4, in a VirTis homogenizer at 20000rev./min. for three 15sec. periods at 0°. The homogenate was centrifuged at $13000g_{av.}$ for 10min., and the pellet was re-extracted three times with 0·1M-sodium phosphate buffer, pH7·4. The supernatants from these extracts were pooled and adjusted to pH3·5 with 0·05N-HCl (Nisizawa & Pigman, 1959). The resulting mucinous clot was removed by centrifuging at $13000g_{av.}$ for 10min. and the supernatant was retained. The clot was washed with water and sedimented again by centrifugation at $13000g_{av.}$ for 10min.; the supernatant was pooled with the previous supernatant. The insoluble mucin was resuspended in water, dissolved by adding 0·05N-NaOH and assayed by the anthrone procedure.

Fractionation with ammonium sulphate. The effluent fractions from Sephadex G-200 shown between the arrows in Fig. 1, containing the bulk of the hyaluronidase activity, were pooled to give fractions S_1 and MLP_1 and fractionated by adding solid $(NH_4)_2SO_4$ at 4° and pH6·0 (Table 1). At each stage the mixture obtained after the $(NH_4)_2SO_4$ had dissolved was allowed to stand for 18hr. at 4° and the suspension was then centrifuged at $16300g_{av.}$ for 10min. in a Servall RC2-B centrifuge with a GSA rotor. The precipitate was redissolved in 4ml. of glass-distilled water to give fraction S_2 or fraction MLP_2 (Table 1) and then dialysed in 1cm.-wide dialysis tubing, with stirring, for 5hr. at 4° against two changes of 3l. of glass-distilled water. The dialysed preparation was centrifuged to remove any precipitated protein(s), and the clear supernatant was freeze-dried to give fraction S_3 or fraction MLP_3 (Table 1).

CM-cellulose chromatography. A portion of fraction S_3 or fraction MLP_3 was chromatographed on a CM-cellulose column (1·5cm. × 16cm.) previously prepared in 0·04M-sodium citrate buffer, pH4·85. The column was eluted by a stepwise procedure (Fig. 2). The three most active effluent fractions shown in Fig. 2 were pooled to give fraction S_4 or fraction MLP_4 (Table 1) and dialysed as described in the preceding section. After dialysis the dialysis residue was centrifuged and the clear supernatant, fraction S_5 or fraction MLP_5, freeze-dried to give fraction S_6 or fraction MLP_6 (Table 1).

RESULTS AND DISCUSSION

Isolation and purification of the enzyme

When the non-sedimentable material from the submandibular gland was subjected to gel filtration (Fig. 1*a*), much carbohydrate material was eluted with protein in the first peak; this was presumably due to the presence of mucin. Sephadex G-200 was found to be better than Sephadex G-100 in separating mucin from the hyaluronidase-active fractions. However, the procedure was time-consuming, as high flow rates with viscous samples tend to shorten the column and decrease subsequent resolution. For this reason a lower flow rate was used during sample application than during subsequent development. A supernatant obtained by homogenizing 1g. of gland with approx. 11ml. of sucrose solution was found to be the most viscous that could be used. The good recovery of activity from the Sephadex G-200 column indicated that hyaluronidase was stable during gel filtration.

An alternative way to remove mucin was to precipitate it by adjusting the pH of the extract to 3·5. By this method it was found that only 0·48% of the hyaluronidase was precipitated in the mucin clot and the rest of the hyaluronidase remained in solution; about 70–72% of the protein and 60–62% of the anthrone-positive material in the extract

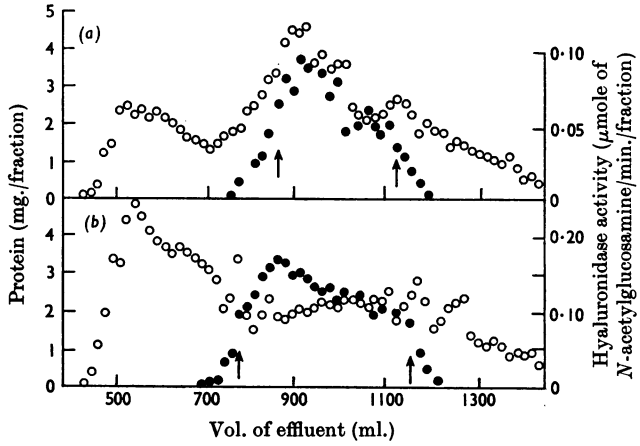

Fig. 1. Chromatography of (*a*) supernatant fraction S (174mg. of assayed protein) and of (*b*) sedimentable fractions MLP (140mg. of assayed protein) on a column (5cm. diam. × 75cm. height) of Sephadex G-200. The effluent fractions between the arrows were pooled for $(NH_4)_2SO_4$ fractionation. ○, Protein; ●, hyaluronidase.

Table 1. *Purification of submandibular-gland hyaluronidase from supernatant fraction S and sedimentable fractions MLP*

Fraction	Step	Total protein (mg.)	Sp. activity (units/mg. of protein)	Hyaluronidase / Hexosaminidase	Recovery (%)
S		174	0·014	1·2	
S_1	Active effluents from Sephadex G-200	55	0·056		126
S_2	0–35%-satd.-$(NH_4)_2SO_4$ ppt.	14	0·140		80
S_3	Freeze-dried supernatant from centrifugation of dialysed S_2	10	0·340	9·2	
S_4	Active effluents from CM-cellulose chromatography of S_3	2	0·720		59
S_5	Dialysis and centrifugation of S_4	0·7	1·28		37
S_6	Freeze-drying of S_5	0·7	1·20	10·4	34
S_7	35–50%-satd.-$(NH_4)_2SO_4$ ppt. from S_1 (dialysed)	3	0·160		
S_8	Dialysed supernatant from 50%-satd.-$(NH_4)_2SO_4$ treatment of S_1	30	0·001		
MLP		140	0·029	1·8	
MLP_1	Active effluents from Sephadex G-200	42	0·079		82
MLP_2	0–35%-satd.-$(NH_4)_2SO_4$ ppt.	12·5	0·250		77
MLP_3	Freeze-dried supernatant from centrifugation of dialysed MLP_2	11·5	0·280	7·0	
MLP_4	Active effluents from CM-cellulose chromatography of MLP_3	2·5	0·75		46
MLP_5	Dialysis and centrifugation of MLP_4	2·3	0·77		44
MLP_6	Freeze-drying of MLP_5	2·0	0·78	7·8	38
MLP_7	35–50%-satd.-$(NH_4)_2SO_4$ ppt. from MLP_1 (dialysed)	2·8	0·102		
MLP_8	Dialysed supernatant from 50%-satd.-$(NH_4)_2SO_4$ treatment of MLP_1	28·8	0·001		

was removed during the precipitation of mucin by acid. The viscosity of the extract after the removal of the mucin clot did not differ greatly from that of water.

The largest fraction of the hyaluronidase in the active effluents from gel filtration was precipitated by ammonium sulphate at 35% saturation at 4° and pH 6·0 (Table 1). Material reacting with anthrone was presumably removed during gel filtration and ammonium sulphate fractionation,

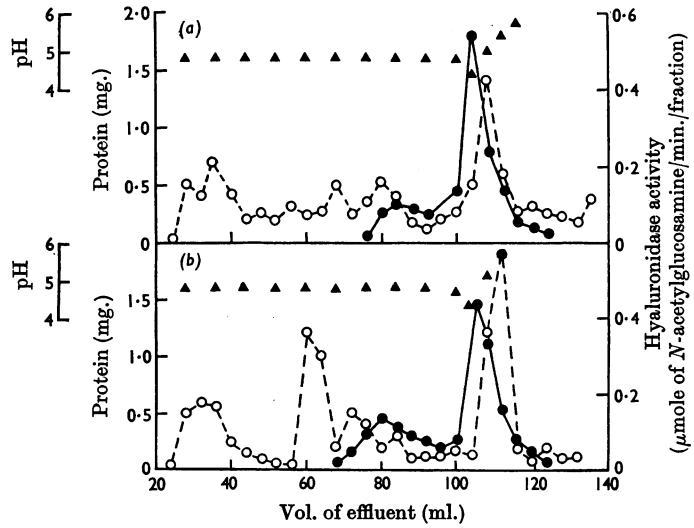

Fig. 2. Chromatography of (*a*) fraction S_3 and of (*b*) fraction MLP_3 on a column (1·5cm. diam. × 16cm. height) of CM-cellulose; 10mg. of assayed protein was applied. The column was eluted by a stepwise procedure at a rate of 60ml./hr. with: (i) 30ml. of 0·04M-sodium citrate buffer, pH4·85; (ii) 50ml. of 0·04M-sodium citrate buffer, pH4·85, containing 0·05M-NaCl; (iii) 0·04M-sodium citrate buffer, pH6·3, containing 0·3M-NaCl. Effluent fractions (4ml.) were collected. ○, Protein; ●, hyaluronidase; ▲, pH of effluent.

as none was detected in enzyme preparation S_3 or subsequently.

CM-cellulose chromatography (Fig. 2 and Table 1) produced a two- to three-fold increase in the specific activity of the hyaluronidase.

There were two dialysis steps during the purification of submandibular-gland hyaluronidase described; the first was immediately after the ammonium sulphate step and the second was carried out on the active effluent fractions from CM-cellulose columns. During dialysis some non-enzymic proteins, presumably globulins, were precipitated. Since submandibular-gland hyaluronidase was relatively stable during dialysis this precipitation provided a further increase in the specific activity of the non-sedimentable hyaluronidase. Little precipitation was observed during the dialysis of hyaluronidase prepared from sedimentable submandibular components (fractions MLP_2).

The content of hyaluronidase in dog submandibular glands was much higher than that in other organs examined so far; although the highest specific activity of the final freeze-dried preparations (fraction S_6 or fraction MLP_6) represented only a 91-fold and a 56-fold increase respectively in specific activity over the unfractionated supernatant fraction, the highest specific activity of 1·28units (fraction S_5) was comparable with the value of 1·29 units reported by Aronson & Davidson (1967*a*) for a preparation of rat liver lysosomal hyaluronidase that represented a 1300-fold increase in specific activity.

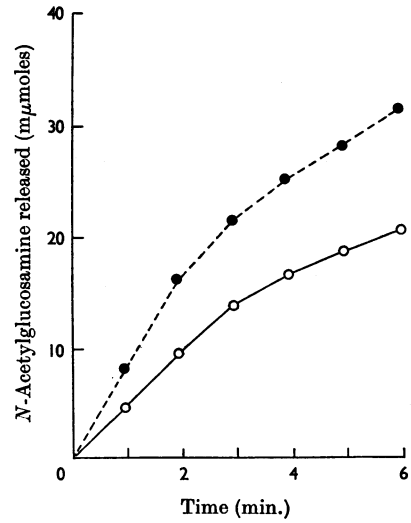

Fig. 3. Progress curve of the hyaluronidase reaction. A sample (16μg.) of preparation S_3 was incubated at 37° and pH3·8 with: ●, 1·2mg. of hyaluronate/ml.; ○, 0·5mg. of hyaluronate/ml.

Properties

Effects of time, protein concentrations and pH. Fig. 3 shows that the release of *N*-acetylglucosamine end groups was linearly related to time only for the first 2min. Aronson & Davidson (1967*b*) found no decrease in rate of *N*-acetylglucosamine release until after 15min. incubation with rat liver lysosomal hyaluronidase. This difference may have several causes apart from a difference in the enzyme protein. For example, the substrate (hyaluronate A) used in the present work may contain more low-molecular-weight hyaluronate, giving rise to a significant back-reaction at an earlier time. An incubation time of 2min. was used to determine the initial velocity for Fig. 6. In the standard assay an incubation time of 5min. was used to obtain adequate colour development for assays with smaller amounts of activity.

Fig. 4 shows the effect of protein concentration on activity. The difference between the two slopes is presumably due only to a higher content of active enzyme in fraction S_3 than in fraction MLP_3. The linearity of the graphs shows that the substrate concentration used was suitable for the standard assay of the enzyme at 5min., and that inhibitor(s) reversibly combining with enzyme and having a different affinity for the enzyme from that of the substrate were absent.

There was no significant difference between the sedimentable and non-sedimentable forms of hyaluronidase. The pH optima and pH–activity profiles of both sedimentable and non-sedimentable hyaluronidase also did not change significantly with variations in incubation times (Fig. 5). This indicates that there is no significant difference between the two fractions in their stability at any of the pH values studied.

Reciprocal plot for effect of substrate concentration (*Fig.* 6). With preparation A as substrate (unfractionated human umbilical-cord hyaluronate), K_m values of 0·240 and 0·242mg. of hyaluronate/ml. of incubation mixture were obtained with hyaluronidase preparation MLP_3 and preparation S_3 (Table 1) respectively. With hyaluronate B (a high-molecular-weight fraction from a commercial umbilical-cord hyaluronate) as substrate, a K_m value of 0·193mg. of hyaluronate/ml. of incubation mixture was obtained with hyaluronidase preparation S_3 (Fig. 6). The difference in K_m values obtained with different substrate preparations may be attributed to differences in the affinity of the substrates for the enzyme (Houck & Pearce, 1957). The slight upturn in the reciprocal plot at concentrations of hyaluronate exceeding 1·2mg./ml. of incubation mixture suggests that there was high-substrate inhibition.

Substrate specificity (*Table* 2). Submandibular-gland hyaluronidase was found to be most active against sodium hyaluronate, of the compounds tested, by either the reductimetric assay or the hexosamine assay after 12min. incubation. With the reductimetric assay, product formation after 12min. was only 18–21% as great for chondroitin 4-sulphate or chondroitin 6-sulphate as for hyaluronate. With the hexosamine assay product formation after 12min. was found to be 20–21% as great for chondroitin 6-sulphate as for hyaluronate. This indicates that the same products were assayed by both procedures with chondroitin 6-sulphate. Little colour development occurred in

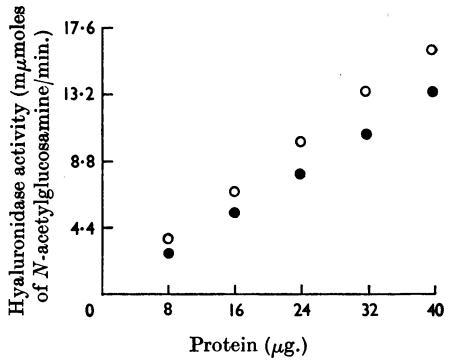

Fig. 4. Velocity and enzyme concentration. Preparation S_3 (○) or preparation MLP_3 (●) was incubated at 37° for 5min. at pH3·8 with 1·2mg. of hyaluronate/ml. of incubation mixture.

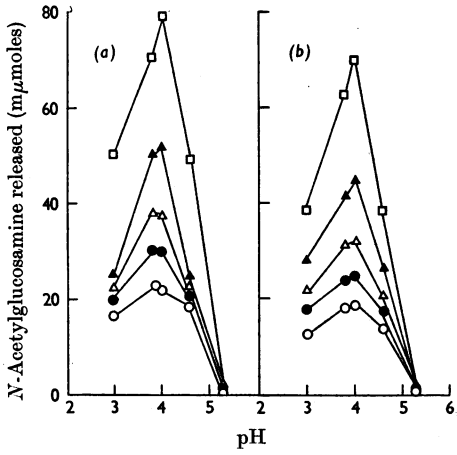

Fig. 5. pH–activity curves of (*a*) preparation S_3 (15μg.) and of (*b*) preparation MLP_3 (15μg.) as measured by product formation at various times at 37° with a substrate concentration of 1·2 mg. of hyaluronate/ml. ○, 5min.; ●, 10min.; △, 15min.; ▲, 30min.; □, 60min.

the hexosamine procedure when chondroitin 4-sulphate was used as substrate. This lack of colour development may be explained by the finding that introduction of an extra substituent at C-4 suppresses the Morgan–Elson reaction (Kuhn, Gauhe & Baer, 1954; Jeanloz & Trémège, 1956; Egge, 1963). After removal of the sulphate group from C-4, as in the desulphated chondroitin 4-sulphate, colour development was found to occur, and the activity by this method of assay corresponded to that found in the reductimetric assay. Of the sulphated acid mucopolysaccharides tested, the various preparations of submandibular-gland hyaluronidase were least active against dermatan sulphate, with about half the product formation at 12min. found for chondroitin 6-sulphate by either the hexosamine or the reductimetric assay. Since dermatan sulphate, like chondroitin 4-sulphate, is sulphated at C-4, no colour development was expected in the hexosamine assay procedure; the fact that colour development did occur suggests the possible presence of hyaluronate or chondroitin 6-sulphate as contaminants in the preparation. That the submandibular-gland hyaluronidase catalyses the breakdown of chondroitin 4-sulphate and chondroitin 6-sulphate was confirmed by the spectrophotometric titration procedure of Bowness (1965). This method measures the breakdown of sulphated acidic glycosaminoglycans by the decrease in metachromasia with toluidine blue.

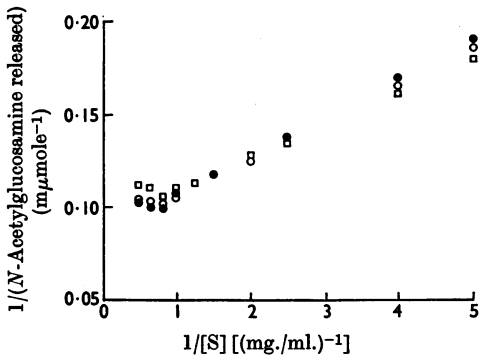

Fig. 6. Reciprocal plot of velocity and substrate concentration for preparation S_3 (16μg.) or preparation MLP_3 (20μg.) incubated at 37° and pH3·8 with two different hyaluronate preparations. ○, Preparation S_3 with hyaluronate A; ■, preparation MLP_3 with hyaluronate A; □, preparation S_3 with hyaluronate B.

Table 2 also shows that canine submandibular-gland hyaluronidase hydrolyses hyaluronate B at a higher rate (10%) than the lower-molecular-weight hyaluronate A. This is in agreement with the finding that the K_m for hyaluronate B is lower than that for hyaluronate A (Fig. 6 and the text above). Houck & Pearce (1957) also found that the affinity of testicular hyaluronidase was greater for longer-chain than for shorter-chain hyaluronate preparations.

Table 2. *Activity of hyaluronidase preparations against different substrates*

The characteristics of the substrates are listed under 'Materials' in the Experimental section. Hyaluronate B showed a higher viscosity than hyaluronate A. Each result is a mean of three replicates. The concentration of each substrate in the incubation mixture was 1·2mg./ml. and the temperature was 37°. The weight (μg. of assayed protein) of each enzyme preparation (lettered as in Table 1) is given in parentheses. These weights were calculated to give the same amount of activity in each case. The standards used were *N*-acetylglucosamine for hyaluronate and *N*-acetylgalactosamine for chondroitin sulphates.

Method of assay	Enzyme preparation (μg.) ... Substrate	Product formation (mμmoles of *N*-acetylhexosamine) in 12min. S_3 (19)	S_6 (6)	MLP_3 (25)	MLP_6 (8)	E* (200)
Reductimetric	Hyaluronate A	47·2	48·7	49·5	45·0	56·2
	Chondroitin 4-sulphate	8·6	9·9	9·9	9·0	12·6
	Chondroitin 6-sulphate	9·0	8·6	10·8	9·5	11·3
	Dermatan sulphate	4·5	4·8	4·5		5·0
	Desulphated chondroitin 4-sulphate	13·7	14·4	15·8		14·4
N-Acetylhexosamine	Hyaluronate A	56·1		62·1		65·2
	Hyaluronate B	59·9		67·2		
	Chondroitin 4-sulphate	0·9		1·6		1·6
	Chondroitin 6-sulphate	11·6		13·2		14·0
	Dermatan sulphate	5·4		6·2		6·3
	Desulphated chondroitin 4-sulphate	16·2		19·8		21·5

* A partially purified hyaluronidase preparation (sp. activity 0·04 unit/mg. of assayed protein).

Though a systematic study of the homogeneity of purified canine submandibular-gland hyaluronidase has not been carried out, Table 2 shows that the ratio of activity against the different substrates remained unchanged at various stages of purification. This supports the hypothesis that only one enzyme is responsible for the activity against the various substrates tested.

Comparison of sedimentable and non-sedimentable hyaluronidase. Results from the purification of sedimentable and non-sedimentable hyaluronidase (Figs. 1 and 2 and Table 1) show that the two forms of hyaluronidase are chromatographically similar on Sephadex G-200 and CM-cellulose columns. The sedimentable and non-sedimentable hyaluronidase were both precipitated at approximately the same percentage saturation with ammonium sulphate in the presence of 2M-sodium chloride–0·1M-sodium phosphate buffer, pH 6·0.

The two subcellular fractions of submandibular-gland hyaluronidase were found to have almost identical K_m values, and both were inhibited by hyaluronate concentrations exceeding 1·2mg. of sodium hyaluronate/ml. (Fig. 6). Results of substrate-specificity studies (Table 2) and pH–activity curves (Fig. 5) also showed no significant differences between the two. In the preceding paper (Tan & Bowness, 1968) it was shown that the end products of the action of sedimentable and non-sedimentable hyaluronidase fractions on hyaluronate were the same. From the properties studied in this paper there is no evidence to suggest that there is a significant difference between the hyaluronidase that sediments with the subcellular particles and the one that is present in the supernatant. It is possible that the hyaluronidase in the supernatant is derived from the sedimented particles either *in vivo* or during the preparative procedure. Alternatively each subcellular compartment of submandibular-gland cells, including the supernatant fraction S, may have associated with it a particular concentration of the same enzyme.

Comparison of submandibular-gland hyaluronidase with other hyaluronidases. Canine submandibular-gland hyaluronidase showed the same properties as rat liver hyaluronidase (Aronson & Davidson, 1967*b*) in product formation, pH optimum (see Tan & Bowness, 1968) and substrate inhibition, but exhibited small differences in pH–activity curves, substrate specificity, activity–time curves and K_m. It also resembled hyaluronidase from infant bone (Vaes & Jacques, 1965) and human gingiva (Goggins, Fullmer & Steffek, 1968) in pH optimum and pH–activity curves. However, the subcellular distribution of canine submandibular-gland hyaluronidase differed somewhat from that found for rat bone hyaluronidase (Vaes, 1967) in having a higher percentage of the enzyme in the supernatant fraction S.

This work was supported by a grant from the Medical Research Council of Canada. We are deeply grateful to Dr Lee Kay Hoon and Mr Chia Chiap Mong of the University of Singapore for the acid mucopolysaccharide preparations. Y.H.T. was the holder of Graduate Studentships from the University of Manitoba and the Medical Research Council of Canada.

REFERENCES

Aronson, N. N., jun. & Davidson, E. A. (1967*a*). *J. biol. Chem.* **242**, 437.

Aronson, N. N., jun. & Davidson, E. A. (1967*b*). *J. biol. Chem.* **242**, 441.

Ashwell, G. (1957). In *Methods in Enzymology*, vol. 3, p. 84. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.

Boas, N. F. (1953). *J. biol. Chem.* **204**, 553.

Bowness, J. M. (1965). *Biochim. biophys. Acta*, **101**, 26.

Dodgson, K. S. & Price, R. G. (1962). *Biochem. J.* **84**, 106.

Egge, H. (1963). In *Comprehensive Biochemistry*, vol. 5, p. 106. Ed. by Florkin, M. & Stotz, E. H. Amsterdam: Elsevier Publishing Co. Ltd.

Goggins, J. R., Fullmer, H. M. & Steffek, A. J. (1968). *Arch. Path.* **85**, 272.

Hashimoto, Y. & Pigman, W. (1962). *Ann. N.Y. Acad. Sci.* **93**, 541.

Houck, J. C. & Pearce, R. H. (1957). *Biochim. biophys. Acta*, **25**, 607.

Jeanloz, R. W. (1965). In *Methods in Carbohydrate Chemistry*, vol. 5, p. 116. Ed. by Whistler, R. L. New York and London: Academic Press Inc.

Jeanloz, R. W. & Forchielli, E. (1950). *J. biol. Chem.* **186**, 495.

Jeanloz, R. W. & Trémège, M. (1956). *Fed. Proc.* **15**, 282.

Kantor, T. G. & Schubert, M. (1957). *J. Amer. chem. Soc.* **79**, 152.

Kuhn, R., Gauhe, A. & Baer, H. H. (1954). *Chem. Ber.* **87**, 1138.

Nisizawa, K. & Pigman, W. (1959). *Arch. oral Biol.* **1**, 161.

Park, J. T. & Johnson, M. J. (1949). *J. biol. Chem.* **181**, 149.

Tan, Y. H. & Bowness, J. M. (1968). *Biochem. J.* **110**, 9.

Vaes, G. (1967). *Biochem. J.* **103**, 802.

Vaes, G. & Jacques, P. (1965). *Biochem. J.* **97**, 380.