Biochem. J. (1968) **110**, 9
Printed in Great Britain

Canine Submandibular-Gland Hyaluronidase

IDENTIFICATION AND SUBCELLULAR DISTRIBUTION

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(*Received* 22 *April* 1968)

1. Submandibular glands from four species of mammal have been shown to contain a hyaluronidase active at acid pH; glands from dog and cat had a much higher content of this enzyme than has been found in other sources. 2. Product formation from hyaluronate after 24hr. incubation was almost the same as with testicular hyaluronidase, indicating that the enzyme is an endo-poly-β-hexosaminidase. 3. When submandibular-gland homogenates were fractionated by the scheme developed for liver by de Duve, Pressman, Gianetto, Wattiaux & Appelmans (1955), all the enzymes assayed, except cytochrome *c* oxidase, were found to occur partly in the soluble fraction and partly in the particulate fractions. Among the particular fractions, the highest specific activity was found in the heavy-mitochondrial fraction for cytochrome *c* oxidase, in the microsomal fraction for alkaline phosphatase and in the light-mitochondrial fraction for acid phosphatase, β-*N*-acetylhexosaminidase and acid-active hyaluronidase. 4. Release of the enzyme activity from the sedimentable fractions occurred in 0·1% Triton X-100 or after high-speed homogenization. 5. Stimulation of dogs by pilocarpine was found to decrease the hyaluronidase content of the submandibular gland by 5% and to cause the occurrence of a corresponding amount of acid-active hyaluronidase in the submandibular saliva. 6. The results are discussed in relation to the subcellular localization of hyaluronidase.

The most potent source of hyaluronidase found so far is the testis of certain animals such as ox and sheep, though the physiological function of the enzyme in this organ is rendered uncertain by the finding that the testes of other animals such as the dog apparently contain no hyaluronidase (Gibian, 1959). The testicular enzyme appears to differ from the hyaluronidase found in other mammalian tissues in possessing greater activity at pH values of 4·5 and above, thus suggesting the name 'acid-active hyaluronidase' for the non-testicular enzyme (de Salegui & Pigman, 1967). Rat liver (Aronson & Davidson, 1965; Hutterer, 1966), human serum (Bollet, Bonner & Nance, 1963; de Salegui & Pigman, 1967), human gingiva (Goggins, Fullmer & Steffek, 1968) and infant rat bone (Vaes, 1967) are known to contain acid-active hyaluronidase, but the activities found in these tissues were low in comparison with the activity extractable from bovine or rabbit testis.

The present investigation was a search for a more potent source of acid-active hyaluronidase that might be used to prepare larger amounts of the purified enzyme than have so far been available. It is also the beginning of a survey of the distribution of the enzyme that may throw light on its physiological function and on the source of the serum hyaluronidase the concentration of which in rats has been shown to be affected by cortisol (Bowness & Harding, 1968).

EXPERIMENTAL

Reagents. Sodium hyaluronate was obtained from human umbilical cords by method IVa of Jeanloz & Forchielli (1950). A commercial preparation of chondroitin sulphate from ox trachea (L. Light and Co. Ltd., Colnbrook, Bucks.) was used in the spectrophotometric titration procedure of Bowness (1965). Hyaluronidase from sheep testis (minimum activity 300i.u./mg.) was obtained from L. Light and Co. Ltd. Hydrolysed gelatin (British Drug Houses Ltd., Poole, Dorset) was prepared in the freeze-dried state by the USP procedure (*U.S. Pharmacopeia*, 1955), broken up by homogenizing it in a blender with ethanol and separated by centrifugation; the ethanol was removed in an oven at 90°. *p*-Dimethylaminobenzaldehyde stock solution was prepared by dissolving 1g. of *p*-dimethylaminobenzaldehyde in 87·5ml. of acetic acid and 12·5ml. of conc. HCl. Dilute *p*-dimethylaminobenzaldehyde

Table 1. *Hyaluronidase extracted from submandibular glands of several species*

Two extracts, each from a separate gland, were each assayed at two pH values for hyaluronidase activity.

Animal	Activity (mμmoles of *N*-acetylglucosamine/min./g. of tissue)	
	pH 3·8	pH 5·3
Cat	1092, 1200	72, 89
Dog	935, 1080	31, 60
Guinea pig	101, 108	0, 10
Rat	55, 60	22, 30
Pig	12, 24	24, 36
Ox	15, 17	30, 34

solution was prepared by diluting the stock solution tenfold with acetic acid.

Assay procedure. Hyaluronidase activity was assayed by a modification of the method of Bonner & Cantey (1966). The incubation mixture (0·3ml.) consisted of 0·1ml. of enzyme extract, 400μg. of sodium hyaluronate, 0·1ml. of buffer, pH 3·8, obtained by mixing 0·3M-Na_2HPO_4 and 0·3M-citric acid, and 0·1ml. of 0·45M-NaCl containing gelatin (1mg./ml.). Incubation was carried out at 37° for 5min. The incubated mixture was boiled with 0·06ml. of 0·8M-$K_2B_4O_7$ for 3min.; after cooling, 2ml. of dilute *p*-dimethylaminobenzaldehyde solution was added and the colour was developed for 12min. at 39°. The tubes were then kept in ice, and if turbidity developed the samples were centrifuged at $400g_{av.}$ for 5min. at 4°; spectrophotometric measurements were then made at 585mμ. One unit of hyaluronidase activity was taken as the release of 1μmole of *N*-acetylglucosamine end group/min. as measured spectrophotometrically with *N*-acetylglucosamine as standard.

Hyaluronidase activity was also assayed by the reductimetric method of Park & Johnson (1949) and the spectrophotometric titration procedure of Bowness (1965).

β-*N*-Acetylhexosaminidase was assayed by the method of Levvy & Conchie (1966*b*) except that one-tenth volumes of all reagents were used.

Acid phosphatase was assayed by the method of Kind & King (1954) with phenyl disodium orthophosphate as substrate. Alkaline phosphatase was assayed by the method of Babson (1965) with the kit available from the General Diagnostics Division of Warner–Chilcott Laboratories (Morris Plains, N.J., U.S.A.).

Cytochrome *c* oxidase was assayed by the method of Cooperstein & Lazarow (1951).

β-Glucuronidase was assayed by the method of Levvy & Conchie (1966*a*) with phenolphthalein glucuronide as substrate.

Protein was assayed by the method of Lowry, Rosebrough, Farr & Randall (1951) with bovine serum albumin powder as standard.

Tissue extraction for total activity. Freshly obtained submandibular gland was chopped into small pieces with a pair of scissors and homogenized in about 4vol. of 0·1M-sodium acetate buffer, pH 5·0, in a VirTis homogenizer at 20000rev./min. for three 15sec. periods at 4°, with a pause of 2min. between operations. The homogenate was centrifuged at $10000g_{av.}$ for 10min. and the supernatant was removed for determination of total hyaluronidase activity (Table 1).

Extraction and separation of subcellular components. A freshly obtained submandibular gland from a 3–10kg. puppy, previously killed by intravenous Nembutal administration at a dose of 50mg./kg. body wt., was stripped of its adhering connective tissues and washed well in 0·15M-NaCl. The gland was then cut into tiny pieces with two scalpel blades on a plastic board. The pieces of gland were divided into three separate portions, each of which was homogenized in a Potter–Elvehjem homogenizer with 10vol. of 0·25M-sucrose. Three up-and-down strokes were used with the Teflon pestle (0·13mm. clearance) rotating at 1000rev./min. during the homogenization at 4°.

The homogenate was centrifuged at $400g_{av.}$ for 5min. to sediment the nuclei and cell debris. The supernatant was retained and the sedimented pellet was homogenized again with 4vol. of 0·25M-sucrose and centrifuged again at $400g_{av.}$ for 5min. This supernatant was pooled with the supernatant of the first centrifugation; the pooled material is referred to as the cytoplasmic fraction E. The sedimented pellet was the nuclear fraction N. Extract E was subsequently fractionated into a heavy-mitochondrial fraction M, a light-mitochondrial fraction L, a microsomal fraction P and a supernatant fraction S by the differential-centrifugation technique of de Duve, Pressman, Gianetto, Wattiaux & Appelmans (1955). Fraction N was resuspended in 4vol. of 0·25M-sucrose and filtered through gauze to remove cellular debris.

Portions of each of the sedimentable fractions N, M, L and P were resuspended in water at 4° and mixed well; 1% Triton X-100 was added to the resuspended fractions and to the extract E such that the final concentration of Triton X-100 was 0·1% in each case. The fractions were left to stand for 15–30min., and centrifuged at $1200g_{av.}$ for 10min. to sediment pieces of insoluble mucin. The supernatant of the various centrifuged fractions was then assayed for hyaluronidase, acid phosphatase and β-*N*-acetylhexosaminidase. Other portions of fractions N, M, L, P, S and E without Triton X-100 were individually ultrasonically treated for three 15sec. periods at 20kcyc./sec. in 5mM-sodium phosphate buffer, pH 7·4, by using an ultrasonic probe (Biosonik) at 4°. Each of the ultrasonically treated fractions was then assayed for cytochrome *c* oxidase activity. The ultrasonic treatment was useful in dispersing the pieces of insoluble mucin present in each of the sedimentable fractions. Ultrasonic treatment for a further two 15sec. periods was not found to increase or decrease the cytochrome *c* oxidase activity, thus indicating that optimum activity was obtained by this procedure. Alkaline phosphatase was assayed in supernatants obtained from fractions N, M, L and P after resuspension in water at 4°, standing for 1hr. at 4° and centrifuging at $1200g_{av.}$ for 10min.

Treatment with 0·1% Triton X-100. Test and control mixtures were prepared each containing 100μl. of resuspended fraction L in 0·15M-NaCl solution and 100μl. of hyaluronate solution in phosphate–citric acid buffer, pH 3·8. Then 30μl. of 1% Triton X-100 was added to each test mixture and the volume made up to 300μl. with water. The tubes were then incubated at 37° and assayed

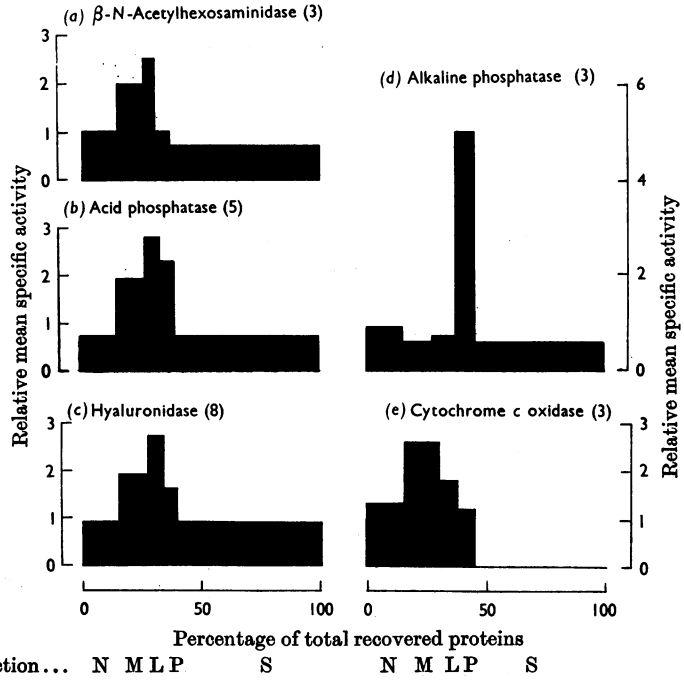

Fig. 1. Distribution patterns of enzymes in subcellular fractions of submandibular-gland homogenates: (*a*) β-*N*-acetylhexosaminidase; (*b*) acid phosphatase; (*c*) hyaluronidase; (*d*) alkaline phosphatase; (*e*) cytochrome *c* oxidase. The results are presented here in the manner used by de Duve *et al.* (1955), the mean values for each fraction being used. The ordinate shows the mean relative specific activity of the fractions. On the abscissa, the fractions are represented by their relative protein content in the order in which they are isolated: from left to right, N (nuclear), M (heavy-mitochondrial), L (light-mitochondrial), P (microsomal) and S (supernatant). Numbers in parentheses refer to numbers of experiments.

for hyaluronidase activity in pairs (test and control) at different times (Fig. 2).

Effects of pH. The effects of pH on the release of *N*-acetylglucosamine end groups from hyaluronate by submandibular-gland hyaluronidase and sheep testicular hyaluronidase were tested in phosphate–citric acid buffers with and without gelatin and in acetate buffers without gelatin (Figs. 3*a* and 3*b*). The maximum time for colour development with *p*-dimethylaminobenzaldehyde in the acetate buffer was 20min. at 37° and 12·5min. in the phosphate–citric acid buffer.

The effect of pH on hyaluronidase activity was also studied by the spectrophotometric titration procedure of Bowness (1965) and by the reductimetric assay (Park & Johnson, 1949) with chondroitin 4-sulphate as substrate in both assays (Fig. 3*c*).

Collection of submandibular-gland saliva. Dogs were anaesthetized by intravenous administration of Nembutal (10–12mg./kg. body wt.). The oral cavity and especially the area around the orifice of the submandibular duct was carefully swabbed with 70% ethanol before insertion of a sterile plastic tube into the submandibular duct. Pilocarpine hydrochloride (1mg.) prepared in 0·15M-NaCl was then given intravenously to each anaesthetized dog. The submandibular-gland saliva was collected in fractions and analysed for hyaluronidase activity, acid phosphatase activity and protein.

RESULTS

Subcellular distribution and latency. The distribution of a number of enzymes in various subcellular fractions of submandibular glands is shown in Fig. 1 and Table 2. Other investigations of these subcellular particles were also carried out. The activity of β-glucuronidase as assayed by the method of Levvy & Conchie (1966*a*) was found to be very low in both the sedimentable fractions and the supernatant fraction.

In a number of fractionations portions of resuspended fractions N, M, L, P and E were treated with 0·1% Triton X-100; this was found to release the sedimentable hyaluronidase, acid phosphatase and β-*N*-acetylhexosaminidase from the particulate fractions. The supernatant fraction S from subcellular fractionation and the supernatant obtained from the extraction for total activity (see the Experimental section) were treated with 0·1% Triton X-100 and the activity was compared with untreated controls. It was found that the Triton X-100 did not inhibit or activate the enzymes studied in the presence of this detergent.

Table. 2. *Subcellular distribution of enzymes*

Results are given as means ± S.D. and show the activity in each fraction as a percentage of the total in fractions E+N. The explanation of the letters and the preparation of the fractions are given in the Experimental section.

		Activity in fraction (% of total in fractions E+N)					
			E				
Enzyme	No. of expts.	N	M	L	P	S	Recovery (%)
Hyaluronidase	8	13·9±2·36	22·5±6·47	15·2±5·39	8·2±5·42	55·4±3·46	115·2±17·54
Acid phosphatase	5	8·8±2·83	14·6±3·36	15·8±3·56	11·7±3·29	46·0±3·81	100·7± 5·16
β-*N*-Acetylhexosaminidase	3	14·3±0·76	20·6±3·00	14·0±2·08	5·1±0·85	42·2±2·47	96·2± 6·33
Alkaline phosphatase	3	16·8±6·87	9·5±1·25	5·1±1·48	52·4±13·9	33·1±7·18	116·9±21·53
Cytochrome *c* oxidase	3	26·2±8·73	49·5±6·55	15·1±5·14	12·5±3·42	2·3±3·91	105·6± 3·81
Protein	8	15·6±5·24	10·9±2·01	6·1±1·59	6·6±2·62	62·7±4·70	101·9± 4·33

After resuspension of fractions N, M, L and P and treatment with Triton X-100 it was found necessary to centrifuge to remove mucin, which otherwise rendered pipetting difficult in some cases. The sedimented mucin was assayed for enzyme activity and was found to contain 0–4·5% of the total activity in each fraction for hyaluronidase, acid phosphatase and β-*N*-acetylhexosaminidase, and almost no alkaline phosphatase activity.

It is possible that some of the activity observed during incubation at pH 3·8 was due to release of hyaluronidase after the break-up of the particles. However, a considerable and consistent increase in activity was found with the light-mitochondrial fraction in the presence of Triton X-100 (Fig. 2).

Effects of pH. The pH–activity curve (Fig. 3*b*) indicated that the pH optimum of both submandibular-gland and testicular hyaluronidase varied with the buffer used. This variation was also observed by Bollet *et al.* (1963) for renal and testicular hyaluronidases.

Submandibular-gland saliva. The intravenous injection of 1 mg. of pilocarpine hydrochloride caused a rapid flow of saliva, beginning about 30 sec. after injection, with maximum rate of flow occurring during the next 1·5 min. (Fig. 4). The first peak of hyaluronidase, acid phosphatase and protein was found in the first 1 ml. fraction of submandibular saliva collected. The concentrations of hyaluronidase, acid phosphatase and protein in the saliva decreased for 10 min. from the time collection was begun, but increased again thereafter. An initial fall in protein concentration, presumably due to the gland secreting fluid faster than protein, followed by a gradual rise in protein concentration concurrent with a decrease in saliva flow rate, was also observed by Dawes (1967) and Shannon (1967).

In another experiment, two 1 mg. doses of pilocarpine hydrochloride were used with the second dose administered 46 min. after the first dose, when the effect of the first dose was wearing off. Saliva collected after both doses was assayed for hyaluronidase and acid phosphatase activity in the presence and absence of 0·1% Triton X-100; no increase in activity was observed in the presence of 0·1% Trixon X-100, thus indicating that the two acid hydrolases were secreted in the free form.

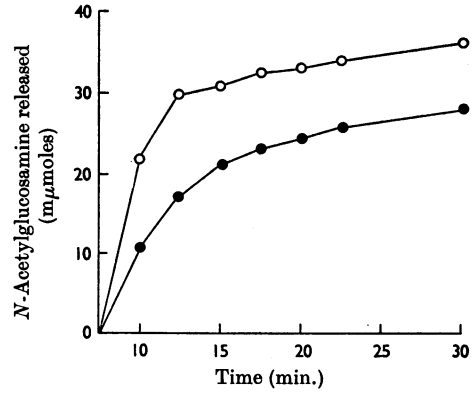

Fig. 2. Effect of Triton X-100 on hyaluronidase in fraction L. For experimental details see the Experimental section. ●, No Triton X-100; ○, 0·1% Triton X-100.

After collection of pilocarpine-stimulated submandibular saliva the submandibular gland was removed, homogenized and fractionated into its subcellular components N, M, L, P and S. A comparison was made with a subcellular fractionation simultaneously carried out on a gland obtained from an untreated control animal of the same sex and from the same litter. No difference between the pilocarpine-stimulated gland and its control was observed in the subcellular distribution of enzymes. It was calculated that the total hyaluronidase activity found in the pilocarpine-stimulated gland was 6·2% less than that in the control gland, and the total activity found in the

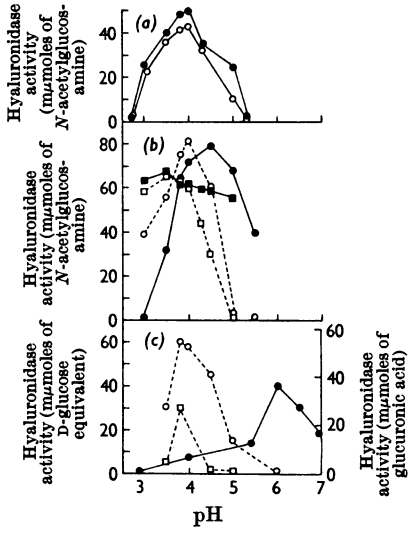

Fig. 3. (*a*) pH–activity curves of submandibular-gland hyaluronidase (○) and submandibular saliva (●) obtained by using the *N*-acetylglucosamine assay; the mixture was incubated for 15min. at 37° in phosphate–citric acid buffers in the presence of gelatin. (*b*) pH–activity curves of submandibular-gland hyaluronidase obtained by using the *N*-acetylglucosamine assay in phosphate–citric acid buffers (○) or in acetate buffers (□), and of sheep testicular hyaluronidase in phosphate–citric acid buffer (●) or in acetate buffer (■); the mixture was incubated for 5min. at 37° in the absence of gelatin. (*c*) pH–activity curve of submandibular-gland hyaluronidase obtained by the spectrophotometric titration procedure (mμmoles of glucuronic acid) (○) and the reductimetric procedure (mμmoles of D-glucose equivalent), (□), and of sheep testicular hyaluronidase also obtained by the spectrophotometric titration procedure (mμmoles of glucuronic acid) (●). The mixture was incubated for 120min. at 37° in the spectrophotometric titration procedure and 30min. at 37° in the reductimetric assay.

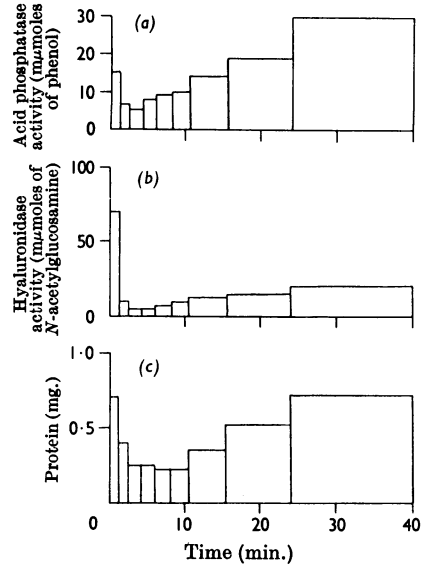

Fig. 4. Enzyme and protein profile in the cannulated flow of submandibular saliva: (*a*) acid phosphatase; (*b*) hyaluronidase; (*c*) protein. Pilocarpine hydrochloride (1mg.) was administered intravenously to stimulate the flow of saliva. The ordinate scales show amounts of activity or protein in 1ml. of saliva and each rectangular section on the abscissa shows the time (min.) required to collect 1ml. of saliva.

submandibular saliva was 5·2% of the total found in the pilocarpine-stimulated gland plus the saliva.

Absence of bacterial hyaluronidase and oxidative–reductive depolymerizing reaction. When samples of unfractionated submandibular-gland homogenates, partially purified submandibular-gland hyaluronidase and unfractionated submandibular saliva were incubated in soya-bean trypticase media, thioglycollate media, blood–agar plates and nutrient–agar plates for 12–48hr. at 37°, no visible turbidity or bacterial growth was observed in the liquid media and agar plates.

Assays for bacterial hyaluronidase by the method of Greiling (1963) were carried out simultaneously with the assays of mammalian hyaluronidase in partially purified submandibular-gland extracts and unfractionated saliva. Though *N*-acetylglucosamine assays showed a high mammalian hyaluronidase activity, no increase in extinction at 230mμ was recorded, thus indicating the absence of bacterial hyaluronidase. The degradation of hyaluronate by bacterial hyaluronidase yields a disaccharide characterized as a 4,5-unsaturated uronide having an absorption maximum near 230mμ (Greiling, 1963).

The oxidative–reductive depolymerizing reaction described by Pigman, Rizvi & Holley (1961) proceeds in the presence of reducing agents such as ascorbic acid and cysteine or Fe^{2+} ion and Cu^{+} ion. In our experiments the incubation solutions for assay of hyaluronidase activity did not contain any of these agents. Further, no oxygen consumption was recorded when hyaluronate was extensively hydrolysed in a Warburg manometric flask by a partially purified submandibular-gland hyaluronidase preparation. Thus it is unlikely that an oxidative–reductive depolymerizing reaction is responsible for the hyaluronidase activity measured in our system.

DISCUSSION

Hyaluronidase activity (Table 1) was found in the submandibular glands of dogs, cats, guinea pigs, rats, pigs and oxen, but only the first two contained

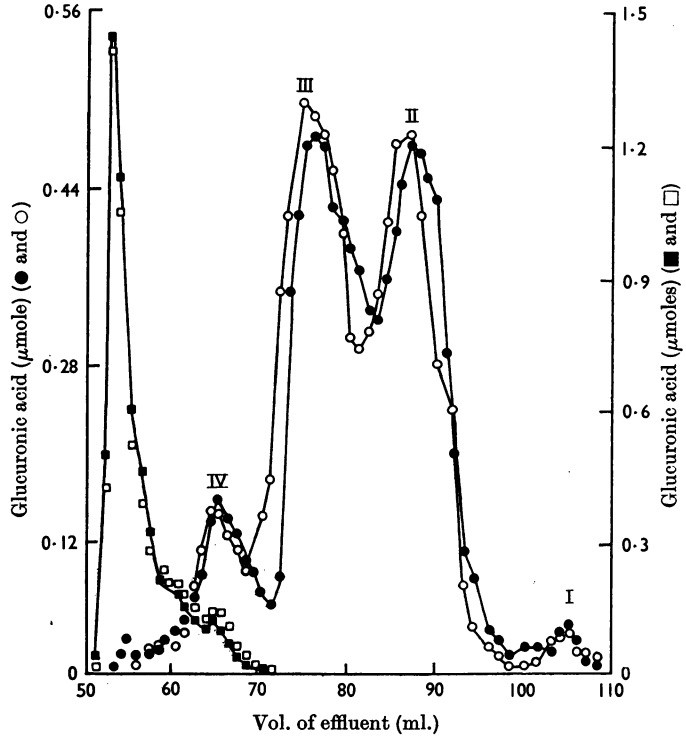

Fig. 5. Gel filtration of submandibular-gland hyaluronidase digest of sodium hyaluronate (10mg.) in 0·1M-NaCl at 35° on a Sephadex G-25 column (2·5cm. diam. × 30cm.). Incubation for 2hr. (□) or 24hr. (○), with 1mg. of non-sedimentable hyaluronidase (sp. activity 0·34unit/mg. of estimated protein); incubation for 2hr. (■) or 24hr. (●), with 1·2mg. of sedimentable hyaluronidase (from fraction MLP; sp. activity 0·28unit/mg. of estimated protein). The hyaluronate preparations were purified as described in the following paper (Tan & Bowness, 1968).

activities comparable with those of hyaluronidase in ox and sheep testes. The range of hyaluronidase activity found per g. of bovine testicular tissue was 750–1000 units (Freeman, Anderson, Oberg & Dorfman, 1949). This unit was stated to be similar to the turbidity reducing unit of Rapport, Meyer & Linker (1950), which was found to be equivalent to about 1·6 μg. of reducing sugar end groups. This being so, the range of activity found per g. of testicular tissue was 230–320mμmoles of *N*-acetylglucosamine end groups/min. Other workers have reported activities two to three times as high for ox testis and very much higher for rabbit testis, though no activity was detected in the testes of dogs and donkeys and relatively low activities were found in other tissues of dogs, oxen, sheep and rabbits (Gibian, 1959). Activity resembling hyaluronidase was found in the saliva and unnamed salivary glands of the dog, but none was found in human and horse saliva (Gibian, 1959). Gibian (1966), however, pointed out that the possibility of degradation by bacterial hyaluronidase or of the oxidative–reductive depolymerizing reaction was not eliminated in most of this work. In the present work no hyaluronidase activity was detected by *N*-acetylhexosamine assay in human parotid or submandibular saliva, and the activity in the dog parotid gland was found to be much lower than in the submandibular gland.

The absence of bacterial growth and of absorption at 230mμ after incubation with sodium hyaluronate indicated the absence of bacterial hyaluronidase from submandibular-gland extracts. The absence of the necessary chemical reagents and of oxygen uptake indicated that the oxidative–reductive depolymerizing reaction did not occur. That the enzyme is in fact a hyaluronidase is shown by the findings on the products. In Table 3 it is shown that the molar ratio of *N*-acetylglucosamine to glucuronate for the four main products of hyaluronate digestion (Fig. 5) increased with the effluent volume at which they were eluted. The ratios for peaks I, II and III agreed closely with

Table 3. *Characterization of oligosaccharide products from a Sephadex G-25 column (Fig. 5)*

The ratios given apply to the single effluent fraction with the highest uronic acid content from each of the numbered peaks of Fig. 5. Glucuronate was determined by the method of Bowness (1957) and *N*-acetylglucosamine end groups were determined by the method of Bonner & Cantey (1966). V_e/V_0 ratios for testicular hyaluronidase were calculated from the disaccharide (I), tetrasaccharide (II), hexasaccharide (III) and octasaccharide (IV) peaks in Fig. 1 of Flodin, Gregory & Rodén (1964). The hyaluronidase preparations were purified as described in the following paper (Tan & Bowness, 1968).

Enzyme	Sample	*N*-Acetylglucosamine end groups (μmoles/μmole of glucuronate)	V_e/V_0
Non-sedimentable hyaluronidase (fraction S_3)	I	1·20	2·18
	II	0·62	1·74
	III	0·42	1·50
	IV	0·18	1·31
Sedimentable hyaluronidase (fraction MLP_3)	I	1·01	2·18
	II	0·60	1·75
	III	0·41	1·52
	IV	0·17	1·33
Testicular hyaluronidase	(I)		2·05
	(II)		1·76
	(III)		1·52
	(IV)		1·36

those found by Aronson *et al.* (1965) for disaccharide, tetrasaccharide and hexasaccharide obtained from hyaluronate by paper chromatography after the action of both liver lysosomal and testicular hyaluronidase. The V_e/V_0 ratios on Sephadex G-25 agreed closely with those found for products of digestion of hyaluronate by testicular hyaluronidase (Flodin *et al.* 1964), thus confirming that peak I was the disaccharide, peak II the tetrasaccharide, peak III the hexasaccharide and peak IV the octasaccharide. Though 4–5% of the total glucosamine in the hyaluronate was liberated in the form of end groups within a 2hr. incubation at 35°, no oligosaccharides of the type found after 24hr. were found at the earlier time. This shows that the enzyme from submandibular glands of the dog is an endohyaluronidase whose product formation at 24hr. is indistinguishable from that of testicular and liver lysosomal hyaluronidases.

Both test and control mixtures were incubated at pH 3·8. Gianetto & de Duve (1955) and Appelmans & de Duve (1955) stressed the fact that lysosomes are broken down under acid conditions. However, the experiments with Triton X-100, which is commonly used to expose lysosomal enzymes (Vaes, 1965), showed that there was considerable latent hyaluronidase activity in the light-mitochondrial fraction (fraction L) from submandibular-gland homogenates, which is released by Triton X-100 (Fig. 2). In the presence of this detergent nearly all the hyaluronidase activity of the subcellular particles in fraction E became non-sedimentable. This indicated that the enzyme was released from particulate material.

Results of the centrifugal fractionation showed that the distribution pattern of submandibular-gland hyaluronidase was similar to that of the other two acid hydrolases studied. In the sedimentable fractions the percentage of hyaluronidase and the two acid hydrolases was highest in the heavy-mitochondrial and light-mitochondrial fractions. This result, together with the finding for hyaluronidase and for the other two acid hydrolases studied that the highest specific activity was in the lysosomal fraction, supports the concept that part of the submandibular-gland hyaluronidase is associated with the lysosomal fraction. However, only 15% of the hyaluronidase of the submandibular-gland homogenate was found in fraction L, as opposed to 28% in fraction L in bone (Vaes, 1967).

The distribution of cytochrome *c* oxidase among the subcellular fractions obtained from submandibular gland was similar to that found for liver (de Duve *et al.* 1955) and the distribution of alkaline phosphatase was similar to that found for bone (Vaes & Jacques, 1965*b*). These results suggest that the techniques originally developed for liver and bone are also applicable to submandibular gland. However, the percentage distribution of hyaluronidase and the two other acid hydrolases studied differed from those described for bone (Vaes & Jacques, 1965*b*; Vaes, 1967) and liver (de Duve *et al.* 1955), mainly in the higher percentage of hyaluronidase (55%), acid phosphatase (42%) and β-*N*-acetylhexosaminidase (46%) found in the supernatant fraction S of submandibular-gland homogenate. For bone only 25% of the hyaluronidase was found in fraction S (Vaes, 1967), but a higher content has been found in other tissues: in thyroid Herveg, Beckers & De Visscher (1966) found that about 50% of the acid phosphatase was non-sedimentable; in lymphoid tissue Bowers, Finkenstaedt & de Duve (1967) found that 51·8% of the acid phosphatase was non-sedimentable; in mixed parotid and submandibular glands of guinea pigs Cirina (1963) found that 48% of the acid phosphatase was in fraction S. Two explanations for the findings are possible: (i) lysosomes are more fragile than mitochondria and microsomes (the difference between the findings for liver and those for submandibular gland may then be due to the greater stress imposed on the subcellular particles during homogenization of the latter tissue); (ii) a high activity of acid hydrolases in the supernatant fraction pre-exists before homogenization of the submandibular gland.

With hyaluronidase and acid phosphatase a further difference between the submandibular-gland enzymes and lysosomal enzymes in other tissues was found. Both enzymes occur in a non-particulate form in a secretion of the gland, namely in pilocarpine-stimulated saliva collected from the submandibular duct (Fig. 4). According to Straus (1967), lysosomal enzymes are confined to intracellular fractions, as opposed to zymogen granules, which are concerned with extracellular secretion. de Duve (1966), in a review, put forward the hypothesis that lysosomes and zymogen granules have a distant but common evolutionary origin in the infolding of a membrane secreting exoenzymes. It is tempting to speculate that there may be a spectrum of digestive particles ranging from the large zymogen granules producing enzymes for extracellular use, such as those found with amylase in the parotid gland by Schramm (1964), to the smaller lysosomes, producing enzymes for purely intracellular use, as exemplified by the liver lysosomes. However, there is no evidence that the hyaluronidase and acid phosphatase found in saliva do in fact have an extracellular use, nor does this hypothesis explain why so high a proportion of the acid hydrolases of the submandibular gland are found in the supernatant fraction of homogenates, since very little salivary amylase is found in this fraction.

All lysosomal enzymes investigated so far have an acid pH optimum (de Duve, 1963); this was also observed in the present work with the submandibular-gland hyaluronidase. The pH optimum and pH–activity curves of dog submandibular-gland hyaluronidase resemble those found for rat liver lysosomal mucopolysaccharidase (Hutterer, 1966), rat liver lysosomal hyaluronidase (Aronson & Davidson, 1967), rat bone lysosomal hyaluronidase (Vaes & Jacques, 1965*a*), human gingival hyaluronidase (Goggins *et al.* 1968) and human serum hyaluronidase (de Salegui & Pigman, 1967; Bowness & Tan, 1968) more than those found for testicular hyaluronidase, which has a broader pH optimum by hexosamine end-group assay and a near-neutral pH optimum (6·0) by the spectrophotometric titration procedure (Fig. 3) and by viscosimetric assay (Cobbin & Dicker, 1962; Gibian, 1959).

The presence of glucuronic acid in canine submandibular-gland mucin was reported by Draus (1963), and a sulphosialopolysaccharide–peptide from canine submandibular gland containing hexosamine but no uronic acid was described by Bignardi, Aureli, Balduini & Castellani (1964). Whether or not these polysaccharides are degraded by canine submandibular-gland hyaluronidase is not known, and at present the physiological role of the enzyme is not clear.

This work was supported by a grant from the Medical Research Council of Canada. We are grateful to Dr C. Dawes for assistance in the collection of saliva, to Dr A. C. van der Muelen for the material from ox and pig, and to Dr Lee Kay Hoon of the University of Singapore for the hyaluronate preparation.

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