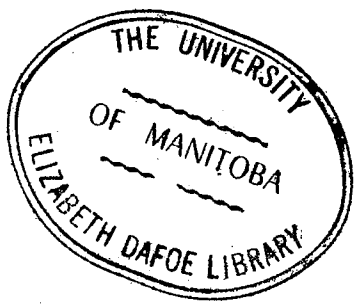
CANINE SUBMANDIBULAR-GLAND HYALURONIDASE

by

Tan Yin-Hwee B.Sc. Hons., M.Sc.

Submitted in partial fulfilment of the
requirements for the degree of Ph.D.

Faculty of Graduate Studies and Research,
The University of Manitoba,
Winnipeg, Canada.



To my parents

and to Celine

ACKNOWLEDGEMENTS

The author is indebted to many persons for their help during the course of this work, especially to:

Dr. J. M. Bowness, for his guidance, patience and encouragement.

Dr. M. C. Blanchaer, for his helpful suggestions and encouragement.

Dr. K. H. Lee and Mr. C. M. Chia, for contribution of hyaluronate preparations.

Dr. F. C. Stevens, for assistance in amino-acid analysis.

Dr. C. Dawes, for advice in the collection of saliva.

Dr. N. L. Stephens, Dr. E. E. Fariday, Dr. B. Sasyniuk, Mr. E. Kroeger and Mr. U. Kromer, for contribution of canine submandibular glands.

Mr. A. H. Tarr, for technical assistance during the last part of this work.

Miss M. Scanlan, for her careful typing of this thesis.

The author also wishes to thank The University of Manitoba for a Graduate Fellowship (1967/1968); the Medical Research Council of Canada for a Graduate Studentship (1968/1970) and for supporting this work.

ABSTRACT

Submandibular glands from four species of mammal have been shown to contain a hyaluronidase active at acid pH; glands from the dog and the cat had a much higher content of this enzyme than the guinea pig and the rat. Product formation from hyaluronate after 24 hr. incubation was almost the same as with testicular hyaluronidase, indicating that the enzyme is an endo-poly-β-hexosaminidase. When submandibular gland homogenates were fractionated by the scheme developed for liver by de Duve et al. (64), all of the enzymes assayed, except cytochrome c oxidase, were found to occur partly in the soluble fraction and partly in the particulate fractions. Of the particulate 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. Release of the enzyme activity from the sedimentable fractions occurred in 0.1% Triton X-100 or after high speed homogenization.

A comparison of some of the properties of the hyaluronidase from the sedimentable particulate and non-particulate portions of homogenates indicated that there was little difference between the two and that they both resemble the acid-active lysosomal hyaluronidase isolated by Aronson and Davidson from rat liver (83). The relationship between these enzymes and testicular hyaluronidase is discussed in the

review of literature and in Section V.5 and V.8.

Stimulation of dogs by pilocarpine was found to decrease the hyaluronidase content of the submandibular-gland by 6% and to cause the occurrence of a corresponding amount of hyaluronidase in the submandibular saliva.

Methods for the purification of dog submandibular-gland hyaluronidase from sedimentable particulate and non-particulate portions of a homogenate and from the whole homogenate are presented. Highly purified hyaluronidase of specific activity of up to 2.7 μmole N-acetylGlu-NH_2 released/min./mg. protein was obtained. This compares favourably with the figure of 1.29 N-acetylGlu-NH_2 released/min./mg. protein which was reported for the hyaluronidase purified from rat liver lysosomes (36).

The purified canine submandibular-gland hyaluronidase was found to be homogeneous by electrophoresis at pH 4.5 on polyacrylamide gels, by chromatography on CM-cellulose columns at pH 5.0 and by gel filtration on Sephadex G-100 in the absence of salt.

The Michaelis-Menten constant (Km), substrate specificity and pH-activity curves for the enzyme were studied at the various stages of enzyme fractionation. Only the pH-activity curves were found to change during the course of enzyme fractionation. The latter change is discussed and may be concluded that it is due to a transition from one active form of the enzyme to another.

A comparison of the composition of the submandibular-

gland hyaluronidase and the bovine testicular hyaluronidase studied by Borders and Raftery (35) indicates some similarity between the two. However there are differences in the content of certain amino-acids. In addition the content of N-acetyl-glucosamine was lower than that found in bovine testicular hyaluronidase. This occurrence of amino-sugar was used by Borders and Raftery as evidence that testicular hyaluronidase is a glycoprotein.

Data on the presence of a material which binds hyaluronidase in submandibular-gland extract is also discussed in relation to the purification of the enzyme.

FIGURE INDEX

Fig. No.	Page No.
1	3
2	9
3	29
4	31
5	38
6	41
7	47
8	57
9	60
10	55
11	63
12	66
13	69
14	70
15	73
16	74
17	75
18	120 (Appendix)
19	78
20	80
21	121 (Appendix)
22a	81
22b	122 (Appendix)
23	86
24	87

FIGURES INDEX

Fig. No.	Page No.
25	93
26	99

TABLE INDEX

Table No.	Page No.
1	4
2	20
3	23
4	30
5	116 (Appendix)
6	45
7	59
8	68
9	117 (Appendix)
10	118 (Appendix)
11	119 (Appendix)
12	83
13	90
14	101
15	102

CONTENTS

	PAGE
Acknowledgements	iii
Abstract	iv
Figure Index	vii
Table Index	ix
Glossary	xiii
I. REVIEW OF LITERATURE	1
II. OBJECTIVES	8
III. MATERIALS	10
IV. GENERAL METHODS	14
1. Standard procedure for hyaluronidase	14
2. Other procedures for hyaluronidase	15
3. Other general assay procedures	15
4. Preparation of CM-cellulose and Sephadex gel for column chromatography	17
5. Analytical disc gel electrophoresis	18
V. IDENTIFICATION AND SUBCELLULAR DISTRIBUTION	21
1. Canine submandibular-gland as a source of hyaluronidase	21
2. Tissue extraction for total enzyme activity	24
3. Test for bacterial contamination and oxidative reductive depolymerizing reaction	25
4. Extraction and separation of subcellular components; treatment with Triton X-100	27
(a) Methods	27
(b) Results	32
(c) Discussion	33

	PAGE
5. Effects of pH	39
(a) Methods	39
(b) Results and Discussion	39
6. Collection of submandibular-gland secretions	42
(a) Methods	42
(b) Results and Discussion	42
7. Identification of products of enzyme action	46
(a) Methods	46
(b) Results and Discussion	46
8. General Discussion and Conclusions	49
VI. PURIFICATION AND PROPERTIES	53
1. Purification of sedimentable particulate and non-particulate submandibular-gland hyaluronidase	53
(a) Methods	53
(b) Results	58
2. Purification of hyaluronidase from whole canine submandibular-gland homogenate	64
(a) Methods	64
(b) Results	71
3. Binding of hyaluronidase to other material in submandibular-gland extracts	76
(a) Methods	76
(b) Results and Discussion	76
4. Some general enzyme properties	79
(a) Methods	79
(b) Results and Discussion	79

PAGE

5. Chromatographic comparison of canine submandibular-gland and bovine-testicular hyaluronidase 88

(a) Methods 88

(b) Results and Discussion 88

6. Composition of canine submandibular-gland hyaluronidase 91

(a) Methods 91

(b) Results and Discussion 91

7. Molecular weight of canine submandibular-gland hyaluronidase 94

(a) Methods 94

(b) Results 94

8. General Discussion and Conclusions 95

VII. CHANGES IN pH ACTIVITY CURVE OF PURIFIED ENZYME PREPARATIONS 100

(a) Methods 100

(b) Results 100

(c) Discussion 103

VIII. CONCLUSIONS 105

Bibliography 108

Appendix 115

GLOSSARY

CS-4	Chondroitin 4-sulphate
CS-6	Chondroitin 6-sulphate
HA	Hyaluronate
N-acetylGlu-NH_2	N-acetyl-glucosamine

I. REVIEW OF LITERATURE

Hyaluronidases are enzymes which catalyze the depolymerization of hyaluronate. Most of these enzymes also attack chondroitin and chondroitin sulphates but not dermatan sulphate. The rates of activity against hyaluronate are higher than against chondroitin sulphates (1, 2). No enzyme which catalyzed the depolymerization of chondroitin sulphates without attacking hyaluronate has been isolated (3). However, evidence for an enzyme which attacks chondroitin sulphate at a faster rate than hyaluronate has been described in crude bovine testicular preparations (4) and in Proteus Vulgaris N.C.T.C. 4636 (5).

Another enzyme known as chondrosulphatase removes sulphate groups from the N-acetylhexosamine units of chondroitin sulphate. This has been found in molluscs (6), moulds (7), bacteria (8, 9), lysosomes of hog kidney (10) and more recently in bovine aorta (11).

Several workers have suggested that there is an interdependence between the actions of chondroitinase and chondrosulphatase against chondroitin sulphate (12, 13, 14). Dodgson and Lloyd separated the chondrosulphatase of Proteus from the associated chondroitinase (14). The chondroitinase-free sulphatase had little or no activity towards the polymerized form of chondroitin 4-sulphate but was able to liberate sulphate groups from the sulphated oligosaccharides prepared by exhaustive digestion of the substrate with

testicular hyaluronidase. It was therefore suggested that chondroitinase action must precede sulphatase action against chondroitin sulphate. Further evidence in support of such a hypothesis comes from the work of Martinez, Wolfe and Nakada (15), and Linker (16).

Although turnover of hyaluronate and chondroitin sulphates has been demonstrated in a number of tissues (17, 18, 19), the only well characterized mammalian enzyme known to degrade these substances in vitro is the hyaluronidase which has been extracted from the testis.

During the in vitro hydrolysis catalyzed by the testicular enzyme the viscosity of hyaluronate solutions decreases very rapidly, especially at the beginning of the reaction (20). The ability of the high molecular weight substrate such as hyaluronate or chondroitin sulphate to form a mucin clot with acidic albumin solution also disappears early; at a later stage in the reaction the release of reducing groups or N-acetylhexosamine end groups can be followed by micromethods of analysis (21)†. As depolymerization of the substrate progresses the turbidity resulting from the addition of acid albumin disappears and only when the reducing values have increased noticeably is it possible to identify the degradation products chromatographically (22). The metachromasia of chondroitin sulphate was also found to decrease when chondroitin sulphate was degraded by the enzyme. It is possible to measure hyaluronidase activity by following the reduction of metachromasia spectrophotometrically (23).

† Whether these two stages correspond with two types of enzyme or two types of enzyme action pattern is discussed on p. 40.

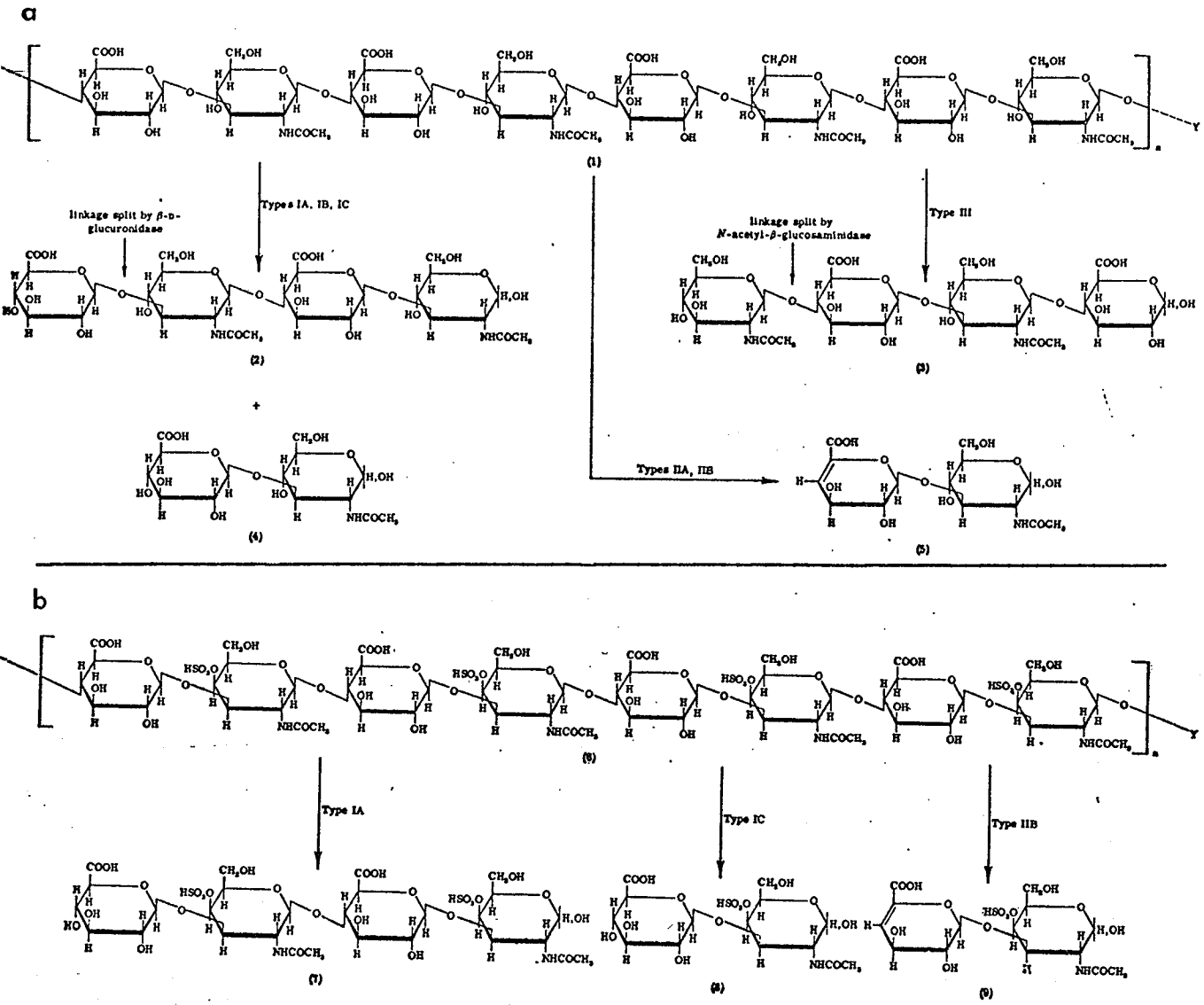


Fig. 1* Action of the three types of hyaluronidase on (a) HA (b) CS-4.

* H. Gibian (1966). In 'The Amino Sugars,' vol. 2B, p. 190-191, Ed. by Balazs, E. A. and Jeanloz, R. W., New York and London : Academic Press Inc.

TABLE 1*

VARIOUS TYPES OF HYALURONIDASES AND THEIR SOURCES

Type	Source	Substrate	Mechanism
IA	Sperm (Mammalian testis)	Hyaluronic acid Chondroitin Chondroitin 4-sulphate Chondroitin 6-sulphate	Endo-poly-β-hexosaminidase with transhexosaminylation
IB	Venom of snakes, spiders scorpions, bees	Hyaluronic acid Chondroitin	Endo-poly-β-hexosaminidase
IC	Proteus vulgaris (Strain 4636NCTC) Clostridium perfringens	Hyaluronic acid Chondroitin sulphate	Poly-β-hexosaminidase
IIA	Micrococcus pyogenes, var. aureus and var. albus Streptococcus mitis Diplococcus pneumoniae Clostridium perfringens Clostridium septicum Clostridium novyi Adapted strains of Escherichia coli	Hyaluronic acid Chondroitin	Endo-poly-β-hexosaminidase with water elimination and formation of $\Delta_{4,5}$ uronides
IIB	Adapted strains of Flavobacterium heparinum and Proteus vulgaris	Hyaluronic acid Chondroitin Chondroitin 4-sulphate Chondroitin 6-sulphate	-
III	Leech	Hyaluronic acid	Endo-poly-β-glucuronidase

* H. Gibian in "The Amino Sugars" vol.IIB eds. E.H. Balazs and R.W. Jeanloz. Academic press, New York and London, 1966 p.185

The methods of qualitative and quantitative determination of hyaluronidases are based on the properties of the substrates and products just described. Recent comprehensive reviews which critically evaluate the various methods of assay of hyaluronidase activity are available (24, 25).

Unfractionated extracts of rat liver lysosomes (26), human serum (27, 28), human gingiva (29), rabbit alveolar macrophages (30), rat kidney and spleen (31) and infant rat bone tissue (32) are known also to contain hyaluronidase activity. The activity extractable per gram of tissue from all these sources is low in comparison with the activity extractable from bovine and rabbit testes (33). 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. This suggests the name acid-active hyaluronidase for the non-testicular enzyme (27).

Hyaluronidases from the different tissues just described together with hyaluronidases from non-mammalian sources may be classified into three distinct types by their mode of action. A summary of the types of hyaluronidase is presented in Table 1 and their mode of action in Fig. 1.

The purification of testicular hyaluronidase has been carried out extensively in several laboratories. However, an objective evaluation of the claims of purity is difficult since assay methods and criteria of homogeneity used were different in different laboratories. Molecular weight values of testicular hyaluronidase determined in different laboratories

also differed considerably. Malgrem reported a figure of 11,000 for testicular hyaluronidase (34) compared to a figure of 61,000 given by Borders and Raftery (35). One possible explanation of such a discrepancy in molecular weight may be the presence of a carrier protein binding testicular hyaluronidase in the preparation of Borders and Raftery. Other possibilities are that the discrepancy is due to the different methods used in molecular weight determinations by the two groups of workers, that active sub-units are present in the higher molecular weight preparation, or that degradation to produce an active sub-fragment occurred in the Malgrem procedure.

The purification of liver lysosomal hyaluronidase was described by Aronson and Davidson (36). Their purified enzyme preparation represented a 1300 fold purification over the unfractionated lysosomal fraction and showed a major band with some contamination on the top of the gel during electrophoresis in polyacrylamide gel. The molecular weight of the purified enzyme, based on sedimentation equilibrium data was 89,000.

The physiological role of hyaluronidases is still uncertain. The testicular enzyme may be related in some as yet unknown manner to the spermiogenic function of testis (37). There is some indication that in certain mammals hyaluronidase acts to facilitate the contact between the male and female gametes by exerting direct liquifying action on the viscous gel which cements the follicule cells around

freshly ovulated eggs (38). The physiological function of the lysosomal hyaluronidases of bone (32) and liver (83) and the source of serum hyaluronidase (27) are also unknown. In liver, hyaluronidase has been shown to play a role in the degradation of injected acid mucopolysaccharides in the lysosomes but the physiological significance of such a potential is obscure (39, 40). Hepatic mucopolysaccharase was increased in reversible fibrosis and decreased in irreversible fibrosis (26).

II. OBJECTIVES

The first objective of this work was to survey the distribution of hyaluronidases in tissues to look for possible alternative sources to bovine or ovine testis. Such a survey might also throw light on the possible physiological functions of the enzyme and provide clues to the investigation of the mechanism by which cortisol increases the serum hyaluronidase activity in rats (41). With the finding of a high concentration of a hyaluronidase-like enzyme in dog submandibular glands it was decided to concentrate on the following points:-

(1) Identification of the enzyme.

(2) Distribution of the enzyme in the different subcellular fractions of the canine submandibular gland.

(3) To find if the enzyme is secreted into the submandibular-gland saliva.

(4) To develop a method for purification of the enzyme from canine submandibular-gland extracts.

(5) To study some properties of the enzyme.

(6) To compare purified canine submandibular-gland hyaluronidase with bovine-testicular hyaluronidase.

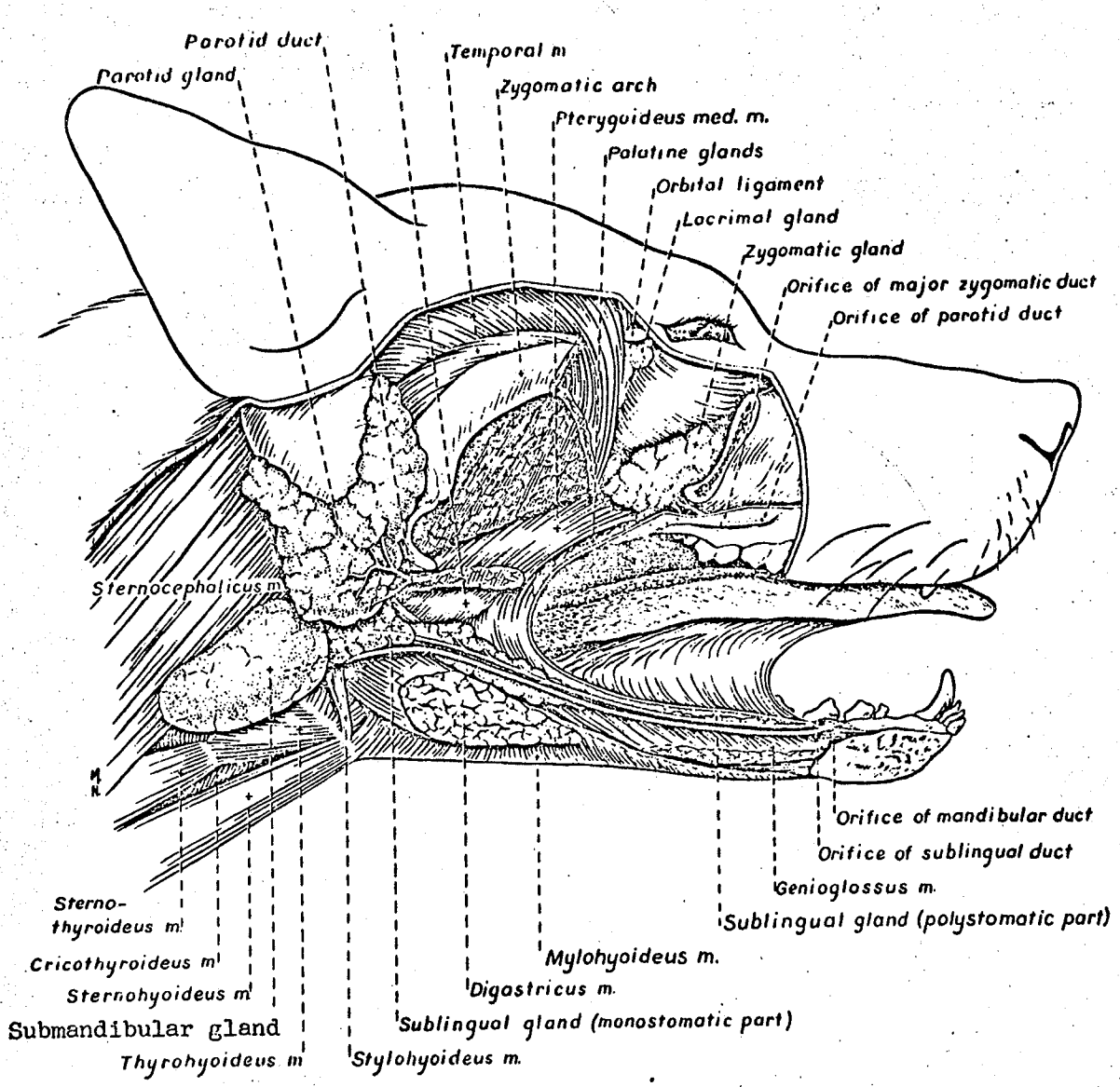


Fig. 2† Salivary glands of dog. (The right half of the mandible is removed.) † From the "Anatomy of the Dog" by M. E. Malcolm.

III. MATERIALS

Submandibular glands and other tissues were obtained from freshly killed dogs of unknown breed. These dogs were donated by staff members and fellow students of the Faculty of Medicine, The University of Manitoba, who routinely killed the animals by intravenous injection of Nembutal, and used some other organs in their experiments. Some of the glands were purchased from Pel Freez (Arkansas, U.S.A.).

The gland was obtained from the dog by making a lateral incision of about 3 to 4 inches at the side of the neck. The incision was pulled apart with the aid of hemostats. This exposed the musculature and connective tissues which partially covered the gland. The gland is easily recognized (Fig. 2). It is an ovoid body situated at the joining of the external and internal maxillary veins to form the external jugular vein. It measures about 3 cm. long, 2.5 cm. wide and 1.5 cm. thick and weighs about 3 to 10 g. in an adult dog. Part of the connective tissues and surrounding musculature covering the gland was dissected to expose the gland. The gland was then firmly held in one hand and easily removed by cutting at its base from the adjoining tissues. In most cases the dogs were bled before beginning this dissection and thus it was unnecessary to avoid damaging the surrounding vessels which otherwise could be extremely messy. Once the gland was removed from the dog, the connective tissues were peeled off using a pair of very fine scissors. The gland

was then washed in cold distilled water to remove bits of adhering tissues and hairs. The washed gland was carefully blotted dry on clean tissue papers and then stored at -20°.

Human white blood cells were prepared from human whole blood by the method of Bertino (42). Submandibular glands from ox and pig were obtained fresh from Winnipeg slaughter houses.

Sodium hyaluronate was obtained from human umbilical cords by method IVa of Jeanloz and Forchielli (43). The dried hyaluronate preparation was redissolved in water and dialyzed for 36 hr. against four changes of water (4 l.) at 4° and then lyophilized. Analysis of the preparation showed a glucuronic acid : hexosamine molar ratio 1:1.08, and the viscosity at 30° for a 1 mg./ml. solution in 0.1 M-sodium phosphate-citric acid buffer, pH 4.0, 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 and Pearce (44). Only the fraction precipitated at the ethanol concentration producing the first cloudiness was collected; 30 mg. of reprecipitated hyaluronate was obtained from 150 mg. of Koch-Light hyaluronate. The viscosity at 30° for a 1 mg./ml. solution in 0.1 M-sodium phosphate-citric acid buffer, pH 4.0, was 1.58 centipoise. The glucuronic acid : hexosamine molar ratio was 1:1.10. This preparation was 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 sulphate was prepared by the method of Kantor and Schubert (45) and dermatan sulphate was obtained by the method of Jeanloz (46). 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.

Hyaluronidase from sheep testis (minimum activity 300 i.u./mg.) was obtained from L. Light and Co. Ltd., Bovine testicular hyaluronidase (1000 T.R.U./mg.) was obtained from Worthington Biochemical Corporation, (Freehold, New Jersey).

Hydrolyzed gelatin (British Drug Houses Ltd., Poole, Dorset) was prepared in the freeze-dried state by the USP procedure described in the U.S. Pharmacopeia (47), 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 1 g. of p-dimethylaminobenzaldehyde in 87.5 ml. of acetic acid and 12.5 ml. of conc. HCl. Dilute p-dimethylaminobenzaldehyde solution was prepared by diluting the stock solution ten fold with acetic acid.

Enzyme-grade $(NH_4)_2SO_4$ was from Mann Research

Laboratories (New York, N.Y., U.S.A.). Sephadex G-25, G-100, G-150 and G-200 were obtained from Pharmacia (Uppsala, Sweden), CM-cellulose (new fibrous Whatman CM-23 and micro-granular Whatman CM-32) was from W. and R. Balston Ltd. (Maidstone, Kent) and Diaflo membranes of UM and XM series were obtained from the Amicon Corporation (Massachusetts, U.S.A.).

Protein standard markers used in the calibration of Sephadex G-150 column were obtained from Sigma and bovine serum albumin was obtained from Armour Pharmaceutical Co.

Triton X-100 was obtained from Hartman-Leddon Co. (Pennsylvania, U.S.A.) and potassium tetraborate was obtained from Sigma Chemical Co. (Missouri, U.S.A.).

4-Dimethylaminobenzaldehyde (p-) of analytical grade was obtained from The British Drug House (Poole, England). However, a number of batches of this reagent had a strong fishy smell suggesting the presence of the free benzaldehyde or the free amine. These batches were therefore not used in the experiments.

IV. GENERAL METHODS

1) Standard Procedure for Hyaluronidase

Hyaluronidase activity was assayed by a modification of the method of Bonner and Cantey (48). The incubation mixture (0.3 ml.) consisted of 0.1 ml. of enzyme extract, 400 µg of sodium hyaluronate in 0.1 ml. of buffer, pH 3.8, obtained by mixing 0.3 M-Na_2HPO_4 and 0.3 M-citric acid, and 0.1 ml. of 0.45 M-NaCl containing gelatin (1 mg./ml.). Incubation was carried out at 37° for 5 min. The incubated mixture was boiled with 0.06 ml. of 0.8 M-$K_2B_4O_7$ for 3 min.; after cooling, 2 ml. of dilute p-dimethylaminobenzaldehyde solution was added and the colour was developed for 12 min. at 39°. The tubes were then kept in ice, and if turbidity developed the samples were centrifuged at $400g_{av.}$ for 5 min. at 4°; spectrophotometric measurements were then made at 585 mµ. One unit of hyaluronidase activity was taken as the release of 1 µmole N-acetylglucosamine end group/min. as measured spectrophotometrically with N-acetyl-glucosamine as standard.

Several modifications of this standard procedure were used for specific purposes. These are described when and where they are used. Bowness and Hardings modification (41) of the standard procedure was used throughout in the latter part of this work. The modification lies in the volume of potassium tetraborate (0.30 ml. of 0.27 M). This modification was adopted because the colour developed was stable for a

slightly longer time than that found for the standard procedure.

2) Other Procedures for Hyaluronidase

Hyaluronidase activity was also assayed by the reductimetric method of Park and Johnson (49), the USP method (50) and the spectrophotometric titration procedure of Bowness (23). The reductimetric method of Park and Johnson was used to assay the activity of hyaluronidase preparations against different substrates (Table 12). The incubation procedure for the reductimetric method was similar to the one described for the N-acetyl-glucosamine assay except that the substrates were prepared in 0.1 M-sodium acetate buffer, pH 3.8; the acetate buffer was neutralized by 0.1 N-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.

3) Other General Assay Procedures

β-N-Acetylhexosaminidase was assayed by the method of Levvy and Conchie (51) except that one-tenth volumes of all reagents were used.

Acid phosphatase was assayed by the method of Kind and King (52) with phenyl disodium orthophosphate as substrate. Alkaline phosphatase was assayed by the method of Babson (53) 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 and Lazarow (54).

β-Glucuronidase was assayed by the method of Levvy and Conchie (55) with phenolphthalein glucuronide as substrate.

Protein was assayed by the method of Lowry, Rosebrough, Farr and Randall (56) with bovine serum albumin powder as standard.

Glucuronic acid was estimated according to the carbazole reaction of Dische (57) as modified by Bowness (58). The volumes of the blanks, standard, test solutions and the concentrated sulfuric acid used were half of those described by the latter worker. A standard curve for glucuronolactone was obtained by the use of a standard solution of concentration 100 mg./100 ml. The glucuronic acid content of tests was calculated from the standard curve.

Inorganic sulphate was assayed by the method of Dodgson and Price (59). Total hexosamine content was assayed by the method of Boas (60); slight modifications were made in the procedure for hydrolysis (5-15 mg. of each material was hydrolyzed in a sealed tube with 2 ml. of 2 N-HCl at 100-105° for 8 hours).

Viscosities were determined at 30° ±0.1°C with a Cannon 100 viscometer previously calibrated with sucrose solution and water.

4) Preparation of CM-cellulose and Sephadex Gel for Column Chromatography

a) CM-cellulose

A weighed amount of CM-cellulose (100 g.) was gently stirred in about 15 vol. of 0.5 N-NaOH at room temperature and left to stand for 30 min. The supernatant liquor was discarded and the cellulose washed with distilled water in a large sintered glass funnel until the pH of the washing was 8. The cellulose was then stirred in about 15 vol. of 0.5 N-HCl and left to stand for 30 min. The acid supernatant liquor was then discarded and the cellulose was washed in a large sintered glass funnel until the pH of the washing was 4. The cellulose was then stirred in another 15 vol. of 0.5 N-HCl and left to stand for another 30 min. The acid supernatant liquor was discarded and the cellulose washed in a large sintered glass funnel until the pH of the washing was about 5.6.

The cellulose treated by the method just described was equilibrated with a volume of the chosen buffer containing at least one equivalent of the eluting counter ion and left for 10 min. before discarding the supernatant liquor. This was repeated 4 times and sometimes 6 times, until the pH of the supernatant liquor was the same as that of the chosen buffer.

The equilibrated cellulose was then stirred in about 2 to 3 times its own volume of the chosen buffer and poured into a column leaving the effluent tubing of the column open.

Further additions of the stirred cellulose was added to the column until the required height of the column was attained. About one column volume of the chosen buffer was passed through the column before it was ready for use.

b) Sephadex G-25, G-100, G-150 and G-200

The swelling and preparation of these gels for column chromatography was carried out according to the procedure given by Pharmacia Fine Chemicals instruction booklet (61).

5) Analytical Disc Gel Electrophoresis

Disc gel electrophoresis at pH 4.5 was carried out by the method of Reisfeld, Lewis and Williams (62) with a 7.5% polyacrylamide gel, omitting the sample gel and applying the samples in 20% sucrose solution. After electrophoresis the gels were removed from their respective glass tubes by rimming the gels out of the tubes with a needle. The gels were stained in 1% Amido Schwarz in 7% acetic acid solution for 30 min. and then destained at 15 ma./tube for 1 hr.

Disc electrophoreses of certain highly purified hyaluronidase samples were carried out in duplicate. One of the two gels was stained with Amido Schwarz and the other immediately frozen on a slab of dry ice to minimize the diffusion of the protein component(s) in the gel. The position of the protein component(s) in the unstained gel was located by the calculated Rf of the protein component found for the stained gel. It was necessary to use the Rf value to locate

the position of protein component(s) in the unstained gel because the frozen and the stained gels were different in length. The gel was then cut up through its radial axis into 0.3 to 0.5 mm. pieces with a sharp razor blade and the pieces homogenized in a small Potter-Elvejhem homogenizer in about 0.5 ml. of 0.1 M-NaCl. The homogenized gel was then centrifuged at $400g_{av.}$ for 10 min. and the supernatant liquor assayed for hyaluronidase activity.

TABLE 2

HYALURONIDASE-LIKE ACTIVITY OF UNFRACTIONATED TISSUE AND TISSUE EXTRACTS

Tissue	Substrates		
	CS-4* mμmole substrate glucuronate	CS-6*	HA** depolymerized /min./g. tissue
Human plasma	1.6-2.1	0.7	6.2
Human white blood cells	0	N.D.	N.D.
Canine kidney	0	N.D.	N.D.
Bovine parotid	5.0-5.2	N.D.	N.D.
Canine submandibular gland	520-600†	260	1050
Canine parotid gland	0	0	0
Canine epiphyseal plates	0.32-0.35	N.D.	N.D.

* assayed by the spectrophotometric titration procedure

** assayed by the USP procedure

† the extract was incubated for 30 min. instead of the usual 120 min. since it was very rich in hyaluronidase-like activity

N.D. No data

V. IDENTIFICATION AND SUBCELLULAR DISTRIBUTION

1) Canine Submandibular Gland as a Source of Hyaluronidase

a) Preliminary methods

Human plasma, human white blood cells and a number of mammalian tissues such as the canine kidneys, bovine parotid glands, canine submandibular glands, canine parotid glands and canine epiphyseal plates were examined for hyaluronidase activity. All these tissues except canine epiphyseal plates were homogenized at 4° by a VirTis homogenizer rotating at top speed for a few seconds. Human white blood cells were homogenized by a Kontes Dual homogenizer. Canine epiphyseal plates were homogenized by ultrasonication. The tissue extracts were separately centrifuged to remove tissue and cellular debris. The supernatants were individually tested for hyaluronidase activity against chondroitin sulphate by the spectrophotometric titration method or against hyaluronate by the USP procedure.

b) Results (Table 2)

By the spectrophotometric titration method of assay, only the extract from canine submandibular gland was found to rapidly decrease the metachromatic reaction of chondroitin sulphate substrate with toluidine blue. This was the first data indicating the gland to be a potent source of hyaluronidase activity.

The canine submandibular-gland extract was also most active against hyaluronate, as assayed by the standard

procedure described in Section IV.1. It appeared from these data that canine submandibular gland might be a useful new source of hyaluronidase activity.

Dialysis of the canine submandibular-gland extract against distilled water indicated the activity was non-dialyzable.

The activity in the submandibular extract was stable in the cold at 4° up to a period of one week. However, storing the extract at 37° caused a loss of at least half of the activity within 24 hr., and at 85° most of the activity was irreversibly destroyed within 10 min.

TABLE 3

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-acetyl-glucosamine /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

2) Tissue Extraction for Total Enzyme Activity

Freshly obtained submandibular gland was chopped into small pieces with a pair of scissors and homogenized in about 4 vol. of 0.1 M-sodium acetate buffer, pH 5.0, in a VirTis homogenizer at 20,000 rev./min. for three 15 sec. periods at 4°, with a pause of 2 min. between operations. The homogenate was centrifuged at $10,000g_{av.}$ for 10 min. and the supernatant was removed for determination of total hyaluronidase activity (Table 3).

3) Test for Bacterial Contamination 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 12-48 hr. 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 (63) were carried out simultaneously with the assays of mammalian hyaluronidase in partially purified submandibular -gland extracts and unfractionated saliva. Though N-acetyl-glucosamine assays showed a high mammalian hyaluronidase activity, no increase in extinction at 230 mμ. was recorded, thus indicating the absence of bacterial hyaluronidase. The degradation of hyaluronate by bacterial hyaluronidase yields a disaccharide characterized by a 4,5 unsaturated uronide having an absorption maximum near 230 mμ. (65).

The oxidative-reductive depolymerizing reaction described by Pigman et al. (66) proceeds in the presence of reducing agents such as ascorbic acid and cysteine or Fe^{++} ions and Cu^{++} ion. In our experiments the incubation solutions for assay of hyaluronidase activity did not contain any of these agents and the controls contained the same materials except that they were boiled. Further, no oxygen consumption was recorded when hyaluronate was extensively hydrolyzed in a Warburg 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.

4) Extraction and Separation of Subcellular Components; Treatment with Triton X-100

a) Methods

A freshly obtained submandibular gland from a 3-10 kg. puppy, previously killed by intravenous Nembutal administration at a dose of 50 mg./kg. body wt., was stripped of its adhering connective tissues and washed well in 0.15 M-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 at 4° in a Potter-Elvehjem homogenizer with 10 vol. of 0.25 M-sucrose. Three up-and-down strokes were used with the Teflon pestle (0.13 mm. clearance) rotating at 1000 rev./min.

The homogenate was centrifuged at $400g_{av.}$ for 5 min. to sediment the nuclei and cell debris. The supernatant was retained and the sedimented pellet was homogenized again with 4 vol. of 0.25 M-sucrose and centrifuged at $400g_{av.}$ for 5 min. 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 and Appelmans (64). Fraction N was resuspended in 4 vol. of 0.25 M-sucrose and filtered

through gauze to remove cellular debris.

Acid hydrolases

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 15-30 min., and centrifuged at $1200g_{av.}$ for 10 min. to sediment material which had become insoluble during Triton X-100 treatment. The supernatants of the various centrifuged fractions were then assayed for hyaluronidase, acid phosphatase and β-N-acetylhexosaminidase.

Cytochrome c Oxidase

Other portions of fraction N, M, L, P, S and E without Triton X-100 were individually treated for three 15 sec. periods at 20 kcyc./sec. in 5 mM-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 for a further two 15 sec. 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

Alkaline phosphatase was assayed in supernatants obtained from fractions N, M, L and P after resuspension in water at 4°, standing for 1 hr. at 4°, and

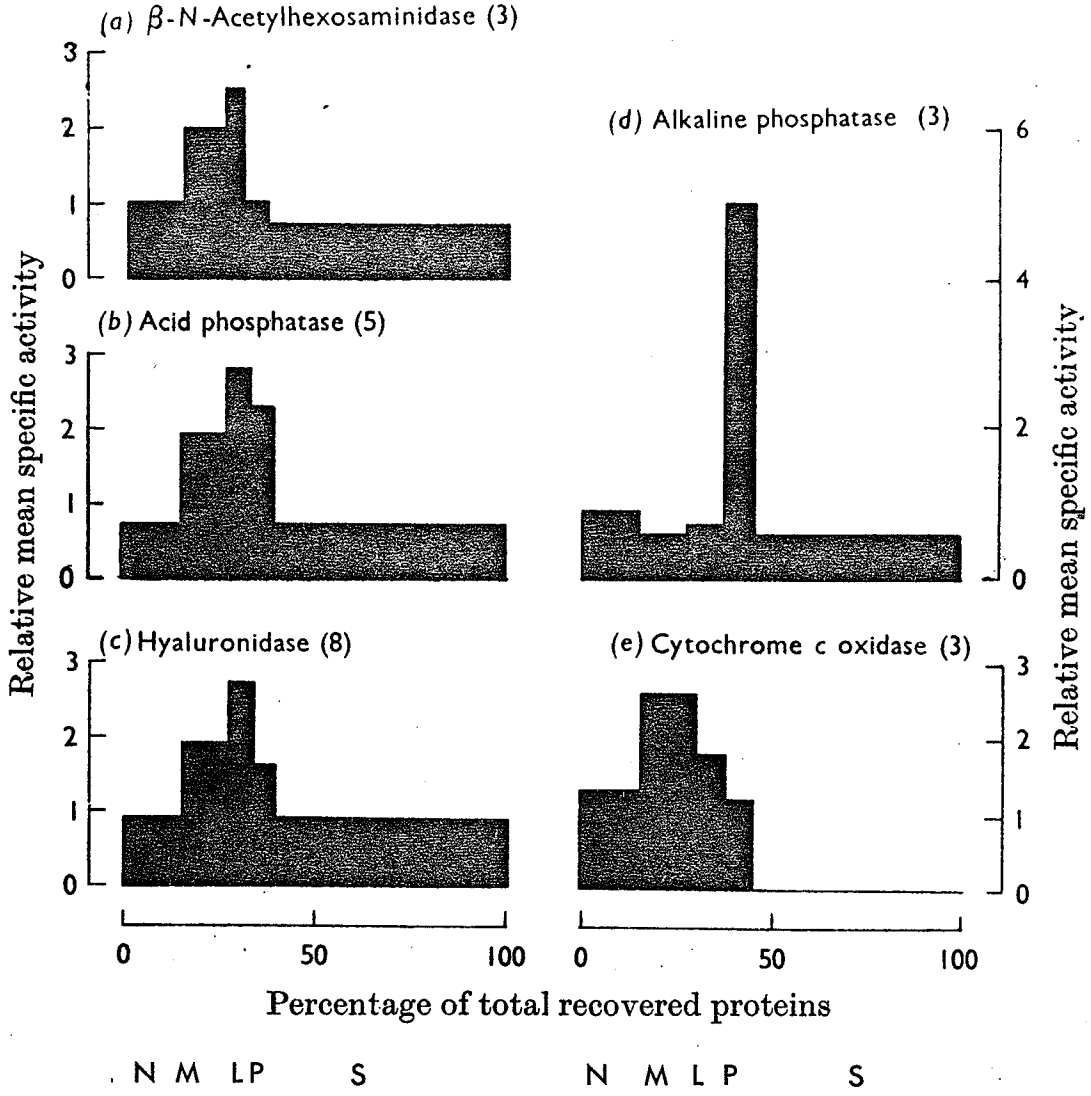


Fig. 3 Distribution patterns of enzymes in subcellular fractions of submandibular-gland homogenates: (a) β-N-acetylhexosaminidase; (b) acid phosphatase; (c) hyaluronidase; (d) alkaline phosphatase; (e) cytochrome c oxidase. The results are presented here in the manner used by de Duve et al. (64), the 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 (lysosomal), P (microsomal) and S (supernatant). Numbers in parentheses refer to numbers of experiments.

TABLE 4

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.

Enzyme	No. of expts.	Activity in fraction (% of total in fractions E+N)					
		N	E				Recovery (%)
			M	L	P	S	
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

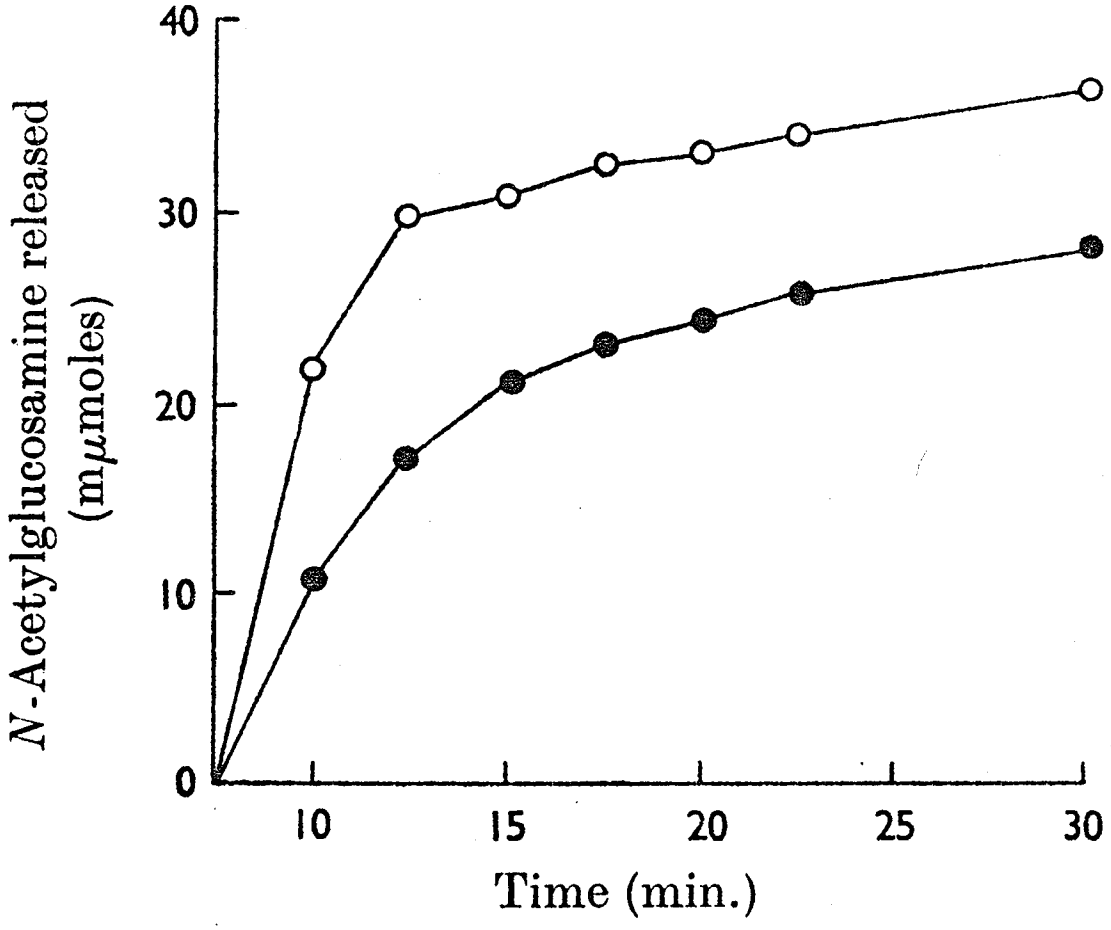


Fig. 4 Effect of Triton X-100 on hyaluronidase in fraction L. ● , No Triton X-100; O, 0.1% Triton X-100.

centrifuging at $1200g_{av.}$ for 10 min.

Release of enzyme activity

Test and control mixtures were prepared each containing 100 µl. of resuspended fraction L in 0.15 M-NaCl solution and 100 µl. of hyaluronate solution in phosphate-citric acid buffer, pH 3.8. Then 30 µl. of 1% Triton X-100 was added to each test mixture and the volume made up to 300 µl. with water. The tubes were then incubated at 37° and assayed for hyaluronidase activity in pairs (Test and control) at different times (Fig. 4).

b) Results

Subcellular distribution

The distribution of a number of enzymes in various subcellular fractions of submandibular gland is shown in Fig. 3 and Table 4. The activity of β-glucuronidase was found to be very low in crude sedimentable particulate and non-particulate fractions and was not assayed in the individual subcellular fractions.

Effects of Triton X-100 (Fig. 4)

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. 4).

In a number of fractionations portions of resuspended fractions N, M, L and P 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. When fraction E was gently stirred in 0.1% Triton X-100 for 1 hr. at 4° 90-95% of the hyaluronidase activity and the two acid hydrolases studied became non-sedimentable.

The supernatant fraction S from subcellular fractionation and the supernatant obtained from the extraction for total canine submandibular hyaluronidase activity (Section V.2) 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 enzyme studied in the presence of this detergent.

c) Discussion

For all the work on subcellular distribution submandibular glands from puppies were used; these were found to be more tender than glands from older dogs and therefore easier to homogenize by the Potter-Elvehjem homogenizer.

Subcellular distribution

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 (32).

The distribution of cytochrome c oxidase among the subcellular fractions obtained from submandibular gland was similar to that found for liver (64) and the distribution of alkaline phosphatase was similar to that found for bone (68). 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 (32, 68) and liver (64), mainly in the higher percentage of hyaluronidase (55%), acid phosphatase (46%) and β-N-acetylhexosaminidase (42%) found in the supernatant fraction S of submandibular-gland homogenate. For bone only 25% of the hyaluronidase was found in fraction S (32), but a higher content has been found in other tissues: in thyroid Herveg, Beckers and De Visscher (69) found that about 50% of the acid phosphatase was non-sedimentable; in lymphoid tissue Bowers, Finkenstaedt and de Duve (70) found that 51.8% of the acid phosphatase was non-sedimentable; in mixed parotid and submandibular glands of guinea pigs Cirina (71) 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. 6). According to Straus (72), lysosomal enzymes are confined to intracellular fractions, as opposed to zymogen granules, which are concerned with extracellular secretion. de Duve (73), 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 exo-enzymes. 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 (75), 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

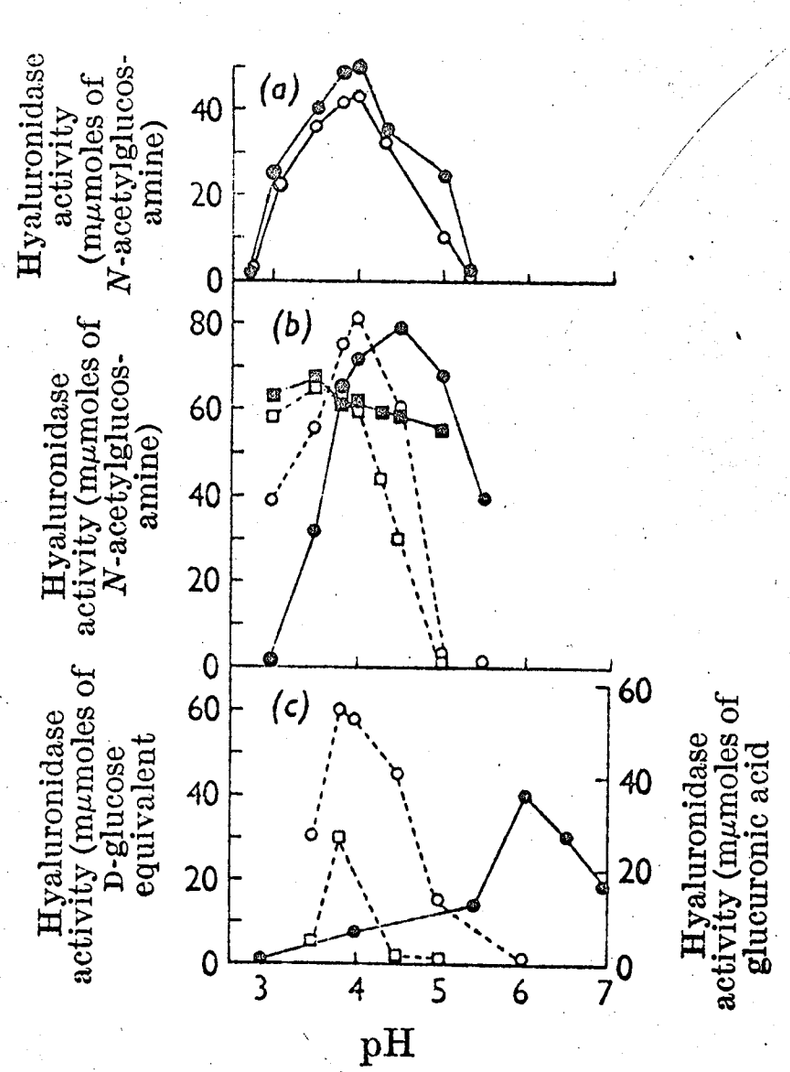
an extracellular use, nor does this hypothesis explain why so high a proportion of the acid hydrolases of the submandibular gland are found in the supernatant fraction of homogenates, since very little salivary amylase is found in this fraction.

All lysosomal enzymes investigated so far have an acid pH optimum (76); 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 (26), rat liver lysosomal hyaluronidase (74), rat bone lysosomal hyaluronidase (67), human gingival hyaluronidase (29) and human serum hyaluronidase (27, 28) 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. 5) and by viscosimetric assay (77, 78).

The presence of glucuronic acid in canine submandibular-gland mucin was reported by Draus on the basis of colorimetry (79), and a sulphosialo-polysaccharide-peptide from canine submandibular gland containing hexosamine but no uronic acid was described by Bignardi, Aureli, Balduini and Castellani (80). 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.

Effects of Triton X-100

In all the enzyme assays described in this work both test and control mixtures were incubated at pH 3.8. Gianetto and de Duve (81) and Appelmans and de Duve (82) 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 (84), showed that there was a considerable latent hyaluronidase activity in the light-mitochondrial fraction (fraction L) from submandibular-gland homogenates, which is released by Triton X-100 (Fig. 4). In the presence of this detergent nearly all the hyaluronidase activity of the subcellular particles in fraction E became non-sedimentable. Release of hyaluronidase from the subcellular particles could also be achieved by high speed homogenization. This indicated that the enzyme was released from the particulate material.



5 (a) pH-activity curves of submandibular-gland hyaluronidase (o) and submandibular saliva (●) obtained by using the N-acetyl-glucosamine assay; the mixture was incubated for 15 min. 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-acetyl-glucosamine assay in phosphate-citric acid buffers (o) or in acetate buffers (□), and of sheep testicular hyaluronidase in phosphate-citric acid buffer (●) or in acetate buffer (■); the mixture was incubated for 5 min. 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) (o) 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 120 min. at 37° in spectrophotometric titration procedure and 30 min. at 37° in the reductimetric assay.

5) Effects of pH

a) Methods

The effects of pH on the release of N-acetyl-glucosamine from hyaluronate by submandibular gland and sheep testicular hyaluronidase were tested in phosphate-citric acid buffers with and without gelatin and in acetate buffers without gelatin (Fig. 5a and 5b). The time for maximum colour development with p-dimethylaminobenzaldehyde in the acetate buffer was 20 min. at 37° and 12.5 min. in the phosphate-citric acid buffer.

The effect of pH on hyaluronidase activity was also studied by the spectrophotometric titration procedure of Bowness (23) and by the reductimetric assay (49) with chondroitin 4-sulphate in both assays (Fig. 5c).

b) Results and Discussion

The pH-activity curve (Fig. 5b) 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. (31) for renal and testicular hyaluronidase.

The pH optimum of dog submandibular-gland hyaluronidase before final purification was found to be 4.0 by either the spectophotometric titration procedure or by the chemical assay of end groups (Fig. 5c). However this was not the case with testicular hyaluronidase which was reported to have a pH optimum of6.0 to 6.6 by the physicochemical methods (77, 78, 85) and 4.5 by N-acetyl-glucosamine end-group assay

(31, 74) and 5.0 by reducing end-group assay (99). In the case of the other acid active hyaluronidases such as serum hyaluronidase the pH optimum was the same at pH 3.8 to 4.0 by either the physicochemical assay methods or the chemical method of assay. This suggests either that the action pattern of testicular hyaluronidase differs from that of the other hyaluronidases or that there are two distinct enzyme activities in testicular hyaluronidase(s); a depolymerase active at pH 6.0 to 6.6 and an oligosaccharase active at pH 4.5 to 5.0. The concept of two enzyme activities was discussed as early as 1947 by Hahn for bovine testicular hyaluronidase (86, 87).

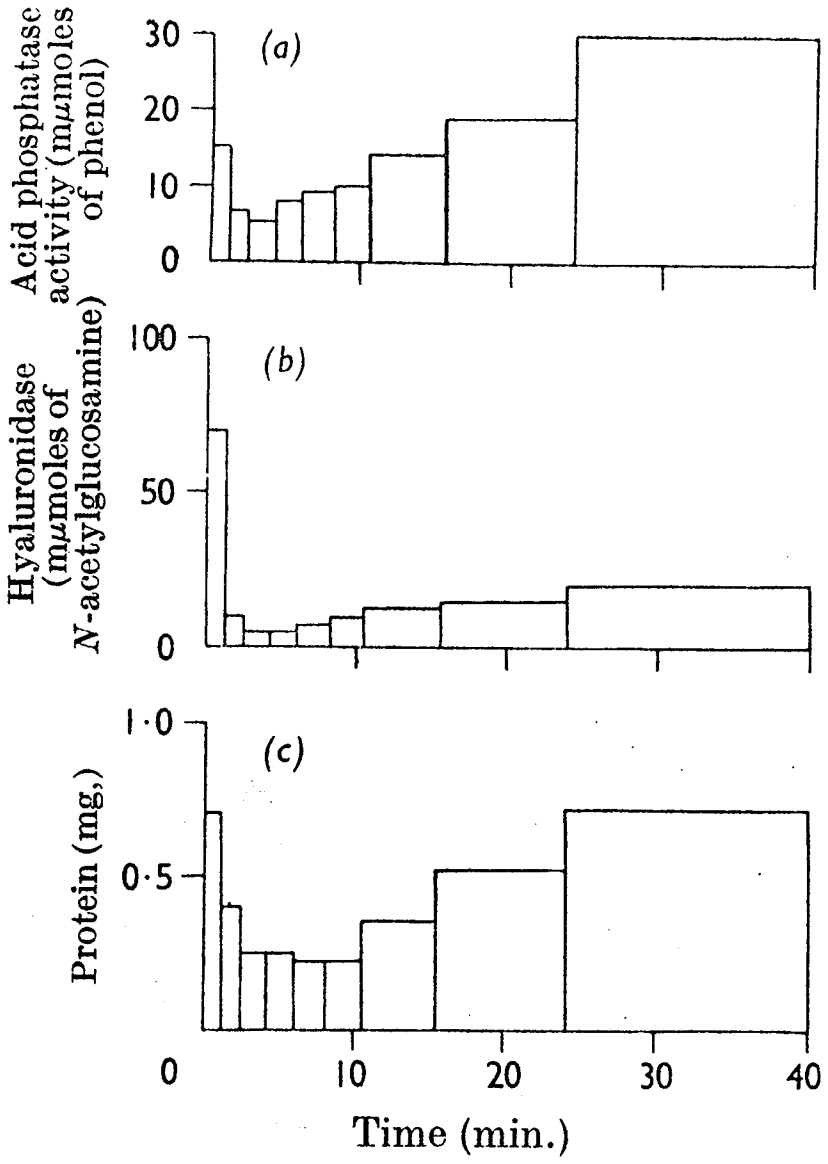


Fig. 6 Enzyme and protein profile in the cannulated flow of submandibular saliva; (a) acid phosphatase; (b) hyaluronidase; (c) protein. Pilocarpine hydrochloride (1 mg.) was administered intravenously to stimulate the flow of saliva. The ordinate scales show amounts of activity or protein in 1 ml. of saliva and each rectangular section on the abscissa shows the time (min.) required to collect 1 ml. of saliva.

6) Collection of Submandibular-gland Secretions

a) Methods

Dogs were anaesthetized by intravenous administration of Nembutal (10-12 mg./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 (1 mg.) prepared in 0.15 M-NaCl was then given intravenously to each anaesthetized dog. The submandibular-gland saliva was collected in fractions and analyzed for hyaluronidase activity, acid phosphatase activity and protein.

b) Results and Discussion

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. 6). 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 (88) and Shannon (89).

.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% Triton X-100, thus indicating that the two acid hydrolases were secreted in the free form.

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 (Table 5 - Appendix). 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 submandibular saliva was 5.2% of the total found in the pilocarpine-stimulated gland plus the saliva.

At present the physiological significance of submandibular gland and submandibular salivary hyaluronidase, if any, is not known. A role as a necessary part of the secretion mechanism is rendered unlikely by the finding of

very low concentration in the submandibular gland of animals other than the dog and cat, but the lack of the enzyme in human submandibular and parotid saliva may suggest a particular digestive function in the dog and cat.

TABLE 6

CHARACTERIZATION OF OLIGOSACCHARIDE PRODUCTS FROM A SEPHADEX G-25 COLUMN (Fig. 7)

The ratios given apply to the single effluent fraction with the highest uronic acid content from each of the numbered peaks of Fig. 7. Glucuronate was determined by the method of Bowness (58) and N-acetylglucosamine end groups were determined by the method of Bonner and Cantey (48). V_e/V_o ratios for testicular hyaluronidase were calculated from the disaccharide (I), tetrasaccharide (II), hexasaccharide (III) and octasaccharide (IV) peaks in Fig. 1 of Flodin, Gregory and Rodén (90). The hyaluronidase preparations were purified as described in Section VI.1.

Enzyme	Sample	N-Acetylglucosamine end groups (μmoles/ μmole of glucuronate)	V_e/V_o
Non-sedimentable	I	1.20	2.18
hyaluronidase	II	0.62	1.74
(fraction S_3)	III	0.42	1.50
	IV	0.18	1.31
Sedimentable	I	1.01	2.18
hyaluronidase	II	0.60	1.75
(fraction MLP_3)	III	0.41	1.52
	IV	0.17	1.33
Testicular	(I)		2.05
hyaluronidase	(II)		1.76
	(III)		1.52
	(IV)		1.36

7) Identification of Products of Enzyme Action

a) Methods

Sodium hyaluronate was left to dissolve in sterilized 0.1 M-sodium acetate buffer, pH 4.0, containing 0.15 M-NaCl, for 48 hr. in the cold room (4°); the concentration of the hyaluronate solution was 5 mg./ml. The hyaluronate solution (2 ml.) was then incubated for 2 hr. or 24 hr. at 35° with 1.2 mg. of hyaluronidase preparation (specific activity of 0.28 units/mg. protein) purified from the sedimentable hyaluronidase activity of the microsomal, lysosomal and mitochrondial fractions from the canine submandibular-gland extract. Similar incubations were also carried out with 1.0 mg. of hyaluronidase preparation (specific activity of 0.34 units/mg. protein) purified from the crude non-sedimentable hyaluronidase activity of the post microsomal supernatant fraction. The purification procedure of the sedimentable and non-sedimentable hyaluronidase is described in Section VI of this thesis.

The oligosaccharide products of the digested hyaluronate were separated on a Sephadex G-25 column (2.5 cm. x 30 cm.) previously equilibrated with 0.1 M-NaCl and eluted by 0.1 M-NaCl at a flow rate of about 20 ml./hr. The effluent was collected in 1 ml. fractions which were analyzed for glucuronic acid and N-acetyl-glucosamine end groups by the standard procedure in hyaluronidase assay.

b) Results and Discussion

In Table 6 it is shown that

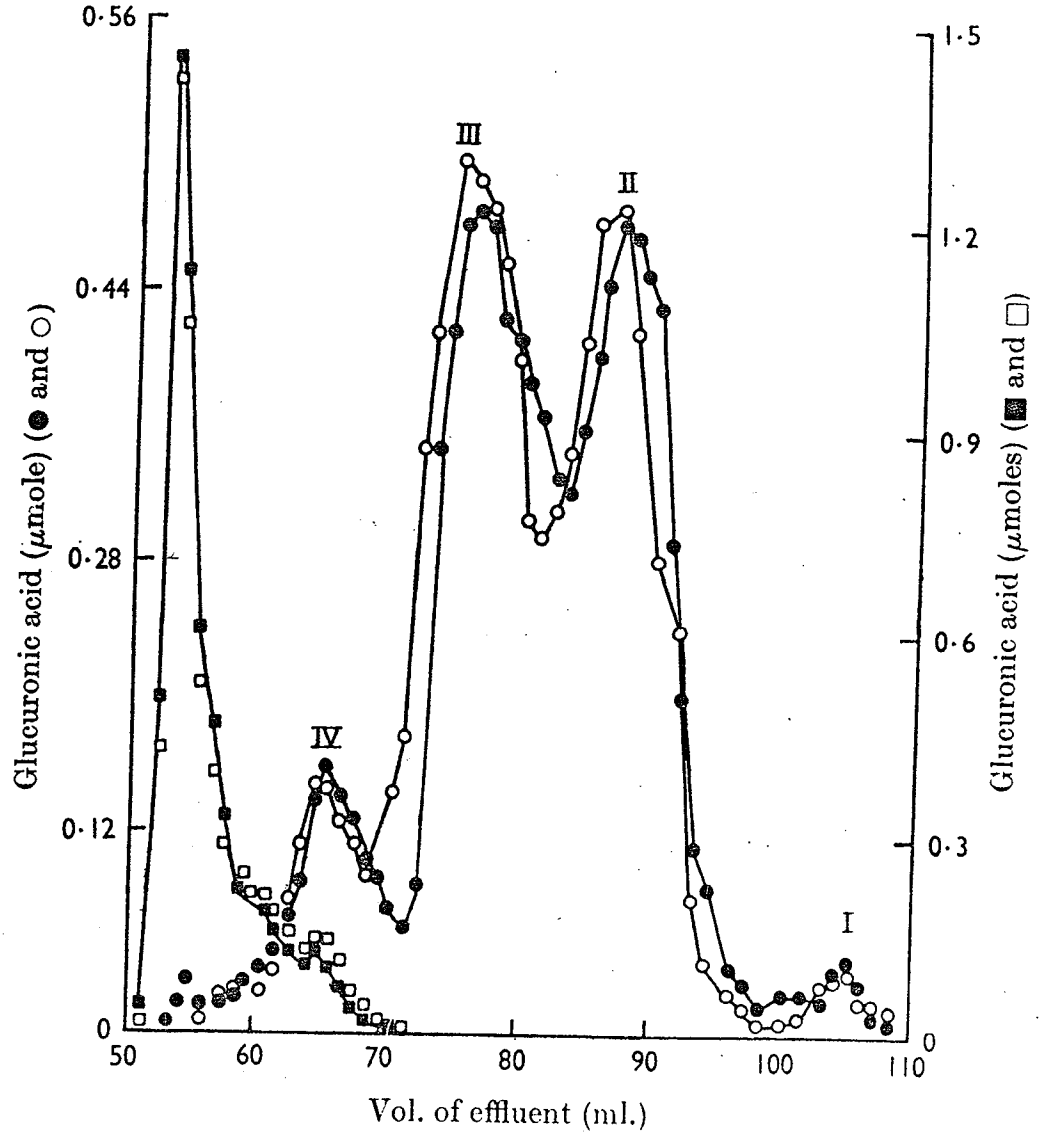


Fig. 7 Gel filtration of submandibular-gland hyaluronidase digest of sodium hyaluronate (10 mg.) in 0.1 M-NaCl at 35° on a Sephadex G-25 column (2.5 cm. diam. x 30 cm.). Incubation for 2 hr. (□) or 24 hr. (○), with 1 mg. of non-sedimentable hyaluronidase (sp. activity 0.34 unit/mg. of estimated protein); incubation for 2 hr. (■) or 24 hr. (●), with 1.2 mg. of sedimentable hyaluronidase (from fraction MLP; sp. activity 0.28 unit/mg. of estimated protein). The hyaluronate preparations were purified as described in Section VI.1.

the molar ratio of N-acetyl-glucosamine to glucuronate for the four main products of hyaluronate digestion (Fig. 7) increased with the effluent volume at which they were eluted. The ratios for peaks I, II and III agreed closely with those found by Aronson et al. (83) 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_o ratios on Sephadex G-25 agreed closely with those found for products of digestion of hyaluronate by testicular hyaluronidase (90), 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 during a 2 hr. incubation at 35°, no oligosaccharides of the type found after 24 hr. were found at the earlier time. This shows that the enzyme from submandibular glands of the dog is an endohyaluronidase whose product formation at 24 hr. is indistinguishable from that of testicular and liver lysosomal hyaluronidase.

8) General Discussion and Conclusions

Hyaluronidase activity (Table 3) was found in the submandibular glands of dogs, cats, guinea pigs, rats, pigs and oxen, but only the first two contained 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 (91). This unit was stated to be similar to the turbidity reducing unit of Rapport, Meyer and Linker (92) 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 230-320 mµmole. of N-acetyl-glucosamine 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 (33). Activity resembling hyaluronidase was found in the saliva and unnamed salivary glands of the dog, but none was found in human and horse saliva (33). Gibian (93), 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 230 mμ. 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 oligosaccharide products.

Results of the centrifugal fractionation show that 15% of the hyaluronidase in canine submandibular-gland extracts is associated with the lysosomal fraction (fraction L) as opposed to 28% in bone (32). Also, unlike liver and bone, a high percentage (50-60%) of the submandibular hyaluronidase was found in the non-sedimentable fraction S. However, practically all of the particle-bound hyaluronidase was found to be liberated by Triton X-100 or high speed homogenization, as is the case with the liver and bone enzymes.

The total amount of hyaluronidase found in the pilocarpine-stimulated gland was 6.2% less than the control gland, and the total activity found in the submandibular secretion was 5.2% of the total found in the pilocarpine-stimulated gland plus the secretion. However the physiological significance, if any, of the enzyme in the saliva is not known.

Results on the effect of pH indicate that canine submandibular-gland hyaluronidase differs from hyaluronidase of sheep testes in possessing greater activity at pH values

of 4 and below. This suggests that the submandibular-gland enzyme can be classed as an acid-active hyaluronidase. However this classification of the submandibular enzyme as an acid-active hyaluronidase and the distinction between this and testicular hyaluronidase should be regarded as arbitrary since little is known of the structure and functions of these enzymes and also there is little information on species and organ variation for hyaluronidases from various mammalian sources.

Although studies of product formation show submandibular-gland hyaluronidase attacks hyaluronate producing the same oligosaccharide products as testicular hyaluronidase little is known of the action pattern of the enzymes in the sense that this term has been used for amylases (94). One may speculate on the use of the Amicon ultrafiltration cell in future as a tool to study the action pattern of hyaluronidases. Briefly, the system could consist of the enzyme incubated with a saturating amount of substrate (chondroitin sulphate) in the ultrafiltration cell using a filter membrane which would only retain the enzyme-substrate complex but not the oligosaccharide product(s). Of the series of membranes currently available commercially only two, the XM-50 and XM-100 appear to be suitable for this purpose. By this technique it may be possible to accomplish the isolation and identification of oligosaccharide products formed during the entire course of enzyme action. Data from this sort of study may also be useful in distinguishing different types

of hyaluronidase through their action patterns.

VI. PURIFICATION AND PROPERTIES

1) Purification of Sedimentable Particulate and Non-Particulate Canine Submandibular-Gland Hyaluronidase

a) Methods

Homogenization and separation into 2 fractions

Freshly obtained canine submandibular glands (33 g.) were chopped with a pair of scalpel blades into tiny pieces; portions of the chopped glands weighing about 2 g. were suspended in 14 ml. of 0.25 M-sucrose and placed in the tube of a Potter-Elvehjem homogenizer with the Teflon pestle rotating at 1000 rev./min.; three up-and-down strokes were made during the homogenization of the chopped glands. The homogenate was centrifuged at $400g_{av.}$ for 5 min., and the supernatant was poured off and retained. The pellet was resuspended in 4 vol. of 0.25 M-sucrose, homogenized again and then centrifuged again at $400g_{av.}$ for 5 min. Both supernatant fractions from all homogenizations were pooled (total volume about 300 ml.) and then centrifuged at $101,000g_{av.}$ for 30 min. in a Spinco model L Centrifuge (no. 40L rotor). The pellet then contained the sedimentable hyaluronidase (fractions M, L and P) and the supernatant fraction S contained the non-sedimentable hyaluronidase. The pellet was resuspended in glass-distilled water (56 ml.) at 4°, homogenized for three 15 sec. periods at 20,000 rev./min. in a VirTis homogenizer to break up the subcellular particles and then centrifuged at $3500g_{av.}$ for 10 min. All the operations described were performed at 4°.

The supernatant (fraction MLP), containing the originally sedimentable hyaluronidase, and the originally non-sedimnetable hyaluronidase (fraction S) were each purified separately by the procedure described in the following sections.

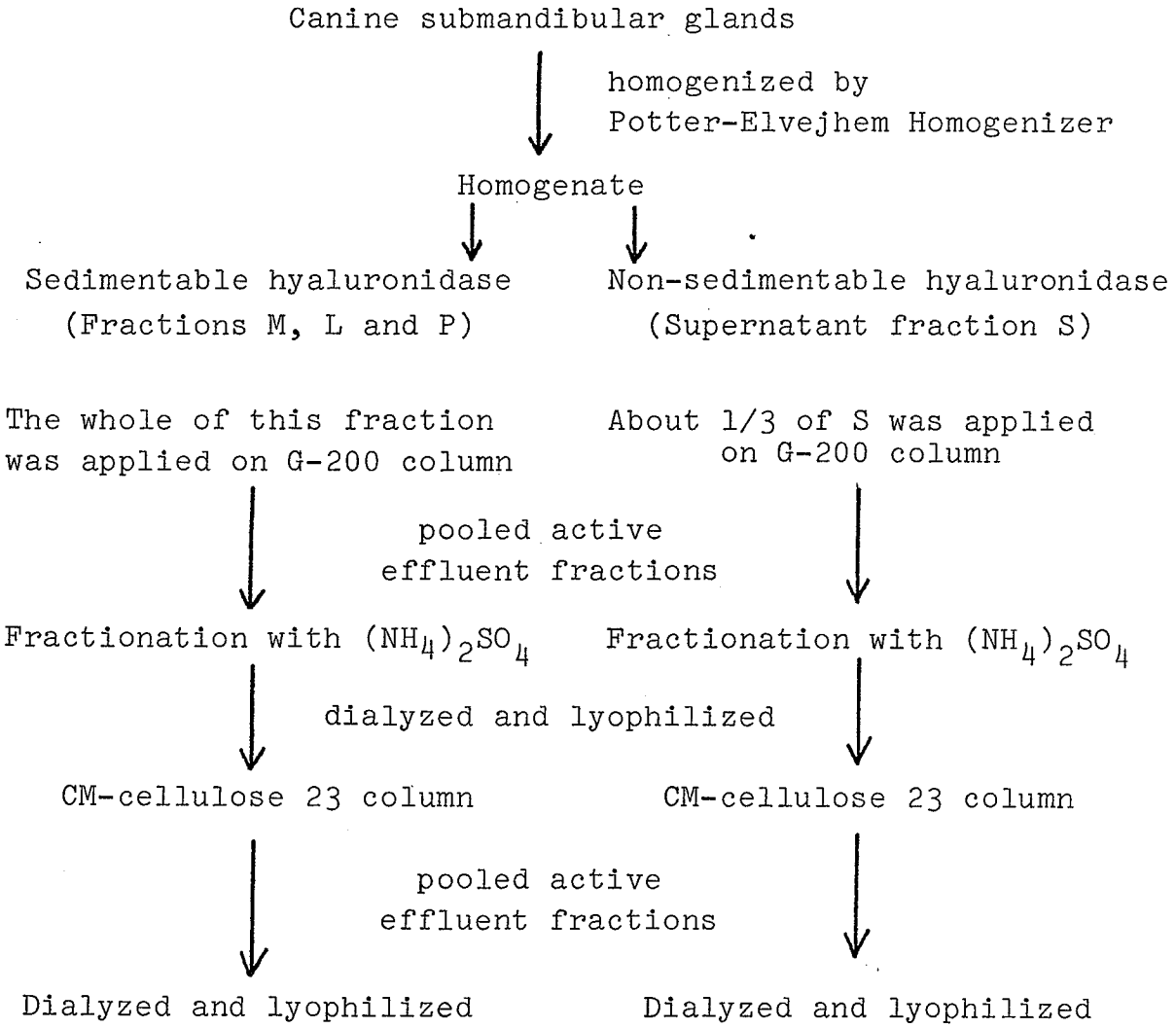
Gel filtration

About one-third of fraction S (88 ml.) or the whole of fraction MLP (45 ml.) was subjected to reverse-flow gel filtration on a Sephadex G-200 column (5 cm. x 75 cm.) prepared in 2 M-NaCl-0.1 M-sodium phosphate buffer, pH 6.0. The flow rate of the applied sample of fraction S in a 0.1 ml. 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 20-30 ml./hr. The column was developed with the buffer used in the preparation of the column at a flow rate of 48 ml./hr., and effluent fractions (16 ml.) were collected. The procedure was repeated twice more to dispose of the remainder of fraction S. This gel filtration is shown in Fig. 8.

Fractionation with ammonium sulphate

The effluent fractions from Sephadex G-200 shown between the arrows in Fig. 8, 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 pH 6.0 (Table 7). At each stage the mixture obtained after the $(NH_4)_2SO_4$ had dissolved was allowed to stand for 18 hr. at 4° and the

Fig. 10 Flow chart for the partial purification of hyaluronidase from the sedimentable and non-sedimentable fractions.



suspension was then centrifuged at 16,300$g_{av.}$ for 10 min. in a Servall RC2-B centrifuge with a GSA rotor. The precipitate was redissolved in 4 ml. of glass-distilled water to give fraction S_2 or fraction MLP_2 (Table 7) and then dialyzed in 1 cm. wide dialysis tubing, with stirring, for 5 hr. at 4° against two changes of 3 l. of glass-distilled water. The dialyzed preparation was centrifuged to remove any precipitated protein(s), and the clear supernatant was freeze-dried to give fraction S_3 or MLP_3 (Table 7).

CM-cellulose chromatography

A portion of fraction S_3 or fraction MLP_3 was chromatographed on a CM-cellulose 23 column (1.5 cm. x 16 cm.) previously prepared in 0.04 M-sodium citrate buffer, pH 4.85. The column was eluted by a stepwise procedure (Fig. 9). The three most active effluent fractions shown in Fig. 9 were pooled to give fraction S_4 or fraction MLP_4 (Table 7) and dialyzed as described in the preceding section. After dialysis the non-diffusate 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 7).

A summary of the purification procedure just described is presented schematically by flow chart in Fig. 10. Some properties of the partially purified hyaluronidase from the sedimentable and non-sedimentable fractions were studied and presented later in Section VI.4 of this thesis.

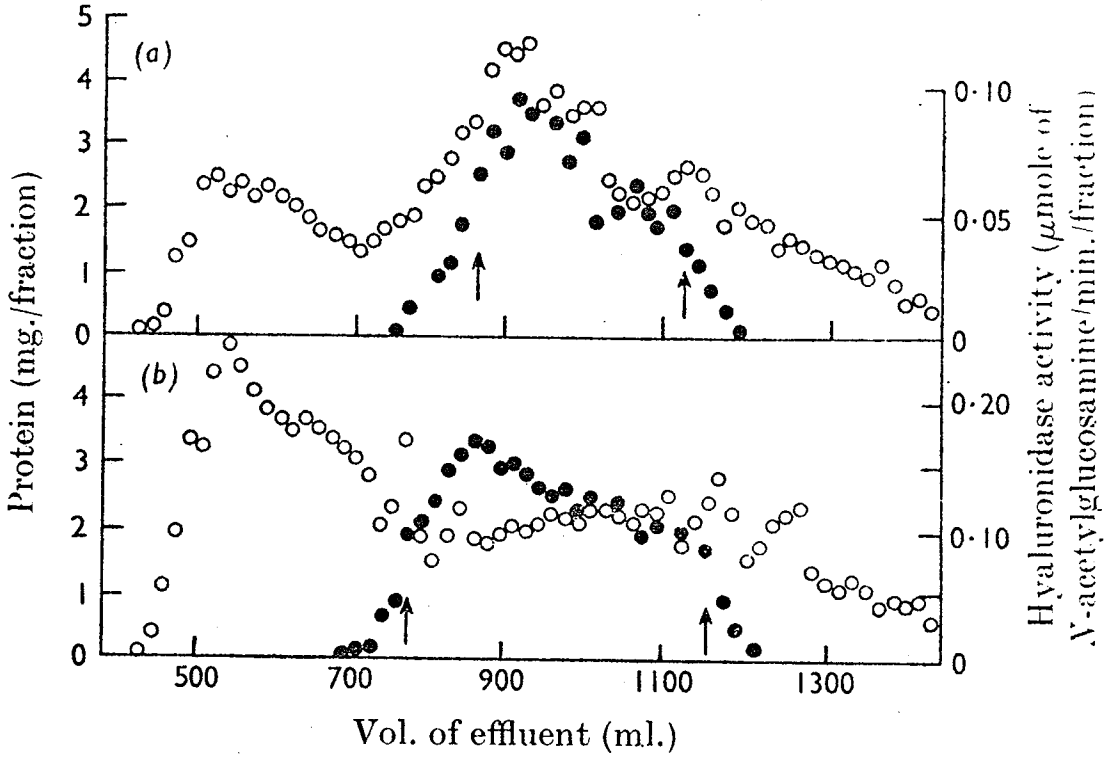


Fig. 8 Chromatography of (a) supernatant fraction S (174 mg. of assayed protein) and of (b) sedimentable fraction MLP (140 mg. of assayed protein) on a column (5 cm. diam. x 75 cm. height) of Sephadex G-200. The effluent fractions between the arrows were pooled for $(NH_4)_2SO_4$ fractionation. O, Protein; ●, hyaluronidase.

b) Results

When the non-sedimentable material from the submandibular gland was subjected to gel filtration (Fig. 8), 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 1 g. of gland with approximately 11 ml. 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 (see Section VI.2). 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 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

TABLE 7

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 dialyzed 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 (dialyzed)	3	0.160		
S_8	Dialyzed 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 dialyzed 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 (dialyzed)	2.8	0.102		
MLP_8	Dialyzed supernatant from 50%-satd.-$(NH_4)_2SO_4$ treatment of MLP_1	28.8	0.001		

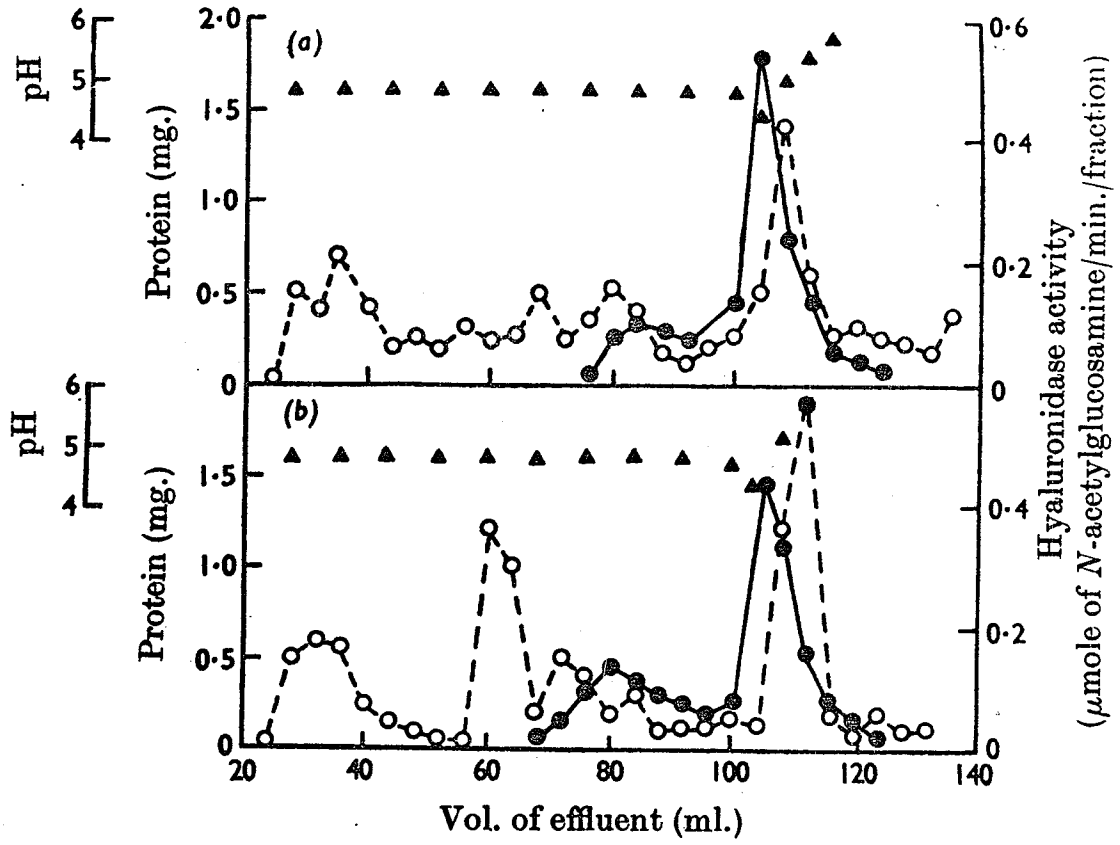


Fig. 9 Chromatography of (a) fraction S_3 and of (b) fraction MLP_3 on a column (1.5 cm. diam. x 16 cm. height) of CM-cellulose; 10 mg. of assayed protein was applied. The column was eluted by a stepwise procedure at a rate of 60 ml./hr. with: (i) 30 ml. of 0.04 M-sodium citrate buffer, pH 4.85; (ii) 50 ml. of 0.04 M-sodium citrate buffer, pH 4.85, containing 0.05 M-NaCl; (iii) 0.04 M-sodium citrate buffer, pH 6.3, containing 0.3 M-NaCl. Effluent fractions (4 ml.) were collected. ○, Protein; ●, hyaluronidase; ▲, pH of effluent.

sulphate at 35% saturation at 4° and pH 6.0 (Table 7). Material reacting with anthrone was presumably removed during gel filtration and ammonium sulphate fractionation, as none was detected in enzyme preparation S_3 or subsequently.

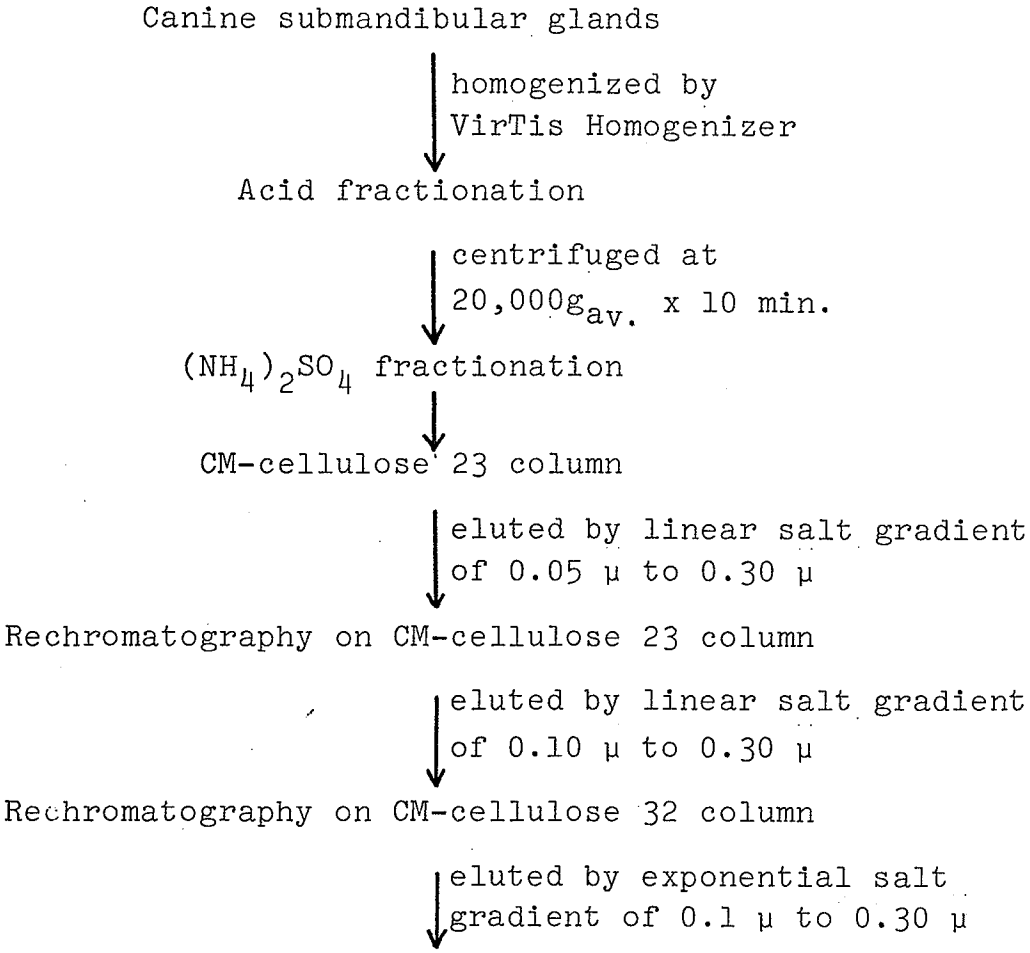
CM-cellulose chromatography (Fig. 9 and Table 7) produced a two-to-three-fold increase in the specific activity of the hyaluronidase.

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 (fraction 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 1.28 units/mg. protein (fraction S_5) was comparable with the value of 1.29 units/mg. protein

reported by Aronson and Davidson (36).

Fig. 11 Flow chart for the purification of canine submandibular-gland hyaluronidase.



2) Purification of Hyaluronidase from Whole Canine Submandibular-Gland Homogenate

a) Methods

The procedure used for the assay of hyaluronidase in the following purification procedure was according to the method described in Bowness and Harding's publication (41) except that an incubation time of 5 min. was used. The extraction and purification procedure is summarized in Fig. 11 and is described in detail below.

Homogenization of glands

Dog submandibular glands (80-150 g.) were sliced into longitudinal strips with a sharp razor, chopped to tiny pieces with a pair of scissors and then homogenized in 30 g. batches in about 4 vol. of 0.1 M-NaCl by a VirTis homogenizer rotating at 20,000 r.p.m. for two 5 sec. periods at 4° with a pause of a few min. between these two periods. The homogenate was then centrifuged at $10{,}000g_{av.}$ for 10 min. and the supernatant liquor collected. The pellet was resuspended in about 4 vol. of 0.1 M-NaCl and homogenized and centrifuged in the manner just described. The supernatant was pooled with the supernatant of the first centrifugation.

Acid precipitation

The pooled supernatants were adjusted to pH 3.6 by addition of 0.25 N-HCl thereby causing the submaxillary mucin to clot. The acidified supernatant was allowed to stand at 4° for 14 to 18 hr. to allow maximum

precipitation of the mucin before removing it by centrifuging at 20,000$g_{av.}$ for 10 min. The supernatant was a clear and non-viscous solution. The pH of the supernatant was adjusted to 5.0 by adding solid sodium acetate.

$(NH_4)_2SO_4$ fractionation at 4°

Solid $(NH_4)_2SO_4$ was added to the solution obtained from the previous step to produce 30% saturation. A fine precipitate was immediately observed in most cases. This was removed by centrifuging the solution at 20,000$g_{av.}$ for 10 min. The clear supernatant was collected and more solid ammonium sulphate was added until the percentage saturation of the solution was about 60%. The precipitate which came down at 30-60% saturation of ammonium sulphate was allowed to aggregate for 16 hr. at 4° before harvesting it by centrifuging the solution at 20,000$g_{av.}$ for 30 min. The pellet was resuspended in minimal amount of cold distilled water and poured into a dialysis bag and dialyzed for 16 hr. at 4° with two changes of water. The dialyzed solution was slightly cloudy and was centrifuged at 100,000$g_{av.}$ for 10 min. in a Beckman Spinco centrifuge at 4° using the 60 Ti rotor to remove the cloudiness. The clear supernatant was retained and applied on a CM-cellulose column.

CM-cellulose chromatography

The whole supernatant obtained from the previous step was applied on a CM-cellulose 23 column (2.5 cm. x 80 cm.), previously equilibrated with 0.02 M-sodium acetate buffer, pH 5.0, containing 0.03 M-NaCl,

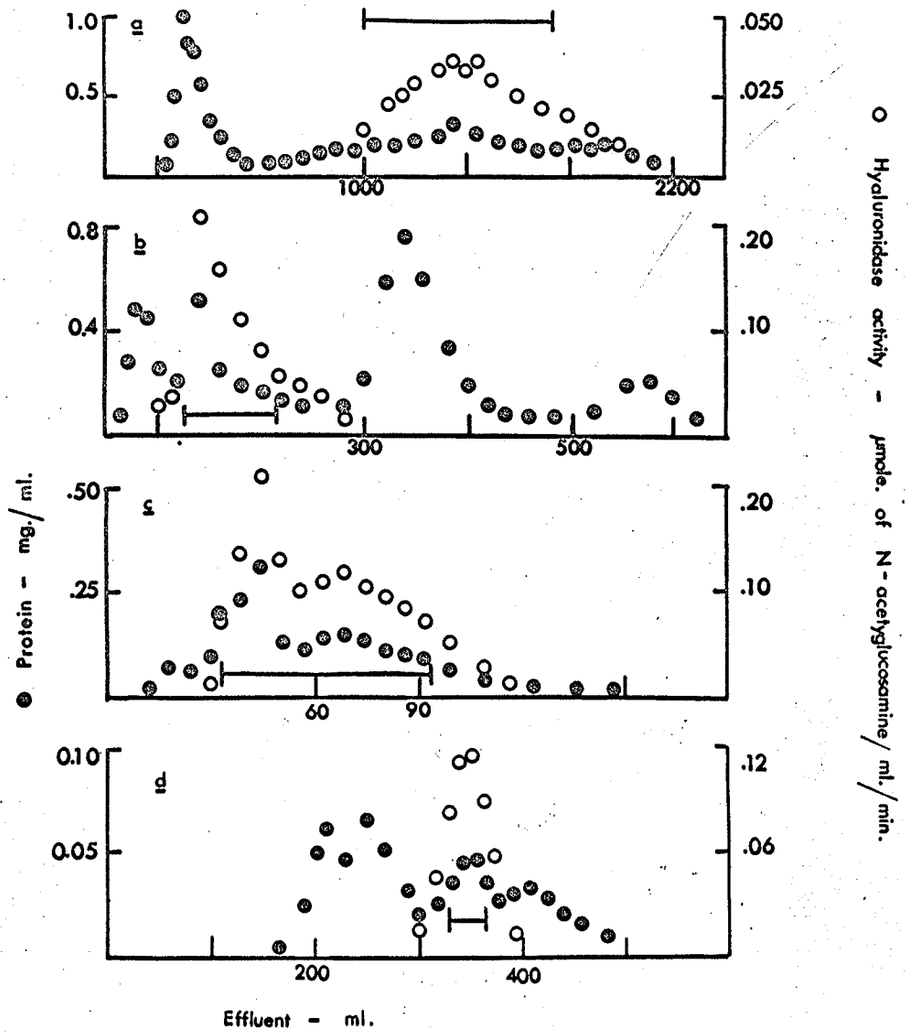


Fig. 12 Purification of canine submandibular-gland hyaluronidase.

The sodium acetate buffer used in these experiments was pH 5.0 and a molarity of 0.02 M. The active effluent fractions which were pooled are indicated between ⊢——⊣.

(a) Chromatography of the enzyme previously fractionated by $(NH_4)_2SO_4$ (440 mg. of assayed protein) on a CM-cellulose 23 column (2.5 cm. x 80 cm.). The column was eluted at a rate of 100 ml./hr. by a linear salt gradient formed by 1 l. each of (i) 0.03 M-NaCl, (ii) 0.28 M-NaCl both in sodium acetate buffer.

(b) Rechromatography of the enzyme from (a) on a CM-cellulose 23 column (2.5 cm. x 19 cm.). The column was eluted at a flow rate of 60 ml./hr. by a linear salt gradient formed by 1 l. each of (i) 0.08 M-NaCl, (ii) 0.28 M-NaCl both in sodium acetate buffer.

(c) Rechromatography of the enzyme from (b) on a CM-cellulose 32 column (1.5 cm. x 20 cm.). The column was eluted at a flow rate of 30 ml./hr. by an exponential salt gradient formed by 125 ml. each of (i) 0.08 M-NaCl, (ii) 0.28 M-NaCl both in sodium acetate buffer.

(d) Chromatography of the enzyme from (c) on Sephadex G-150 column (2.5 cm. x 90 cm.). The column was eluted by 0.2 M-NaCl at a flow rate of 26 ml./hr.

and the column then eluted by a linear salt gradient (Fig. 12a). The active effluent fractions were pooled and the volume of the pooled fractions was reduced to about 6 ml. in an Amicon Model 401 ultrafiltration cell using a UM-10 membrane. The concentrated enzyme solution was stirred and then removed by a pasteur pipette. A few ml. of cold distilled water was added to the cell and swirled a few times in the cell. This washing was added to the concentrated enzyme. The enzyme and washings were then diluted with cold distilled water to give an ionic strength (μ) of about 0.08 to 0.10. The diluted enzyme solution was applied on a CM-cellulose 23 column (2.5 cm. x 19 cm.) previously equilibrated with 0.02 M-acetate buffer containing 0.08 M-NaCl. The column was subsequently eluted by a linear salt gradient and the effluent collected in 5 ml. fractions (Fig. 12b). The active effluent fractions were pooled as shown in Fig. 12b, and concentrated in a Model 50 ultrafiltration cell with an XM-50 membrane to a volume of about 5 ml. Cold distilled water was added to the cell such that the ionic strength of the concentrated enzyme solution was reduced to about 0.1. The enzyme solution was then removed from the cell and applied on a CM-cellulose 32 column (1.5 cm. x 20 cm.). The enzyme was then eluted from the column by an exponential salt gradient as shown in Fig. 12c. The active effluent fractions shown in Fig. 12c were then concentrated to about 2 ml. by ultrafiltration as described above.

TABLE 8

PURIFICATION OF CANINE SUBMANDIBULAR-GLAND HYALURONIDASE

Step	Specific activity* µmole N-acetylGlu-NH_2/ min./mg. est. protein	mg. est. protein	% Yield of extracted activity
Crude extract	0.014	2010	100
Acid ppt.	0.024	1100	94
$(NH_4)_2SO_4$ ppt.	0.040	440	63
CM-23 cellulose column	0.070	220	55
CM-23 cellulose column	0.37	16.5	22
CM-32 cellulose column	0.42	9.8	15
Sephadex G-150 column	2.40	1.4	12

* at pH 3.8

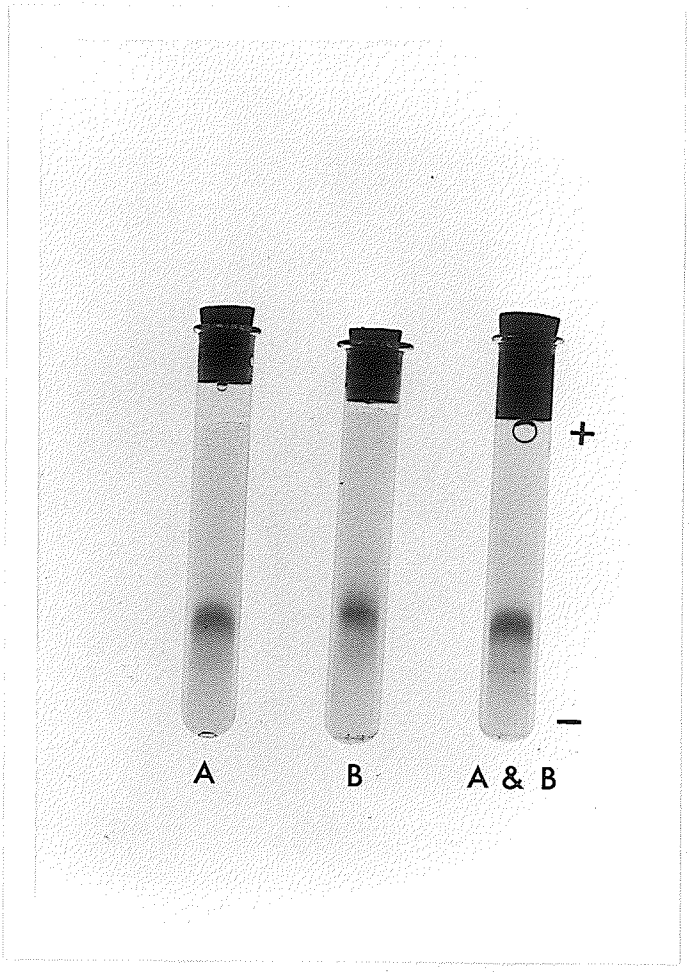


Fig. 13 Disc electrophoresis of canine submandibular-gland hyaluronidase preparations in polyacrylamide gel at pH 4.5. The current was 6 ma. per tube for 90 min. A is the hyaluronidase preparation (80 µg. est. protein) obtained by the method described in Fig. 11. B is the hyaluronidase preparation Peak IIC (80 µg. est. protein) obtained by the method described in Fig. 15. A & B is an equal mixture of the two preparations (40 µg. est. protein each).

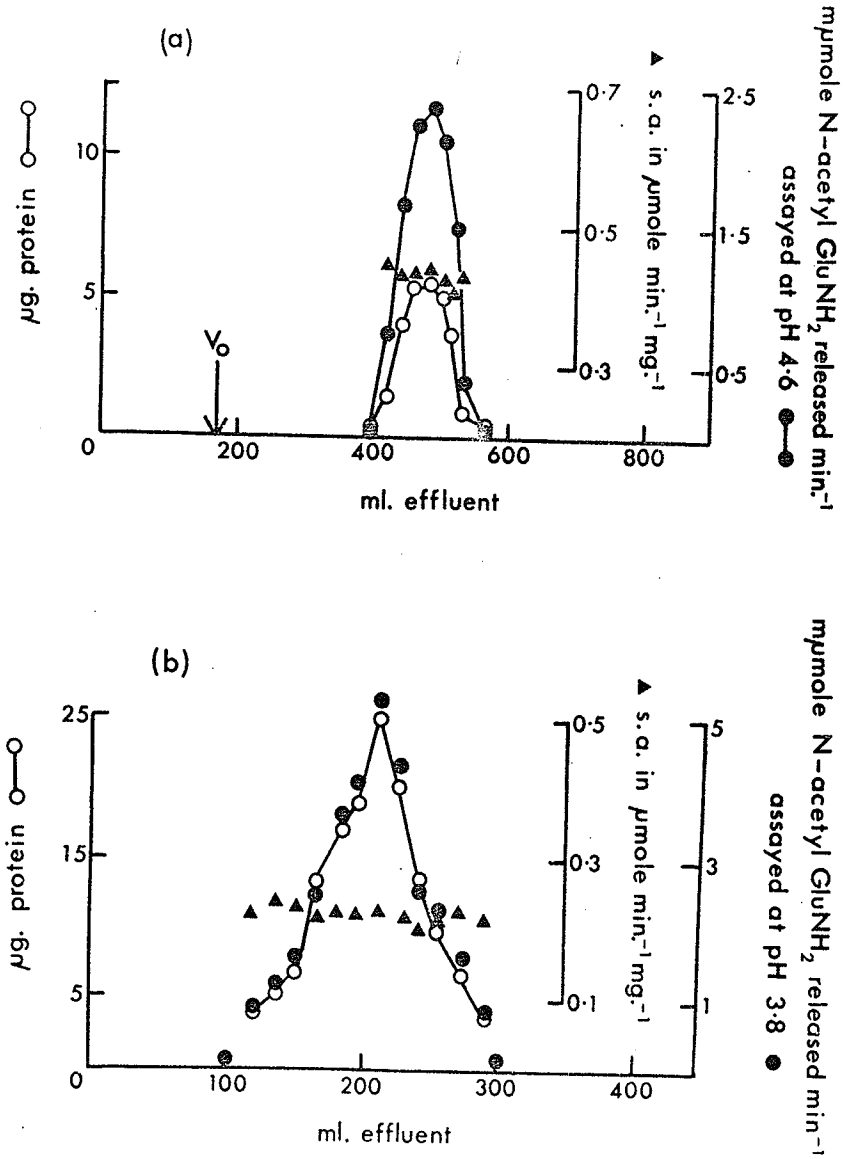


Fig. 14 (a) Chromatography of 0.5 mg. submandibular -gland hyaluronidase on Sephadex G-100 column (2.5 cm. x 80 cm.) in the absence of salt. (b) Chromatography of 2.3 mg. submandibular-gland hyaluronidase on CM-cellulose 23 column (1.5 cm. x 14 cm.). The column was previously equilibrated to pH 5.0 with 0.02 M-sodium acetate buffer, pH 5.0 containing 0.05 M-NaCl and was eluted by a linear salt gradient formed by 150 ml. each of (a) the equilibrating buffer, (b) 0.02 M-sodium acetate buffer containing 0.14 M-NaCl.

Gel filtration on Sephadex G-150

The concentrated enzyme solution from the CM-cellulose 32 column was applied on a Sephadex G-150 column (2.5 cm. x 90 cm.) previously equilibrated with 0.2 M-NaCl. The active effluents were pooled as shown in Fig. 12d and then concentrated and desalted in a Model 50 ultrafiltration cell with an XM-50 membrane.

b) Results

When canine submandibular-gland hyaluronidase was purified on five separate occasions according to the method just described, each preparation had a specific activity within the range of 2.4 to 2.7 μmole N-acetyl-glucosamine /min./mg. protein (Fig. 12 and Table 8). This represents a 171 to 193 fold increase in specific activity over that of the unfractionated submandibular-gland extract.

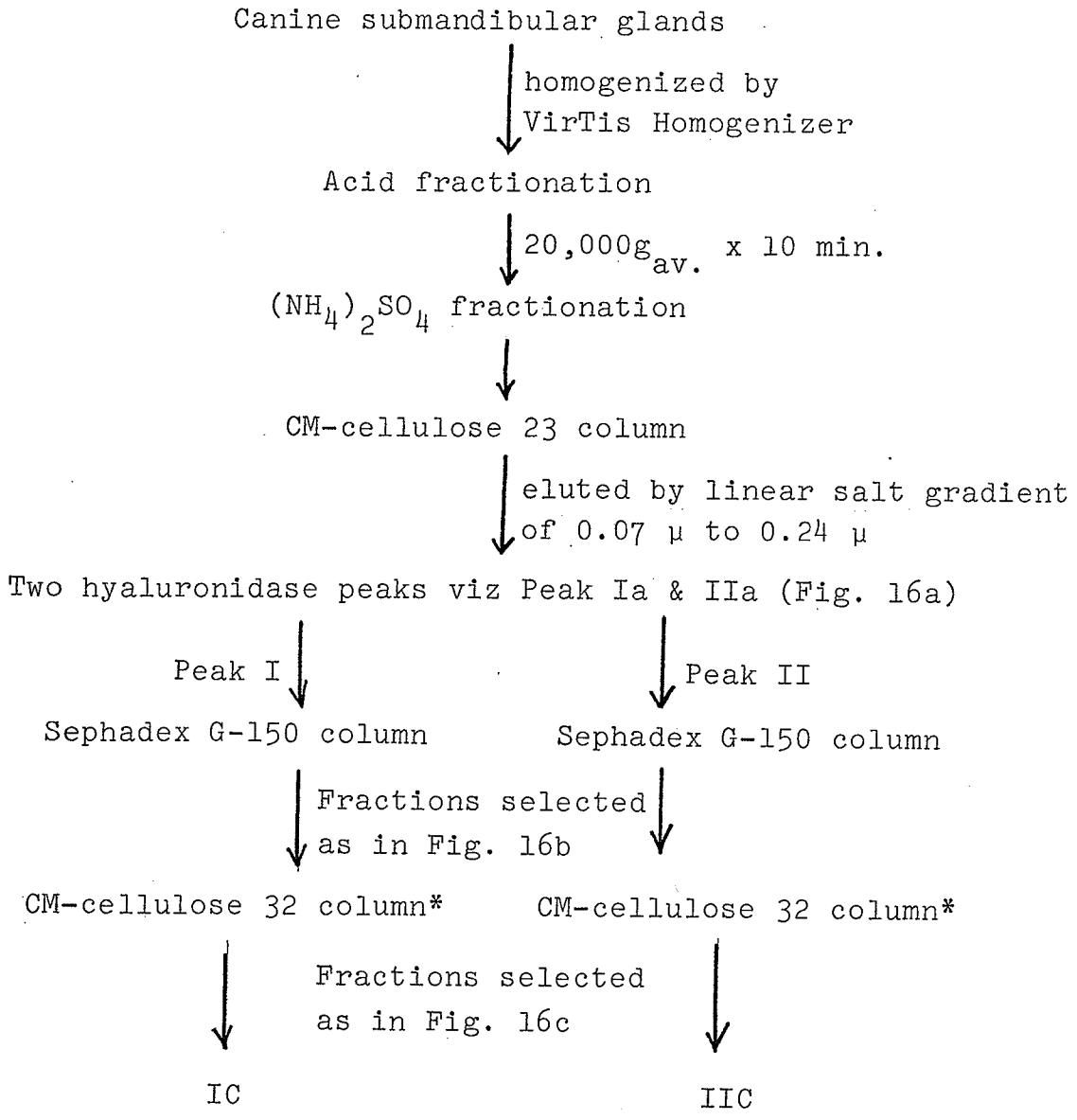
Electrophoresis of three of these preparations on polyacrylamide gel showed one band staining intensely for protein plus a very faint extra band (Fig. 13). That this band which stains intensely for protein is in fact hyaluronidase is discussed in the following page. The purity of a pooled sample of two of these three preparations was also tested by chromatographing it on a Sephadex G-100 column in the absence of salt (Fig. 14a) and on a CM-cellulose column (Fig. 14b). The protein recovery from these chromatograms was quantitative in each case. The specific activity values in all the effluent fractions where activity could be measured were fairly constant (in Fig. 14a and Fig. 14b). This is also a good indication of enzyme homogeneity.

That the single intense protein band obtained during electrophoresis of the three enzyme preparations just described was submandibular-gland hyaluronidase was indicated by the recoveries of 8 to 15% of the total hyaluronidase applied from the analogous region of their respective duplicate gels (refer Section IV.5) and by the absence of any activity in the rest of the gel. This low recovery of enzyme activity may possibly be due to the loss of enzyme activity by heat denaturation of the enzyme during electrophoresis.

The other two batches of purified enzyme when examined on disc-gel electrophoresis were shown to contain one or two more minor components.

It can be seen from Fig. 14a that canine submandibular-gland hyaluronidase is adsorbed on a Sephadex G-100 column, in the absence of salt. In this case the V_e/V_o ratio calculated for the enzyme was 2.8, whereas if the enzyme was eluted by 0.2 M-NaCl the V_e/V_o ratio was 1.33. It is interesting to note that bovine-testicular hyaluronidase was also found to be adsorbed to Sephadex G-75 columns in the absence of salt (35). This may indicate that similar ionic groups are present in both the testicular and submandibular hyaluronidase and that these are responsible for the interaction of the enzyme(s) with the carboxyl groups of Sephadex. This interaction is thought to be responsible for the adsorption of proteins to Sephadex in the absence of salt (61).

Fig. 15 Flow chart for the purification of canine submandibular-gland hyaluronidase in which a more gradual salt gradient was used during CM-cellulose chromatography.



* Eluted by a linear salt gradient of 0.07 μ to 0.16 μ

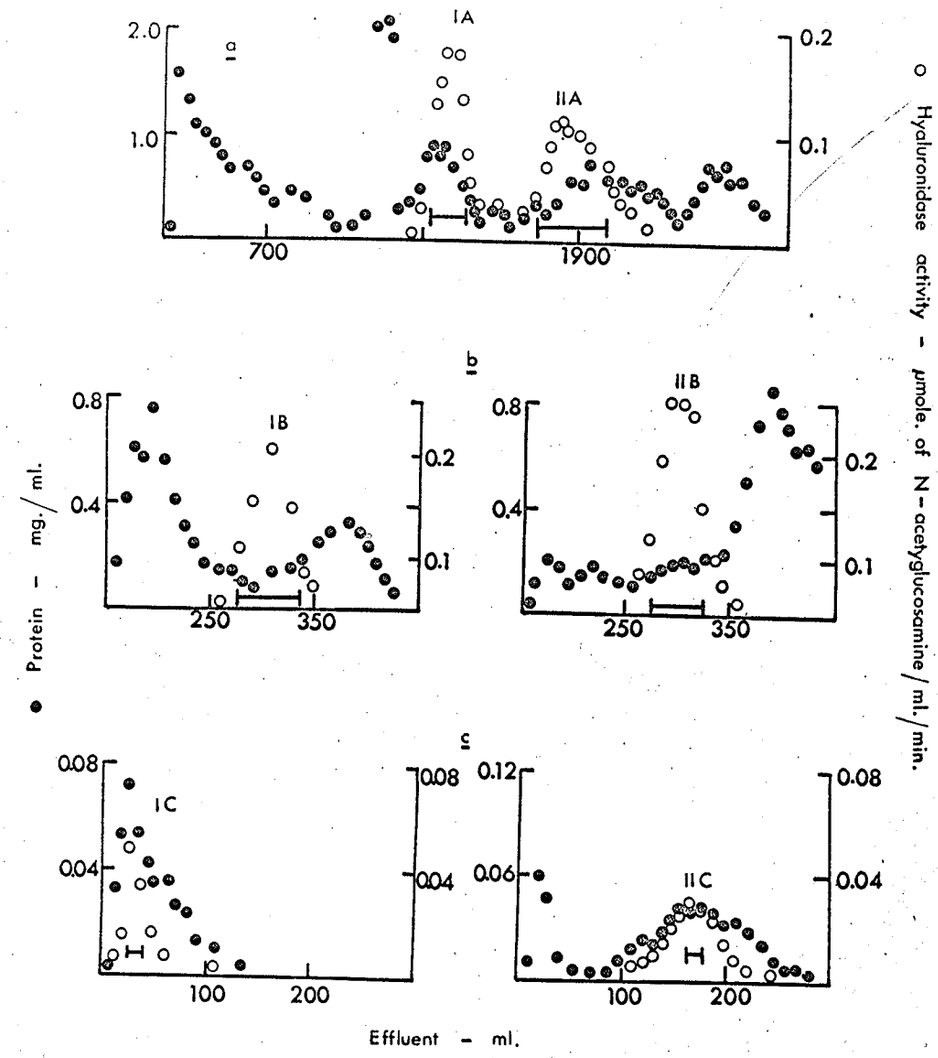


Fig. 16 Purification of canine submandibular-gland hyaluronidase.

The sodium acetate buffer described in these experiments was pH 5.0 and 0.02 M. The active effluent fractions which were pooled are indicated between ⊢——⊣.

(a) Chromatography of the enzyme previously fractionated by $(NH_4)_2SO_4$ (1 g. of assayed protein) on CM-cellulose 23 column (2.5 cm. x 80 cm.). The column was eluted first by a stepwise procedure at a rate of 100 ml./hr. with 300 ml. each of (i) 0.03 M-NaCl (ii) 0.05 M-NaCl both in sodium acetate buffer and then by a linear salt gradient formed by 1 l. each of (i) 0.05 M-NaCl (ii) 0.22 M-NaCl, both in sodium acetate buffer.

(b) Chromatography of peak IA and peak IIA active material on Sephadex G-150 column (2.5 cm. x 90 cm.). The column was eluted by 0.2 M-NaCl at a flow rate of 26 ml./hr.

(c) Rechromatography of peak IB and peak IIB material on CM-cellulose 32 column (1.5 cm. x 14 cm.). The column was eluted at a flow rate of 30 ml./hr. by a linear salt gradient formed by 130 ml. each of (i) 0.05 M-NaCl (ii) 0.14 M-NaCl, both containing sodium acetate buffer.

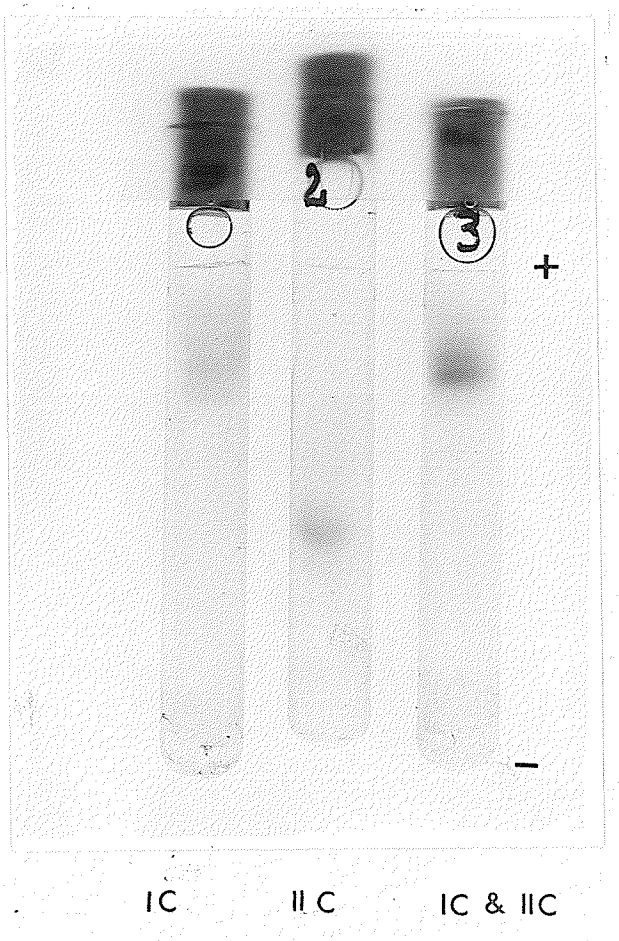


Fig. 17 Disc gel electrophoresis of Peak IC (38 µg. protein), Peak IIC (32 µg. protein) and Peak IC & IIC (38 µg. protein) at pH 4.5. In each case the current was 6 ma. for 70 min.

3) Binding of Hyaluronidase to Other Material in Submandibular-Gland Extracts

a) Methods

A few batches of canine submandibular glands were fractionated by a modification of the method described in the preceding section. A summary of the modified procedure is given in a flow chart (Fig. 15).

b) Results and Discussion

Using this modified fractionation procedure the hyaluronidase of canine submandibular-gland extract was separated into two peaks from the CM-cellulose column (Fig. 16a and Table 9 - Appendix). Only peak II on further fractionation yielded a homogeneous enzyme preparation on polyacrylamide gel electrophoresis (Fig. 13).

Electrophoresis of preparations IC and IIC (Fig. 17) on polyacrylamide gel indicates that I was still heterogeneous although the specific activity was as high as peak II material prior to the final chromatography on CM-32 cellulose (Table 9 - Appendix). All components of peak IC were slower in electrophoretic mobility than the single component of peak IIC. The difference in electrophoretic mobility of peak IC and IIC may be explained by the binding of peak IIC hyaluronidase to one of the components of peak IC during electrophoresis. This is clearly evident in Fig. 17 which shows electrophoresis of an equal mixture of peak IC and IIC. Further evidence that peak I and II contain the same enzyme, comes from the pH vs. activity curves at various times of incubation

(Fig. 18 - Appendix) and Km studies (Table 10 - Appendix) in which no differences were detected between the two types of material. Substrate specificity studies of the two types of material (Table 11 - Appendix) also indicated no differences. Further evidence that peak I and II contain the same enzyme comes from the fractionation procedure in which a steeper salt gradient was used on CM-cellulose 23 column (cf. Fig. 12a and Fig. 16a). This produced a single enzyme peak which, on further fractionation on CM-cellulose columns and finally on Sephadex G-150 column gave a single band during polyacrylamide gel electrophoresis (Fig. 13).

Although the modified fractionation procedure gives a lower yield of purified enzyme (cf. Table 8 and Table 9 - Appendix), it is nevertheless useful in showing the presence of a material binding hyaluronidase in canine submandibular-gland extracts.

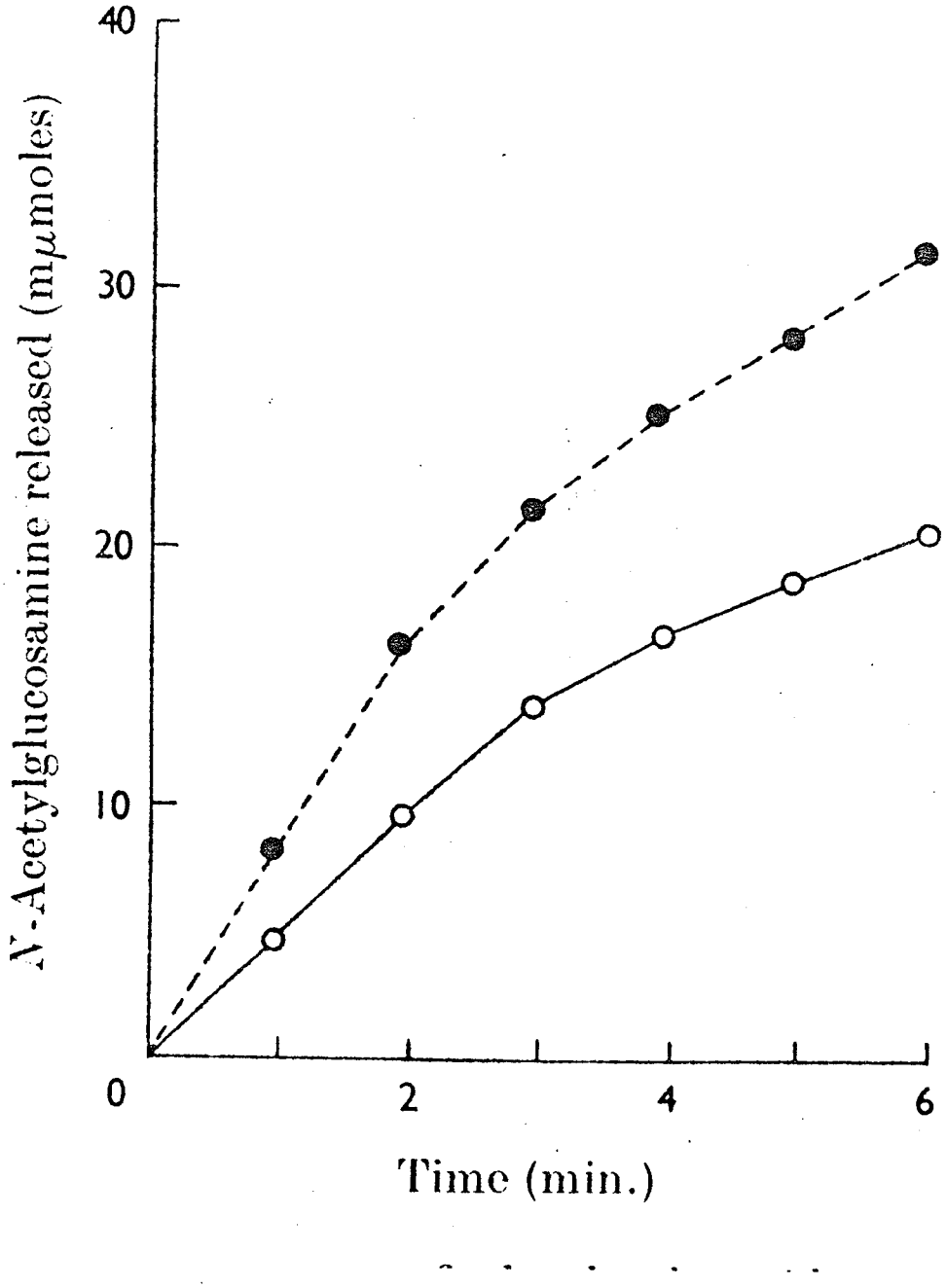


Fig. 19 Progress curve of the hyaluronidase reaction. A sample (16 µg.) of preparation S_3 was incubated at 37° and pH 3.8 with: ●, 1.2 mg. of hyaluronate/ml.; ○, 0.5 mg. of hyaluronate/ml.

4) Some General Enzyme Properties

a) Methods

Effects of time, protein concentration and pH were studied on several preparations of hyaluronidase of varying degree of purity. The substrate specificity of canine submandibular-gland hyaluronidase was determined by incubating the enzyme with one of the following polysaccharides: chondroitin 4-sulphate, chondroitin 6-sulphate, dermatan sulphate, desulphated chondroitin 4-sulphate and hyaluronate. In the studies of Km values for canine submandibular-gland hyaluronidase an incubation time of 2 min. was used to determine the initial velocity of enzyme action against various concentrations of hyaluronate (Fig. 22a & b).

b) Results and Discussion

Effects of time, protein concentrations and pH

Fig. 19

shows that the release of N-acetylglucosamine end groups was linearly related to time only for the first 2 min. Aronson and Davidson (74) found no decrease in rate of N-acetylglucosamine release until after 15 min. 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 2 min. was used to determine the initial velocity for Fig. 22. In the standard assay an incubation

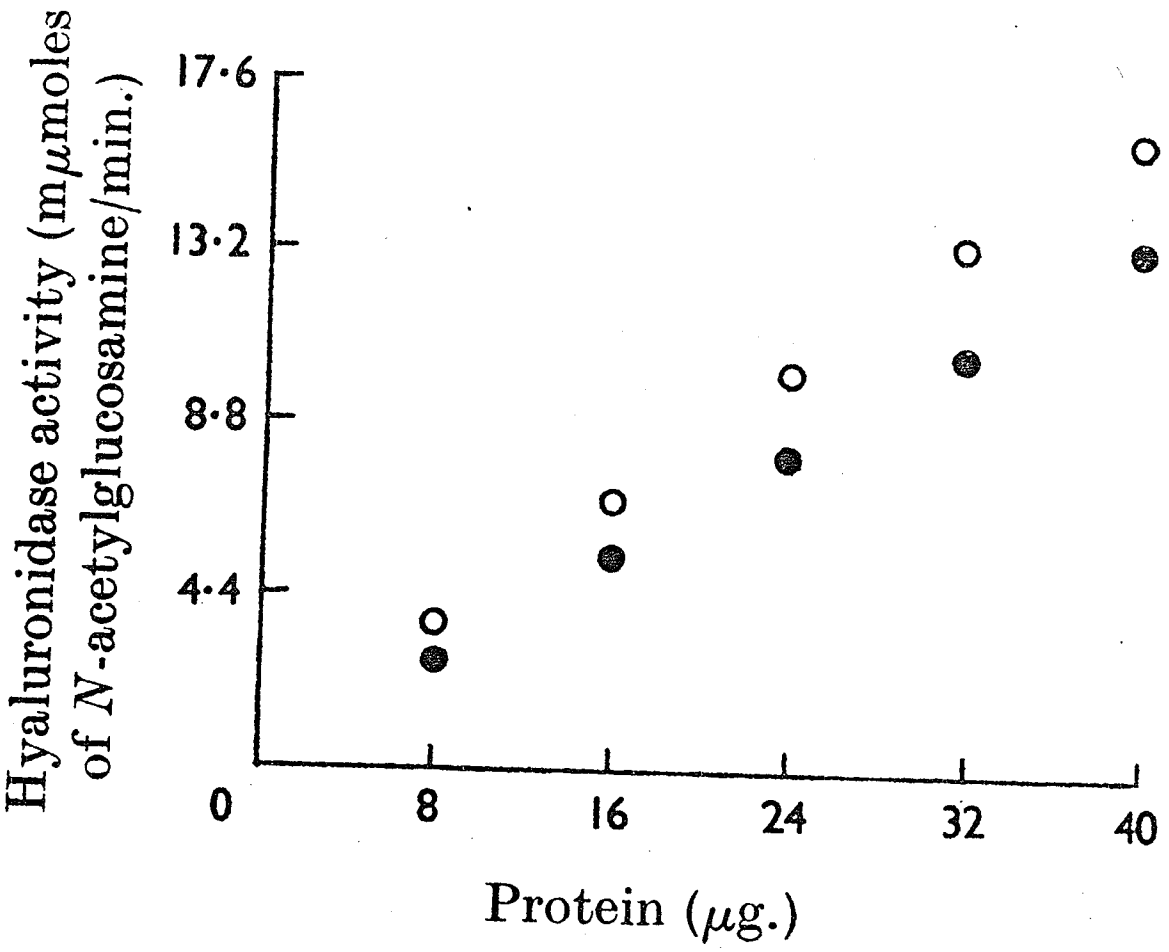


Fig. 20 Velocity and enzyme concentration. Preparation S_3 (O) or preparation MLP_3 (●) was incubated at 37° for 5 min. at pH 3.8 with 1.2 mg. of hyaluronate/ml. of incubation mixture.

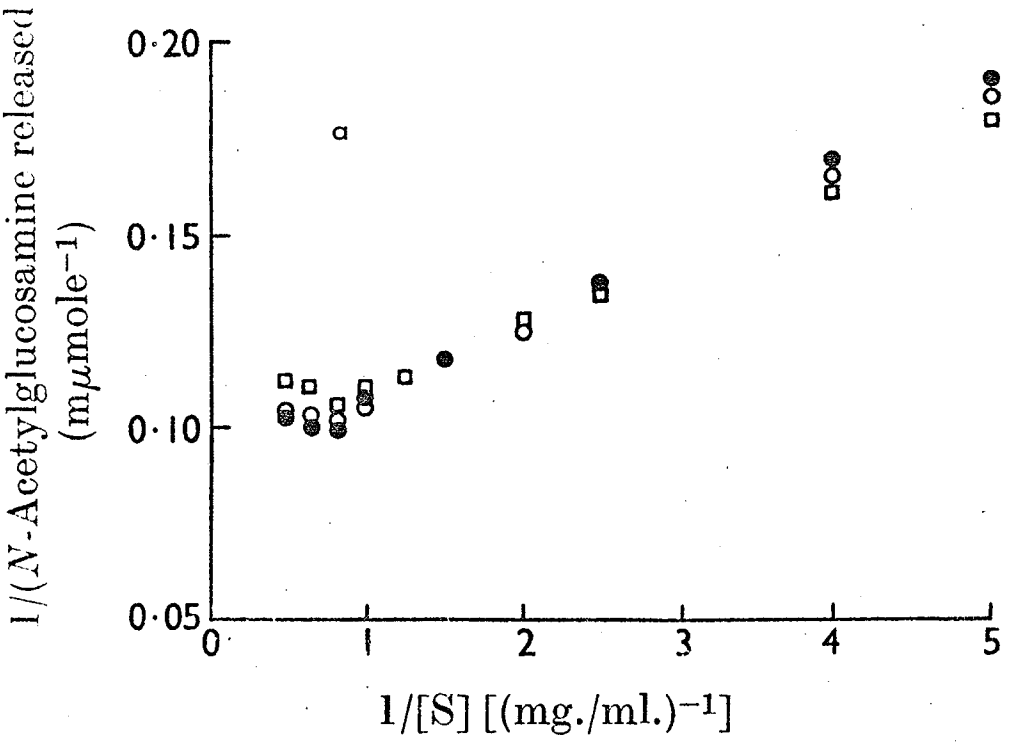


Fig. 22a Reciprocal plot of velocity and substrate concentration for preparation S_3 (16 µg.) or preparation MLP_3 (20 µg.) incubated at 37° and pH 3.8 with two different hyaluronate preparations. ○, Preparation S_3 with hyaluronate A; ■, preparation MLP_3 with hyaluronate A; □, preparation S_3 with hyaluronate B.

time of 5 min. was used to obtain adequate colour development for assays with smaller amounts of activity.

Fig. 20 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 5 min., and that inhibitor(s) reversibly combining with the 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. 21 - Appendix). 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. 22a and Fig. 22b*)

With preparation A as substrate (unfractionated human umbilical-cord hyaluronate), Km values of 0.240, 0.242 and 0.265 mg. of hyaluronate/ml. of incubation mixture were obtained with hyaluronidase preparation MLP_3, preparation S_3 (Table 7) and the homogeneous hyaluronidase preparation respectively. With hyaluronate B (a high-molecular-weight fraction from a commercial umbilical-cord

* in Appendix

TABLE 12

ACTIVITY OF HYALURONIDASE PREPARATIONS AGAINST DIFFERENT SUBSTRATES

The characteristics of the substrates are listed under "Materials" in Section III. 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 1.2 mg./ml. and the temperature was 37°. The weight (µg. of assayed protein) of each enzyme preparation (lettered as in Table 7) is given in parentheses. These weights were calculated to give the same amount of activity in each case. The standards used were N-acetyl-glucosamine for hyaluronate and N-acetyl-galactosamine for chondroitin sulphates.

Method of assay	Enzyme preparation (µg.) ... Substrate	Product formation (mµmoles of N-acetylhexosamine) in 12 min.				
		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).

hyaluronate) as substrate, a Km value of 0.193 mg. of hyaluronate/ml. of incubation mixture was obtained with hyaluronidase preparation S_3 (Fig. 22a). The difference in Km values obtained with different substrate preparations may be attributed to a greater affinity of the enzyme for higher-molecular-weight substrates (44). The slight upturn in the reciprocal plot at concentrations of hyaluronate exceeding 1.2 mg./ml. of incubation mixture suggests that there was inhibition at high substrate concentration.

Substrate specificity (Table 12)

Submandibular-gland hyaluronidase was found to be most active against sodium hyaluronate of the compounds tested, by either the reductimetric assay or the hexosamine end-group assay after 12 min. incubation. With the reductimetric assay, end-group product formation after 12 min. was only 18-21% as great for chondroitin 4-sulphate or chondroitin 6-sulphate as for hyaluronate. With the hexosamine assay, product formation after 12 min. 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 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 (95, 96, 97). 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 12 min. 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 (23). This method measures the breakdown of sulphated acidic glycosaminoglycans by the decrease in metachromasia with toluidine blue.

Table 12 also shows that canine submandibular-gland hyaluronidase hydrolyses hyaluronate B at a higher rate (7%) than the lower-molecular-weight hyaluronate A. This is in agreement with the finding that the Km for hyaluronate B is lower than that for hyaluronate A (Fig. 22a).

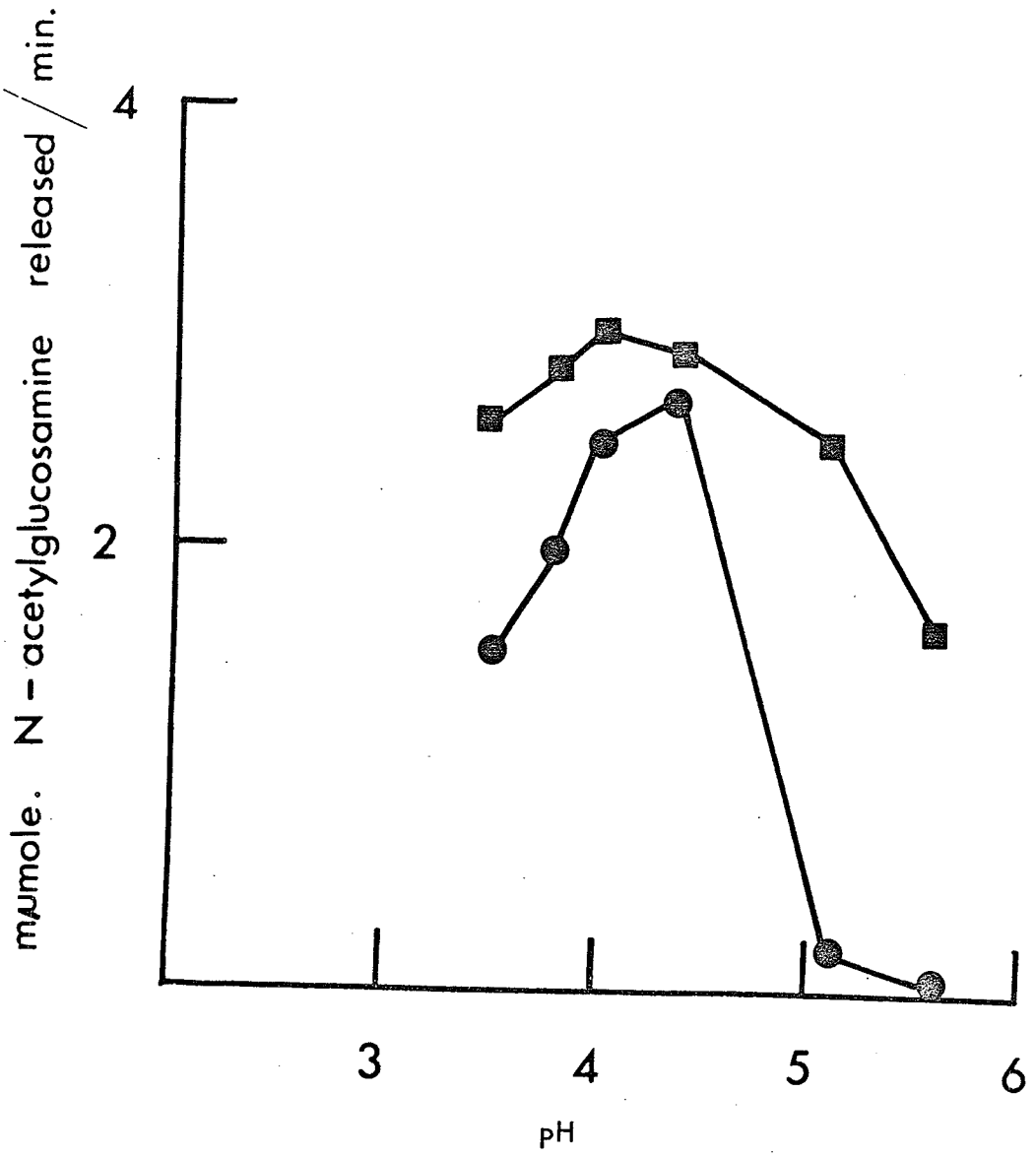


Fig. 23 pH-activity curve of ●, canine submandibular-gland hyaluronidase (sp. activity of 1.4 units/mg. protein) and ■, bovine testicular hyaluronidase (sp. activity of 1000 TRU/mg. protein).

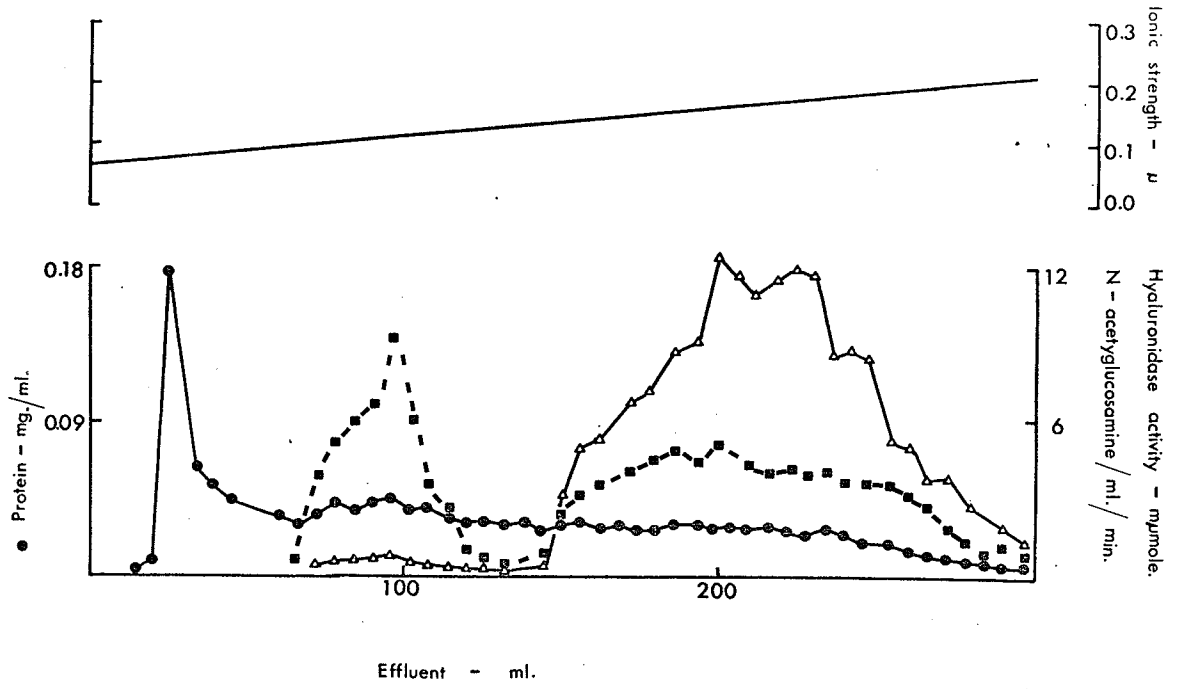


Fig. 24 Chromatography of canine submandibular-gland hyaluronidase (3.2 mg. est. protein; sp. activity of 1.4 units/mg. protein) and bovine-testicular hyaluronidase (4.6 mg.; sp. activity of 1000 TRU/mg. protein) on CM-cellulose 23 column (1.5 cm. x 12 cm.). Elution with a linear salt gradient formed by 270 ml. each of (i) 0.045 M-NaCl (ii) 0.28 M-NaCl, both in 0.02 M-sodium acetate buffer, pH 5.0. ■, hyaluronidase activity at pH 4.0, △, hyaluronidase activity at pH 5.1.

5) Chromatographic Comparison of Canine Submandibular-Gland and Bovine-Testicular Hyaluronidase

a) Method

A weighed amount of commercial bovine-testicular hyaluronidase (4.6 mg.) was dissolved in a solution containing about 3.2 mg. of highly purified canine submandibular hyaluronidase. The mixture was applied to a CM-cellulose 23 column (1.5 cm. x 12 cm.) previously equilibrated with 0.02 M-acetate buffer, pH 5.0, containing 0.045 M-NaCl. The column was eluted by a linear salt gradient and the effluent was collected in 3 ml. fractions. The collected fractions were assayed for hyaluronidase activity at pH 4.0 and 5.1 (Fig. 24).

b) Results and Discussion

At pH 5 canine submandibular-gland hyaluronidase was eluted earlier than bovine-testicular hyaluronidase from a CM-cellulose column. Bovine-testicular hyaluronidase was eluted by an ionic strength of about 0.16 μ. and submandibular-gland hyaluronidase by 0.12 μ. The separation of the two hyaluronidases on CM-cellulose column was followed by using the different ratios of activity pH 4.0 : activity pH 5.1 which they exhibit (Fig. 23). For the highly purified canine submandibular-gland hyaluronidase the ratio of activity at pH 4.0 to 5.1 was 14 whereas that of bovine-testicular hyaluronidase was 1.2.

In Fig. 24 the ratio was 11 to 15 for the first activity peak and 0.3 to 0.7 in the second activity peak, thus indicating

that canine submandibular-gland hyaluronidase is less strongly retained on CM-cellulose than bovine-testicular hyaluronidase.

From these findings it would appear that the active protein from submandibular-gland preparation has a lower positive charge under this range of conditions than the active protein from bovine-testicular preparation. On the assumption that the same charge distribution is involved in the binding of enzyme to negatively charged hyaluronate molecules this would fit with the finding that the submandibular hyaluronidase possesses much less activity than testicular hyaluronidase at pH 5.0 (Fig. 23).

TABLE 13

AMINO ACID ANALYSES OF HYALURONIDASE

Enzyme preparation weighing 2.34 mg. after vacuum drying at 20° and containing 2.13 mg. estimated protein was hydrolyzed in 6 N-HCl at 105° for 20 hr.

		Canine submandibular gland	Testicular (35)
Residue	mμmole residue/g. estimated protein	g. residue/100 g. dry enzyme	
Lys	480	7.72	4.77
His	100	1.71	1.82
Arg	235	4.50	4.37
Asp	640	9.35	7.83
Thr	285	3.72	3.34
Ser	360	4.15	4.38
Glu	838	13.6	6.55
Pro	318	4.00	3.58
Gly	355	2.93	2.13
Ala	605	5.95	2.49
Half Cys	74	0.99	1.89
Val	305	3.94	4.37
Met	18	0.30	1.04
Ile	116	1.68	2.87
Leu	570	8.20	6.13
Tyr	183	3.65	3.98
Phe	253	4.60	3.61
Try	-	-	3.48
Glucosamine	40	0.79	2.17
Galactosamine	0	0	0
Mannose	-	-	5.0
Total		81.78 g.	75.80 g.

6) Composition of Canine Submandibular-Gland Hyaluronidase

a) Methods

The homogeneity of the highly purified hyaluronidase preparations was tested by disc-gel electrophoresis before pooling them for chemical analysis. The pooled homogeneous enzyme was dialyzed at 4° against a thousand times its own volume of glass distilled water for 3 days with three changes of water. The dialyzed enzyme was lyophilized in a Thunberg vessel; 1 ml. of glass distilled water was added to dissolve the dried enzyme and 0.85 ml. of the dissolved enzyme removed from the vessel and placed in a hydrolysis tube. The fluid remaining in the vessel was lyophilized. The weight of the vacuum dried enzyme removed for acid hydrolysis was obtained by the difference in weight of the Thunberg vessel after the first and second lyophilization.

Concentrated hydrochloric acid (0.86 ml.) was added to the hydrolysis tube containing the dissolved enzyme (0.85 ml.) so that the final normality of the acidified solution was 6 N. The hydrolysis tube was evacuated, sealed and left in the oven at 105° for 20 hr. The hydrolysis tube was then unsealed and the hydrochloric acid removed by placing the tube in a vacuum desiccator using sodium hydroxide pellet as the desiccant.

The amino acid and amino sugar composition of the acid hydrolyzed enzyme was then determined on a Beckman Spinco Model 120C Amino Acid Analyzer.

b) Results and Discussion (Table 13)

A comparison of the

composition of canine submandibular-gland hyaluronidase and bovine-testicular hyaluronidase shows that there is a resemblance between the two enzymes except for their glu, ala, met and ileu residues. The content of N-acetyl-glucosamine of the submandibular enzyme was lower than found in bovine-testicular hyaluronidase. This occurrence of amino sugar and also the occurrence of a neutral sugar (mannose) was used by Borders and Raftery as evidence that the bovine-testicular enzyme is a glycoprotein (35). The absence of galactosamine in the submandibular-gland hyaluronidase analysis is a good indication that canine submandibular salivary mucin, which contains galactosamine (98) to the extent of 12.4% is absent from the preparation.

The data in Table 13 was obtained from the analysis of a single submandibular hyaluronidase sample. Thus it can only be regarded as an approximate composition and it would probably be unwise to draw firm conclusions from the observed differences between the canine submandibular-gland hyaluronidase and testicular hyaluronidase.

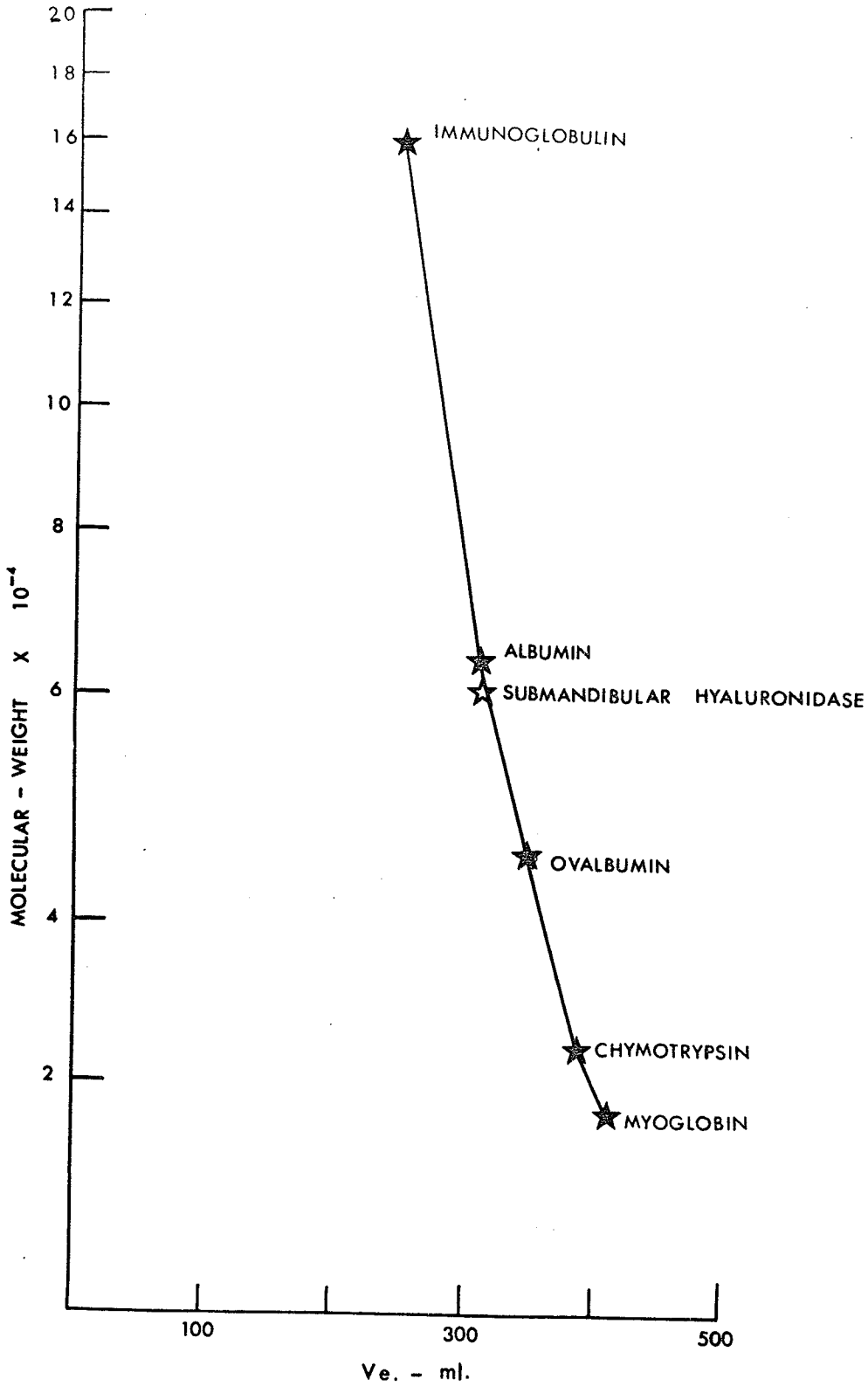


Fig. 25 A plot of the molecular weight of marker proteins against their respective elution volume (V_e).

7) Molecular Weight of Canine Submandibular-Gland Hyaluronidase

a) Methods

The molecular weight of canine submandibular-gland hyaluronidase was determined by gel filtration on a Sephadex G-150 column (2.5 cm. x 94 cm.) using 0.2 M-NaCl as eluant. The purified enzyme was applied on the column together with immunoglobulin, bovine serum albumin, ovalbumin, chymotrypsin and myoglobin. The effluent was collected in 2.5 ml. fractions. The marker proteins were detected in the collected fractions by standard protein assay and the position of hyaluronidase in the effluent was located by its activity.

The Ve values of the marker proteins were then plotted against the log. of their corresponding molecular weight. The molecular weight of hyaluronidase was then obtained graphically from this plot (Fig. 25).

b) Results

Hyaluronidase was eluted soon after the elution of bovine serum albumin from Sephadex G-150 column. A log. plot of molecular weights of the standard proteins against their corresponding Ve values indicates the molecular weight of canine submandibular-gland hyaluronidase to be 60,000 (Fig. 25) on the assumption that the enzyme is a globular protein.

8) General Discussion and Conclusions

It is apparent from the subcellular distribution of hyaluronidase (Section V) that about 40% of the enzyme in submandibular-gland extract was sedimentable and 60% was in the soluble supernatant fraction. The submandibular-gland extract was viscous; this is presumably due to the content of mucin (98). The purification procedure described for the sedimentable and non-sedimentable hyaluronidase was designed to remove the mucin or mucin plus sucrose of the extraction media, before fractionation of the remaining proteins to obtain an active hyaluronidase fraction. However this procedure is only useful for the fractionation of a small amount of dog glands (5 to 30 g.). Only a limited volume of crude extract (not exceeding 6% of the bed volume of the Sephadex G-200 column used) could be applied on the Sephadex G-200 column in the first step of enzyme fractionation.

Results from the purification of sedimentable and non-sedimentable hyaluronidase (Fig. 8 and Fig. 9 and Table 7) 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 2 M-sodium chloride—0.1 M-sodium phosphate buffer, pH 6.0.

The two subcellular fractions of submandibular-gland hyaluronidase were found to have almost identical Km values, and both were inhibited by hyaluronate concentrations exceeding

1.2 mg. of sodium hyaluronate/ml. (Fig. 22a). Results of substrate specificity studies (Table 12) and pH-activity curves (Fig. 2f*) also showed no significant differences between the two. In the preceding section (Section V.7) of this thesis it was shown that end products of the action of sedimentalbe and non-sedimentable hyaluronidase fractions on hyaluronate were the same (Fig. 7 and Table 6). From the properties studied of the enzyme from these two subcellular situations 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 S, may be associated with a particular concentration of the same hyaluronidase.

In view of the limitation on the volume of extract that could be fractionated at a time by the purification method just discussed another purification procedure described in Section VI.2 was designed to fractionate a larger amount of dog glands (100 to 200 g.). In this method, the use of Sephadex G-200 column as the first step to remove mucin was replaced by precipitation of the mucin by acidifying the extract to a pH of 3.5 - 3.6. The acid fractionation of mucin was found to be quicker and simpler than gel filtration on Sephadex G-200 in the removal of submandibular mucin.

* Appendix

When dog submandibular glands were fractionated by this method homogeneous submandibular hyaluronidase of high specific activity was obtained (Table 8).

An attempt was made to reduce the number of CM-cellulose column chromatography steps in the fractionation procedure. This was done by using a more gradual salt gradient than the one previously used to elute the first CM-cellulose column (Fig. 16a and Table 9*). This produced two active hyaluronidase peaks instead of the single peak found when a steeper salt gradient was employed. The data on the electrophoresis of IC, IIC and an equal mixture of IC and IIC indicated the presence of a material binding hyaluronidase in active peak IC material (Fig. 17). Combination of peak I and II active material did not result in a significant change in the average activity of the mixture thus indicating that the enzyme activity is not effected by the binding process which retards the hyaluronidase on disc-gel electrophoresis.

The V_e/V_o ratios calculated for peak I and peak II on Sephadex G-150 during purification of the two peaks did not show any significant difference between the peaks (Fig. 16b). It is possible that the material binding hyaluronidase may be a low molecular weight component. If this was so the V_e/V_o ratios of the two peaks would not show any significant difference. Alternatively the material binding hyaluronidase may be attached only at the low pH and ionic strength used in disc-gel electrophoresis and not at the high ionic strength of 0.2 M-NaCl used during gel filtration on Sephadex G-150

* Appendix

column.

Although the nature of the material binding hyaluronidase has not been investigated it is possible that it may be related to the concept of a carrier protein described by Malgrem (34) for testicular hyaluronidase. It is also possible that the material binding to hyaluronidase may have a non-protein component.

The attempt to reduce the number of steps in enzyme fractionation by using a more gradual salt gradient on CM-cellulose column did not result in any improvement in recovery or simplification of the purification procedure. Instead the recovery of enzyme activity was lower and the recovery of a homogeneous enzyme preparation even less than the original method. However the method was nevertheless useful in indicating the presence of a material binding to hyaluronidase in canine submandibular-gland extracts.

A comparison of the Km values of the partially purified sedimentable, non-sedimentable hyaluronidase and homogeneous hyaluronidase preparations (Section VI.4) shows that there is no significant difference between the three. This indicates that the use of an acid pH in the first fractionation step and the pooling of sedimentable and non-sedimentable hyaluronidase fractions did not significantly effect the Km (see also SectionVI.4 for a comparison of sedimentable and non-sedimentable fractions).

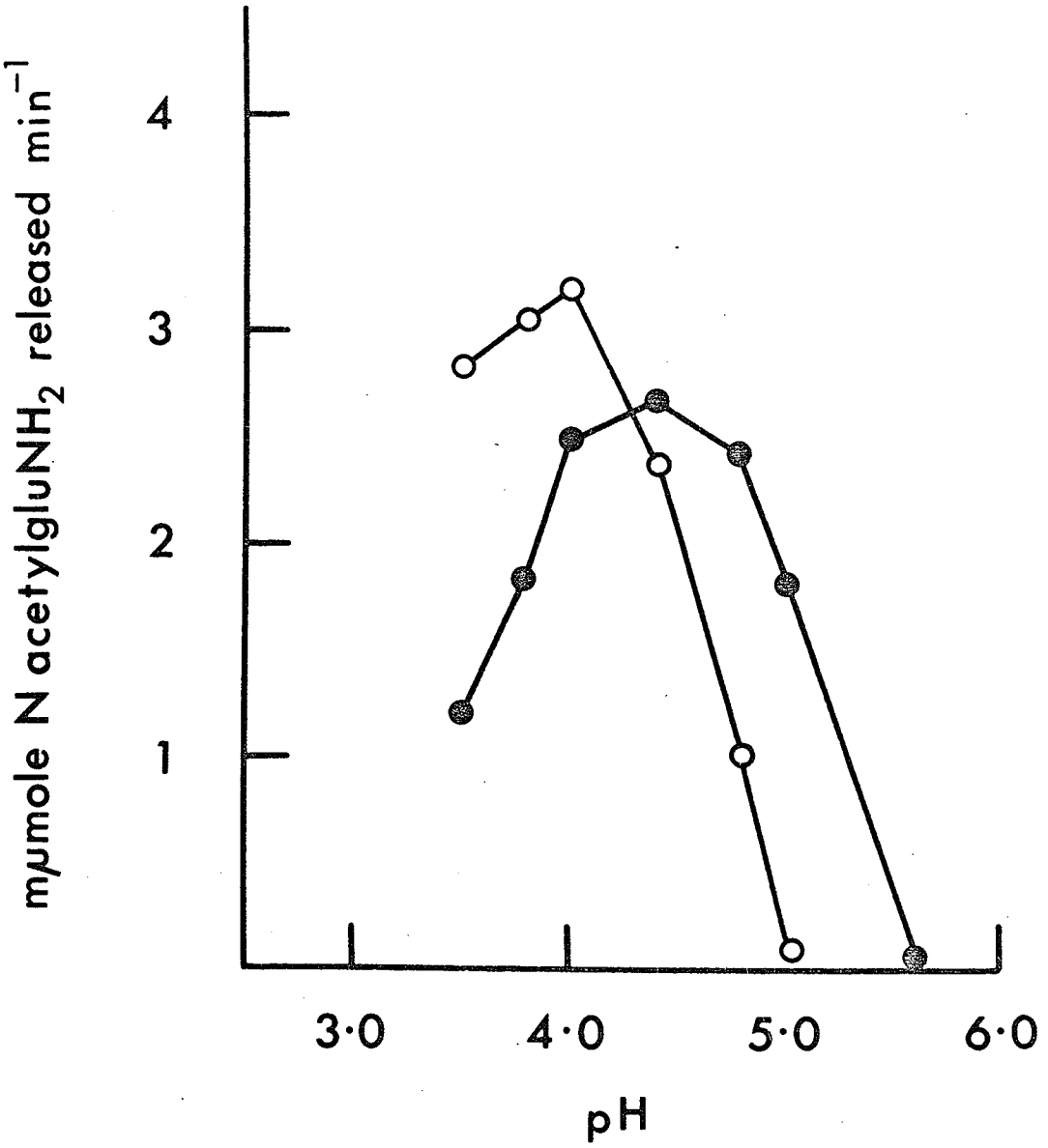


Fig. 26 pH-activity curves of unfractionated submandibular -gland extract ○ (750 μg. est. protein) and of ●, purified submandibular hyaluronidase (9 μg. est. protein), homogeneous on disc gel electrophoresis. The mixture was incubated for 15 min. at 37° in sodium acetate buffer.

VII. CHANGES IN pH ACTIVITY CURVE OF PURIFIED ENZYME PREPARATIONS

a) Methods

The pH vs. activity curve for an unfractionated canine submandibular-gland hyaluronidase was compared with that of a homogeneous submandibular hyaluronidase (Fig. 26). The activity was measured by the modified standard procedure (Section IV.1) using sodium acetate buffers without gelatin.

The effects of incubation in the presence of substrate was studied by varying the time of incubation during the assay of the purified enzyme at pH 3.8 and pH 4.4. The effect of incubation of the enzyme in the absence of substrate was also studied by preincubating the purified enzyme for 1 hr. in 0.01 M-sodium acetate buffer at pH 3.5, 3.8, 4.0, 4.4, 5.0 and 5.6 respectively or for 16 hr. in 0.01 M-sodium acetate buffer at pH 4.0. The preincubated enzyme was then assayed for hyaluronidase activity at pH 3.8 and pH 4.4.

b) Results

The pH optimum and pH-activity curve of the crude hyaluronidase and purified hyaluronidase (Fig. 26 and see also Fig. 5, 18 and 23) shows that there is a shift in the pH optimum from pH 3.8-4.0 to 4.4 in the case of the purified enzyme. The data in Table 14 shows firstly that this shift occurs as the enzyme is purified. This can be seen by comparing the ratios of activity at pH 4.4 over pH 3.8 for the various enzyme preparations of increasing purity. Secondly, increasing the time of incubation during

TABLE 14

RATIO OF HYALURONIDASE ACTIVITY AT pH 3.8 AND pH 4.4

The concentration of hyaluronate used was 1.2 mg./ml.

Sample	O.D. at 585 mμ. Test minus Blank at pH 3.8	Specific activity μmole N-acetylGlu-NH_2 /min./mg. at pH 3.8	Ratio of activity at pH $\frac{4.4}{3.8}$	Incubation time min.
Crude extract †	0.159	0.008	0.78	15
Fraction 1 †	0.165	0.080	0.88	15
Fraction 2 †	0.159	0.144	1.08	15
Fraction 3 †	0.162	0.792	1.20	15
Homogeneous preparation †	0.168	0.440	1.67	15
Fraction 3	0.162	0.792	1.20	15
	0.276	Not applicable	1.31	30
	0.316	Not applicable	1.93	60
	0.541	Not applicable	1.83	120
Fraction 3 preincubated 37° for:-				
1 hr.	0.107	0.520	2.10	15
16 hr.	0.076	0.375	1.77	15

† The $\frac{\text{Test - Blank}}{\text{Conc. of Hyaluronate}}$ ratios of these preparations are fairly constant.

TABLE 15

EFFECTS OF PREINCUBATION AT DIFFERENT pH

Canine submandibular-gland hyaluronidase (specific activity of 0.792 µmole N-acetylGlu-NH_2 /min./mg. est. protein) was preincubated without substrate for 1 hr. at 37° in 0.01 M-sodium acetate buffer. Hyaluronidase activity was then assayed at pH 3.8 and 4.4 using an incubation time of 15 min.

pH of preincubation	Specific activity µmole N-acetylGlu-NH_2/ min./mg. est. protein at pH 3.8	Ratio of activity at pH $\frac{4.4}{3.8}$
3.5	0.642	2.44
3.8	0.542	2.00
4.0	0.520	2.10
4.4	0.520	1.77
5.0	0.530	1.97
5.6	0.582	1.51

assay of the purified enzyme causes an increase in the ratio. Thirdly, preincubation of the enzyme without substrate causes an increase in the ratio.

c) Discussion

The change in ratio of activities $\left(\frac{\text{activity at pH 4.4}}{\text{activity at pH 3.8}}\right)$ appears to be due to a transformation of one active form to another at 37°, since the specific activity at pH 4.4 measured after preincubation for 1 hr. at 37°, was slightly higher than the specific activity at pH 3.8 before incubation (Table 14). Also this transformation was shown to occur faster at a more acid pH of 3.5-4.0 than at 4.0-5.6 (Table 15).

The shift in pH optimum in Fig. 26 from 3.8-4.0 to 4.4 only occurs after enzyme purification. Although a material binding to hyaluronidase was shown to be present in the canine submandibular-gland extract (Section VI.3) it is unlikely that the removal of this particular material could have caused the shift in pH optimum during purification, since a comparison of the pH-activity curve at different times of incubation (Fig. 18 - Appendix) of peak IC (4 bands on electrophoresis) and peak IIC (one band) shows that there is no difference between the two.

A shift in pH optimum from 6.0 to 5.0 was observed by Houck (99) for bovine-testicular hyaluronidase when the concentration of hyaluronate was increased from 1/4 to full saturation (1.6 mg./ml.). He also reported a shift and

broadening of the pH optimum for the testicular enzyme when the concentration of active enzyme was increased from 0.40 to 40 TRU/ml. These changes in pH optimum thus appeared to be associated with a change in the $\frac{\text{enzyme}}{\text{substrate}}$ ratio. This is not the case with the shift in pH-activity curve described in this section of the present thesis. The (Test-Blank) values in Table 14 show that the $\frac{\text{enzyme}}{\text{substrate}}$ ratio was fairly constant in the assays carried out at various stages of enzyme purification. In addition, it was found (Table 14) that the shift occurred at 37° after preincubation in the absence of substrate.

It is not known whether this shift in pH optimum has any physiological significance but it was found that the pH optimum of an unfractionated submandibular-gland hyaluronidase previously prepared by homogenizing a fresh dog gland at room temperature and kept at room temperature was at pH 4.0. This suggests that at 37° in the presence of materials such as those in the crude glandular extract the pH optimum would be at pH 4.0, but it is not known whether there are circumstances in the gland (such as compartmentation) which might produce the same effect as purification in vitro.

VIII. CONCLUSIONS

An enzyme which attacks hyaluronate producing the same oligosaccharide products as hyaluronidase of mammalian testes was found to occur in the submandibular glands of dogs. The amount of hyaluronidase activity extractable from the dog gland was comparable to that from the bovine testis which is one of the most potent source of mammalian hyaluronidase.

It was found that 15% of the hyaluronidase in the dog submandibular-gland extract was associated with the lysosomal fraction (L) as opposed to 28% in bone (32). A high percentage (50-60%) of the enzyme was found in the non-sedimentable fraction S. Practically all of the particulate-bound hyaluronidase was found to be liberated by Triton X-100 or high speed homogenization, as in the case with liver and bone lysosomal enzymes.

The hyaluronidase activity could also be detected in dog submandibular saliva. The physiological significance of the hyaluronidase if any is not known; however, the lack of the enzyme in human submandibular and parotid saliva may suggest a particular digestive function in the dog.

Comparison of the properties of the enzyme from the sedimentable fractions and the supernatant fraction (S) did not reveal any significant differences between the two. It is possible that hyaluronidase in the supernatant fraction (S) is derived from the sedimentable particles either in vivo or during the preparative procedure. Alternatively each subcellular compartment of the submandibular gland cells may be associated

with a particular concentration of the same hyaluronidase.

Methods for the purification of the hyaluronidase from dog submandibular-gland extracts were presented. Highly purified hyaluronidase of specific activity of up to 2.7 units/mg. protein was obtained. This compares favourably with the figure of 1.29 units/mg. protein which was reported for the hyaluronidase purified from rat liver lysosomes (36). The purified enzyme was found to be homogeneous by electrophoresis at pH 4.5 on polyacrylamide gel and by chromatography on CM-cellulose and Sephadex G-100 columns. The molecular weight of the purified submandibular hyaluronidase was determined by gel filtration on Sephadex G-150 column and found to be 60,000 on the assumption that the enzyme is a globular protein.

During the course of enzyme fractionation evidence for the presence of a material which binds hyaluronidase in submandibular-gland extracts was observed. Although the nature of such a material was not investigated, it may be related to the concept of a carrier protein described by Malgrem (34) for testicular hyaluronidase.

A comparison of the composition of canine submandibular-gland hyaluronidase and bovine-testicular hyaluronidase indicated that there were some similarities between the two enzymes. However, differences in their content of certain amino acids and N-acetyl-glucosamine were also noted.

During chromatography on CM-cellulose at pH 5.0 (Fig. 24) the active material from canine submandibular-gland

hyaluronidase was more weakly adsorbed than that of bovine-testicular hyaluronidase. Thus it appears that the active protein from submandibular gland has a lower positive charge at pH 5.0 than the active protein from bovine testicular preparations.

The Km and substrate specificity of the submandibular hyaluronidase remained fairly constant at various stages of enzyme fractionation, only the pH-activity curves were found to change with enzyme fractionation. Changes in the ratio of activity at $\frac{\text{pH } 4.4}{\text{pH } 3.8}$ were also observed when the purified hyaluronidase was preincubated at 37° without substrate or when the incubation time for enzyme assay was increased. These changes may be due to a transition from one active form of the enzyme to another. It is not known whether these changes in pH-activity curves have any physiological significance.

BIBLIOGRAPHY

1. Humphrey, J. H. (1946). Biochem. J. 40, 442.
2. Meyer, K. & Rapport, M. (1950). Arch. Biochem. 27, 287.
3. Gibian, H. (1966). In 'The Amino Sugars,' vol. 2B. p. 185, Ed. by Balazs, E. A. & Jeanloz, R. W., New York and London : Academic Press Inc.
4. Houck, J. C. & Pearce, R. H. (1957). J. Biol. Chem. 226, 267.
5. Dodgson, K. S., Lloyd, A. G. & Spencer, B. (1957). Biochem. J. 65, 131.
6. Soda, T. & Egami, F. (1938). J. Chem. Soc. Japan 59, 1202.
7. Pincus, P. (1950). Nature 166, 187.
8. Hartles, R. L., McLean, N.D. (1952). Brit. Dental J. 93, 147.
9. Neuberg, C. & Hofmann, E. (1931). Biochem. Z. 234, 345.
10. Mehl, E. & Jafzkewitz, H. (1963). Z. Physiol. Chem. 331, 292.
11. Held, E. & Buddecke, E. (1967). Hoppe Seyler's Z. Physiol. Chemie. 348, 1047.
12. Dodgson, K. S. (1966). In 'The Amino Sugars,' vol. 2B, p. 202, Ed. by Balazs, E. A. & Jeanloz, R. W., New York and London : Academic Press Inc.
13. Konetzka, W. A., Pelczar, M. J., Jr. & Burnett, G. W. (1954). Bacteriol. Proc. p. 106.
14. Dodgson, K. S. & Lloyd, A. G. (1957). Biochem. J. 66, 532.

15. Martinez, R. J., Wolfe, J. B. & Nakada, H. I. (1959). J. Bacteriol. 78, 217.

16. Linker, A., Hoffman, P., Meyer, K., Sampson, P., & Korn, E. D. (1960). J. Biol. Chem. 235, 3061.

17. Muir, H. (1964). Int. Rev. Connective Tissues Res. 2, 101.

18. Rich, C. (1959). J. Lab. Clin. Med. 54, 381.

19. Bowness, J. M. (1963). Biochim. Biophys. Acta 78, 295.

20. Anderson, S. O. & Graae, J. (1955). Acta Chem. Scand. 9, 1431.

21. Gibian, H. (1966). In 'The Amino Sugars,' vol. 2B. p. 186, Ed. by Balazs, E. A. & Jeanloz, R. W., New York and London : Academic Press Inc.

22. Rapport, M. M., Meyer, K. and Linker, A. (1951). J. Am. Chem. Soc. 73, 2416.

23. Bowness, J. M. (1965). Biochim. Biophys. Acta 101, 26.

24. Meyer, K., Hoffman, P. & Linker, A. (1960). In 'The Enzymes,' vol. 4. p. 447, Ed. by Boyer, P. D., Lardy, H. & Myrback, K., New York and London : Academic Press Inc.

25. Tolksdorf, S. (1954). In 'Methods of Biochemical Analysis,' vol. 1. p. 425, Ed. by Glick, D., London : Interscience publishers, Ltd.

26. Hutterer, F. (1966). Biochim. Biophys. Acta 115, 312.

27. de Salegui, M. & Pigman, W. (1967). Arch. Biochem. Biophys. 120, 60.

28. Bowness, J. M. & Tan, Y. H. (1968). Biochim. Biophys. Acta 151, 288.

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

30. Goggins, J. F., Lazarus, G. S. & Fullmer, H. M. (1968). J. Histochem. Cytochem. 16, 688.

31. Bollet, A. J., Bonner, W. M., jun. & Nance, J. L. (1963). J. Biol. Chem. 238, 3522.

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

33. Gibian, H. (1959). Mucopolysaccharide und Mucopolysaccharidasen, p. 132, Vienna : Franz Deuticke.

34. Malgrem, H. (1953). Biochim. Biophys. Acta 11, 524.

35. Borders, C. L., jun. & Raftery, M. A. (1968). J. Biol. Chem. 243, 3756.

36. Aronson, N. N., jun. & Davidson, E. A. (1967). J. Biol. Chem. 242, 437.

37. Mann, T. (1964). In 'The Biochemistry of Semen & the Male Reproductive Tract,' p. 135, London : Methuen & Co. Ltd.

38. Yamane, J. (1956). Proc. 2nd. World Congr. Fertil. Steril. Naples.

39. Aronson, N. N., jun. & Davidson, E. A. (1968). J. Biol. Chem. 243, 4494.

40. Aronson, N. N., jun & de Duve, C. (1968). J. Biol. Chem. 243, 4564.

41. Bowness, J. M. & Harding, G. (1968). Canad. J. Biochem. 46, 489.

42. Bertino, J. R. (1964). Methods in Med. Research 10, 264.

43. Jeanloz, R. W. & Forchielli, E. (1950). J. Biol. Chem. 186, 495.

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

45. Kantor, T. G. & Schubert, M. (1957). J. Am. Chem. Soc. 79, 152.

46. 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.

47. U. S. Pharmacopeia (1955). 15, 330.

48. Bonner, W. M., jun. & Cantey, E. Y. (1966). Clin. Chim. Acta 13, 746.

49. Park, J. T. & Johnson, M. J. (1949). J. Biol. Chem. 181, 149.

50. U. S. Pharmacopeia (1955). 15, 329.

51. Levvy, G. A. & Conchie, J. (1966). In 'Methods in Enzymology,' vol. 8, p. 577, Ed. by Neufeld, E. F. & Ginsburg, V., New York and London : Academic Press Inc.

52. Kind, P. R. N. & King, E. J. (1954). J. Clin. Path. 7, 322.

53. Babson, A. L. (1965). Clin. Chem. 11, 789.

54. Cooperstein, S. J. & Lazarow, A. (1951). J. Biol. Chem. 189, 665.

55. Levvy, G. A. & Conchie, J. (1966). In 'Methods in Enzymology,' vol. 8, p. 575, Ed. by Neufeld, E. F. & Ginsburg, V., New York and London : Academic Press Inc.

56. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). J. Biol. Chem. 193, 265.

57. Dische, Z. (1955). J. Biol. Chem. 169, 189.

58. Bowness, J. M. (1957). Biochem. J. 67, 295.

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

60. Boas, N. F. (1953). J. Biol. Chem. 204, 553.

61. Sephadex - Gel filtration in Theory and Practice. (1966). Pharmacia Fine Chemicals, Uppsala, Sweden : Printed in Sweden.

62. Reisfeld, R. A., Lewis, U. J. & Williams, D. E. (1962). Nature 195, 281.

63. Greiling, H. (1963). In 'Methods of Enzymatic Analysis,' p. 80, Ed. by Bergmeyer, H. U., New York and London : Academic Press Inc.

64. de Duve, C., Pressman, B. C., Gianetto, R., Wattiaux, R. & Appelmans, F. (1955). Biochem. J. 60, 604.

65. Linker, A. (1966). In 'Methods in Enzymology,' vol. 8, p. 650, Ed. by Neufeld, E. F. & Ginsburg, V., New York and London : Academic Press Inc.

66. Pigman, W., Rizvi, S. & Holley, H. L. (1961). Arthr. & Rheum. 4, 240.

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

68. Vaes, G. & Jacques, P. (1965). Biochem. J. 97, 389.

69. Herveg, J. P., Beckers, C. & De Visscher, M. (1966). Biochem. J. 100, 540.

70. Bowers, W. E., Finkenstaedt, J. T. & de Duve, C. (1967). J. Cell Biol. 32, 325.

71. Cirina, F. (1963). Minerva Stomat. 12, 309.

72. Straus, W. (1967). In 'Enzyme Cytology,' p. 258, Ed. by Roodyn, D. B., London and New York : Academic Press Inc.

73. de Duve, C. (1966). Annu. Rev. Physiol. 28, 435.

74. Aronson, N. N., jun. & Davidson, E. A. (1967). J. Biol. Chem. 242, 441.

75. Schramm, M. (1964). In 'Salivary Glands and their Secretion,' p. 315, Ed. by Sreebny, L. M. & Meyer, J., New York : Pergamon Press Inc.

76. de Duve, C. (1963). In 'Ciba Found. Symp. : Lysosomes,' p. 1, Ed. by de Reuck, A. V. S. & Cameron, M. P., London : J. and A. Churchill Ltd.

77. Cobbin, L. B. & Dicker, S. E. (1962). J. Physiol. 163, 168.

78. Dicker, S. E. & Elliott, A. B. (1963). J. Physiol. 165, 89.

79. Draus, F. J. (1963). Arch. Oral Biol. 8, 219.

80. Bignardi, C., Aureli, G., Balduini, C. & Castellani, A. A. (1964). Biochem. Biophys. Res. Commun. 17, 310.

81. Gianetto, R. & de Duve, C. (1955). Biochem. J. 59, 433.

82. Appelmans, F. & de Duve, C. (1955). Biochem. J. 59, 426.

83. Aronson, N. N., jun. & Davidson, E. A. (1965). J. Biol. Chem. 240, PC 3222.

84. Vaes, G. (1965). Biochem. J. 97, 393.

85. Meyer, K. (1947). Physiol. Rev. 27, 343.

86. Hahn, L. (1946). Ark. Kemi Mineral. Geol. 22A, 1.

87. Hahn, L. (1947). Biochem. Z. 318, 123.

88. Dawes, C. (1967). Arch. Oral Biol. 12, 783.

89. Shannon, I. L. (1967). J. Oral Med. 22, 75.

90. Flodin, P., Gregory, J. D. & Rodén, L. (1964). Analyt. Biochem. 8, 424.

91. Freeman, M. E., Anderson, P., Oberg, M. & Dorfman, A. (1949). J. Biol. Chem. 180, 655.

92. Rapport, M. M., Meyer, K. & Linker, A. (1950). J. Biol. Chem. 186, 615.

93. Gibian, H. (1966). In 'The Amino Sugars,' vol. 2B. p. 196, Ed. by Balazs, E. A. & Jeanloz, R. W., New York and London : Academic Press Inc.

94. Greenwood, C. T. & Milne, E. A. (1968). Adv. Carbohydrate Chem. 23, 337.

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

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

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

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

99. Houck, J. C. (1956). Ph.D. thesis, p.69

APPENDIX

TABLE 5

SUBCELLULAR DISTRIBUTION OF ENZYMES

The activity in each fraction is given as a percentage of the total in fractions E+N. The explanation of the letters and the preparation of the fractions are given in Section V.4.

	% of total activity in fractions E+N					Total hyaluronidase activity mμmole /g./min.
		E				
	N	M	L	P	S	
Pilocarpine treated gland						760
Hyaluronidase	9.8	21.6	13.2	9.8	58.1	
Acid phosphatase	8.2	18.2	11.2	7.4	44.3	
Protein	10.5	12.0	9.6	5.8	68.0	
Control gland						810
Hyaluronidase	12.5	24.1	14.4	7.8	56.0	
Acid phosphatase	6.7	16.4	15.0	10.0	44.5	
Protein	10.8	12.8	6.7	6.8	69.0	

TABLE 9

PURIFICATION OF CANINE SUBMANDIBULAR-GLAND HYALURONIDASE

Step	Specific activity μmole N-acetylGlu-NH_2/ min./mg. est. protein	mg. est. protein	% Yield of extracted activity
Crude extract	0.020	5230	100
Acid ppt.	0.027	3130	80
$(NH_4)_2SO_4$ ppt.	0.050	1020	49
CM-23 cellulose column			
peak IA	0.22	60	12
peak IIA	0.28	90	24
Sephadex G-150 column			
peak IB	1.49	6.2	9
peak IIB	1.69	8.8	15
CM-32 cellulose column			
peak IC	0.51	0.40	0.2
peak IIC	1.02	0.32	0.3

TABLE 10

Km OF PEAK IC AND IIC HYALURONIDASE

An incubation time of 2 min. was used in these experiments. The enzyme activity was assayed by the standard procedure for hyaluronidase. The weight (in µg. estimated protein) of each enzyme preparation is given in brackets.

Preparation	Specific activity µmole N-acetylGlu-NH_2 /min./mg. protein	Km in mg. HA/ml. incubation mixture
Peak IC (8)	0.50	0.255
Peak IIC (4)	1.02	0.268

TABLE 11

ACTIVITY OF PEAK IC AND IIC HYALURONIDASE AGAINST HA AND CS-6

The concentration of each substrate in the incubation mixture was 1.2 mg./ml. and the temperature was 37°. The weight (µg. of assayed protein) of each enzyme preparation is given in parentheses. The enzyme activity was assayed by the modified standard assay procedure for hyaluronidase described in Section IV.1. The standards used were N-acetyl-glucosamine for HA and N-acetyl-galactosamine for CS-6.

Enzyme preparation	Specific activity units/mg. protein	Substrate HA mµmoles N-acetylhexosamine released in 10 min.	Substrate CS-6 mµmoles N-acetylhexosamine released in 10 min.
PEAK IB (5)	1.50	62.5	12.4
PEAK IIB (5)	1.70	68.9	15.5
PEAK IC (15)	0.50	52.8	10.8
PEAK IIC (8)	1.02	56.8	11.4

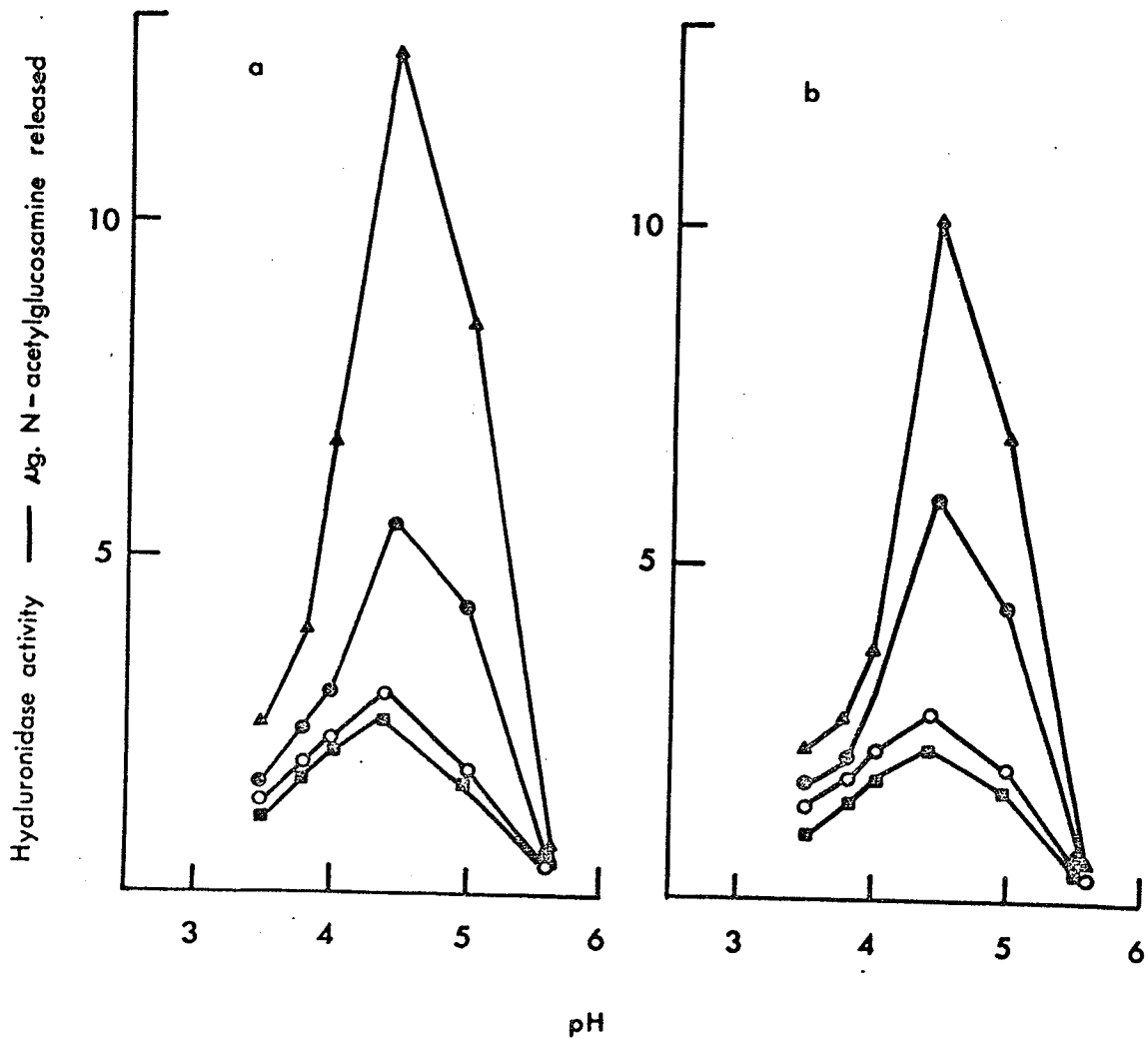


Fig. 18 pH activity curves of (a) Peak IC and of (b) Peak IIC as measured by product formation at various times at 37° with a substrate concentration of 1.2 mg. HA/ml. ▣, 15 min.; ○, 30 min.; ◉, 60 min.; ▲, 120 min.

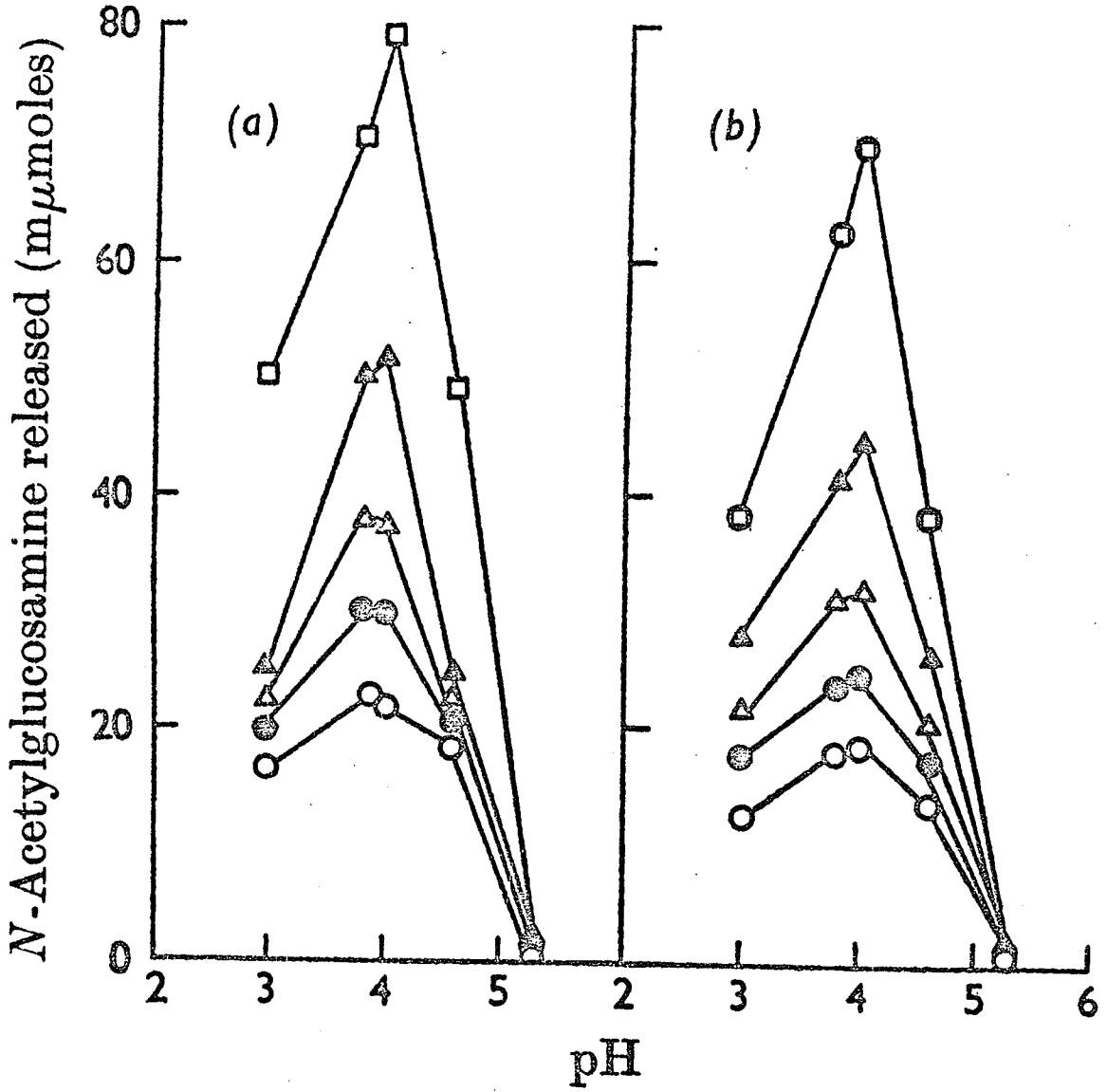


Fig. 21 pH-activity curves of (a) preparation S_3 (15 µg.) and of (b) preparation MLP_3 (15 µg.) as measured by product formation at various times at 37° with a substrate concentration of 1.2 mg. of hyaluronate/ml. ○, 5 min.; ◉, 10 min.; △, 15 min.; ▲, 30 min.; □, 60 min.

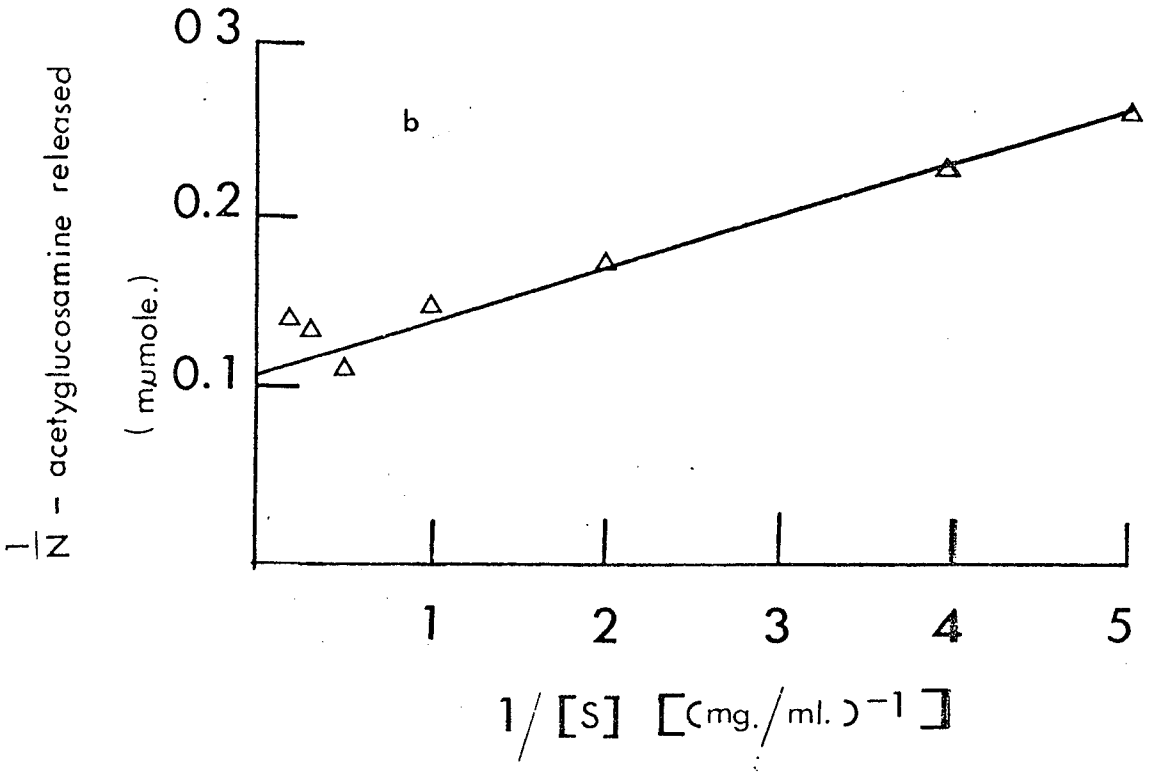


Fig. 22b Reciprocal plot of velocity and substrate concentration for a homogeneous submandibular -gland hyaluronidase preparation (sp. activity of 2.7 units/mg. protein) with hyaluronate A as substrate.