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Isolation and Partial Characterization of Hyaluronidase and Exo-polysaccharidases in Rat Carrageenin Granuloma

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Hyaluronidase, N-acetylglucosaminidase and β-glucuronidase of rat carrageenin granuloma were isolated and partially characterized, and changes in these enzyme activities during development of granuloma were also examined in order to clarify the role in metabolism of mucopolysaccharides of the granulomatous tissue.

The exo-polysaccharidases, N-acetylglucosaminidase and β-glucuronidase, were present in both the granuloma and the pouch fluid, but hyaluronidase was dected only in the pouch fluid.

After the gel filtration of pouch fluid on Sephadex G-200, hyaluronidase was separated completely from the two exo-polysaccharidases, and its enzymatic properties were examined. Optimum pH for the activities of hyaluronidase and N-acetylglucosaminidase was different from that of β-glucuronidase. The hyaluronidase fraction obtained was capable of degrading macromolecular hyaluronate and this fraction also depolymerized chondroitin 4-sulfate and 6-sulfate, but not dermatan sulfate.

Appearance of these enzymes in the pouch fluid was periodically independent of one another; maximum hyaluronidase activity was shown at 8 days after carrageenin injection and at initial experimental stage, higher N-acetylglucosaminidase was detected. On the other hand, β-glucuronidase was almost constant during the experimental period.

The degradation of hyaluronic acid and the granuloma slices by hyaluronidase was not increased by the addition of N-acetylglucosaminidase and β-glucuronidase.

Keywords——hyaluronidase; N-acetylglucosaminidase; β-glucuronidase; rat carrageenin granuloma; degradation of hyaluronate and proteoglycans

The exo-polysaccharidases, N-acetylglucosaminidase and β-glucuronidase, were markedly increased in rheumatoid as compared to osteoarthritic synovial fluid,[2] but hyaluronidase known as an endo-polysaccharidase, was similarly active in both rheumatoid and osteoarthritic synovial fluid.[3] Although some enzymatic properties of hyaluronidase and β-glucuronidase[3] and N-acetylglucosaminidase[4] were already examined, the role of these enzymes in the process of inflammation and metabolism of mucopolysaccharides has not been established.

On the other hand, enzymatic studies concerned with experimental model of inflammation, carrageenin granuloma, have been reported; Robert, *et al.*[5] found the catheptic activity in the granuloma. Shubin and Thomas[6] also reported that acid exo-polysaccharidases increased dramatically during the resorption of carrageenin granuloma in physiological condition. They suggested that these enzymes play a major role in this process. However, hyaluronidase activity has not yet been found.

In the present work, the presence of hyaluronidase in the pouch fluid was confirmed and enzymatic properties were examined. Change in hyaluronidase activity of the pouch fluid was also examined together with N-acetylglucosaminidase and β-glucuronidase, and the role

1) Location: *1432, Horinouchi, Hachioji, Tokyo, 192–03, Japan.*
2) B.A. Barthlomew, *Scand. J. Rheumatol.*, **1**, 69 (1972).
3) R.W. Stephens, P. Ghosh, and T.K.F. Taylor, *Biochim. Biophys. Acta*, **399**, 101 (1975).
4) B.A. Barthlomew and S.E. Dudek, *Scand. J. Rheumatol.*, **2**, 43 (1973).
5) B. Robert, D. Cambier, and L. Robert, "Structure and Function of Connective and Skeletal Tissue," ed by S.F. Jackson, Butherworth, London, 1965, p. 453.
6) J.A. Shubin and D.W. Thomas, *Proc. Soc. Exp. Biol. Med.*, **147**, 300 (1974).

of these enzymes in the degradation of mucopolysaccharides of granulomatous tissue was discussed.

Materials and Methods

Substrates and Reagents Used——Seakem 202 carrageenin was a product of Marine Colloid Inc., Springfield, N.J. U.S.A. Hyaluronic acid (Na-salt), chondroitin 4-sulfate and 6-sulfate (Na-salt), dermatan sulfate (Na-salt) and heparin (Na-salt) were obtained from Seikagaku Kogyo, Co., Ltd. Tokyo. The hyaluronic acid and dermatan sulfate were from pig skin. The chondroitin 4-sulfate (super special grade) was from whale cartilage and the chondroitin 6-sulfate (super special grade) was from shark cartilage. Analyses of these compounds supplied by Seikagaku Kogyo indicated a molar ratio of 4-sulfate to 6-sulfate of 80: 20 and 10: 90 for the chondroitin 4-sulfate and the chondroitin 6-sulfate, respectively. *p*-Nitrophenyl-2-acetamido-2-deoxyglucopyranoside and *p*-nitrophenyl-2-acetamido-2-deoxygalactopyranoside for N-acetylhexosaminidase assays were purchased from BDH Chemicals Ltd. Poole, England. *p*-Nitrophenyl-glucuronide was obtained from Koch-Light Laboratories, Ltd. Colnbrook Bucks, England. Sephadex G-100 and 200 were purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. Other reagents used were of analytical reagent grade.

Induction of Carrageenin Granuloma——Carrageenin granuloma was induced according to the method of Fukuhara and Tsurufuji.[7] Male rats of Donryu strain with body weight of about 120 g were employed. Six ml of air was injected subcutaneously on the back of animals 1 day before the injection of 2% (w/v) carrageenin solution sterilized in an autoclave. Then 5 ml of the solution was injected into the air pouch formed. From the sacrified animals, the entire fluid in the granuloma pouch was collected and the capsule of a granulomatous tissue was removed. These samples were kept at $-20°$ until used. Under the condition, the enzyme activities assayed in these experiments were stable for several months. For the estimation of enzyme activities, these samples were treated as follows; the pouch fluid was centrifuged at $9500 \times g$ for 10 min to remove blood cells, *etc.*, and the supernatant was used as the enzyme preparations. The granuloma was washed 3 times in cold saline and homogenized in citric acid-phosphate buffer (McIlvaine buffer, pH 4.0) by VirTis 45 at a top speed for 1 min and 5% (w/v) of the homogenate was finally prepared.

Hyaluronidase Assay——Hyaluronidase activity was assayed as follows; the enzyme solution (0.1 ml) and 200 μg of pig skin hyaluronate were incubated at 37° for 3 hr in a total volume of 0.5 ml made up with McIlvaine buffer (pH 4.0). After the incubation, 0.2 ml of 0.4M NaOH was added to neutralize the reaction mixture and N-acetylhexosamine end groups were determined by the method of Reissig, *et al.*[8] One unit of hyaluronidase activity was defined as the amount of the enzyme which produced reducing N-acetylhexosamine in an equivalent to 1 μmole of N-acetylglucosamine per 3 hr. Optical density was measured by a Hitachi 101 spectrophotometer.

N-Acetylglucosaminidase, N-Acetylgalactosaminidase and β-Glucuronidase Assays——For the assay of hexosaminidases, 0.5 ml of the enzyme solution was added to the same volume of 1 mM *p*-nitrophenyl-hexosaminide in McIlvaine buffer (pH 4.0) and the mixture was incubated at 37° for 30 min. A 0.25M NaOH (2 ml) was then added, and *p*-nitrophenol liberated was calculated from the absorption at 410 nm β-Glucuronidase assay was also carried out as above except for the use of *p*-nitrophenylglucuronide as a substrate. One unit of enzyme was calculated as the amount of the enzyme that produced 1 μmole of *p*-nitrophenol per 30 min under these conditions.

Protein Determination——Protein determination was carried out by the method of Lowry, *et al.*[9] using bovine serum albumin as a standard. If the samples contained Triton X-100, the estimation was modified according to the procedure of Wang and Smith.[10]

Results and Discussion

Distribution of Polysaccharidases in Rat Carrageenin Granulomatous Tissues

Distribution of acid polysaccharidases were examined with the granulomatous tissues, 8 days after carrageenin injection. Results are presented in Table I. Hyaloruonidase as an endo-polysaccharidase was detected only in the pouch fluid and not in the granuloma. N-Acetylglucosaminidase and β-glucuronidase as the exo-enzymes were confirmed in both granuloma and pouch fluid. In the granuloma, specific activities of the both enzymes were higher

7) M. Fukuhara and S. Tsurufuji, *Biochem. Pharmacol.*, **18**, 475 (1969).
8) J.L. Reissig, J.L. Strominger, and L.F. Leloir, *J. Biol. Chem.*, **217**, 959 (1955).
9) O.H. Lowry, N.J. Rosebrough, A.L. Farr, and R.J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
10) C.S. Wang and R.T. Smith, *Anal. Biochem.*, **63**, 414 (1975).

Table I. Distributions of Polysaccharidases in Rat Carrageenin Granuloma

Sample	Hyaluronidase μmole as N-acetyl glucosamine /mg protein/3 hr ($\times 10^{-2}$)	N-Acetylglucosaminidase μmole as PNP[a] /mg protein/30 min ($\times 10^{-2}$)	β-Glucuronidase μmole as PNP[a] /mg protein/30 min ($\times 10^{-2}$)
Granuloma	not detectable	159 ±15	26.9 ±5.2
Pouch fluid	2.90±0.51	5.33± 0.38	0.27±0.03

The animals were sacrified 8 days after the carrageenin injection and the samples were immediately prepared as described in the text. Enzyme activities were estimated under the standard method, respectively. All values are shown as mean of 5 samples ± S.E.
a) *p*-nitrophenol

Table II. Effect of Triton X-100 on Polysaccharidases in Rat Carrageenin Granuloma

Granuloma	Hyaluronidase μmole as N-acetyl-glucosamine /mg protein/3 hr	N-Acetylglucosaminidase μmole as PNP[a] /mg protein/30 min	β-Glucuronidase μmole as PNP[a] /mg protein/30 min
Control day 6	not detectable	1.18	1.81
8a		1.63	2.69
8b		1.57	3.24
10		1.05	4.07
Triton X-100 treated day 6		1.89	2.77
8a		3.00	3.02
8b		3.17	3.59
10		3.69	5.33

Two ml of homogenate (5%) was treated with same volume of 2% Triton X-100 (in McIlvaine buffer, pH 4.0) for 30 min at 0° and then diluted with the same buffer for enzyme assays.
a) *p*-nitrophenol

than those in the pouch fluid. After the treatment of the granuloma homogenate with Triton X-100, the enzyme activities released were also examined. As shown in Table II, hyaluronidase activity was not detected after the treatment. On the other hand, N-acetylglucosaminidase and β-glucuronidase exhibited an increase in activity of 60—250% and 10—50%, respectively. In order to investigate the inhibitory effect of carrageenin taken up by granulomatous macrophages on hyaluronidase, the effect of carrageenin on the activity was also examined *in vitro*. The presence of carrageenin in the standard assay system had, however, no effect on the activity. These results suggested that the exo-polysaccharidases detected in the granuloma were lysosomal enzymes and that hyalorunidase was not contained in the granuloma. Stephens, *et al.*[3] described that hyaluronidase in human synovial fluid was derived from a remote source *via* diffusion from serum. Our results and Stephens' indicated that hyaluronidase in the pouch fluid was not derived from the cells of the granuloma but was diffused from the serum. In fact, hyaluronidase activity in rat sera was about 3 times as high as that in the pouch fluid (unpublished data).

Gel Filtration of the Pouch Fluid on Sephadex G-200

To examine the role of polysaccharidases, especially of hyaluronidase, in the destruction of mucopolysaccharides and the enzymatic properties in the pouch fluid, the fractionation of the enzyme in the pouch fluid was performed on Sephadex G-200. The pouch fluid, 8 days after carrageenin injection, was fractionated between 0.3—0.6 saturation of $(NH_4)_2SO_4$. The precipitate obtained was dissolved in 100 mM phosphate buffer (pH 7.0) and applied on a Sephadex G-200 column. The elution profile is presented in Fig. 1. β-Glucuronidase was

first eluted at a higher molecular weight fraction and then N-acetylglucosaminidase associated with a part of β-glucuronidase fraction appeared. N-acetylgalactosaminidase activity was also eluted in the same fraction as N-acetylglucosaminidase. At lower molecular weight fraction, hyaluronidase activity was eluted late and isolated completely from the above three enzymes, Stephens, *et al.*[3] reported the fraction of mucopolysaccharidases in human synovial fluid on Sepharose 4B. Their elution pattern was very similar to that of the pouch fluid, but they were not able to isolate hyaluronidase from the exo-polysaccharidases. Each enzyme peak was collected and used in the following experiments.

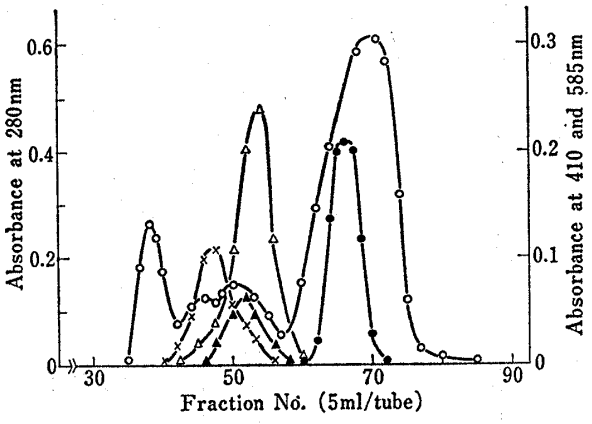

Fig. 1. Gel Filtration of Rat Carrageenin Granuloma Pouch Fluid on Sephadex G-200

The enzyme preparation fractionated with $(NH_4)_2SO_4$ was dissolved in 8 ml of 100 mM phosphate buffer (pH 7.5) and applied on a column (3×95 cm) of Sephadex G-200 equilibrated with the same buffer. Flow rate was 12 ml/hr. ○: absorbance at 280 nm, ●: hyaluronidase activity, ×: β-glucuronidase activity, △: N-acetylglucosaminidase activity, ▲: N-acetylgalac tosaminidase activity

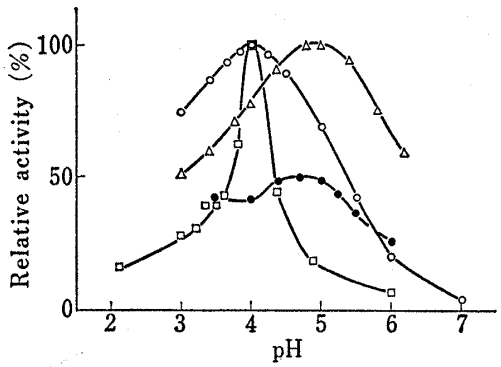

Fig. 2. Effect of pH on the Activities of Polysaccharidases in the Granuloma Pouch Fluid

Each enzyme preparation was fractionated on Sephadex G-200 and the enzyme activity was estimated as described in the text except for buffers used. In McIlvaine buffer, □: hyaluronidase, ○: N-acetylglucosaminidase, △: β-glucuronidase. In 100 mM acetate buffer, ●: N-acetylglucosaminidase

Effect of pH on the Activities of Polysaccharidases

pH dependent activity curves of polysaccharidases fractionated on Sephadex G-200 are shown in Fig. 2. Both hyaluronidase and N-acetylglucosaminidase were most active at pH 4.0. Active pH range of hyaluronidase was, however, very narrow; 50% of the maximum activity was observed over the pH range from 3.6 to 4.4. Aronson and Davidson[11] reported pH activity curves of rat hyaluronidases; the liver lysosomal enzyme was most active at pH 3.5 and inactive above 4.5, whereas the testicular enzyme showed its maximum activity above pH 3.9 and 50% of the maximum was observed at pH 5.0. Optimum pH of hyaluronidase from the granuloma pouch fluid was similar to that of liver lysosomal enzyme.

Stephens, *et al.*[3] described that human synovial N-acetylglycosaminidase was inhibited by the acetate ion, and so was the enzyme in carrageenin granuloma pouch; *e.g.* in acetate buffers the optimum pH for the enzyme was observed at 4.7 and the maximum activity was shown only 49% of that in McIlvaine buffer.

Maximum activity of β-glucuronidase was detected at pH 4.8—5.0, and at pH 4.0, best pH for hyaluronidase and N-acetylglucosaminidase, only 42% of its maximum activity of β-glucuronidase was observed.

Inactivation of hyaluronidase by NaCl was examined, It is known that estimation of hyaluronidase activity, in general, is carried out under the presence of NaCl in the reaction medium. With rat liver hyaluronidase, however, NaCl was not required for maximum enzyme activity except at a high substrate concentration.[11] In Fig. 3, the effect of NaCl concentration on hyaluronidase activity is presented under the standard assay system. Maximum

11) N.N. Aronson, Jr. and E.A. Davidson, *J. Biol. Chem.*, **242**, 441 (1967).

activity was observed in the absence of NaCl and the salt also acted as an inhibitor of hyaluronidase with concentration dependency. This fact is different from those of other hyaluronidase such as ram sperm acrosomes[12] and bull sperm,[13] except for the enzyme of rat liver.

Substrate Specificity of Hyaluronidase

Substrate specificity of the hyaluronidase in the granuloma pouch fluid was investigated. Each substrate was incubated with hyaluronidase and the reaction mixture was then centrifuged at 9500 $\times g$ for 10 min. The supernatant was applied on Sephadex G-100 and the elution pattern was monitored by the estimation of uronic acid in the effluent. As shown in Fig. 4, hyaluronidase can depolymerize hyaluronate with a high affinity and also chondroitin-4-sulfate and 6-sulfate, but not dermatan sulfate. The substrate specificity is the same as that of rat liver lysosomal hyaluronidase.[11]

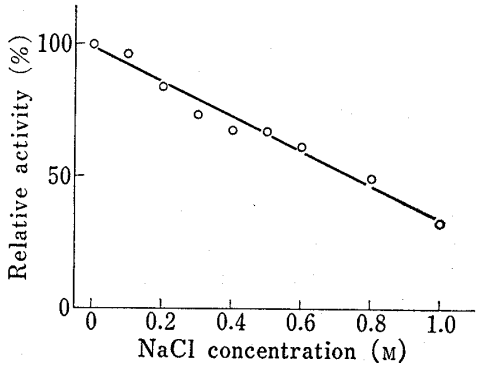

Fig. 3. Inhibition of Hyaluronidase in the Granuloma Pouch Fluid by NaCl

The enzyme preparation fractionated on Sephadex G-200 was used for the experiments. The activity was determined under the standard assay system except for the presence of NaCl.

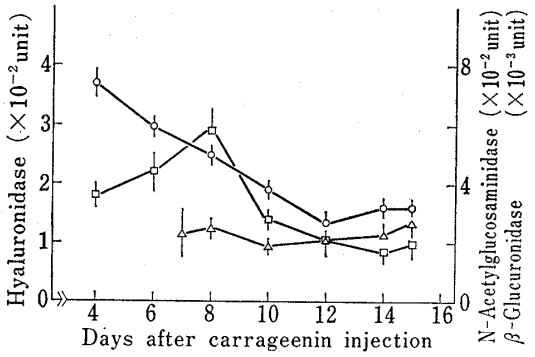

Fig. 5. Changes in Polysaccharidases of the Granuloma Pouch Fluid

After the carrageenin injection, animals were sacrificed at indicated days and the pouch fluid was collected, and prepared for the enzyme assays as described in the text. All values are shown as mean of five animals ±S.E.
□: hyaluronidase, ○: N-acetylglucosaminidase, △: β-glucuronidase

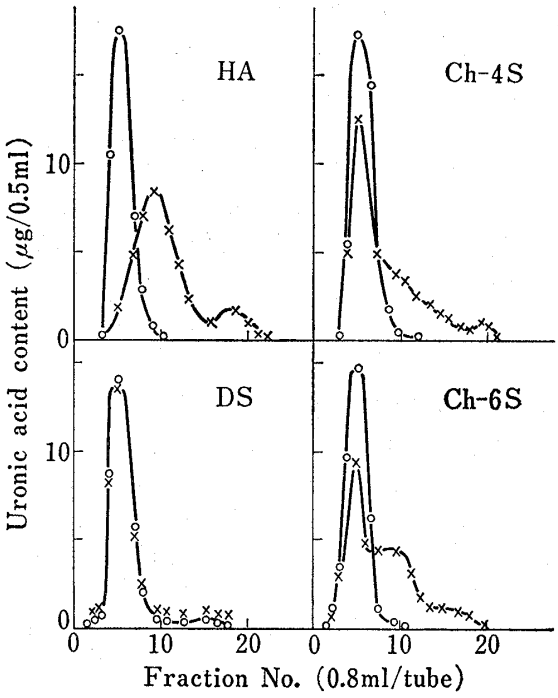

Fig. 4. Substrate Specificity of Hyaluronidase of Rat Carrageenin Granuloma Pouch Fluid

Hyaluronidase fractionated on Sephadex G-200 was incubated with 400 μg of each substrate in a total volume of 0.5 ml of McIlvaine buffer (pH 4.0) at 37° for 16 hr. Then the mixture was fractionated on a Sephadex G-100 column (1×18 cm) equilibrated with 50 mM LiCl, and uronic acid content in the effluent was determined. HA: Hyaluronic acid, Ch-4S: chondroitin 4-sulfate, DS: dermatan sulfate, Ch-6S: chondroitin 6-sulfate ○: before incubation, ×: after incubation

12) C.-H. Yang and P.N. Srivastava, *J. Reprod. Fert.*, **37**, 17 (1974).
13) C.-H. Yang and P.N. Srivastava, *J. Biol. Chem.*, **250**, 79 (1975).

Role of Hyaluronidase, N-Acetylglucosaminidase and β-Glucuronidase in Degradation of Hyaluronate and Carrageenin Granuloma

To examine the significances of polysaccharidases in the pouch fluid on the degradation of mucopolysaccharides contained in the granuloma, activities of the enzymes were determined daily. As shown in Fig. 5, hyaluronidase was increased gradually till 8 days after the carrageenin injection and then tended to decrease. On the other hand, β-glucuronidase was almost constant during the experimental period, and N-acetylglucosaminidase was found to be highest at initial stage and decreased gradually; in the pouch fluid, hyaluronidase, N-acetylglucossaminidase and β-glucuronidase appeared independently of one another.

Stephens, *et al.*[3] and Linker, *et al.*[14] described that degradation of hyaluronate was carried out by the concerted action of hyaluronidase, N-acetylglucosaminidase and β-glucuronidase; their experiments were performed under the following conditions. Hyaluronate degradation was examined with and without saccharo-1,4-lactone as a β-glucuronidase inhibitor and then the significances of the exo-polysaccharidases in the depolymerization process were discussed. In the present series of experiments, hyaluronidase, N-acetylglucosaminidase and β-glucuronidase of the granuloma pouch fluid isolated from one another by Sephadex G-200 were used as the enzyme preparation, and the effect of addition of the exo-enzymes to the endo-enzyme on the degradations of hyaluronate and the granuloma slices was examined. To examine the granuloma degradation, uronic acid and N-acetylhexosamine released in the incubation medium were estimated. For a monitoring of hyaluronate degradation, reducing N-acetylhexosamine was determined. Results are shown in Table III. At

Table III. Effect of Hyaluronidase, N-Acetylglucosaminidase and β-Glucuronidase on the Granuloma Slices and Hyaluronic Acid *in Vitro*

Assay system	Incubation time (hr)	N-Acetylglucosamine released μg/ml medium	Uronic acid released μg/ml medium
Slice+HAase[a]	18	10.2	52.3
Slice+HAase+NAGase[b]+βGase[c]	18	9.7	52.7
Slice+HAase+NAGase+βGase	0	0	37.8
HA[d]+HAase	3	28.8	
	24	33.6	
HA+HAase+NAGase+βGase	3	28.4	
	24	34.7	

The sliced granuloma (50 mg), 6 days after the carrageenin injection, was suspended and incubated in a total volume of 1.4 ml of McIlvaine buffer (pH 4.0) at 37°. After 18 hr, the incubation mixture was centrifuged at $9500 \times g$ for 10 min, and the supernatant was analized. Degradation of hyaluronate was examined as described in the text. Enzyme activity added; 2.16×10^{-2} unit of hyaluronidase, 1.73×10^{-1} unit of N-acetylglucosaminidase and 1.6×10^{-2} unit of β-glucuronidase, respectively.
a) hyaluronidase, *b*) N-acetylglucosaminidase, *c*) β-glucuronidase, *d*) hyaluronic acid

pH 4.0, optimum pH for the hyaluronidase activity, the enzyme fraction was confirmed to release reducing N-acetylhexosamine and some substances containing uronic acid, probably proteoglycans, from the granuloma slices, but the release of these substances was not increased by the addition of N-acetylglucosaminidase and β-glucuronidase fractions to the hyaluronidase. The similar phenomenon was observed with hyaluronate degradation; *i.e.* the release of reducing N-acetylhexosamine was not affected with or without exo-enzymes in the reaction mixture.

In the rat carrageenin granuloma, we confirmed that true hyaluronidase activity was detected only in the pouch fluid, but that the changes in the enzyme and exo-polysaccharidases, N-acetylglucosaminidase and β-glucuronidase, were independent of one another. The specific

14) A. Linker, K. Meyer, and B. Wessmann, *J. Biol. Chem.*, **213**, 237 (1955).

activities of the exo-enzymes in the granuloma were also higher than those in the pouch, and optimum pH of hyaluronidase and N-acetylglucosaminidase was different from that of β-glucuronidase, too. Although hyaluronidase degraded macromolecular hyaluronate and released some substances containing uronic acid from the granuloma slices, the additional action of exo-polysaccharidases, which was generally known as the concerted action, was not confirmed *in vitro*. In addition, pH values of the pouch fluid were confirmed to be constant and indicated the range of 7—7.5 during the experiment (unpublished data). From the results of the present experiments, it seems rather unlikely that these enzymes in the pouch fluid have positive roles in degradation of mucopolysaccharide of the granulomatous tissue.