Inhibition of Leucocytic Lysosomal Enzymes by Glycosaminoglycans *in vitro*

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1. A lysosomal fraction was separated by density-gradient centrifugation from a highly purified human polymorphonuclear leucocyte suspension. 2. Some 23 different lysosomal enzymes were assayed for activity in the presence of various concentrations of glycosaminoglycans. 3. The 21 acid hydrolases assayed were strongly inhibited to different degrees by low (0–12 mmol/l) concentrations of glycosaminoglycans in a pH-dependent manner. Thus inhibitions were stronger below pH 4.5, with activity returning to control values at about pH 5.0. 4. On a molar basis, the inhibitory activity for the several glycosaminoglycans studied was: heparin > chondroitin sulphate > hyaluronic acid. 5. Once the glycosaminoglycan-acid hydrolase complex was formed, it was partially dissociated by slight elevations in the pH of the incubation medium, by increasing the ionic strength of the incubation medium, or by adding several cationic proteins (e.g. histone, protamine). 6. As leucocytic lysosomes contain large amounts of chondroitin sulphate, and have a strongly acid intragranular pH, we suggest that glycosaminoglycans may modify lysosomal function through the formation of complexes with lysosomal enzymes, by inhibiting the digestive activity of the acid hydrolases when the intralysosomal pH is below their pI.

Human polymorphonuclear leucocytes contain glycosaminoglycans, mainly chondroitin sulphate (Olsson & Gardell, 1967), and are capable of synthesizing these polysaccharides (Olsson *et al.*, 1968).

Several histochemical or biochemical approaches have revealed that anionic glycosaminoglycans are present in the cytoplasmic granules of rabbit (Horn & Spicer, 1964; Fedorko & Morse, 1965; Zeya & Spitznagel, 1971) and human polymorphonuclear leucocytes (Dunn & Spicer, 1969; Olsson, 1969), being especially concentrated in the primary granules or lysosomes.

The primary granules of human polymorphonuclear leucocytes contain numerous soluble hydrolytic enzymes with acid or neutral pH optima, all confined together in the same osmotic sacs delimited by an impervious lipoprotein membrane (Avila & Convit, 1973*b*). The leucocytic lysosomal enzymes appear to be unreactive toward one another, although the physical or chemical reasons for this phenomenon are not yet well established (de Duve, 1963).

The present study describes the reversible inhibition *in vitro* of 21 lysosomal acid hydrolases. An attempt was made to delineate the biochemical factors that regulate these inhibitions *in vitro*. Our results suggest the possibility that the lysosomal glycosaminoglycans function as regulators of the hydrolytic capacity of polymorphonuclear leucocyte lysosomes. A preliminary account of some of this work has been given (Avila & Convit, 1973*c*).

Experimental

Materials

Chondroitin sulphate (grade III; lot 22C-2100), hyaluronic acid (grade I), heparin (grade I, lot 52C-2160), protamine sulphate (grade I) and calf thymus histone (type II) were from Sigma Chemical Co., St. Louis, Mo., U.S.A. The reported mol.wt. of chondroitin sulphate was 25000; that of hyaluronic acid was 230000, and that of heparin, 23000. Chemical analysis revealed 1 sulphate group per disaccharide residue for chondroitin sulphate and 2 groups per disaccharide residue for heparin. All other materials were obtained as described (Avila & Convit, 1973*e*, 1974).

Preparation of leucocyte lysosomal fractions

These experiments were performed according to the principles of the declaration of Helsinki. Informed consent was obtained from all blood donors.

Leucocytes were isolated from the blood of normal fasting human subjects and processed as described by Avila & Convit (1973*a*); 93–97% of the cells in the final suspension were polymorphonuclear leucocytes. A batch (400 mg) of these leucocytes was homogenized for 3 min in a Teflon-glass homogenizer kept in ice. After homogenization, the suspension was centrifuged at 4°C in an International PR-6 centrifuge at 500g (rotor 259) for 10 min. The

supernatant was then centrifuged through a linear 21–56% (w/w) sucrose density gradient for 2.5 h at 4°C and 75 500g in the SW 25.2 rotor of a Beckman L2-65B centrifuge. Gradients were sampled by aspiration from the bottom of the tubes and the lysosomal fraction was identified by its peroxidase and acid hydrolase content (Avila & Convit, 1973d). The lysosomal fractions showed a 12–18-fold enrichment in acid hydrolases and peroxidase over the homogenate and contained a maximum of 2–8% contamination of secondary granules and plasma membranes, as shown by the assay of marker enzymes (Avila & Convit, 1973d; Avila, 1974). Once separated, this lysosomal fraction was centrifuged for 1 h at 4°C at 106 000g (rotor 40) in a Beckman L2-65B centrifuge. The pellet thus obtained was resuspended in a small volume of 0.34 M-sucrose for the enzyme-inhibition experiments.

Enzyme assays

The methods used for the enzyme assays were essentially those of Avila & Convit (1970, 1973a,e; 1974). β -Glucosidase was assayed as described by Beutler & Kuhl (1970), sialidase by the method of Yeh *et al.* (1971), ribonuclease by the method of Anfinsen *et al.* (1954), phospholipase A₂ by the method of Franson *et al.* (1974), α -glucosidase, arylsulphatase A and β -xylosidase by the method of Van Hoof & Hers (1968), and β -fucosidase and N-acetyl- β -galactosaminidase as described by Hindman & Cotlier (1972). Cathepsins A, B and C were assayed as described by Peters *et al.* (1972). Suitable enzyme and substrate blank assays were performed.

Studies on the influence of glycosaminoglycans on enzyme activities

The incubation conditions used in these assays are described in each individual set of experiments, but in general enzyme assays were carried out in the pH range 4.0–5.5, this despite the fact that lysozyme, ribonuclease and cathepsin C showed optimum pH values at 6.2, 6.8 and 6.6 respectively, with 40, 10 and 30% respectively of the maximum activity at pH 4.0, and 62, 86 and 70% of the maximum activity at pH 5.5. Incubation times were the longest that insured a linear relationship of time versus activity for each individual enzyme, so as to use the minimum lysosomal protein concentration. On the other hand, previous experiments had revealed that under our experimental conditions the inhibitory reaction of the several glycosaminoglycans studied was immediate.

For a few enzymes, in which activity was very low or negligible at pH 4.0, experiments

were carried out with higher lysosomal protein concentration or at pH 4.4 in order to increase enzyme activity. In all experiments, enzyme assays were initiated by adding the substrate to the incubation mixture, which had been preincubated for 10 min at 37°C with the enzyme and glycosaminoglycan.

Table 1. *Comparative study on the degree of glycosaminoglycan-induced inhibition of leucocytic lysosomal enzyme activities*

Highly purified lysosomal fractions obtained from human polymorphonuclear leucocytes, and containing about 45–150 μ g of total proteins/ml, were preincubated for 10 min at 37°C in 50 mM-sodium acetate buffer, pH 4.0, with 6 mmol of each of the several glycosaminoglycans studied/l. The enzymic reactions were always initiated by adding the respective substrate to the incubation mixture. Incubations were in all cases for periods longer than 1 h (varying between 1 and 10 h according to the enzyme), except for peroxidase and lysozyme which were assayed after 6 and 12 min respectively. Results are the means of six different experiments, carried out with separate lysosomal preparations.

Enzyme	Activity (% of control) in the presence of:		
	Heparin	Chondroitin sulphate	Hyaluronic acid
β -Galactosidase (EC 3.2.1.23)	28	28	48
α -Galactosidase (EC 3.2.1.22)	46	46	65
β -Glucuronidase (EC 3.2.1.31)	18	15	66
N-Acetyl- β -glucosaminidase (EC 3.2.1.30)	43	44	85
α -Mannosidase (EC 3.2.1.24)	26	30	79
α -Fucosidase (EC 3.2.1.51)	21	33	69
β -Fucosidase (EC 3.2.1.38)	22	32	72
Lysozyme (EC 3.2.1.17)	50	69	70
Ribonuclease (EC 3.1.4.22)	21	38	51
α -Glucosidase (EC 3.2.1.20)	28	43	69
β -Glucosidase (EC 3.2.1.21)	36	47	72
Sialidase (EC 3.2.1.18)	51	66	89
N-Acetyl- β -galactosaminidase (EC 3.2.1.53)	63	60	83
β -Xylosidase (EC 3.2.1.37)	42	49	76
Arylsulphatase A (EC 3.1.6.1)	80	80	96
Acid β -glycerophosphatase (EC 3.1.3.2)	55	87	95
Phospholipase A ₂ (EC 3.1.1.4)	48	62	76
Cathepsin A (EC 3.4.2.-)	61	52	81
Cathepsin B (EC 3.4.22.1)	53	41	92
Cathepsin C (EC 3.4.14.1)	60	71	92
Cathepsin D (EC 3.4.23.5)	48	64	90
Peroxidase (EC 1.11.1.7)	99	100	100
Neutral proteinase (EC 3.4.24.4)	129	104	106

Results

Studies on the degree of glycosaminoglycan-induced inhibition of acid hydrolases

Table 1 shows that, when assayed at pH4.0, the various glycosaminoglycans inhibited all of the 21 acid hydrolases measured. Table 1 also shows that, on a molar basis, the inhibitory capacity was in general heparin > chondroitin sulphate > hyaluronic acid. When comparative experiments were carried out to test the inhibitory strength of commercial chondroitin sulphate against the isolated human leucocytic glycosaminoglycan, it was found that the commercial preparation was slightly more inhibitory than the human product per unit of weight. Table 1 also demonstrates that the only lysosomal enzymes that were not inhibited in the concentration range

used of glycosaminoglycans were peroxidase and neutral proteinase activities; further, the latter enzyme increased its activity in the presence of heparin.

Particularly important is the fact that the inhibitions obtained for the various acid hydrolases measured were very strong for some (β -glucuronidase, α -fucosidase, β -galactosidase) and moderate for others (arylsulphatase A, acid β -glycerophosphatase). This may be due to the existence of isoenzymes of lysosomal acid hydrolases (Touster, 1973), some having relatively high pI values (basic forms: pI > 5.0) (Goldstone & Koenig, 1974) and thus being extremely sensitive at pH values lower than their pI to the inhibitory effects of anionic glycosaminoglycans (Kint, 1973); however, any further interpretation is difficult, as little is known about the chemical

Fig. 1. Effect of the concentration of exogenous glycosaminoglycans present in the incubation mixture on human polymorphonuclear acid hydrolase activities

In these experiments a constant protein concentration (10–150 μ g, according to the enzyme studied) was preincubated for 15 min in 50 mm-acetate buffer, pH4.0, with several different concentrations of exogenous glycosaminoglycans at 37°C. The incubation period was always initiated by the addition of substrate to the incubation mixture, and varied from 1 to 10 h according to the activity displayed by each enzyme. Results are expressed as percentage of the activity obtained with the same preparation treated as described, except that exogenous glycosaminoglycan was absent. (a) β -Galactosidase; (b) α -galactosidase; (c) β -glucuronidase; (d) *N*-acetyl- β -glucosaminidase; (e) α -mannosidase; (f) α -fucosidase; (g) β -fucosidase; (h) ribonuclease; (i) α -glucosidase; (j) phospholipase A₂; (k) cathepsin C; (l) cathepsin D. ●, Heparin; ▲, chondroitin sulphate; ■, hyaluronic acid. Results are the mean values from three identically treated enzyme preparations.

Table 2. Degree of inhibition of leucocytic acid hydrolases as a function of the glycosaminoglycan/lysosomal-protein ratio

These experiments were initiated by the addition of the corresponding substrate to the incubation mixture which contained 50mM-sodium acetate buffer, pH4.0, and different glycosaminoglycan/lysosomal-protein ratios (obtained by changing the glycosaminoglycan concentration). Incubation periods varied between 1 and 10h according to the enzyme. The conditions of the enzyme assays were as described in the Experimental section. Values are percentages of the control activity and are means of seven different experiments, each carried out with a distinct lysosomal preparation.

Glycosaminoglycan/lysosomal-protein ratio (w/w) ...	Activity (% of control)			
	0.5	1	2	3
Enzyme				
(a) Glycosaminoglycan:				
heparin				
α -Mannosidase	62	26	20	19
β -Galactosidase	64	49	25	17
α -Galactosidase	89	69	56	33
β -Glucuronidase	43	20	19	12
<i>N</i> -Acetyl- β -glucosaminidase	73	45	40	38
α -Fucosidase	49	23	20	16
Cathepsin A	72	61	54	50
Cathepsin B	61	49	41	34
Cathepsin C	72	58	43	41
Cathepsin D	74	56	54	51
(b) Glycosaminoglycan:				
chondroitin sulphate				
α -Mannosidase	62	52	50	49
β -Galactosidase	39	28	16	15
α -Galactosidase	49	42	40	39
β -Glucuronidase	21	17	16	15
<i>N</i> -Acetyl- β -glucosaminidase	50	45	42	40
α -Fucosidase	52	49	43	43
Cathepsin A	56	50	42	32
Cathepsin B	62	49	45	40
Cathepsin C	75	66	52	43
Cathepsin D	68	59	47	46

structure of lysosomal enzymes. Further, the degree of inhibition found for the same acid hydrolase in the presence of glycosaminoglycans at a constant glycosaminoglycan/protein ratio varied between the different lysosomal preparations used (variation range: 4–9%), this perhaps being due to the percentage of basic isoenzymes related to the total enzyme activity present in a given lysosomal preparation.

As shown in Fig. 1, in the concentration range of glycosaminoglycans used (0–12mmol/l), the maximum inhibition was in general obtained with 6mmol of glycosaminoglycan/l as the absolute concentration of inhibitor, although in subsequent experiments we used the glycosaminoglycan/protein ratio as the controlling factor of inhibition.

Fig. 2. Influence of a fixed pH value of the incubation mixture on the glycosaminoglycan-induced inhibition of human polymorphonuclear acid hydrolases

Acid hydrolase activities were determined as described in the Experimental section. Incubations were carried out in 50mM-sodium acetate buffers at fixed pH values in the presence of a glycosaminoglycan/lysosomal-protein ratio of about 1. Results are expressed as percentages of the activity obtained with the same preparation under similar experimental conditions, except that exogenous glycosaminoglycan was absent. ●, α -Mannosidase; ▼, β -glucuronidase; ○, *N*-acetyl- β -glucosaminidase; ■, α -fucosidase; □, β -galactosidase; ▲, cathepsin C; ▽, α -galactosidase; △, cathepsin B. The result is a typical example from a series of five experiments, each carried out with a distinct lysosomal preparation.

Table 2 shows that a ratio as low as 0.5 μ g of chondroitin sulphate or heparin/ μ g of lysosomal proteins was inhibitory for the various acid hydrolases studied, when experiments were carried out at pH4.0. In addition, a ratio of about 1 μ g of glycosaminoglycan/ μ g of lysosomal proteins seems optimum, as higher concentrations of glycosaminoglycans did not significantly further increase the acid hydrolase inhibition.

Dependence on pH of the glycosaminoglycan-induced inhibition of acid hydrolases

Fig. 2 shows that the glycosaminoglycan-induced inhibition of acid hydrolases is absolutely dependent on the pH of the incubation mixture in which the glycosaminoglycan is in contact with the enzyme; thus there was almost complete inhibition at pH values lower than pH4.5, whereas at higher pH values the inhibition of acid hydrolase progressively decreased, reaching the control activity value at pH5.0 or above.

Table 3. *Reversibility of the effects of heparin on leucocytic acid hydrolases mediated by changes in the ionic strength of the medium*

Lysosomal proteins (240 µg) were preincubated for 40 min at 37°C with 240 µg of heparin in sodium acetate buffer, pH 4.0 ($I = 0.05$ mol/l) in a final volume of 6 ml. Six different 1 ml portions were separated, and to each was added a known volume of 0.3 M-NaCl to give a final I value between 0.05 and 0.3 mol/l (final vol. 4 ml). The samples were then preincubated again for another 20 min at 4°C and immediately used for enzyme assays. These were carried out in each of the samples with exactly the same I value in the incubation media. Results are expressed as a percentage of the activity obtained for the same enzyme preparation, treated exactly as described, except that no heparin was present in the preincubation mixture and that I was maintained at the initial value in which the glycosaminoglycan-enzyme interaction took place. Values are means of four different experiments, each carried out with a distinct lysosomal preparation.

Enzyme	<i>I</i>	...	Activity (% of control)				
			0.05	0.10	0.15	0.20	0.30
<i>β</i> -Glucuronidase			26	35	46	52	79
<i>α</i> -Mannosidase			18	29	37	61	70
Sialidase			36	48	59	71	74
<i>α</i> -Fucosidase			20	37	49	63	76
<i>N</i> -Acetyl- <i>β</i> -glucosaminidase			32	51	64	75	81
<i>β</i> -Galactosidase			29	40	52	61	75
Ribonuclease			27	34	46	53	68
Cathepsin B			41	52	69	75	80
Cathepsin C			48	54	63	71	87
Phospholipase A ₂			40	56	65	76	79
<i>α</i> -Galactosidase			28	35	43	56	61
<i>α</i> -Glucosidase			30	40	56	68	77
Cathepsin D			40	54	62	70	83

Blocking of the glycosaminoglycan-acid hydrolase interaction by the ionic strength of the preincubation medium

As the previous experiments suggest that there is some type of electrostatic action between anionic groups of glycosaminoglycans and cationic groups of lysosomal acid hydrolases, we tested the possibility of blocking this interaction by increasing the ionic strength of the incubation medium in which the enzyme and the glycosaminoglycan came into contact. Table 3 shows that the inhibition of lysosomal enzymes by heparin was effectively blocked by this procedure. Similar results were also found when chondroitin sulphate was used instead of heparin.

Blocking of the glycosaminoglycan-acid hydrolase interaction by small changes in the pH of the preincubation medium

Table 4 shows that once a lysosomal hydrolase had been inhibited by previous contact with

Table 4. *Effect of small changes in the pH of the preincubation medium once the glycosaminoglycan-acid hydrolase interaction had taken place*

Lysosomal proteins (2000 µg) were preincubated for 20 min at 37°C with glycosaminoglycans (at a glycosaminoglycan/protein ratio of about 1:1, w/w) in a final volume of 3.5 ml of 50 mM-sodium acetate buffer, pH 4.0. At the end of preincubation, 1.5 ml of ice-cold deionized water was added and immediately four 1 ml portions were separated from this mixture. The pH of one of the portions was maintained at pH 4.0, and the other three portions were carefully adjusted to pH 4.5, 5.0 and 5.5 respectively by the addition of small volumes of 2 M-NaOH. The final volume of each of the four portions separated was adjusted to 2 ml with deionized water, and enzyme assays were carried out in each of the portions at exactly the final pH of each portion. Results refer to the activity found for each pH value in the same enzyme preparation, treated exactly as described, except that no glycosaminoglycan was present in the preincubation mixture. Values are means of seven different experiments, each carried out with separate lysosomal preparations.

Enzyme	pH	...	Activity (% of control)			
			4.0	4.5	5.0	5.5
(a) Glycosaminoglycan: heparin						
β -Glucuronidase			39	60	70	83
α -Mannosidase			33	57	57	86
Sialidase			46	53	68	84
α -Fucosidase			27	34	56	86
<i>N</i> -Acetyl- β -glucosaminidase			33	58	93	100
α -Galactosidase			46	70	89	97
β -Galactosidase			28	39	56	72
Cathepsin B			42	57	78	86
Cathepsin C			57	62	84	99
(b) Glycosaminoglycan: chondroitin sulphate						
β -Glucuronidase			22	26	56	88
α -Mannosidase			37	45	87	89
Sialidase			54	62	89	100
α -Fucosidase			30	41	67	84
<i>N</i> -Acetyl- β -glucosaminidase			31	59	72	83
α -Galactosidase			51	70	81	96
β -Galactosidase			24	39	76	83
Cathepsin B			43	54	78	84
Cathepsin C			53	69	81	92

glycosaminoglycans at pH 4.0, simply raising the pH of the mixture slightly could partially reverse the inhibition.

Blocking of the glycosaminoglycan-acid hydrolase interaction by cationic proteins added to the preincubation medium

As it is known that leucocytic primary lysosomes are also rich in cationic proteins (Zeya &

Table 5. *Reversibility of the effects of heparin on leucocytic acid hydrolases mediated by the presence of histone and protamine*

Lysosomal proteins (180 μ g) were preincubated for 20 min at 37°C with 180 μ g of heparin in 50mM-sodium acetate buffer, pH 4.0, in a final volume of 6 ml. Six different 1 ml samples were separated and to each was added a different concentration of calf thymus histone or protamine to give a final concentration range of cationic proteins between 0 and 120 μ g/ml (histone/heparin ratio between 0 and 4:1, w/w). The samples were then preincubated again for another 10 min at 4°C and immediately used for enzyme assays. Results refer to the activity obtained for the same enzyme preparation, treated exactly as described, except that initially ice-cold water was added to the preincubation mixture instead of heparin. Values given represent percentage of the control activity, and are means of four different experiments, each carried out with a distinct lysosomal preparation.

Cationic protein/ heparin ratio (w/w) ...	Activity (% of control)				
	0	1.0	2.0	3.0	4.0
Enzyme					
(a) Cationic protein used: histone					
β -Glucuronidase	38	42	55	66	78
<i>N</i> -Acetyl- β -glucosaminidase	32	40	49	56	67
α -Mannosidase	20	31	46	65	79
α -Fucosidase	32	50	66	74	83
α -Galactosidase	41	62	75	84	91
β -Galactosidase	22	36	45	70	72
Phospholipase A ₂	36	42	51	68	70
β -Glucosidase	30	40	48	61	76
Cathepsin B	31	52	63	68	79
Cathepsin C	44	51	57	71	89
(b) Cationic protein used: protamine					
β -Glucuronidase	38	47	50	56	68
<i>N</i> -Acetyl- β -glucosaminidase	32	42	51	59	76
α -Mannosidase	20	26	31	55	64
α -Fucosidase	32	48	71	76	83
α -Galactosidase	41	58	70	81	90
β -Galactosidase	22	39	58	76	88
Phospholipase A ₂	36	44	59	77	80
β -Glucosidase	30	39	47	66	84
Cathepsin B	31	50	68	72	77
Cathepsin C	54	59	67	74	86

Spitznagel, 1963) we tested the influence of some cationic proteins on the glycosaminoglycan-induced inhibition of leucocytic acid hydrolases.

Table 5 shows that cationic proteins such as protamine or histone added to the preincubation mixture partially reversed the inhibition previously induced on acid hydrolases by contact with heparin at pH 4.0. Similar results were also found in the case of chondroitin sulphate.

The results given above emphasize the reversibility

of the inhibition of acid hydrolases by glycosaminoglycans and also suggest that there may exist in leucocytic primary lysosomes a competition for complexing with glycosaminoglycans between acid hydrolases and cationic proteins. Finally, other experiments have revealed that in most of the acid hydrolases, the glycosaminoglycan-induced inhibition is of the non-competitive type (J. L. Avila & J. Convit, unpublished work).

Discussion

In previous papers (Avila & Convit, 1973a,c; Avila *et al.*, 1973; Avila, 1974) we have reported the inhibition of certain leucocytic acid hydrolases by low concentrations of exogenous heparin added together with the homogenizing medium. In the present paper, we have extended these observations to 21 different human leucocytic acid hydrolases. All these enzymes were strongly inhibited *in vitro* by exogenous commercial as well as native glycosaminoglycans in a pH-dependent manner. Similar results have been reported for the phospholipase A₂ of rabbit polymorphonuclear leucocytes (Franson *et al.*, 1974). This fact tends to support in part the idea that it is a generalized phenomenon particular perhaps to leucocytic acid hydrolases; although Kint & Huys (1973) have reported a potent inhibition of human liver β -galactosidase by exogenous commercial mucopolysaccharides, they did not find inhibition of α -mannosidase, α -glucosidase or *N*-acetyl- β -hexosaminidase activity. Further, Kint *et al.* (1973) have reported 15% and 25% inhibition respectively for human liver α -galactosidase and β -glucuronidase incubated in the presence of various mucopolysaccharides, chondroitin sulphate being most potent. Caygill (1966) and Robinson & Stirling (1968) have also found strong inhibition by mucopolysaccharides of *N*-acetyl- β -glucosaminidases from several sources, including human spleen. Heijlman (1974) reported the inhibition of calf brain sialidase by glycosaminoglycans, of which heparin was as inhibitory as chondroitin sulphate, whereas hyaluronic acid showed no effect. However, it must be emphasized that in all these experiments no care was taken with the pH of the incubation medium in which the contact between glycosaminoglycan and lysosomal protein took place.

The present paper is therefore the first systematic study of the influence of glycosaminoglycans on lysosomal hydrolase activities.

Of the 23 leucocytic lysosomal enzymes studied, only peroxidase and neutral proteinase were not inhibited in the concentration range used for the glycosaminoglycans (0–12 mmol/l). Further, neutral proteinase activity (the only lysosomal

hydrolase assayed at a pH different from our normal working range of 4.0–5.5) increased in the presence of heparin at pH 7.4, a value that perhaps did not allow a strong enzyme–glycosaminoglycan interaction. Similar results were reported by Lieberman & Gawad (1971).

Our results suggest that, at low pH, the ionized anionic groups of glycosaminoglycans might strongly interact with cationic groups of the acid hydrolases, perhaps inducing a change in the tertiary structure of the proteins, leading to alterations at or near the active centre and consequently to enzyme inhibition (Bernfeld, 1963). Thus the stronger inhibitory effect of heparin than of chondroitin sulphate, as noted in our experiments, might be a consequence of the higher number of sulphate groups/disaccharide unit observed in our heparin preparation. Stone (1972) has reported a helical conformation of various acidic glycosaminoglycans in solution, with conformational differences between heparin and chondroitin sulphate, these depending on the number of sulphate groups per molecule. It can then be supposed that the conformational differences between these glycosaminoglycans may influence their affinity for lysosomal hydrolases. Evidence that these stable complexes may exist *in vivo* was given by Kint *et al.* (1973), thus explaining the lower β -galactosidase, α -galactosidase and arylsulphatase A activities found in patients with type 1, 2 and 3 mucopolysaccharidosis (Van Hoof & Hers, 1968; Van Hoof, 1972).

That the glycosaminoglycan–enzyme interaction is in general partially reversible under our conditions *in vitro* was demonstrated by modifying certain experimental conditions of the preincubation medium wherein the initial glycosaminoglycan–enzyme contact was carried out at pH 4.0. Thus the inhibition could be partially blocked by slightly raising the pH of the medium, by the addition of histone or protamine, or by raising the ionic strength of the medium. These results are significant, since several authors have found evidence that the internal lysosomal milieu is acid; reported values vary widely from pH 6.0–6.5 (Mandell, 1970; Reijngoud & Tager, 1973) to as low as pH 4.5 (Sprick, 1956; Pavlov & Soloviev, 1967; Jensen & Bainton, 1973; Goldman & Rottenberg, 1973).

Since polymorphonuclear leucocyte lysosomes contain high concentrations of chondroitin sulphate, and since their internal milieu is acid, it is evident that the reversible glycosaminoglycan–acid hydrolase interaction might be interpreted as an intralysosomal control mechanism, capable of explaining the mutual unreactiveness of the lysosomal enzymes in the living cells. Thus the primary lysosomes with a strong intragranular acid pH should have their lysosomal enzymes in the inhibited (latent) form, and secondary lysosomes would activate or inactivate

hydrolytic enzymes according to the pH prevailing in the digestive vacuole at that time.

To be sure, this mechanism would need to postulate: (a) inhibition of lysosomal enzymes by glycosaminoglycans at the normal intragranular pH of primary granules, (b) total or partial block of this inhibition by changes in the preincubation pH in the range of pH values prevailing inside secondary lysosomes and (c) that the intralysosomal pH changes *in vivo* would be great enough to include the value where dissociation of the glycosaminoglycan–enzyme complex would occur.

Direct evidence for the first two conditions have been presented here. As for the third, Jensen & Bainton (1973) have demonstrated wide changes in the pH of the digestive vacuoles of rat polymorphonuclear leucocytes phagocytosing yeast cells.

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