96. THE β-GLUCOSAMINASE ACTIVITY OF TESTICULAR EXTRACTS AND ITS BEARING ON THE PROBLEM OF DIFFUSING FACTORS

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DIFFUSING FACTOR concentrates from testicular extracts, bacterial filtrates and snake venoms are known to decrease rapidly the viscosity of aqueous solutions of mucopolysaccharides of the hyaluronic acid type. Prolonged action of these concentrates leads to hydrolysis of the mucopolysaccharide by rupture of some of the glycosidic linkages [Chain & Duthie, 1939; 1940; McClean & Hale, 1940; 1941]. Hyaluronic acid is believed to contain equal numbers of *N*-acetylglucosamine and glucuronic acid residues [Meyer, 1938], and since it has virtually no reducing properties it probably contains equal numbers of glucosaminidic and uronidic linkages, either or both of which must be ruptured in the hydrolysis brought about by diffusing factor preparations.

It has been shown that diffusing factor concentrates from testes contain a β-glucosaminase which catalyses the hydrolysis of β-phenyl-*N*-acetylglucosaminide [East *et al.* 1941]. The same is true of snake venom and the enzyme in both cases appears to be, like the glucosaminase in emulsin [Helferich *et al.* 1933; 1934; Grassmann *et al.* 1934], specific for the β-glycosides of *N*-acetylglucosamine. Testicular extracts containing glucosaminase are unable to bring about hydrolysis of the β-glucuronides of menthol [East *et al.* 1941], borneol or pregnanediol [Meyer *et al.* 1941]. Since it is not known whether the uronidic linkages in hyaluronic acid are of the α or β type this evidence is not strong enough to rule out the possibility that an enzyme capable of hydrolysing the uronidic linkages in hyaluronic acid may also exist in testicular extracts. The methods available for the determination of *N*-acetylglucosamine residues and of the total reducing power in the partially hydrolysed hyaluronic acid are insufficiently accurate to permit of their being used to establish or disprove the existence of such an enzyme.

It has already been suggested that the effect of testicular extracts on hyaluronic acid may be due to a complex system of enzymes rather than to one enzyme only [Madinaveitia & Quibell, 1940; McClean & Hale, 1940; Meyer *et al.* 1941]. The above-mentioned specific β-glucosaminase of testis has now been shown to be distinct from the enzyme or enzymes responsible for decreasing the viscosity of hyaluronic acid solutions. There is no parallelism between the glucosaminase activity and viscosity-reducing properties of testicular extracts, and the behaviour of the glucosaminase on adsorption differs from that of the viscosity-reducing material. Crude emulsin preparations contain a specific oligo-β-glucosaminase [Zechmeister & Tóth, 1939]: it is of interest to note that a sample of emulsin containing this enzyme (B.D.H. emulsin) failed to decrease the viscosity of hyaluronic acid solutions and had no diffusing properties.

It may well be that the decrease in viscosity of hyaluronic acid solutions by testicular extracts is a process which does not involve hydrolysis of glycosidic

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linkages. In addition to such extracts, ascorbic acid [McClean & Hale, 1940], some diazo-compounds [Favilli, 1940], phenylhydrazine and H_2O_2 [Madinaveitia & Quibell, 1941, 1] all have a similar effect. It is difficult to ascribe hydrolytic properties to some of these reagents. Moreover, treatment with alkali irreversibly reduces the viscosity of hyaluronic acid [East *et al.* 1941] although glycosidic linkages are as a rule unaffected by this reagent.

Experimental

Estimation and properties of β-glucosaminase

The substrate used, β-phenyl-*N*-acetylglucosaminide, was incubated with the enzyme preparation to be tested and the degree of hydrolysis determined by means of the Folin & Ciocalteu [1927] phenol reagent. This technique has been successfully applied in the study of glucosaminases from other sources [Neuberger & Pitt Rivers, 1939]. The alternative procedure, estimation of liberated *N*-acetylglucosamine by the method of Morgan & Elson [1934] was found to be somewhat unreliable.

Quantitative estimation. A solution of β-phenyl-*N*-acetylglucosaminide (1 ml., containing 10 mg.) in *M*/6 acetate buffer *p*H 4·6 mixed with an aqueous solution (1 ml.) of the enzyme preparation is incubated at 37° for 18–24 hr. To the incubated mixture Folin & Ciocalteu's phenol reagent (5 ml.) is added and the precipitated proteins removed. To a known volume (5 ml.) of the clear solution saturated Na_2CO_3 (5 ml.) is added, the colour allowed to develop for 30 min. or more and the liberated phenol determined colorimetrically by comparison with standard solutions of phenol treated in the same way. The blanks obtained using dialysed and moderately active enzyme preparations (containing not more than 10 mg. solids per ml.) are negligible.

Good recovery is obtained when known amounts of phenol are mixed with a dialysed testicular extract (10 mg. solid per ml.):

Phenol present (mg./ml.)	0·346	0·277	0·241	0·138
Phenol found (mg./ml.)	0·345	0·266	0·238	0·134

Up to 10% the degree of hydrolysis of the substrate (24 hr. at 37°) is practically proportional to the concentration of enzyme.

Dialysed testis extract

mg. enzyme/ml.	0·4	0·8	1·2	1·6	2·0
% hydrolysis	3·9	5·7	8·7	10·0	11·2
Ratio	0·10	0·14	0·14	0·16	0·18

Crude Crotalus atrox *venom*

mg. enzyme/ml.	3	5	10	20
% hydrolysis	0·6	1·0	1·6	3·7
Ratio	5·0	5·0	6·2	5·3

The degree of hydrolysis is virtually a linear function of time, and therefore the length of incubation is not critical, provided that it is the same for all samples compared in each particular experiment.

Time (hr.)	1	2	3·5	5	7	20
% hydrolysis	0·8	1·0	1·6	2·3	2·5	8·8
Ratio	1·25	2·0	2·5	2·2	2·8	2·3

pH optimum. The *p*H chosen for the quantitative estimation is the optimum for glucosaminase activity. To determine it constant amounts of a dialysed testicular extract (2 mg.) and of substrate (10 mg.) were incubated for 18 hr. at 37° in the presence of acetate buffers (2 ml.) of varying *p*H.

*p*H	3·6	3·8	4·0	4·2	4·4	4·6	4·8	5·8
% hydrolysis	2·7	2·7	4·0	4·0	4·9	6·8	6·7	5·5

Effect of NaCl. This salt has a great effect on the reaction by which the viscosity of vitreous humour is decreased [Madinaveitia & McClean, 1940; McClean & Hale, 1940; Madinaveitia & Quibell, 1941, 2], but very little on the action of glucosaminase. Constant amounts (2 mg.) of a dialysed testicular extract were dissolved in NaCl solutions of various concentrations (1 ml.) and mixed with the buffered standard substrate solution. After 18 hr. incubation at 37° the degrees of hydrolysis attained were:

Molarity NaCl	0·000	0·001	0·005	0·01	0·05	0·1	0·5	1·0
% hydrolysis	10·6	12·6	13·0	13·0	11·1	10·4	10·7	8·0

Effect of substrate concentration. Solutions of β-phenyl-*N*-acetylglucosaminide (2 ml.) of various concentrations in *M*/6 acetate buffer were incubated for 18 hr. at 37° with a solution of dialysed testicular extract (0·5 ml., 6 mg./ml.).

Concentration of substrate (mg./ml.)	4	3	2	0·8	0·6	0·4	0·2
% hydrolysis	13	17·5	24	30	35	40	50

Comparison of the viscosity-reducing and glucosaminase activities of various testicular diffusing factor preparations

The viscosity-reducing powers [Madinaveitia & Quibell, 1940] and the glucosaminase activities of various diffusing factor preparations were compared with those of the standard diffusing factor preparation:

	Viscosity reducing power	Glucosaminase activity
Standard [Madinaveitia, 1938]	1	1
Standard [Bacharach *et al.* 1940]	4·0	0·1
Preparation DF 8B (Glaxo Laboratories, Ltd.) dialysed testis extract	2·7	0·7
$(NH_4)_2SO_4$ purified [Madinaveitia, 1939]	4·4	0·6
$(NH_4)_2SO_4$ and Pb acetate purified	20·3	0·74

It is clear that no parallelism exists between the two kinds of activity and that $(NH_4)_2SO_4$ treatment followed by Pb acetate fractionation [Madinaveitia, 1941] yields a mucinase preparation practically free from glucosaminase.

Kaolin adsorption. The mucinase from dialysed testicular extracts is more readily adsorbed than the glucosaminase from this source. A solution of dialysed testicular extract (5 ml., 7 mg./ml.) was adsorbed using various amounts of kaolin (B.D.H. acid-washed) for 30 min. at room temperature. After removing the adsorbate the N content, the glucosaminase activity and the viscosity-reducing power of the clear supernatants were estimated.

	Kaolin, g.				
	0·05	0·1	0·2	0·4	0·8
	% adsorbed				
Mucinase	0	5	15	69	100
Glucosaminase	0	0	0	0	60
Nitrogen	6·6	—	27·6	50·5	93·4

With more dilute enzyme solutions this effect is more marked. Solutions of the same preparation (4 ml., 2 mg./ml.) were treated with kaolin as above.

	Kaolin, g.				
	0·019	0·037	0·075	0·15	0·3
	% adsorbed				
Mucinase	28·5	47·5	65·7	100	100
Glucosaminase	0	0	0	90·7	96

Comparison of kaolin with other adsorbents. Solutions of the above enzyme preparation in *M*/5 acetate buffer *p*H 5·0 (4 ml., 2 mg./ml.) were treated with the adsorbent under examination (0·75 g.). After centrifuging, the mucinase and the glucosaminase contents of the supernatants were determined.

	% adsorbed		
Adsorbent	Mucinase	Glucosaminase	Ratio
Kaolin	60	14	4·3
Fuller's earth	68	24	2·8
Bauxite	45	18	2·5
Kieselguhr	51	8	6·4
Frankonite	84	38	2·2

Action of HCl and of NaOH on the viscosity of vitreous humour preparations

Two samples (40 ml. each) of a solution in *M* NaCl of a vitreous humour preparation (0·25 %) were mixed with the same volume (10 ml.) *M* HCl and *M* NaOH respectively and kept in a thermostat at 49·2°. Samples (5 ml.) of each solution were withdrawn at intervals. To those containing HCl, Na acetate (2 ml., *M*) was added and those containing NaOH were buffered with acetic acid (2 ml., *M*). The specific viscosity of the buffered solutions was then determined at 25°. The comparison was thus carried out at about the same *p*H and in the presence of comparable concentrations of NaCl.

	Sample kept in	
Time of reaction min.	HCl	NaOH
	Specific viscosity	
0	0·295	0·249
5	0·254	0·203
10	0·240	0·183
15	0·222	0·170
20	0·210	0·162
30	0·207	0·145
40	0·177	0·137
80	0·147	0·095
160	0·109	0·070
1140	0·055	0·012

SUMMARY

Diffusing factor concentrates from testicular extracts contain a specific β-glucosaminase. This enzyme is different from the agent present in these concentrates which rapidly decreases the viscosity of mucopolysaccharides of the hyaluronic acid type.

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