46. DIFFUSING FACTORS

9. THE EFFECT OF SALTS ON THE ACTION OF TESTICULAR EXTRACTS ON THE VISCOSITY OF VITREOUS HUMOUR PREPARATIONS

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THE reduction of the viscosity of certain mucins by testicular extracts and by mucinases from other sources of diffusing factors has been associated by Chain & Duthie [1939] with the characteristic spreading properties of these factors. Comparison of the relative diffusing and mucolytic activities of diffusing factor preparations [Madinaveitia *et al.* 1940] requires a relatively accurate quantitative estimation of the two effects. Methods are now available which permit such a comparison to be carried out; the diffusing activity can be determined with enough accuracy by the technique of Bacharach *et al.* [1940] and the available viscosimetric method [Madinaveitia & Quibell, 1940] accurately measures mucolytic activity. The results obtained by these methods do not quite agree with the view that the effect of diffusing factors is solely due to the mucolytic activity of the assayed preparations.

The viscosimetric method for the estimation of mucinases is based on the fact that the rate of decrease of the viscosity of vitreous humour preparation is proportional to the concentration of enzyme present in the reacting mixture, provided that the reaction is carried out under certain conditions. The mucolytic activities of a number of diffusing factor preparations have now been compared by this method. The results obtained are in good agreement when comparison is made at different concentrations of enzyme (c) after the original viscosity of the substrate (ηsp_0) has been reduced to a given value ($\% \eta sp_0$). The product of the concentration of enzyme and the time (t) required to reach the chosen viscosity level ($c \times t$) is practically constant for each preparation whatever is the decrease in the viscosity, provided that it is the same for all the samples compared (Table I).

Table I

Preparation	c mg./6 ml.	$\% \eta sp_0$			
		64.8	57.7	48.7	40.8
		$c \times t$			
Glaxo standard	0.49	122	196	343	538
	0.37	140	229	370	573
	0.245	134	238	368	587
Madinaveitia's [1938] standard	2.55	1120	1730	2670	4450
	1.915	950	1650	2680	4600
Crude <i>Crotalus atrox</i> venom	4.84	1350	2410	3950	5550
	2.41	1570	2340	3740	6620
Purified <i>Crotalus atrox</i> venom	1.08	194	335	540	840
	0.54	232	365	567	890
	0.36	254	397	630	950

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Using one preparation as a standard of reference, the relative mucolytic activity of each of the others is the same irrespective of the viscosity level at which comparison is carried out (Table 2).

Table 2

Preparation	% ηsp_0			
	71.5	63.5 Ratio to Glaxo Standard	45.0	35.0
From testicle:				
Glaxo standard	100	100	100	100
Madinaveitia standard	23.5	24.7	23.2	24.2
M/10 acetic acid extract	84	85	79	67?
(NH ₄) ₂ SO ₄ -purified	150	152	147	147
From <i>Crotalus atrox</i> :				
Crude venom	19.9	17	15.7	15.9
(NH ₄) ₂ SO ₄ -purified venom	51.5	57.5	67	69

All comparisons were carried out under the conditions originally suggested [Madinaveitia & Quibell, 1940], i.e. in 0.66 *M* NaCl and in the presence of 0.18 *M* citrate buffer pH 4.7. However, on testing by this method a mucinase preparation from *Clostridium welchii* prepared by Dr D. McClean of the Lister Institute we found that contrary to his indications it was practically inactive. This apparent discrepancy was due to the fact that Dr McClean's estimation of the mucolytic activity of this preparation had been carried out in the absence of NaCl. In view of this an investigation has now been carried out on the effect of salts on the reaction in which the viscosity of vitreous humour preparations is destroyed by the action of testicular extracts. A summary of the results has already been published [Madinaveitia & McClean, 1940]. The substrate used was prepared from an acetone powder of bovine vitreous humour (1 g.) by extraction with *M*/50 NaOH (50 ml.) and precipitation of the centrifuged and acidified (10 ml. *M* acetic acid) extract with 6 vol. acetone. After washing the precipitate with ether and drying *in vacuo* a white powder was obtained which was almost completely soluble in water but which still contained some salts. The source of enzyme was a preparation obtained by (NH₄)₂SO₄-fractionation from an acetic acid (2 l. *M*/10) extract of dry testicle powder (100 g.). The material precipitated between 30 and 70 % of saturation was redissolved, dialysed, subsequently electro-dialysed until the conductivity was practically constant and finally dried whilst frozen. It was salt-free. The techniques used for estimating the viscosity and for determining the half-life time of the viscous substrate were those already described [Madinaveitia & Quibell, 1940].

As previously reported addition of salts has the effect of decreasing the specific viscosity (ηsp_0) of the substrate (Tables 3 and 5). However, the relationship between specific viscosity and the concentration of the substrate is the same at any given NaCl concentration as indicated by the fact that the ratio of the viscosity of the substrate (ηsp_0) to that of the substrate diluted with 1 vol. of the corresponding NaCl solution (ηsp_1) is constant (Table 3). The only ratio which differs significantly from the others is that in which there is no NaCl present but this can be easily accounted for by the fact that the vitreous humour preparation used was not absolutely free of electrolytes. Reduction of the concentration of electrolyte on dilution with distilled water would render the more diluted substrate somewhat more viscous than expected. Electro-dialysis throws the viscous materials out of solution.

When a series of samples of electro dialysed enzyme (0.5 mg. in 1 ml. water) are allowed to react with a substrate solution (5 ml. 2 %) in NaCl of increasing concentration the half-life time (t) of the viscous material decreases as the concentration of NaCl in the reacting mixture becomes larger and after reaching a minimum it increases again (Table 3). Therefore there is an optimum salt concentration for the activity (t^{-1}) of the enzyme.

Table 3

NaCl <i>M</i>	ηsp_0	ηsp_t	$\frac{\eta sp_0}{\eta sp_t}$	t min.	t^{-1}
0.00000	1.44	0.752	1.92	60	0.012
0.00083	1.28	0.596	2.14	30	0.033
0.00167	1.12	0.513	2.18	15.5	0.064
0.00334	0.94	0.443	2.13	12.5	0.080
0.00500	0.850	—	—	12.5	0.080
0.00667	0.775	—	—	9.0	0.112
0.00833	0.725	0.339	2.14	6.0	0.167
0.01677	0.593	0.279	2.13	4.2	0.238
0.0334	0.491	0.234	2.10	3.3	0.303
0.0500	0.441	—	—	2.3	0.425
0.0667	0.417	—	—	0.7	1.43
0.0833	0.400	—	—	0.7	1.43
0.167	0.339	—	—	0.7	1.43
0.334	0.308	—	—	3.0	0.333
0.500	0.289	—	—	3.2	0.312
0.667	0.283	—	—	4.7	0.213
0.833	0.270	—	—	14.5	0.069
1.670	0.259	—	—	30	0.033

The same effect was observed when a similar experiment was carried out in the presence of 0.2 *M* citrate buffer pH 4.7 and with concentrations of substrate and of enzyme different from those in the previous experiment. The same picture is obtained at whatever fraction of the original viscosity the comparison is carried out (Table 4). In addition to NaCl certain other salts in dilute solution

Table 4

NaCl <i>M</i>	% ηsp_0					
	65	60	55	50	45	40
	Sec. required to reach % ηsp_0					
0.00	300	380	900	1580	2400	3500
0.01	190	240	310	440	700	1050
0.02	160	240	350	530	880	1450
0.05	100	190	320	500	750	1050
0.10	130	210	370	550	820	1250
0.16	110	240	330	480	700	1050
0.21	130	240	370	550	820	1250
0.53	150	290	500	850	1320	2100
1.56	170	1050	1900	—	—	—
2.08	1300	2900	5000	—	—	—

also enhance the action of the enzyme and all the salts examined inhibit at relatively high concentrations. In Table 5 the effect of increasing amounts of NaCl is compared with that of other salts. The reaction was carried out in the presence of 0.2 *M* Na citrate buffer pH 4.7.

The method suggested [Madinaveitia & Quibell, 1940] for the comparison of the activities of mucinase preparations depends on the half-life time of the substrate being inversely proportional to the concentration of enzyme. This relationship does not hold good when working in an unbuffered solution at low

Table 5

	t	ηsp_0	t	ηsp_0	t	ηsp_0
Without salt	15	0.515	15	0.515	15	0.515
Molarity of salt		0.0083		0.083		0.83
NaCl	7.5	0.430	7	0.392	29.5	0.330
KCl	7.5	0.435	7	0.402	29.5	0.326
NH ₄ Cl	5.5	0.472	5.5	0.468	45	0.310
NaI	11.5	0.404	13.5	0.382	100	0.325
NaBr	9.5	0.418	9.5	0.398	95	0.320
Na ₂ SO ₄	15	0.422	40	0.407	200	0.450

NaCl concentrations. Under these conditions, when the concentration of enzyme is increased, the half-life time of the substrate decreases much more rapidly than expected (Fig. 1). This effect is most marked in the absence of NaCl and it becomes less noticeable as the concentration of salt increases. After a certain level of salt concentration has been reached the half-life time of the substrate becomes inversely proportional to the concentration of enzyme and thereafter this relationship is maintained although the reaction velocity decreases.

Fig. 1. Effect of the concentration of NaCl on the dose-response curve of testicular mucinase.

Testicular mucinase is not the only enzyme whose action is enhanced by the presence of salts. The effect of neutral salts on the action of different amylases is well known and has been studied in some detail [cf. Myrbäck, 1926; Sherman *et al.* 1928]. It has been found that some ions increase the activity of amylases, changing the *pH* optimum. An alteration of the kinetics of the reaction between amylase and starch due to the presence of NaCl, such as observed with testicular mucinase, has never been recorded as far as we are aware.

SUMMARY

The time required to reduce the viscosity of vitreous humour preparations to half its original value is inversely proportional to the concentration of testicular mucinase present in the reacting mixture, provided that the reaction is carried out in the presence of NaCl. At low concentrations of salt this time decreases very rapidly as the concentration of enzyme increases.

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