44. DIFFUSING FACTORS

7. CONCENTRATION OF THE MUCINASE FROM TESTICULAR EXTRACTS AND FROM *CROTALUS ATROX* VENOM

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FRACTIONATION of testicular extracts by lead acetate at different *p*H [Morgan & McClean, 1932; Madinaveitia, 1938] and by $(NH_4)_2SO_4$ at various concentrations [Madinaveitia, 1939, 1] are convenient methods for the concentration of diffusing factors from these sources. There is not complete parallelism between the mucolytic and diffusing activities of concentrates obtained by either of these two methods [Madinaveitia *et al.* 1940]. However, by a combination of both procedures highly active preparations of mucinase have now been obtained. The relatively accurate viscosimetric assay method [Madinaveitia & Quibell, 1940] for mucolytic activity has made it possible to determine with some exactitude the optimal *p*H range for lead acetate purification. The best mucinase preparations obtained by this method were about 1000 times more active than the dry testicle powder used as a starting material.

From a dry testicle powder *M* NaCl in *M*/10 acetic acid extracts about the same amount of mucinase as *M*/10 acetic acid alone. The precipitate formed on dilution of the NaCl extract with water contains the whole of the mucinase and the diffusing factor present in the original extract. This is probably due to adsorption of the active constituents on the protein precipitate which occurs as the electrolyte concentration is decreased. The mucinase from preparations purified with $(NH_4)_2SO_4$ does not precipitate in this way and the purest preparations of mucinase and of diffusing factor are soluble in distilled water. In this precipitation we again observe the mucinase and the diffusing factor going together in a fractionation.

The substrate used throughout this paper for testing the mucinase activity [Madinaveitia & Quibell, 1940] was a preparation of vitreous humour. Following the original idea of Chain & Duthie [1940] that diffusing factors are mucinases, Mayer *et al.* [1940, 1, 2] have suggested that the substrate on which these factors act in the skin is similar to, if not identical with, the polysaccharide from the cornea. However, intracorneal injections of diffusing factor preparations into the isolated eyes of slaughtered cattle do not increase the area of spread relative to control injections.

There is evidence that the diffusing factor and the neurotoxic constituents of *C. terrificus* are different [Madinaveitia, 1939, 2]. The mucolytic action of *C. atrox* venom has now proved to be due to a more thermolabile constituent than either the protease or the lecithinase of this venom. Fuller's earth readily adsorbs the mucinase from dialysed testicular extracts, but a relatively large amount of adsorbent is required for removal of the enzyme from solutions of *C. atrox* venom. In this case it is possible to remove part of the inactive material by adsorption at suitable *p*H with a small amount of fuller's earth and to produce

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a further inactive precipitate by readjustment of the *p*H. $(NH_4)_2SO_4$ fractionation can also be used for purification of the mucinase from the snake venom, the active constituents precipitating at about half-saturation.

Experimental

Purification of testicular mucinase

$(NH_4)_2SO_4$ fractionation. Dry testicle powder [Madinaveitia, 1938] (500 g.) mixed with sand (1 kg.) was extracted in a mortar with acetic acid (5 lots of 500 ml. *M*/10). The extract was filtered through paper and the residue twice extracted with acetic acid (2 lots of 1 l. *M*/10). To the pooled filtered extracts (4 l.; *p*H 3·7) $(NH_4)_2SO_4$ was added to 27 % of saturation. The inactive precipitate was removed and the filtrate made 70 % saturated with $(NH_4)SO_4$. The precipitate now formed contained practically the whole of the mucinase. It was dissolved in water (200 ml.) and dialysed in a cellophane bag for 24 hr. against running tap water. The dialysed mucinase solution (1 l.) was concentrated *in vacuo* (37°) and the concentrated solution (100 ml.) evaporated to dryness whilst frozen. Yield: 22 g. having 85 % of the mucinase of the original material.

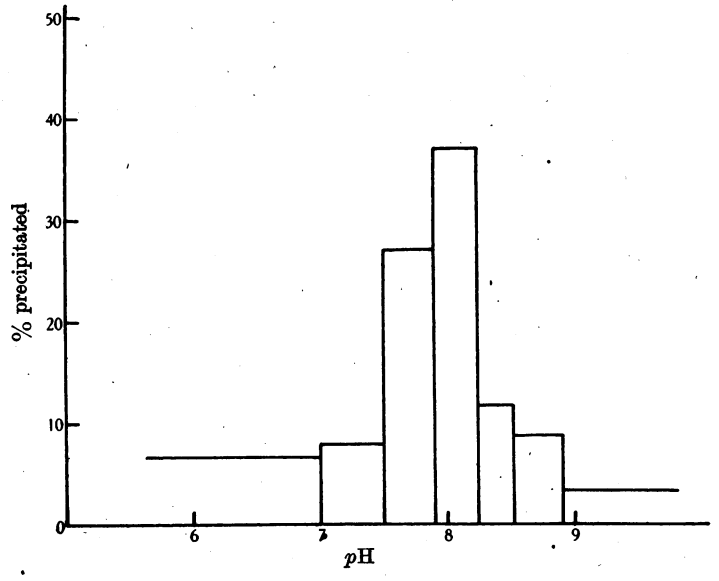

Fig. 1. Lead acetate precipitation of testicular mucinase at different *p*H.

Lead acetate fractionation. To a solution (*p*H 4·37) of $(NH_4)_2SO_4$-purified mucinase (2 g.) in water (5 ml.) a solution of neutral lead acetate (2 ml. 10 %) was added. To this opalescent mixture (*p*H 5·2) dilute ammonia (*M*/5) was added in small portions; the *p*H was determined electrometrically and the precipitate removed before addition of further amounts of ammonia. Each of the precipitates was dissolved in acetic acid (2 ml. *M*/10) and all of them made up to the same volume (50 ml.) with water. Fig. 1 represents the relative mucolytic activities of these precipitates.

A larger amount (10 g.) $(NH_4)_2SO_4$-purified mucinase was dissolved in water (250 ml.) and to the solution neutral lead acetate (100 ml. of 10 %) was added.

The *p*H was then adjusted to 7·37 with ammonia (2*N*) and the precipitate discarded. The filtrate was adjusted to *p*H 8·55 with further amounts of ammonia. The precipitate now formed was collected, dissolved in acetic acid (50 ml. *M*/10) and dialysed for 18 hr. in a cellophane bag against running tap water. After filtering from an inactive precipitate the dialysed solution was evaporated to dryness whilst frozen. The resulting preparation (0·7 g.) had 80 % of the mucinase of the already purified starting material. Lead acetate in the concentrations used in the test has no effect on the action of the mucinase on the substrate.

NaCl fractionation. Dry testicle powder (1 g.) mixed with sand (2 g.) was extracted in a mortar with a mixture of NaCl (18 ml. *M*) and acetic acid (2 ml. *M*). After 15 min. the insoluble constituents were centrifuged off. The slightly turbid supernatant (1 ml.) was diluted with water (19 ml.) and kept for 1 hr. Precipitate and supernatant were now separated and the former dissolved in NaCl (20 ml. *M*). Dilutions (1/20) of each of the fractions were tested for mucolytic and diffusing activities [Madinaveitia, 1938, 1939, 1].

	Mucolytic activity. % of original solution	Diffusing activity. Area of spread in cm.²	Increase of the area of spread
Original extract	100	8·31	4·29
Supernatant	5	4·81	0·79
Precipitate	75	7·58	3·56
Control	—	4·02	—

To a solution of $(NH_4)_2SO_4$-purified mucinase (300 mg.) in water (12 ml.) solid NaCl (2 g.) was added. On addition of acetic acid (0·5 ml. *M*) a precipitate was formed (precipitate i) which, after 10 min., was collected by centrifuging. In the supernatant a further amount of NaCl (1 g.) was dissolved and the precipitate now formed (precipitate ii) collected as before. Each of the precipitates was dissolved in water (10 ml.), their N contents established and dilutions in water of each precipitate (1/10) and the final supernatant (1/50) were tested for mucolytic activity, no precipitate appearing on dilution.

	% of original solution	
	N	Mucinase
Precipitate i	28·5	5
Precipitate ii	38·5	8
Supernatant	24·5	86

Heat-inactivation of Crotalus atrox *venom*

Crude dried *C. atrox* venom (0·5 g.) dissolved in water (25 ml.) was heated for 10 min. in thermostats at different temperatures. Each sample was then made isotonic by addition of the appropriate volume of 8·5 % NaCl. The lecithinase activity was estimated by the method of Slotta & Szyszka [1938]. Proteolytic activity was then estimated by allowing the venom solution (1 ml.) to react with gelatin (10 ml. 5 %) at 37° and determining the degree of hydrolysis by formaldehyde titration.

Temperature heated ° C.	Lecithinase activity. Dilution of enzyme (Haemolysis) 1/100	1/200	1/400	1/800	1/1600	Proteolytic activity increase ml. *M*/50 KOH 4 hr.	18 hr.	Mucinase half life-time min.
Unheated	+	+	+	+	−	0·14	0·25	7
45°	+	+	+	+	−	0·15	0·25	14·5
55°	+	+	+	+	−	0·12	0·25	∞
65°	+	±	±	−	−	0·14	0·25	—

Purification of the mucinase of Crotalus atrox *venom*

Solutions of the venom (12 mg.) in water (12 ml.) are turbid. The insoluble material is brought into solution by addition of acetic acid (*M*/10), Na_2HPO_4 (*M*/15) and to a less extent by NaCl (*M*/3). The water-insoluble constituents are inactive and readily flocculated at *p*H 5·1 (*M*/15 acetate buffer).

$(NH_4)_2SO_4$ fractionation. A solution of the dry crude venom (1·65 %) was centrifuged and to the supernatant (4 ml.) acetic acid (0·2 ml. *M*) were added. To this solution (2 ml.) increasing amounts of saturated $(NH_4)_2SO_4$ solution were added, the precipitate being removed after each addition. The precipitates were dissolved in *M* NaCl (10 ml.) and tested for mucolytic activity:

% saturation in $(NH_4)_2SO_4$	0–33	33–50	50–67
Mucolytic activity % of original solution	21	62	25

Dry *C. atrox* venom (1 g.) dissolved in water (50 ml.) was acidified with acetic acid (2·5 ml. *M*); saturated $(NH_4)_2SO_4$ (25 ml.) was added and the precipitate discarded. To the clear solution more $(NH_4)_2SO_4$ (75 ml. saturated solution) was added and the active precipitate collected. This precipitate was extracted with water (20 ml.), the extract acidified with acetic acid (0·2 ml. *M*) and again precipitated with $(NH_4)_2SO_4$ (5 ml. saturated solution). The inactive precipitate was discarded and the clear solution treated with more $(NH_4)_2SO_4$ (30 ml. saturated solution). The active precipitate now formed was collected, dissolved in water (25 ml.) dialysed in a cellophane bag against running tap water overnight and dried whilst frozen. Yield: 0·07 g. of a preparation having 40 % of the mucinase content of the starting material (1 g. crude venom).

Adsorption of C. atrox *mucinase.* A solution (0·5 %) of crude venom was centrifuged. To part of the supernatant (20 ml.) acetic acid (1 ml. *M*) was added (*p*H 3·5) and another portion (20 ml.) was diluted with water (1 ml.) (*p*H 6·05). Samples (0·5 ml.) of each of these two solutions were shaken with different adsorbents (0·2 g.). After 30 min. the adsorbates were centrifuged and the N and mucinase contents of the supernatant estimated:

		% of original solution	
*p*H	Adsorbent	N	Mucinase
3·5	Kaolin	28	39
	Kieselguhr	34	18
	Fuller's earth	48	12
6·0	Kaolin	41	0
	Kieselguhr	34	10
	Fuller's earth	48	10

Adsorption with fuller's earth. A centrifuged solution (50 ml. 0·5 %) of crude *Crotalus* venom was acidified with acetic acid (1·25 ml. *M*). Samples of the solution (5 ml.) were shaken with various amounts of fuller's earth and kept for 30 min. with occasional stirring. After removal of the adsorbates the N and mucinase contents of the supernatants were estimated:

g. Fuller's earth	0·8	0·4	0·2
% N adsorbed	96	77	48
% Mucinase adsorbed	100	66	25

To samples (5 ml.) of a solution of crude venom (5 mg./ml.) acetate buffers (1 ml. *M*) of different *p*H were added and each sample adsorbed with fuller's

earth (0·25 g.). The adsorbate was removed and dilutions (1/3) of the supernatant in *M* citrate buffer *p*H 4·7 tested for mucolytic activity. The N contents of the supernatants were also determined. The results are recorded in Fig. 2.

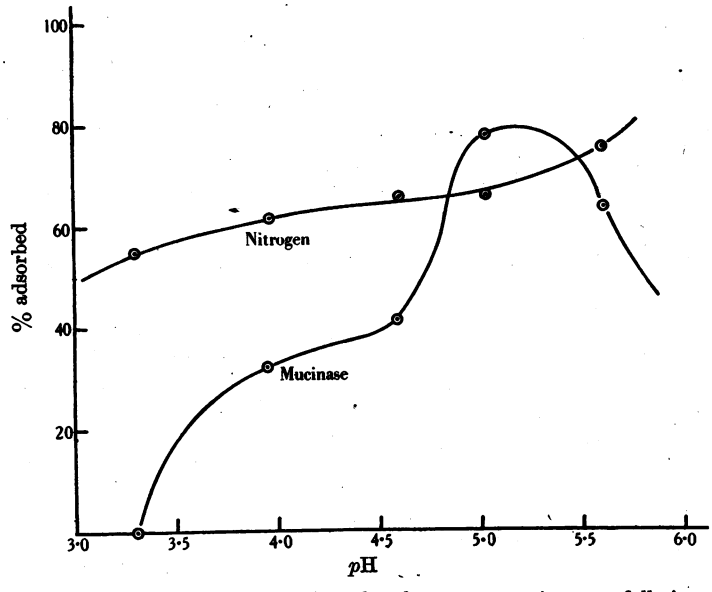

Fig. 2. Effect of *p*H on the adsorption of snake venom mucinase on fuller's earth.

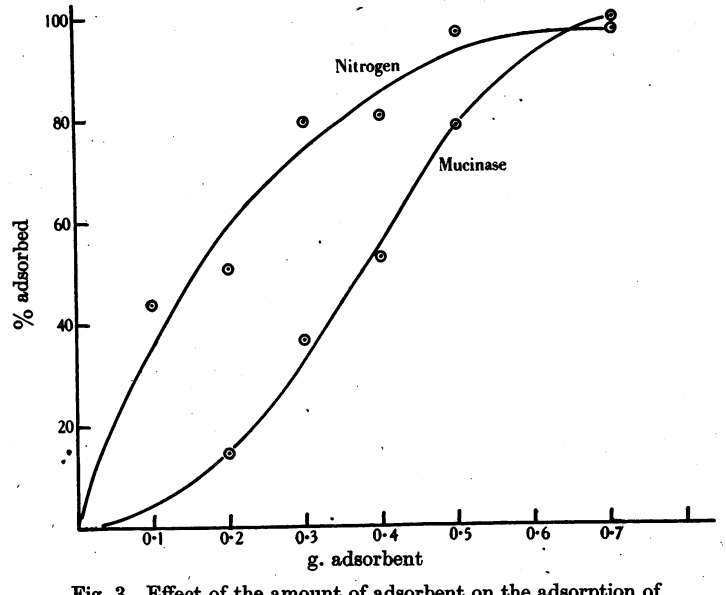

Fig. 3. Effect of the amount of adsorbent on the adsorption of snake venom mucinase on fuller's earth.

A solution (5 mg./ml.) of crude venom was mixed with acetate buffer (*M*). Samples of the buffered solution (*p*H 3·7) were adsorbed with various amounts of

fuller's earth and after 30 min. the adsorbates were removed. Dilutions (1/3) of the supernatant in *M* citrate buffer *p*H 4·7 were tested for mucolytic activity, and the N contents of the supernatants determined. The results are recorded in Fig. 3.

A solution of crude venom (60 ml., 5 mg./ml.) was mixed with acetate buffer (5 ml. *M*). The mixture (*p*H 3·5) was shaken with fuller's earth (2·5 g.) and filtered after 15 min. Samples (10 ml.) of the filtrate were partially neutralized with NaOH (1 ml.) of increasing concentration. The precipitate now formed was centrifuged after 1 hr. and the amounts of mucinase and N precipitated were determined.

	*p*H ...	4·50	5·25	6·00
% precipitated:	Mucinase	0	0	20
	N	50	50	70

Summary

$(NH_4)SO_4$-fractionation of aqueous testicular extracts, followed by lead acetate precipitation under controlled *p*H, gives a convenient purification of mucinase.

This enzyme can also be purified by dilution of NaCl extracts of testicle with water. Both mucolytic and diffusing activities are in the precipitate. The lecithinase and protease of *Crotalus atrox* venom are more thermostable than the mucinase. Concentration of the mucinase from this venom has been carried out by $(NH_4)_2SO_4$-fractionation and by adsorption methods.

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