CLXXXI. STUDIES ON DIFFUSING FACTORS. III

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The presence of diffusing factors in snake venoms has been demonstrated by Duran-Reynals [1936, 1939]. The immediate effect of intracutaneous injection of indicator solutions containing rattlesnake venom is similar to that of solutions containing testicular diffusing factor [Madinaveitia, 1939], namely, an almost instantaneous flattening of the resulting bleb with rapid spread of the indicator through the skin tissue. About 1 hr. after injection, however, a difference is observed between the two; the rate at which the area of spread increases becomes extremely slow in the case of testicular diffusing factor while it is still relatively rapid with the rattlesnake venom solution (Fig. 1). This secondary

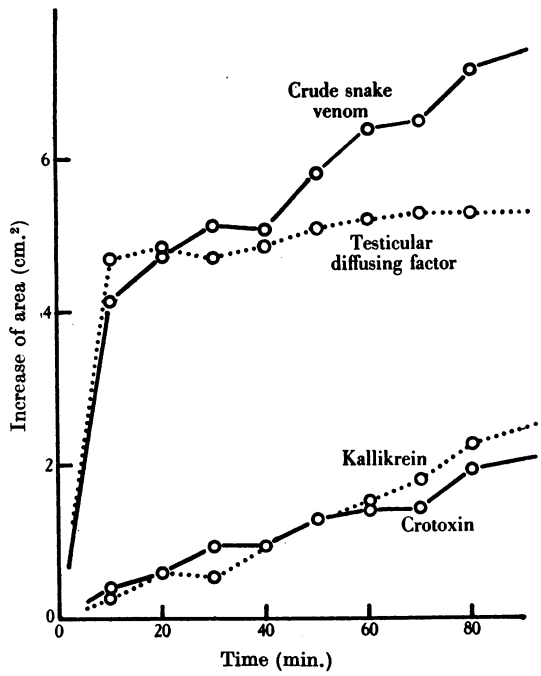

Fig. 1. Comparison of 1 : 3 dilutions in isotonic haemoglobin solution of solutions of 1 mg./ml. of crude snake venom and crystalline crotoxin, testicular diffusing factor 300 units/ml. and kallikrein ("Padutin" Bayer).

spreading effect has never been observed with testicular diffusing factor and it suggests that the diffusing factor in rattlesnake venom is accompanied by another substance capable of causing a slow spread of indicator through skin tissue. Substances giving rise to a slow spread are known to exist, e.g. kallikrein [Christensen, 1939; Madinaveitia, 1939]. Intracutaneous injection of snake

venom produces local oedema and eventually necrosis, and it is possible that the slow spread above-mentioned might arise as a result of these effects. The occurrence of local reactions is much reduced when, instead of working with normal rabbits, animals immunized against the venom are employed.

Duran-Reynals [1939] has compared the increases in area of spread due to intracutaneous injections of rattlesnake venom in normal and immune rabbits. From his results he deduced that immunization inhibits or suppresses the effect both of the diffusing factor and of the substance responsible for the local reactions. If, however, his experimental results are plotted graphically (Fig. 2)

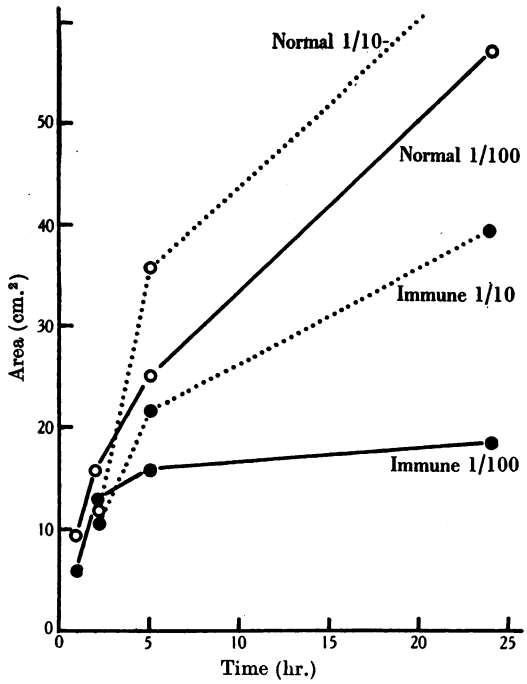

Fig. 2. Time-response curve of rattlesnake venom in normal and immune rabbits. Experimental results of Duran-Reynals [1939]. Indian ink as an indicator.

it is observed that the time-response curves obtained with immune rabbits are like those given by testicular diffusing factor preparations and show a very rapid initial spread followed by a very slow one. Immunization has suppressed not the initial rapid spread, but only the secondary one. Since some local reaction occurs when relatively strong solutions of venom are injected into immune animals, a certain degree of secondary spread is observed in such cases.

Slotta & Fraenkel-Conrat [1938] have isolated in crystalline form crotoxin, the neurotoxic constituent of rattlesnake venom. Through the kindness of Prof. Slotta, to whom my thanks are due, a sample of the crystalline toxin was placed at my disposal for examination. Intracutaneous injection of crotoxin produces a slow spread of the injected fluid but the initial rapid spread characteristic of diffusing factors is entirely absent (Fig. 1). It therefore appears that the neurotoxic constituent and the diffusing factor in rattlesnake venom are distinct from each other. Crotoxin possesses the lecithinase properties of the

crude venom but the blood-clotting properties of the latter are absent [Slotta & Fraenkel-Conrat, 1938]. Whether or not the blood-clotting properties are related to the diffusing factor is not yet known. The blood-clotting power of snake venoms has been attributed by Eagles [1937] to their proteolytic activity.

Some bacteria of the gas gangrene group secrete proteolytic enzymes in culture media. Maschmann [1938] showed that one of these enzymes, collagenase, is specific for gelatin and he suggested that it might be responsible for the changes in tissue following invasion by bacteria of the gas gangrene group. It was therefore of interest to investigate the possibility that diffusing factor activity might be associated with an enzyme of the type of collagenase or the proteolytic enzymes of snake venom. This does not appear to be the case; testicular extracts with a high diffusing activity appear to be without action on gelatin. To conclude from this experiment that diffusing activity is not associated with a proteolytic enzyme involves the assumption that diffusing factors from different sources are identical or at least very closely related in their mode of action. Such an assumption seems reasonable on account of the apparent identity of their effects and the close similarity in their chemical properties as far as they have been investigated.

The methods hitherto used for the purification of diffusing factors from various sources depend on the fact that these factors are not precipitated by neutral lead acetate or by half-saturation with $(NH_4)_2SO_4$.

The inaccuracy of the biological test method makes it very difficult to determine the relative activities of different fractions obtained during concentration, since it only indicates with certainty whether any one fraction is ten times more of less active than another. This fact together with the instability of diffusing factors, limits the choice of concentration methods. The possibility of concentration by filtration through a column of aluminium oxide has been previously reported [Madinaveitia, 1939]. The results indicated that the testicular diffusing factor is to some extent adsorbed on the alumina used. It has now been found that *ortho* aluminium hydroxide C (alumina C_γ) quantitatively adsorbs the active constituents both from testicular extracts previously purified by $(NH_4)_2SO_4$ fractionation and from dialysed *Clostridium Welchii* filtrates. Of the various eluents tried Na_2CO_3 and Na_2HPO_4 were most satisfactory and removed most of the adsorbed material. Removal of most of the material is necessary on account of the above-mentioned inaccuracy of the assay method; the non-eluted material cannot be tested and it is impossible to decide whether or not the eluate contains the main bulk of the active material. By two successive adsorptions and elutions a preparation was obtained from bull testicle which has, as far as could be judged from the biological test, most of the activity of the starting material with only 0·3% of its N content. This preparation appears from its chemical properties to be entirely protein in character.

Experimental

Adsorption of testicular diffusing factor on alumina C_γ

The starting material was a concentrate obtained by $(NH_4)_2SO_4$ fractionation of crude testicular extract. 75 ml. of this concentrate containing about 1 mg. N/ml. were buffered with 25 ml. of an $M/3$ acetate buffer pH 5·5.

To 20 ml. portions of this buffer solution varying amounts of the alumina gel were added and the volume made up to 25 ml. in each case. After 15 min. at room temperature with frequent shaking the alumina was centrifuged off and

the N contents of each of the supernatants determined. Fig. 3 represents the percentages of N adsorbed by different amounts of adsorbent. Between *p*H 3 and 6 (acetate buffer) variation of the *p*H at which adsorption takes place has practically no influence on the amount of material adsorbed by a given quantity of adsorbent.

Besides alumina C_γ other adsorbents have also been tried in the following way: to 10 ml. of the buffer solution 0·5 g. of adsorbent was added and, after 15 min., the solution was centrifuged and the N content of the supernatant estimated. The results thus obtained are given in Table I.

Table I

Adsorbent	% of N adsorbed
Kaolin	45
Merck alumina	13·5
Fuller's earth	51
Bauxite	23
Frankonite	43

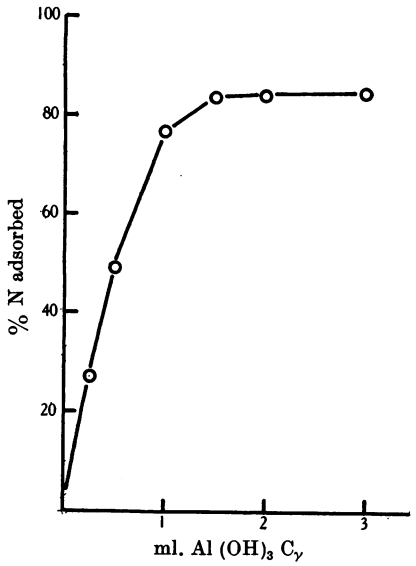

Fig. 3.

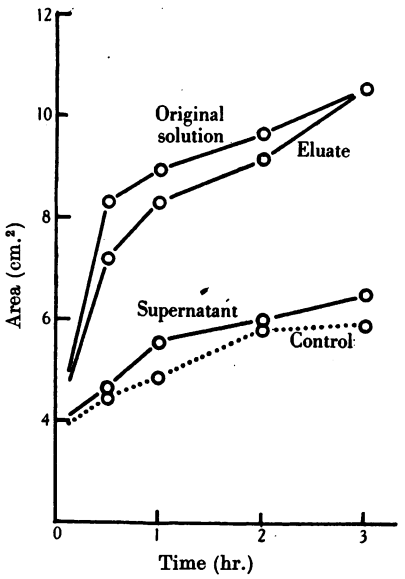

Fig. 4.

Fig. 3. Adsorption of purified testicular diffusing factor on alumina C_γ.

Fig. 4. Adsorption of testicular diffusing factor on alumina C_γ and elution with *M*/15 Na_2HPO_4. Activity of the different fractions.

In order to find the best eluent for the alumina gel adsorbates, portions of 10 ml. of the buffered solution used in the previous experiment were treated with 2 ml. of alumina C_γ. The adsorbate was centrifuged after 15 min. and eluted by shaking for 10 min. with 10 ml. of a *M*/15 solution of the eluent. The results thus obtained are recorded in Table II.

Table II

M/15 eluent	CH_3COOH	KH_2PO_4	$NaHCO_3$	Na_2HPO_4	Na_2CO_3	H_2O
% of adsorbed N eluted	59	17·5	11·5	48	45	1

Although $M/15$ acetic acid appears on this basis to be the best eluent it is unwise to use it since the acidity may partially destroy the activity.

Biological assay. To 75 ml. of the unbuffered solution used in previous experiments (original solution) 25 ml. of alumina gel were added and kept for 2 hr. with frequent shaking. The adsorbate was then centrifuged and washed with 20 ml. of water. After centrifuging again the washing water was added to the supernatant of the adsorption (supernatant). The adsorbate was eluted 4 times with 20 ml. of $M/15$ Na_2HPO_4 and the mixed eluates made up to 100 ml. (eluate). The relative amounts of N in each fraction are shown in Table III.

Table III

Fraction	N content
Original solution	100
Supernatant	18·5
Eluate	69
Non-eluted (by difference)	12·5

For testing purposes each of these fractions was diluted 1 : 10 with an isotonic solution of haemoglobin. Fig. 4 represents the spread of haemoglobin due to each fraction. The technique used for injection and measurement of area was that already described [Madinaveitia, 1938]. Three rabbits were used and each of them was given two injections of each of the solutions under test. The results plotted are the average of the six determinations.

Preparation of concentrates of testicular diffusing factor by adsorption methods

An aqueous extract of dry testicle powder (100 g.) was purified by $(NH_2)_2SO_4$ fractionation [Madinaveitia, 1939]. The resulting solution (3 l. containing 140 mg. N) was adsorbed on 100 ml. alumina C_γ. After stirring for 1 hr. the adsorbate was allowed to sediment and the supernatant readsorbed with a further 50 ml. alumina gel. The adsorbates were separated from the supernatant (which contained 38 mg. N) by centrifuging, and were washed by shaking with 50 ml. water which removed 38 mg. of the adsorbed N. They were then eluted by shaking with two portions of 100 ml. 1 % Na_2CO_3. Elution was completed by shaking with two further portions of 50 ml. of the same eluent. The combined eluates which contained 57 mg. N were adjusted to *p*H 5·5 with $3N$ acetic acid. This solution was adsorbed on 50 ml. alumina gel, the adsorbate separated and the supernatant readsorbed with a further 25 ml. alumina. After separating the supernatants (which contained 7 mg. N), the adsorbate was washed with water which removed 0·7 mg. N. The active material was then eluted by shaking three times with portions of 20 ml. $M/15$ Na_2HPO_4. The eluates were dialysed against running tap water for one day and then against frequent changes of distilled water for two further days (ice box). The volume of the solution was finally made up to 100 ml. It had 26·4 mg. N, about 0·3 % of the N content of the original crude testicular extract. Biological assay indicated that this preparation had the whole activity of the crude extract and that no fraction having any appreciable diffusing power had been rejected.

The final preparation thus obtained gave precipitates with nitric acid, trichloroacetic acid, salicylsulphonic acid, tannic acid, metaphosphoric acid and uranium acetate. The biuret, xanthoproteic and glyoxylic reactions were positive. After alkaline hydrolysis the test for sulphur was positive. The ash content of the dried material was 1 % and it contained 17·2 % N.

Determination of the Hausmann numbers of this protein according to Thimann [1926] gave the following results: amide-N, 10·1 %, humin-N, 2·2 %, basic N, 27·5 %, amino- and non-amino-N, 63·7 %. These figures are within the limits of those obtained for other proteins.

Concentration of the diffusing factor filtrates of cultures of Clostridium Welchii

Cultures of *Cl. Welchii* were made in a meat broth by Dr D. McClean of the Lister Institute (Elstree) to whom the author is greatly indebted. 2 l. of a culture filtrate having 6 ml. N/ml. were reduced to 250 ml. by evaporation *in vacuo* (23°). In order to find whether or not the active constituents would dialyse this concentrated solution was dialysed against 500 ml. of distilled water (cellophane membrane). After 24 hr. the liquid outside the bag had been reduced to 200 ml. and was completely devoid of any diffusing activity. Dialysis was then continued against running tap water for 2 days. The volume inside the bag increased to 1300 ml. After keeping it for some days in the ice box an inactive precipitate separated. This was filtered off and the clear filtrate had 2·25 mg. N/ml., i.e. about 25 % of the N content of the starting material. This preparation gave copious precipitates with tannic acid and uranium acetate. Lead acetate (basic or neutral) trichloroacetic acid and salicylsulphonic acid only produced slight precipitates.

Adsorption of Cl. Welchii *diffusing factor on alumina* C_γ

To 5 ml. quantities of the dialysed culture filtrate 1, 5 and 10 ml. of alumina gel were added and in each case the volume was made up to 15 ml. with water. After 30 min. the precipitate was centrifuged. The N contents and spreading activities of the supernatants are recorded in Table IV.

Table IV. *Average of the results obtained in double determinations in two rabbits*

ml. of dialysed culture filtrate	ml. $Al(OH)_3$	ml. H_2O	% N adsorbed	Area of spread cm.²	Increase of area (cm.²)
5	0	10	—	5·43	2·34
5	1	9	38	4·83	1·74
5	5	5	54	3·36	0·27
5	10	0	56	3·34	0·25
Control	—	—	—	3·09	—

The whole of the active constituents have been adsorbed by 5 ml. of alumina gel, but it is doubtful whether 1 ml. is enough to adsorb the bulk of active material.

When working with bacterial diffusing factors there is always the danger that the results of the biological assay are complicated by secondary spread due to the toxin or some other constituents of the filtrate. In order to avoid this difficulty a slight variation of the method of assay has been made. The substance under test is suitably diluted with an isotonic solution of haemoglobin. 0·3 ml. of this dilution is intracutaneously injected into the shaven flank of a rabbit. The area of spread is measured once every minute during the first 5 min. following injection and the figures so obtained are averaged. Comparison can be made between the results obtained with dilutions of any two given preparations in a fashion similar to that previously described [Madinaveitia, 1938]. The comparison can be made either between the whole areas or between the increase of area relative to a control injection without diffusing factor.

In order to determine whether the adsorbed material could be eluted by Na_2HPO_4 as in the case of the testicular diffusing factor, a sample of 5 ml. of the dialysed filtrate was adsorbed with 5 ml. of alumina gel. After 15 min. at room temperature the precipitate was removed by centrifuging. The adsorbate was washed with 5 ml. of water, again centrifuged and then eluted by two portions of 5 ml. of $M/15$ Na_2HPO_4. The activities of each of these fractions are shown in Table V where the figures represent the average of double determinations in two rabbits.

Table V

Fraction	Area of spread cm.²	Increase of area cm.²
Original solution diluted 1/1	7·22	3·95
Supernatant of the adsorption	3·42	0·15
Water washings	3·46	0·19
Eluates	7·30	4·03
Control	3·27	—

Comparison of the proteolytic and diffusing activities of Cl. Welchii *filtrates and of testicular extracts*

The substrate used for determining proteolytic activity was a 5% aqueous solution of gelatin. Solutions containing 8 ml. substrate and 2 ml. enzyme were incubated at 38° for 24 hr., toluene being added as a preservative. The increase in carboxyl groups as determined by titration of 2 ml. quantities of these solutions with $M/20$ KOH [Willstätter & Waldschmidt-Leitz, 1921] was taken as a measure of proteolytic activity. Controls without gelatin and of gelatin without enzyme were uniformly negative.

The crude testicular extract used was prepared by grinding up 1 g. dry testicle powder with sand and water in a mortar. The volume was made up to 20 ml. and the insoluble material separated. The supernatant was coagulated by addition of one drop of $M/10$ acetic acid and filtered. The preparation of the other solutions tested is described elsewhere. The diffusing activity was determined in 1/3 dilutions of the different materials. The results obtained are recorded in Table VI.

Table VI

	Proteolytic activity increase of ml. $M/20$ KOH	Diffusing activity increase of area of spread (cm.²)
Cl. Welchii filtrate	2·43	1·89
Cl. Welchii dialysate	1·60	2·13
Crude testicular extract	0·02	2·37
Purified testicular extract	0·01	2·69

Summary

1. Crude rattlesnake venom contains a factor similar to testicular diffusing factor. Accompanying it is another factor which causes a slow spread of indicators through skin. Rabbits immunized against the venom respond only to the diffusing factor.

2. Crystalline crotoxin does not show the characteristic spreading properties of diffusing factors although it causes a slow spread of indicators through skin.

3. Although crude rattlesnake venom and *Cl. Welchii* filtrates have proteolytic activity it is unlikely that this is associated with the diffusing factors since testicular extracts have no such activity.

4. The diffusing factors from testicle and *Cl. Welchii* are strongly adsorbed by alumina C_γ from which they may be conveniently eluted with sodium carbonate or disodium hydrogen phosphate. The similar behaviour of the factors from these sources suggests that they are closely related or even identical proteins.

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