XLIV. STUDIES ON DIFFUSING FACTORS
II. COMPARISON OF DIFFUSING FACTORS FROM DIFFERENT SOURCES AND PREPARATION OF CONCENTRATES FROM BULL TESTICLE

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It has been recently suggested [Christensen, 1938] that kallikrein, the non-dialysable vasodilator principle from pancreas and urine, is a diffusing factor similar to that in mammalian testicle. This suggestion was based on the fact that intracutaneous injections of kallikrein, mixed with a suitable indicator, result in the spread of the latter over a much larger area than in the case of control injections of indicator alone. The areas were compared 24 hr. after injection.

In endeavouring to compare the relative spreading activities of kallikrein preparations and diffusing factor concentrates, some differences between the modes of action of the two substances have been observed. The technique used for the comparison was that recently described [Madinaveitia, 1938] in which haemoglobin is used as an indicator of the area of skin penetrated by the diffusing factor. The characteristic effect of the testicular diffusing factor is the immediate flattening of the blebs formed by intracutaneous injection of the solution. When haemoglobin is present in the injected fluid the visible effect is a rapid increase of the area of skin coloured by haemoglobin (Figs. 1, 2, 3, curve *DF*).

The effect of intracutaneous injections of kallikrein is different. The skin first becomes oedematous at the site of injection and the oedema slowly spreads. The weal takes a long time to disappear. When haemoglobin is present in the injected fluid it slowly diffuses through this oedematous area (Figs. 1, 2, 3, curve *K*). The area of skin over which the haemoglobin spreads eventually becomes equal to, and even larger than, that following injection of testicular diffusing factor. After some hours the oedema disappears leaving a patch of skin coloured by haemoglobin, identical in appearance with that obtained by injection of testicular diffusing factor. Although the final result as regards the spread of haemoglobin is identical in both cases, the ways in which it is obtained are different. It seems therefore that the testicular diffusing factor and kallikrein are not identical in their biological actions. The available data on diffusing factors, other than the testicular one, are mainly based on measurements taken at least 24 hr. after injection. It has been found desirable to establish whether their actions are like that of the testicular diffusing factor or like that of kallikrein. Leech extracts, rattlesnake venom, solutions of diazobenzenesulphonic acid-serum globulin, *Clostridium Welchii* filtrates, *Vibrion septique* filtrates and staphylococcal toxoid behave, in different degrees of intensity, in a manner similar to the testicular diffusing factor (Figs. 2 and 3).

The mechanism by which the diffusing factors induce water to spread so rapidly through the skin tissues is not yet known. This spread is observed when injections are made in the isolated skin of a rabbit (Fig. 4; see also McClean [1931]). This would indicate that the mechanism by which the diffusion of water takes place through the skin tissues is independent of the circulation of

blood through the skin capillaries. A further support for this view might perhaps be found in the fact that the presence of adrenaline or of histamine in the injected fluid does not interfere with the action of testicular diffusing factor. Neither of these substances has any spreading activity.

Purified preparations of diffusing factor are devoid of haemolytic properties [Favilli & McClean, 1934]. This, together with the fact that substances like saponin, bile salts [McClean, 1930], triethanolamine, soap, allylamine and octyl alcohol do not show the characteristic spreading properties of the testicular diffusing factor, indicates that the spreading is not due to a surface tension effect.

It seems that the diffusing factor is able to increase the porosity of the skin tissues. The increase in the diameter of the pores appears to be substantial, since through them can pass particles as large as those of Indian ink.

Most of the available evidence on the chemical constitution of the testicular diffusing factor suggests that it is a protein, or an active constituent intimately associated with a protein carrier. Nevertheless, some earlier observations did not agree with this point of view. In the preparation of concentrates by fractional lead acetate precipitation [Morgan & McClean, 1932; Madinaveitia, 1938] most of the proteins are removed by precipitation with neutral lead acetate, the active material remaining in the filtrate. Since most proteins precipitate under these conditions it seemed doubtful whether the diffusing factor was a protein or not. Another point which did not seem to be in agreement with the diffusing factor being a protein is the fact that some of the preparations made by Aylward [1937] did not coagulate on heating.

The fractionation of aqueous testicular extracts with $(NH_4)_2SO_4$ has shown that these two facts do not exclude the possibility of the diffusing factor being a protein or being intimately associated with one. Only a small amount of active material precipitates at half saturation with $(NH_4)_2SO_4$, and under the conditions used precipitation is not complete at full saturation (Figs. 5 and 6). From a half-saturated solution the $(NH_4)_2SO_4$ is easily removed by dialysis. The resulting solution retains the full activity of the original extract and contains only 1·6 % of the N present in the desiccated bull testicle powder. Half saturation with $(NH_4)_2SO_4$, removal of the precipitate and dialysis of the filtrate give a purification of the same order as that obtained by the fractional lead precipitation method.

McClean [1936], working with diffusing factor from bacterial filtrates, found that it was completely precipitated at full saturation with $(NH_4)_2SO_4$; Claude & Duran-Reynals [1937] came to the same conclusion. These authors, before treatment with $(NH_4)_2SO_4$, precipitated the active material with acetone and extracted this precipitate with water. This procedure denatures the proteins; only 1–10 % of the original activity is recovered from the acetone precipitate.

Preparations obtained by $(NH_4)_2SO_4$ fractionation do not give a precipitate with lead acetate, basic or neutral. They readily precipitate with other protein precipitants. The peculiar failure of these proteins to precipitate with lead acetate explains the mechanism of the purification of diffusing factor by means of the fractional lead precipitation. Half saturation with $(NH_4)_2SO_4$ and subsequent dialysis of the filtrate yields a solution of proteins which are not precipitated by lead acetate.

The heat-coagulation of purified $(NH_4)_2SO_4$ diffusing factor is greatly dependent on the *p*H at which the solution is heated. Between *p*H 5 and 6 coagulation was observed, but at other *p*H the solution remained clear after heating. This would indicate that the isoelectric point of this preparation is somewhere between *p*H 5 and 6. No indication is given by previous workers

about the *p*H at which they heated their solutions and it might very well be that some of them were not in the optimum range.

The active constituents of crude testicular extracts cannot be separated from proteins by chromatographic analysis. Some of the proteins present in the extracts submitted to adsorption were less readily adsorbed than the active constituents, and others much more. The separation thus achieved was not so complete as that attained by $(NH_4)_2SO_4$ precipitation.

Experimental

Comparison of testicular diffusing factor with kallikrein

As a source of kallikrein the commercial preparation "Padutin" (Bayer) was used. One unit is stated to correspond to 0·003 mg. of a standard preparation. 9 ml. of "Padutin for oral administration" (1 ml. corresponds to 7 units) were concentrated *in vacuo* over H_2SO_4 to 5·5 ml. A thick syrup resulted. In the shaved back of each of a group of three rabbits two injections of 0·3 ml. of each

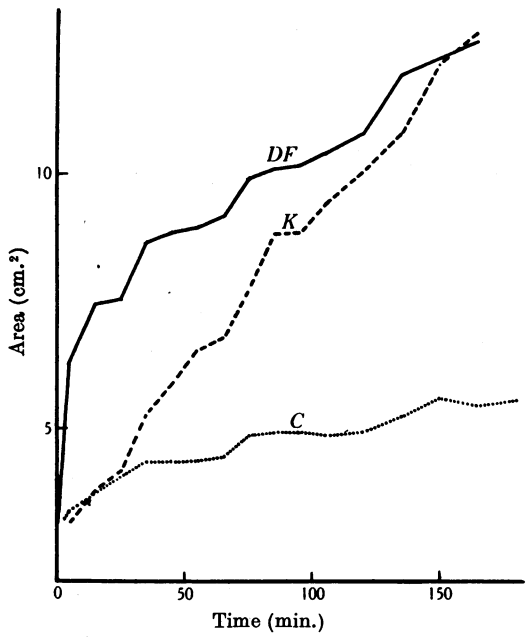

Fig. 1. Comparison of diffusing factors from different sources. *C*, control; *W*, *Clostridium Welchii*; *DF*, testicular diffusing factor; *S*, *Vibrion septique*; *K*, kallikrein; *R*, rattlesnake venom; *T*, *Staphylococcus*; *L*, leech extract; *G*, diazobenzenesulphonic acid-serum globulin.

of the following solutions were made: the concentrated kallikrein solution, a solution of a standard preparation of testicular diffusing factor [Madinaveitia, 1938] having 300 units/ml. and isotonic saline. All three solutions were diluted with 2 vol. of isotonic haemoglobin solution.

Measurements of the size of the coloured areas of skin were made every 10 min. during the first 2 hr. after injection and every 15 min. during the 3rd hr. The size recorded in every instance represents the average of three consecutive readings, the taking of which occupied not more than 5 min. The average of the results obtained in the three rabbits is shown graphically in Fig. 1.

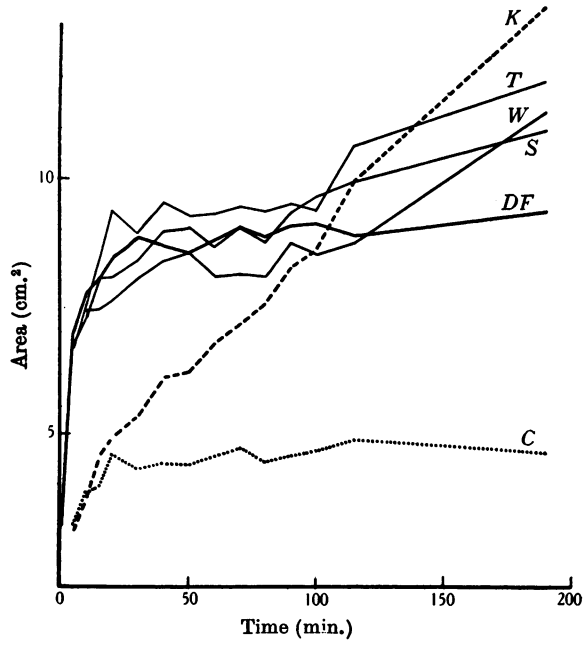

Fig. 2. For description see Fig. 1.

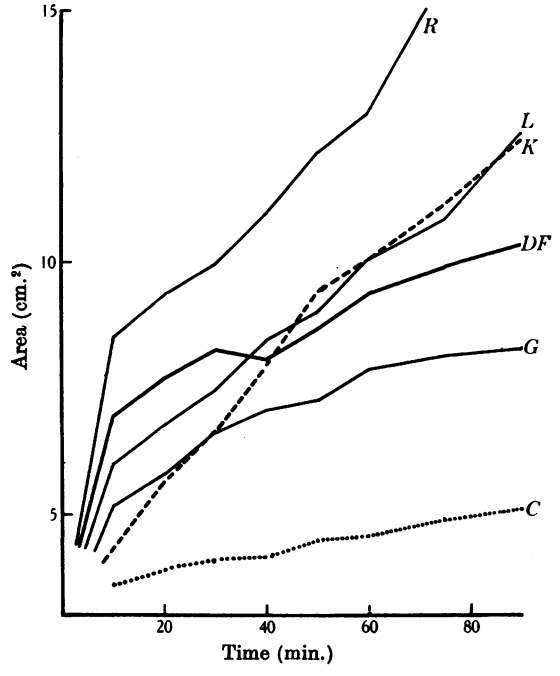

Fig. 3. For description see Fig. 1.

Comparison of diffusing factors from different sources

The bacterial diffusing preparations compared were filtrates of cultures of *Clostridium Welchii*, *Vibrion septique* and *Staphylococcus* toxoid, diluted with 2 vol. of an isotonic solution of haemoglobin. 0·3 ml. of each solution was injected into the shaved back of two rabbits. Injections were also made of 1 : 3 dilutions in isotonic haemoglobin solution of concentrated kallikrein solution (see above), a solution of testicular diffusing factor (100 units/ml.) and isotonic saline. Fig. 2 shows the rate of spread of haemoglobin in each case. The measurements were made as described in the previous experiment.

In another group of three rabbits the following solutions were compared in a similar way: diazobenzenesulphonic acid-serum globulin (kindly supplied by Prof. C. R. Harington) containing about 0·8% protein, a filtered leech extract prepared by grinding up four leeches in a mortar with 25 ml. of water, and a solution of rattlesnake venom containing 30 mg. dry venom per ml. Injections of solutions of diffusing factor (1000 units/ml.) and of a concentrated solution of kallikrein were also made, as well as a control injection of isotonic saline. The average of the results obtained in the three rabbits is represented in Fig. 3.

Action of testicular diffusing factor on the isolated skin of a rabbit

The back of a rabbit was shaved and the animal killed and skinned. The skin was fixed on a board over wet cotton wool. Into this prepared skin injections of 0·3 ml. of serial tenfold dilutions (in isotonic haemoglobin solution) of the

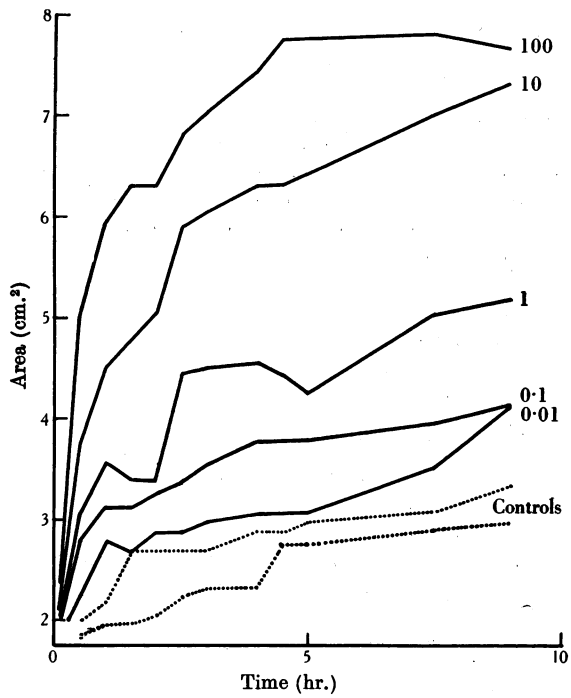

Fig. 4. Increased spread of haemoglobin in the isolated skin of a rabbit.

standard preparation were made. The most concentrated had 100 units/ml. and the most dilute 0·01 unit/ml. Two control injections of saline were also made.

The increase in the area of skin in each case stained by haemoglobin is represented in Fig. 4. Since only one animal was used in the experiment the curves are not so continuous as when the average of results in several animals is represented. The temperature of the skin during the experiment was 18–20°.

Fractionation of aqueous extracts of bull testicle with $(NH_4)_2SO_4$

1 g. of dry testicle powder mixed with the same bulk of silver sand was extracted in a mortar with 20 ml. of water. After centrifuging the turbid supernatant liquor was flocculated by addition of 0·15 ml. 2*N* acetic acid. Centrifuging now yielded a clear solution with the full activity of the original material.

Increasing amounts of saturated $(NH_4)_2SO_4$ were added to this solution as indicated below:

% of saturation	20	33	50	75	90
ml. testicular extract	4	2	2	2	1
ml. sol. sat. $(NH_4)_2SO_4$	1	1	2	2	9

The precipitate in each case was separated by centrifuging and washed with 5 ml. of $(NH_4)_2SO_4$ of the corresponding concentration. Both the precipitates and supernatants of each precipitation were made up to ten times the original volume of testicular extract used. All solutions were diluted with 10 vol. isotonic haemoglobin solution. Their spreading activities were compared with that of serial tenfold dilutions of the testicular extract. A group of two rabbits was used for the precipitates and another group for the supernatants. The average of the results is plotted in Figs. 5 and 6. The precipitate obtained at 50 % saturation with $(NH_4)_2SO_4$ has less than 10 % of the original activity. At 75 and 90 % of saturation the precipitation is still incomplete, both precipitate and supernatant having more than 10 % of the activity of the original solution.

For the preparation of purified concentrates of diffusing factor the following procedure has been used: 250 g. of a dry testicle powder (5 % moisture, 12·5 % ether-soluble materials 11 % N) were extracted overnight in a ball mill with 1 l. of water. A small amount of toluene was added as an antiseptic. After dilution with 1 l. of water 10 ml. 3*N* acetic acid were added and the mixture filtered after 1 hr. The residue was extracted during 4 hr. with 1 l. of water, filtered, and the volume of the united filtrates reduced *in vacuo* at 20° to 250 ml. This concentrated solution contains 58 mg. N/ml. (53 % of the N present in the dry testicle powder). To it 1 vol. of saturated $(NH_4)_2SO_4$ was added and after 1 hr. the precipitate removed. The clear solution was then dialysed against running tap water for 1 day, concentrated *in vacuo* at 20° at about 300 ml. and again dialysed for a day. After making up the volume of the solution to 400 ml. it contained 2·2 mg. N/ml. (i.e. 1·6 % of the N present in the 250 g. dry testicle powder).

The solution so obtained has the full activity of the original testicular extract. It gives all the usual precipitation reactions of proteins, except that it is not precipitated by lead acetate, basic or neutral.

The effect of heat on the coagulation of this solution is recorded in the table below, 2 ml. of solution were heated with 10 ml. of buffer in a water bath.

M/3 acetate buffer of *p*H	3	4	5	6		
M/15 phosphate buffer of *p*H	.	.	.	6	7	8
Temp. ° C.						
70	C	C	C	C	C	C
75	C	C	T	T	C	C
80	C	C	T	T	C	C
90	C	C	T	F	C	C
100	C	C	F	F	C	C

C, clear; T, turbid; F, flocculation.

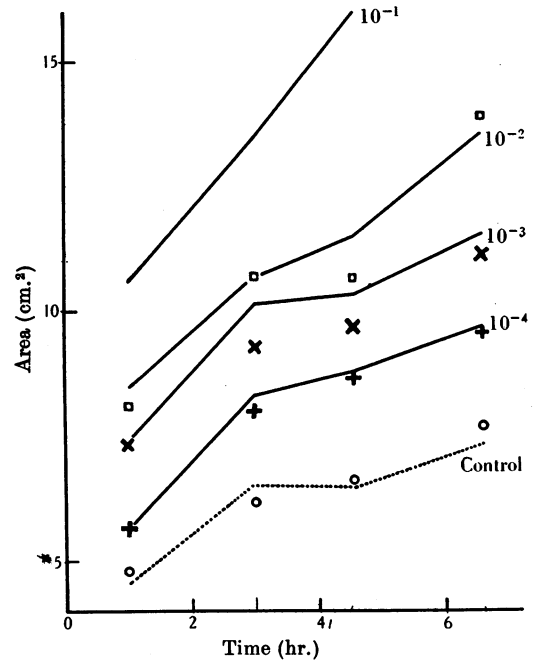

Fig. 5. Fractionation with $(NH_4)_2SO_4$. Activity of precipitates.

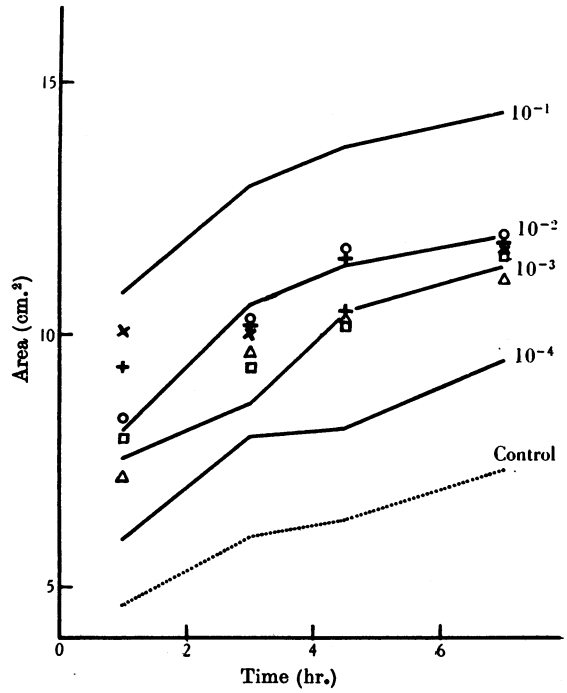

Fig. 6. Fractionation with $(NH_4)_2SO_4$. Activity of supernatants.
o 20%; + 33%; × 50%; □ 75%; △ 90%

Adsorption of crude testicular extracts on aluminium oxide

250 g. dry testicle powder were extracted (mechanical stirring for 1 hr.) three times with 4 l. of water. The insoluble material was separated each time

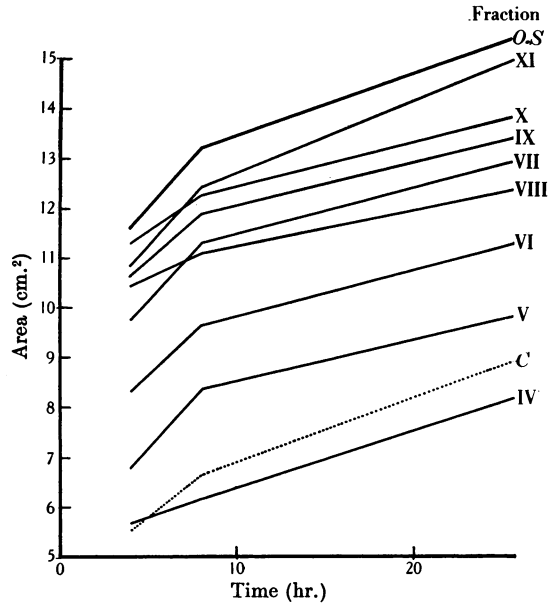

Fig. 7. Adsorption of crude testicular extract on aluminium oxide.

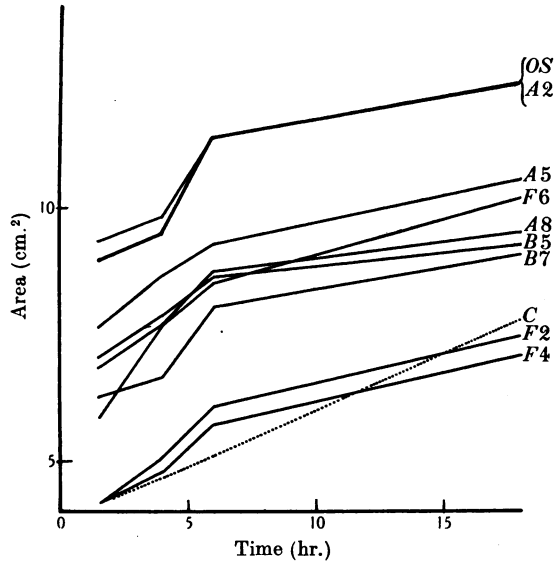

Fig. 8. Adsorption and elution of crude testicular extracts.

by decantation, and the supernatants mixed. To these 100 ml. $N/10$ acetic acid were added and the precipitate allowed to sediment in the cold room. The clear

supernatant was passed through a column (10 × 4·5 cm.) of aluminium oxide (Merck) (100 g.) previously wetted with water. The receiver was changed every 250 ml. (30 min.). After the first four fractions were collected the vacuum was increased and the receiver changed every 500 ml. (20 min.). Two yellowish bands were observed, the first reaching the bottom of the column when 3 l. of solution had passed through. In the table below some of the properties of the resulting fractions are recorded. Tenfold dilutions (in isotonic haemoglobin solution) of fractions iv–xi were injected into the back of three rabbits. The spread of haemoglobin through the skin is recorded in Fig. 7. Fraction vi giving tests for protein has not the full activity of the original solution (*OS*) while fraction ix, although it does not give a precipitate with lead acetate, has almost the same activity as the original solution.

Fraction no.	*OS*	i	ii	iii	iv	v	vi	vii	viii	ix	x	xi
Vol. of fraction in ml.	.	250	250	250	250	550	500	400	500	475	500	600
Vol. of liquid through column in l.	.	0·25	0·5	0·75	1	1·5	2	2·5	3	3·5	4	4·5
Precipitation by trichloroacetic acid	+	−	−	−	−	−	+	+	+	+	+	+
Precipitation by neutral lead acetate	+	+	±	±	±	±	+	+	±	−	+	+
Heat-coagulation	+	−	−	−	−	+	+	+	+	+	+	+
Biuret test	+	−	−	−	±	+	+	+	+	+	+	+

In another experiment a similar column of aluminium oxide (Hopkins and Williams pure anhydrous) was prepared. 300 ml. of the testicular extract used in the previous experiment (2·63 mg. *N*/5 ml.) were filtered through it, changing the receiver every 50 ml. The column was then washed with 400 ml. *M*/500 acetic acid and subsequently with 1 % $NaHCO_3$ (400 ml.). The behaviour towards trichloroacetic acid and the N content of some of the fractions collected are shown in the following table.

Filtrates (*F*)

Fractions of 50 ml.	1	2	3	4	5	6
Trichloroacetic acid	−	−	−	+	+	+
mg. *N*/5 ml.	.	0·014	.	0·45	.	0·94

Acetic acid washings (*A*)

Fractions of 50 ml.	1	2	3	4	5	6	7	8
Trichloroacetic acid	+ +	+ +	+	+	+	+	±	±
mg. *N*/5 ml.	.	2·48	.	0·384	.	.	.	0·128

Bicarbonate washings (*B*)

Fractions of 50 ml.	1	2	3	4	5	6	7	8
Trichloroacetic acid	±	?	+	+	±	±	±	?
mg. *N*/5 ml.	.	.	0·331	.	.	.	0·069	

The area of skin over which haemoglobin is induced to spread by injection of 1 : 10 dilutions (in isotonic haemoglobin solution) of some of these fractions is shown in Fig. 8. After most of the active materials have been eluted with acid, substantial amounts of inactive protein are eluted from the column by $NaHCO_3$.

Summary

1. Comparison of testicular diffusing preparations with kallikrein indicate that, contrary to the statement of Christensen [1938], the two materials are quite unlike one another in their effects on the rate of spread of injected fluids through rabbit skin.

2. Rattlesnake venom, filtrates of *Clostridium Welchii* and *Vibrion septique*, staphylococcal toxoid, leech extracts and diazobenzenesulphonic acid-serum globulin all resemble the testicular diffusing factor in producing a very rapid increase in the rate of spread of fluids through skin.

3. Purification of the testicular diffusing factor by means of fractional precipitation with ammonium sulphate and dialysis is described. This method yields products of similar activity to those previously described [Madinaveitia, 1938].

4. The main bulk of evidence indicates that the active material has protein properties and that its biological action, which does not depend on circulation, is not due to a surface tension effect.

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