STUDIES ON SPREADING FACTORS

I. The Importance of Mechanical Factors in Hyaluronidase Action in Skin*

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It is generally agreed that there is a close relationship between spreading factors (S. F.) from testis, bacteria, venoms, and other sources with the mucolytic enzyme, hyaluronidase (*cf.* the review of Duran-Reynals (1) for references). While it is known that hyaluronidase removes a dermal barrier (presumably hyaluronic acid) to the diffusion of fluids in skin certain aspects of hyaluronidase spreading activity are little understood. For example, there is no explanation for the flat upper portion of the sigmoid dosage-response curve of S. F. (2). To state that this result is simply due to saturation of the skin substrate with hyaluronidase, ignores the fact that the entire skin is potentially available for the enzyme and that the amount of substrate is not constant in this system.

The fact that above a maximal concentration of hyaluronidase increasing enzyme concentration has so little effect in increasing the area of spread might be attributed to the operation of one of the following mechanisms: (*a*) Normal skin may contain an active inhibitor of hyaluronidase, which inactivates the enzyme at a rate dependent upon the hyaluronidase concentration. (*b*) Hyaluronidase may be adsorbed on some constituent of skin, so that it is retained close to the site of injection. This possibility, suggested by Evans and Madinaveitia (3) has not been tested experimentally. (*c*) Intradermally injected hyaluronidase may be rapidly inactivated as in (*a*) by a circulating antihyaluronidase which can readily pass through the capillary wall. Normal serum is known to contain antihyaluronidase activity (4–7), although no information is available concerning the rate of diffusion of this factor through the capillary wall. (*d*) Hyaluronidase may be rapidly absorbed directly into the circulatory system, or indirectly *via* the lymphatics, and thus be eliminated from the skin. (*e*) The diffusion of hyaluronidase in skin may be controlled by the pressure associated with the injection of fluid, so that the enzyme can diffuse only to an area determined by the volume injected. The above possibilities have been examined in this study.

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Methods

General.—Albino rabbits, derived from various strains, approximately 3 months old, were used in these studies. The abdomens of these rabbits were shaved approximately 24 hours prior to experimentation. Rabbits were selected for study whose skin was sufficiently thin to permit visualization of the colored indicator throughout the entire course of the spreading reaction. No rabbit was used in more than one experiment. Control experiments confirmed the finding (2) that in the spreading of the various agents used there were no statistically significant differences between one area of the abdominal skin and any other. The area of spread at various time intervals after injection was obtained using the formula $\frac{D \times d \times \pi}{4}$ (assuming an elliptical shape for the bleb) where D is the longest diameter and d the smallest diameter. D and d were measured with calipers and care was taken to exert no external pressure on the blebs.

Indicator Solution.—In most of the experiments to be reported twice recrystallized bovine hemoglobin (Welker and Williamson (8)) was used as the indicator. The hemoglobin concentration after adjustment was 18 per cent and the NaCl concentration was 0.9 per cent. On standing in the cold room the hemoglobin was spontaneously converted to methemoglobin. This change, however, did not affect the indicator qualities of the solution. Sterile solutions of the methemoglobin indicator at 2°C. have kept well for as long as 6 months. In other experiments the usual Madinaveitia (9) hemoglobin indicator was employed. No differences between these hemoglobin indicators were observed.

Spreading Factors.—Hyaluronidase prepared from bull testicles by the method of Madinaveitia (10) was obtained through the courtesy of Dr. Erwin Schwenk of the Schering Corporation. In contrast to other testis S. F. reported in the literature (11, 12), this preparation did not produce an inflammatory reaction accompanied by capillary damage when injected intradermally in amounts as high as 1 mg. per cc. Thus, the intradermal administration of hyaluronidase did not lead to localized accumulation of trypan blue (intravenously administered) at the site of the intradermal injection; nor did these dosages produce erythema and edema. The finding that S. F. activity can be dissociated from any effect on capillary permeability is not new (13).

In addition to purified bovine testis hyaluronidase three poisonous snake venoms were also studied. These were supplied by Ross Allen, Silver Springs, Florida, and were Florida diamondback rattlesnake (*Crotalus adamanteus*), moccasin (*Agkistrodon piscivorus*), and copperhead (*Agkistrodon mokasen*). The crude venoms were obtained as dry preparations and were kept at room temperature for approximately 6 months before use. In marked contrast to the testis hyaluronidase, these venoms uniformly produced marked inflammation characterized by capillary damage, hemorrhagic erythema, and edema when injected by the intradermal route. Intense dye accumulation at the site of intradermal administration of 5 to 10 μg. of these venoms resulted when trypan blue was administered intravenously.

Solutions for Intradermal Injection.—The solutions of S. F. were made up in physiological saline shortly before injection. To each dilution an equal volume of hemoglobin or methemoglobin solution was added so that the final dilution of the S. F. was one-half of the original concentration. Once the S. F. and the indicator solutions were mixed, the final solution was injected within 2 to 3 minutes to minimize reactions between possible inhibitory materials in the indicator solution and S. F. Although no evidence of reactions of this type was observed, this precaution was uniformly adhered to in all experiments.

Rate of Spreading.—The area colored by the indicator at 1, 2, 5, 10, 20, and 40 minutes after injection was measured unless otherwise stated. The time required to take the area measurements was approximately 10 to 15 seconds. All time intervals during the first 5 minutes of the reaction were measured with a stop-watch. The area at zero time with S. F. solutions was

not measurable because the solution spreads as it is being injected. Since saline blebs spread very slowly, area at zero time with S. F. was arbitrarily taken as that equal to the initial area of the saline-control bleb as measured immediately after injection. Each concentration of S. F. was injected intradermally into at least two areas of skin. In 100 successive tests where two areas injected with the same material were compared to each other (over a wide range of S. F. concentrations) the variation averaged ± 4.7 per cent with a range of 0 to ± 11.5 per cent.

Assay of S. F.—In the experiments designed to test whether hyaluronidase was destroyed or adsorbed it was necessary to assay S. F. activity after varying treatments. This was accomplished in the following manner: Varying concentrations of hyaluronidase (in a constant volume and indicator concentration) were injected in duplicate sites of a rabbit and the area of spread 1, 2, 5, and 10 minutes after injection was determined. From the log-dose response curves obtained, concentrations of hyaluronidase which produced spreads intermediate between minimal and maximal, as well as those which produced maximal responses were selected. These enzyme concentrations were then allowed to react with the system under investigation, and then retested for S. F. activity in the same animal. Using this technic, the factor of individual animal variation was eliminated. To check the results obtained in the single animal, all experiments were repeated at least three times with three different rabbits. In the following sections the expression "various concentrations of hyaluronidase" indicates that the above procedure was followed.

EXPERIMENTAL

Effect of Hyaluronidase Concentration on the Spreading Reaction.—The log-dosage response curve of hyaluronidase has been determined by Bacharach *et al.* (2) at only one time interval after injection, *e.g.* 20 minutes, at a time when the spreading reaction is complete. In our first experiments the dosage-response curve of S. F. was determined during the initial, as well as later, stages of the spreading reaction.

Table I shows the results obtained in a representative experiment where the effect of hyaluronidase concentration (administered intradermally in a constant volume of 0.2 cc.) upon area of spreading at varying time intervals has been studied. Fig. 1 illustrates the log-dosage response curves plotted at various time intervals during the experiment. Fig. 2 illustrates the log-dosage response curve in two different rabbits indicating the extent of variation encountered from animal to animal.

These data show that (*a*) the log-dosage response curve is best fitted by a sigmoid curve at all time intervals after injection; (*b*) there is a maximal area of spread attained 10 to 20 minutes after injection; (*c*) the rate at which this maximal area is attained varies with the enzyme concentration; and (*d*) the effect of hyaluronidase concentration upon spreading is most evident during the initial phase of the reaction; *i.e.*, during the first minute.

It may be seen from Table I that the spreading obtained with 10 μg. per cc. is not increased by a 100-fold increase of hyaluronidase concentration either as regards the rate of spreading during the first minute or the final area of spread. This finding, however, should not be interpreted as indicating that actually no differences exist between the initial rates of spreading with these

TABLE I

*The Effect of Hyaluronidase Concentration on the Spreading of Indicator in Rabbit Skin**

Hyaluronidase	Area							
	0 min.	1 min.	2 min.	5 min.	10 min.	20 min.	30 min.	60 min.
μg. per cc.	*cm.²*	*cm.²*	*cm.²*	*cm.²*	*cm.²*	*cm.²*	*cm.²*	*cm.²*
1000	—	4.05	4.20	4.87	5.05	5.55	5.55	5.55
100	—	4.02	4.14	5.07	5.32	5.32	5.32	5.32
10	—	4.08	4.54	4.93	5.18	5.20	5.20	5.20
1	—	3.08	4.28	5.10	5.10	5.10	5.10	5.20
0.5	—	2.82	3.88	5.00	5.05	5.05	5.05	—
10^{-1}	—	1.95	2.20	3.70	3.97	5.05	5.05	—
5×10^{-2}	—	1.86	2.02	2.64	3.38	4.12	4.25	—
2.5×10^{-2}	—	1.64	1.92	2.36	2.80	3.42	3.42	—
1.2×10^{-2}	—	1.64	2.01	2.50	—	3.48	3.48	—
0	1.25	1.51	1.73	2.39	2.64	2.76	2.93	3.25

* Administered intradermally in a constant volume of 0.2 cc.

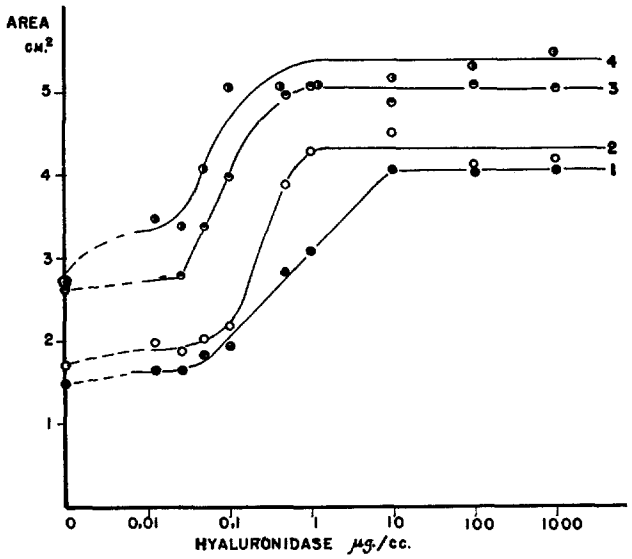

FIG. 1. Log dosage-response curve of hyaluronidase administered intradermally in a volume of 0.2 cc. at varying time intervals after injection. Curves 1, 2, 3, and 4 represent the values obtained 1, 2, 5, and 20 minutes after injection respectively.

enzyme concentrations. It appeared more likely from observation of the bleb that the first reading in our rabbits could not be taken soon enough after the injection to establish accurately the actual initial rate.

Effect of Skin and Dermal Breis and Extracts upon S. F.—In an attempt to test the possibility that skin contains an active antihyaluronidase, extracts of whole shaven skin and of dermis from rabbits were prepared in a variety of ways; they were incubated *in vitro* for 2, 10, 30, or 60 minutes at 25°C. with varying concentrations of hyaluronidase and the solutions were then retested for S. F. activity. Since the results were almost uniformly negative, only brief mention will be made of the extraction procedures employed.

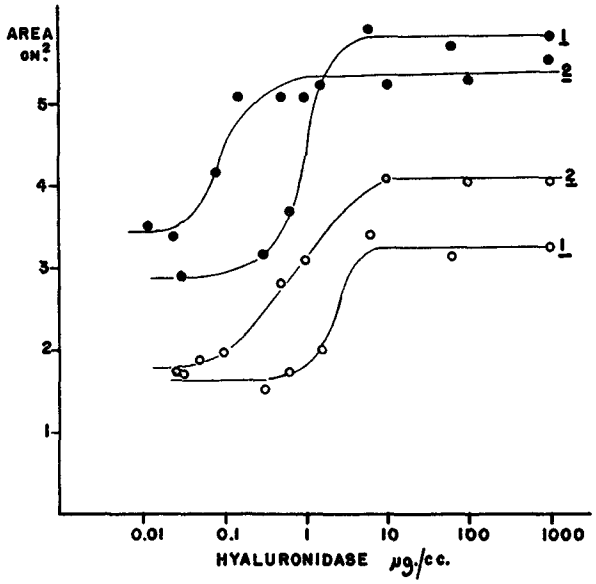

FIG. 2. Degree of variation observed in two rabbits injected with 0.2 cc. hyaluronidase solutions. The log dosage-response curves of the first rabbit are labeled 1; those of the second rabbit 2. The open circles (○-○) show the curves obtained 1 minute after injection; the closed circles (●-●) those obtained 10 minutes after injection.

Whole shaven skin or dermis was finely minced and ground with either saline or water, in proportions varying from one part of tissue to 1–4 parts fluid. These extracts were set into the refrigerator at 2–4°C. and allowed to extract for periods ranging from 6 to 48 hours. Of ten extracts prepared, only one showed very slight inhibitory activity. When the extraction procedure which gave this possible positive result was repeated on two subsequent occasions, the results were completely negative as regards S. F. inactivation. The skin extracts tested by themselves were devoid of spreading activity.

The following experiments were performed to test the possibility that hyaluronidase is readily adsorbed by a skin constituent. The insoluble residues obtained from the above extraction procedures were incubated with varying concentrations of hyaluronidase for intervals ranging from 2 to 60 minutes. In these experiments the residues were suspended in solutions of hyaluronidase (1 part residue to 1 part fluid) and shaken for intervals ranging from 15 to 60 minutes at room temperature. It was consistently observed that hyaluronidase spreading activity was not affected by this treatment.

In four experiments using breis (two with shaven skin and two with dermis) no inactivation of hyaluronidase (using varying concentrations) as regards spreading activity was demonstrable. In these experiments one part of brei was mixed with one volume of solution, and shaken for 60 minutes. Samples were taken after 15, 30, and 60 minutes' incubation at room temperature.

From the results of the above experiments it appears unlikely that skin contains a highly active S. F. inhibitor. While the absence of adsorption of S. F. by brei or residues of skin is clear, the possibility that S. F. may be adsorbed on skin *in vivo* is not entirely ruled out by these experiments.

Hyaluronidase Spreading Activity and Skin Blood Flow.—In the next experiments, the influence of skin blood flow upon hyaluronidase was studied. If there is either rapid circulatory absorption of hyaluronidase or a readily diffusible antihyaluronidase circulating in blood, then as blood flow is decreased hyaluronidase should exhibit greater spreading activity. Contrariwise, if either of these postulated mechanisms is operative, the spreading effect of hyaluronidase should decrease as skin blood flow increases.

The effect of skin blood flow was evaluated by studying the spreading response of hyaluronidase in normal as compared to hyperemic or ischemic skin. These experiments were performed in the following manner. Varying doses of hyaluronidase were administered to a normal rabbit and the response noted; thereafter, other areas of the skin were either rendered hyperemic by the application of xylol, or ischemic by producing hemorrhagic shock in the rabbit. The dosages of hyaluronidase previously used were then retested in the skin whose circulation had been altered by the above means. In other experiments, the responses produced by varying doses of hyaluronidase were compared before and after exsanguination of rabbits. All experiments wherein the effect of xylol hyperemia on hyaluronidase spreading was studied were performed by injecting the area 5 minutes after xylol had been applied topically to the skin.[1]

Table II shows representative experiments in which hyaluronidase spreading activity was compared in hyperemic, ischemic, and normal skin. The results demonstrate that the spreading activity of hyaluronidase is not influenced by changes in skin blood flow. It therefore appears that blood does not contain a readily diffusible antihyaluronidase. In addition, these results indicate that intradermally injected hyaluronidase is not removed from skin by absorption into the vascular or lymphatic system during the time interval of these experiments.

[1] The experiments using xylol as a hyperemic agent require special mention. If one applies xylol to the surface of the skin, hyperemia occurs almost immediately, and if hyaluronidase plus hemoglobin indicator is injected intradermally the spread may be visualized for approximately 20 to 25 minutes without great difficulty. If, however, one injects hyaluronidase 20 to 30 minutes after xylol application, in some animals the indicator tends to disappear or to "fade out" leaving the injected area almost colorless in a short time. This phenomenon also occurs when a solution containing saline plus hemoglobin indicator is injected, although somewhat more slowly and less dramatically. When the skin in which the "fading out" has ocurred is sectioned, it is noted first that the skin thickness is increased as the result of edema; and second that the indicator which seemed to disappear as visualized through the surface of the skin, has spread through the lower layers of the dermis and is clearly evident there. The phenomenon of "indicator disappearance" in skin treated with xylol 20 to 30 minutes prior to injection therefore seems to be due to the thickening of skin by edema fluid associated with a diffusion of the hemoglobin indicator in lower dermal layers which makes visualization of the indicator difficult or impossible.

TABLE II

Spreading Activity as Related to Blood Flow

Experiment No.	Hyaluronidase	Blood flow	Increase in area over control* at				
			1 min.	2 min.	5 min.	10 min.	20 min.
	μg. per cc.		*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
1‡		Normal	16	25	39	56	—
	1	Decreased	15	19	42	56	—
		Normal	65	77	84	65	—
	2	Decreased	53	65	53	36	—
		Normal	111	90	92	65	—
	5	Decreased	76	85	81	60	—
		Normal	119	119	112	82	—
	10	Decreased	104	96	80	61	—
2§		Normal	—	27	25	30	20
	0.25	Absent	—	24	24	32	21
		Normal	—	—	47	60	50
	0.5	Absent	—	—	52	62	47
		Normal	—	117	97	97	83
	2.5	Absent	—	110	90	101	81
		Normal	—	117	100	105	89
	250	Absent	—	119	107	107	90
3‖		Normal	47	68	64	81	—
	0.2	Increased	45	59	60	78	—
		Normal	65	88	73	80	—
	1	Increased	61	80	84	70	—
		Normal	109	95	92	90	—
	2	Increased	104	96	85	80	—

* The control was saline-indicator solution, administered to skin the blood flow of which had been altered as indicated, and to normal skin. No significant differences in the spreading produced by saline were noted as blood flow was altered. However, there did appear to be a tendency for saline to spread more rapidly as blood flow was increased, and more slowly when blood flow was absent.

‡ In Experiment 1, hyaluronidase in varying concentrations but constant volume was injected before and after the induction of hemorrhagic shock in a single rabbit.

§ In Experiment 2, hyaluronidase (as in Experiment 1) was administered before and after exsanguination of a rabbit.

‖ In Experiment 3, hyaluronidase (as in Experiment 1) was administered before and after xylol treatment of skin.

The Effect of Volume of Fluid on the Spreading Reaction.—In the previous section evidence for rapid hyaluronidase removal from skin as the result of (*a*) inhibitors present in skin or blood, (*b*) adsorption by a skin constituent, or (*c*) elimination from skin by absorption into the vascular system has been examined and found insufficient to account for the lack of correspondence between dosage of hyaluronidase and spreading. These negative findings suggest that the spreading of hyaluronidase solutions might be in part influenced by simple mechanical pressure of the injection. The pressure required to make the injection may be considered as being transferred to the bleb which results, and is expressed as a localized increase of interstitial pressure and volume of the skin. This pressure within the bleb is a force which tends to accelerate the diffusion of material within the injected area into the adjacent non-injected areas of skin. As the bleb of injected fluid spreads, the local increase of interstitial pressure diminishes and eventually a point is reached when the localized pressure of the bleb is no longer higher than the normal interstitial pressure of the skin. When this point is reached, spreading of injected material depends entirely upon the physiological forces which control diffusion in tissues and the diffusion characteristics of the substance under consideration. If one assumes that hyaluronidase diffuses slowly through skin in the absence of local pressure and volume effects produced by injection, then one might expect hyaluronidase solutions to spread rapidly only when local interstitial pressure and volume of the skin are increased over a certain minimal value. On this basis, once the bleb injected with a maximal amount of hyaluronidase has spread sufficiently to produce this minimal value of interstitial pressure and volume, the presence or absence of excess hyaluronidase should should have no further significance as regards spreading, inasmuch as the excess hyaluronidase could no longer diffuse to remove dermal barriers to fluid diffusion.

According to this view hyaluronidase administered intradermally with minimal pressure should exhibit little or no spreading activity. To test this point, solutions of hyaluronidase (ranging in concentrations from 3.3 to 333 μg. per cc.) plus indicator were placed on superficial epidermal incisions. A vaseline rim was placed around each incision to form a cup into which the solutions were placed, thus insuring that the incision was constantly bathed with fluid being tested. Care was taken not to allow the fluid to dry out. When properly cut, the amount of bleeding in the incision was nil. At intervals ranging from 20 to 90 minutes the fluid within the cup was removed by absorbent cotton, the hemoglobin on the surface of the skin was removed with water, and the distance of penetration of the solutions into each portion of skin adjacent to the longitudinal incision measured. Table III illustrates a typical experiment where concentrations of hyaluronidase as high as 333 μg. per cc. failed in 90 minutes to exhibit significant spreading activity as compared to the saline control. Since 0.2 cc. of a 1 μg. per cc. solution of hyaluronidase injected intradermally increases the area spread *ca.* 3 cm.^{2} over the saline control in a few minutes (see Table I) the absence of a spreading effect with hyaluronidase administered through skin incisions is very striking.

In the next experiments, varying interstitial pressures and volumes were obtained by varying the volume of hyaluronidase solution administered in-

TABLE III

The Spread of Indicator in Skin through Epidermis in the Absence and Presence of Hyaluronidase

Hyaluronidase	Spread from incision into skin at 90 min.
μg. per cc.	*mm.*
0	1.6
3.3	1.8
33.3	1.6
333.3	2.0

TABLE IV

The Effect of Hyaluronidase Administered in Varying Volume

(Enzyme concentration constant)

Volume	Hyaluronidase	Time after injection, *min.*									
		0		1		2		5		10	
		A*	T‡	A	T	A	T	A	T	A	T
cm.²	*μg./cc.*	*cm.²*	*mm.*	*cm.²*	*mm.*	*cm.²*	*mm.*	*cm.²*	*mm.*	*cm.²*	*mm.*
0.02	3.3	—	—	0.67	0.30	0.85	0.24	0.85	0.24	0.92	0.22
	0	0.40	0.50	0.49	0.41	0.51	0.39	0.52	0.38	0.59	0.34
0.10	3.3	—	—	2.18	0.46	2.26	0.44	2.64	0.38	2.64	0.38
	0	0.79	1.30	1.04	0.96	1.22	0.82	1.32	0.76	1.63	0.62
0.25	3.3	—	—	4.58	0.54	4.90	0.51	5.36	0.47	5.36	0.47
	0	1.32	1.90	1.51	1.65	1.94	1.29	2.20	1.13	2.60	0.96
0.50	3.3	—	—	9.03	0.56	11.40	0.44	11.62	0.43	11.62	0.43
	0	1.53	3.26	2.08	2.40	2.36	2.12	2.80	1.78	3.25	1.54
1.00	3.3	—	—	10.39	0.91	13.20	0.76	15.20	0.68	15.56	0.64
	0	2.98	3.36	3.80	2.63	4.32	2.32	5.08	1.97	5.70	1.76

* A, area of spread in cm.².

‡ T, average thickness of the bleb in millimeters calculated from volume injected per area of spread.

tradermally while the enzyme concentration (3.3 μg./cc.) was kept constant. Table IV shows these results in a representative experiment. Fig. 3 illustrates the relationship between the volume of fluid administered, the rate of spread during the first minute, and the area of spread at 10 minutes. It will be seen that as the volume is increased from 0.02 to 0.5 cc. both values increase

in linear fashion and thereafter level off. These results demonstrate that the spreading of a hyaluronidase solution is directly related to the volume of the injection. In an attempt to evaluate the changes in the interstitial pressure of the bleb as it was spreading, an indirect method was employed. It was assumed that the "thickness" or the height of the bleb was proportional to the increased interstitial pressure. Knowing the volume administered and the area of spread, an "average thickness" T of the bleb at varying time intervals

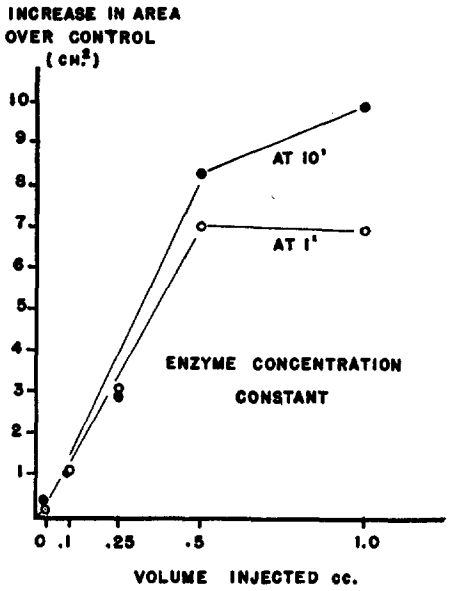

FIG. 3. Spreading effect produced by a constant hyaluronidase concentration (3.3 μg. per cc.) intradermally administered in volumes ranging from 0.02 to 1.0 cc. at 1 and 10 minutes after injection. It will be seen that the initial rate of spreading during the first minute, and the area of spread at 10 minutes are directly related to the volume injected in the range from 0.02 to 0.5 cc.

was calculated from T = volume of fluid administered/area of spread (assuming that there is neither gain nor loss from the injected volume) and these values are shown in Table III. Fig. 4 shows the change of T with respect to time with various volumes of fluid, in the absence and presence of hyaluronidase. It will be seen that the initial T values increase with volume increase. With hyaluronidase T and presumably the interstitial pressure of the bleb rapidly fall off and tend to reach a similar low value independent of the volume injected.

Another experiment shown in Table V answers possible objections that the results of Table IV are due to differences in the total amount of hyaluronidase administered. Here the total amount of enzyme was kept constant (10 μg.)

but the volumes were varied from 0.05 to 0.5 cc. As will be seen, the solutions administered in largest volume (but lowest concentration) spread to a greater extent than did the solutions injected in smaller volume and higher concen-

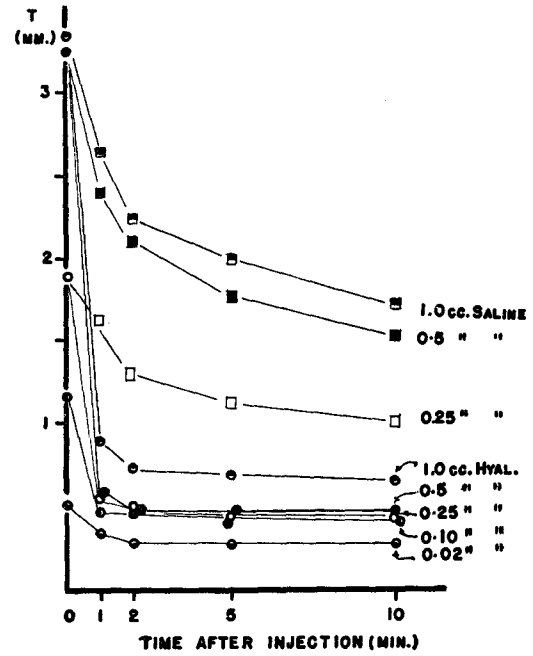

FIG. 4. Change in T, the calculated average thickness of the bleb (regarded as an index of the local increase of interstitial pressure produced by the injection) with respect to time, in the presence and absence of a constant hyaluronidase concentration (3.3 μg. per cc.) administered in varying volumes. It will be seen that the initial T values increase as the volume administered is increased from 0.02 to 0.5 cc. and then levels off so that 1 cc. produces a T value only slightly higher than that achieved with 0.5 cc. In general with hyaluronidase, the greater the initial thickness of the bleb, the faster the rate of decrease of T; thus the T values of the hyaluronidase blebs, independent of the volume injected, tend to rapidly decrease to the same minimal value and thereafter show little change. This is taken to indicate that the local increase of interstitial pressure produced by the injection, in the presence of hyaluronidase is rapidly decreased to a minimal value as spreading occurs. The change of T with respect to time in the absence of hyaluronidase is entirely dissimilar.

tration. Fig. 5 shows the relationship between the effect of volume upon the rate of spread during the first minute and the final area of spread of these hyaluronidase solutions. Here again, as in the experiment where the enzyme concentration was kept constant but the volume varied, there is linear relationship between volume and spreading.

When it had been demonstrated that the volume of administered fluid determines to a great extent the characteristics of the spreading phenomenon, it

TABLE V

The Effect of 10 μg. Hyaluronidase Administered in Varying Volume

Volume	Hyaluronidase	Time after injection, *min.*									
		0		1		2		5		10	
		A*	T‡	A	T	A	T	A	T	A	T
*cm.*³	*μg./cc.*	*cm.*²	*mm.*	*cm.*²	*mm.*	*cm.*²	*mm.*	*cm.*²	*mm.*	*cm.*²	*mm.*
0.05	200	—	—	1.43	0.35	1.63	0.31	1.66	0.30	1.70	0.29
	0	0.67	0.75	0.79	0.63	0.91	0.55	0.98	0.51	1.32	0.38
0.10	100	—	—	2.12	0.47	2.42	0.41	2.51	0.40	2.67	0.38
	0	0.91	1.10	1.08	0.93	1.17	0.86	1.41	0.71	1.91	0.52
0.20	50	—	—	3.42	0.59	3.72	0.54	4.26	0.47	4.53	0.44
	0	1.43	1.40	1.63	1.22	1.76	1.14	1.98	1.01	2.26	0.89
0.30	33.3	—	—	4.25	0.71	4.60	0.65	6.22	0.48	6.48	0.46
	0	1.65	1.82	2.01	1.50	2.14	1.40	—	—	3.65	0.98
0.50	20	—	—	5.95	0.84	6.32	0.79	7.95	0.63	9.45	0.53
	0	2.07	2.42	2.64	1.89	2.94	1.70	3.74	1.33	4.20	1.19

*, area of spread, cm.²

‡, average thickness of the bleb in millimeters calculated from volume injected per area of spread.

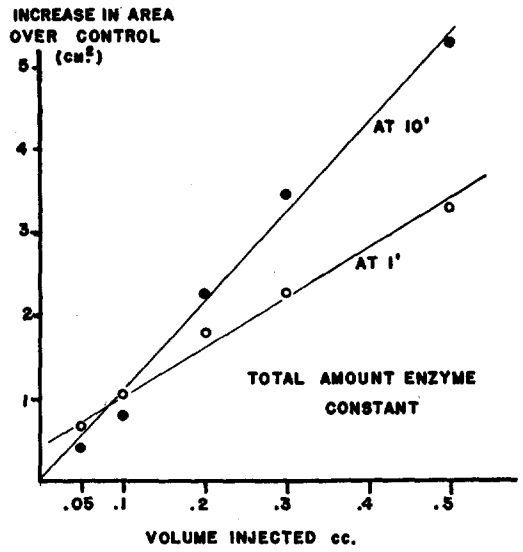

FIG. 5. Spreading effect produced by a constant amount of hyaluronidase (10 μg.) administered intradermally in volumes ranging from 0.05 to 0.5 cc., at 1 and 10 minutes after injection. The initial rate of spreading at 1 minute and the final area of spread are directly related to the volume injected, despite the fact that the enzyme concentration is decreased as the volume is increased.

was of interest to compare the log dosage-response curves with hyaluronidase administered in two different volumes. Fig. 6 illustrates the effect of hyaluronidase administered in 0.1 cc. and 0.2 cc. volumes on the rate of spread during the first minute and the area of spread at 10 minutes. It will be seen that the curves are similar in shape, but that the employment of the lower injection volume reduces the height of the curves.

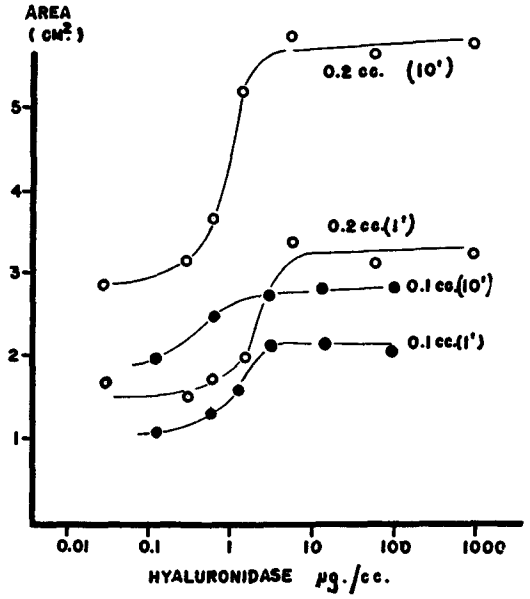

FIG. 6. Log dosage-response curve of hyaluronidase administered in 0.1 cc. and 0.2 c volumes, at 1 and 10 minutes after injection. The hyaluronidase administered in greate volume spreads to a greater area, but the shape of the curves are otherwise not greatly dissimilar.

The Spreading of Crude Snake Venoms.—Since spreading produced by hyaluronidase depends so greatly upon mechanical factors of interstitial fluid pressure and volume, agents which induce inflammatory edema and thereby influence interstitial pressure may be expected to influence spreading. In this connection, it is of interest to note that rattlesnake venom which contains hyaluronidase as one of its constituents and which produces a marked inflammatory reaction in skin, induces initially a typical hyaluronidase spreading reaction in skin but in contrast to purified hyaluronidase, there is a slower secondary spreading which persists for an hour or more (14). The following experiments were performed in an attempt to discover whether the slower secondary spreading reaction observed with venom is explicable on the basis that the inflammatory edema produced by the venom maintains local increase

of interstitial pressure and volume in the injected area and thus allows the hyaluronidase to continue to act.

Spreading Effects of Testis S. F. and of Snake Venoms in Skin of Living and Dead Rabbits.—In preliminary experiments, it was observed that diamondback rattlesnake, moccasin, and copperhead venoms all produced the secondary spreading described for rattlesnake venom by Madinaveitia (14). To determine whether the continued spreading observed with the snake venoms was

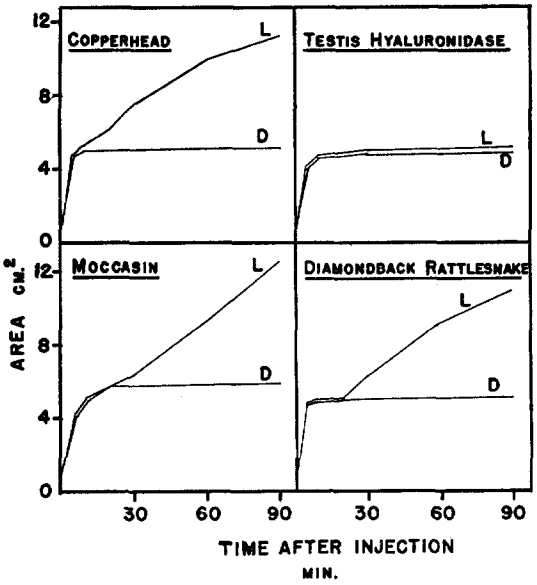

FIG. 7. Effect of 200 μg. of testis hyaluronidase, and three snake venoms administered intradermally in a volume of 0.2 cc. in the skin of a rabbit when living (curve *L*) as compared to the effect produced in the skin of the same rabbit after exsanguination (curve *D*). In these experiments, saline injected into the skin before and after exsanguination produced similar spreading responses.

associated with the inflammation produced by these agents, the spreading effects of testis hyaluronidase and snake venoms were compared in the skin of dead animals where inflammation cannot occur. The experimental procedure utilized was the following: Solutions containing spreading factors were injected intradermally and spreading was measured for 90 minutes in a living rabbit. The animal was then exsanguinated, and immediately thereafter, the S. F. in the same concentration and volume was injected into other areas of the skin of the dead rabbit and spreading was followed for another 90 minutes.

Fig. 7 illustrates a typical experiment comparing the effect of testis hyaluronidase, copperhead, diamondback rattlesnake, and moccasin venoms admin-

istered intradermally (0.2 cc. of a 1 mg./cc. solution). These data show that while negligible differences are observed in the rate and final area of spread with testis hyaluronidase as tested in skin of living and dead rabbits, the spread of the venoms is uniformly and markedly affected. It will be seen that after death the secondary spreading response observed in living skin with all of the venoms studied is abolished and the spreading reaction becomes identical with that obtained with hyaluronidase alone.

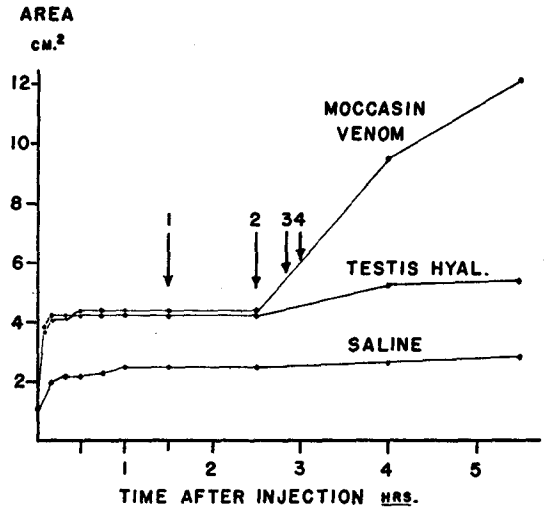

FIG. 8. Spread of testis hyaluronidase and moccasin snake venom in the skin of a rabbit in shock, with peripheral vasoconstriction, before and after fluid treatment was instituted. At 0 time, 200 μg. of testis hyaluronidase and moccasin venom were injected intradermally in a volume of 0.2 cc. into a rabbit which had been placed in the cold room to produce skin ischemia. At the time indicated by arrow 1, the rabbit was removed from the cold room and transferred to a normally heated room. At the times indicated by arrows 2, 3, and 4, saline and whole blood were intravenously administered as described in the text. This treatment relieved the shock state and it will be seen, that upon return of blood flow to the skin, moccasin venom spreads significantly, while hyaluronidase increases in area only slightly.

Spreading Effects of Testis S. F. and of Venoms in Ischemic Skin.—The abolition of the continued secondary spreading of snake venoms in the skin of dead rabbits could result because inflammatory edema cannot be produced in the absence of blood flow, or it might be due to some immediate change in the skin which develops after the death of the animal. In order to distinguish between these possibilities, testis hyaluronidase or moccasin venom was injected into the ischemic skin of a living rabbit and spreading measured during ischemia and after skin blood flow was increased.

Fig. 8 illustrates an experiment of this type.

A rabbit anesthetized with pentobarbital was placed in the cold room (2–4°C.), and a damp cloth was placed on the shaved abdomen for 5 to 10 minutes. This treatment produced marked vasoconstriction, as evidenced by skin coloration and temperature. Approximately 30 minutes later, 0.2 cc. of a 1 mg. per cc. solution of testis hyaluronidase or moccasin venom was injected intradermally in separate areas and saline control injections were similarly made. The spreading obtained with these solutions during the next 90 minutes, while the animal was in the cold room, is shown in Fig. 8. It will be seen that the secondary prolonged spreading obtained with moccasin venom in normal skin does not occur in the ischemic skin of the living rabbit. At the point indicated by arrow 1 on Fig. 8, the rabbit was removed from the cold room, and brought into a normally heated room. It was observed that the rabbit was in

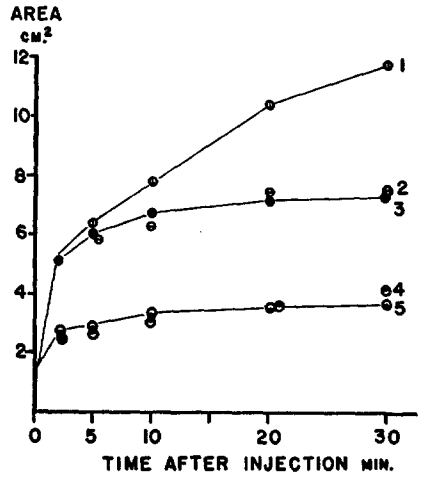

FIG. 9. Effect of alkali-treated moccasin venom to increase the spreading produced by a maximal dose of hyaluronidase, in the skin of a living animal. All injections were in a volume of 0.25 cc. Curve 1 shows the effect of 33.3 μg. per cc. alkali-treated venom plus 3.3 μg. per cc. of testis hyaluronidase; curve 2, 33.3 μg. per cc. testis hyaluronidase; curve 3, 3.3 μg. per cc. testis hyaluronidase; curve 4, 33.3 μg. per cc. alkali-treated venom; and curve 5, shows the saline control.

a critical state of shock; the heart beat was barely detectable, respiration was extremely shallow, and peripheral vasoconstriction was generalized. No therapy was given during the next 60 minutes, and the rabbit remained in shock. No further spreading of the injected areas occurred throughout this time interval when the blood flow to the skin was minimal. Having demonstrated that moccasin venom fails to exhibit its secondary spreading activity in ischemic skin, it was of interest to determine whether secondary spreading with venom but not with hyaluronidase, would result when blood flow to the skin was increased. Accordingly, at the times indicated by arrows 2, 3, and 4 in Fig. 8, 100 cc. saline, 50 cc. heparinized rabbit blood, and 100 cc. saline were administered intravenously. This therapy relieved the state of shock as evidenced by onset of regular deep respiration, strong heart beat, and a decreased peripheral vasoconstriction (increase in skin temperature and coloration). One hour after the last injection of intravenous saline, the moccasin venom-treated area exhibited a marked increase in area, while the hyaluronidase-treated area increased only slightly (Fig. 8).

This experiment demonstrates that the secondary spreading produced by snake venoms requires the presence of normal skin blood flow in order to

manifest itself. Taken together, with the results on snake venoms in the skin of dead animals, it seems likely that the secondary spreading occurring with venoms in normal skin is associated with the inflammation produced by these agents.

Augmentation of the Spreading Effect of Hyaluronidase with S. F.-Inactivated Venom.—To provide further evidence for the view that inflammatory agents affect the rate and area of spread of S. F. solutions injected intradermally, the effect of moccasin venom (inactivated as regards S. F.) upon the spreading produced by a maximal dosage of testis hyaluronidase was next studied. In these experiments the S. F. in moccasin venom was inactivated by bringing the solution to pH 10.5 with NaOH. After standing at 25°C. for 20 minutes the pH was readjusted to 7.0. This treatment with alkali affects only slightly the ability of the venom to increase capillary permeability as evidenced by the trypan blue test, but completely destroys the ability to produce hemorrhagic erythema. Fig. 9 is a representative experiment showing the effect of 3.3 μg. per cc. hyaluronidase (intradermally administered in a volume of 0.25 cc.). This dose of hyaluronidase produces a maximal spreading response since a tenfold increase of hyaluronidase has no further effect. It will be seen that the alkali-treated moccasin venom by itself has no significant spreading activity. However, this alkali-treated venom augments the spreading response produced by a maximal dose of hyaluronidase. Repetition of this experiment, in a dead animal, failed to show this enhancing effect of the alkali-treated venom on purified hyaluronidase.

DISCUSSION

The importance of mechanical pressure-volume factors, associated with intradermal injection of fluids in the spreading activity of hyaluronidase has been shown by the following findings:

(*a*) The initial rate and final area of spreading produced by a constant total amount of hyaluronidase administered in varying volume is directly proportional to the volume of fluid injected intradermally.

(*b*) Hyaluronidase administered to skin in high concentrations, but minimal volume and pressure (*e.g.*, by diffusion *via* epidermal incisions) fails to promote spreading.

(*c*) The spreading activity of hyaluronidase is rapid only during a stage when the local interstitial pressure is high and falls off when the mechanical factors involved decrease to minimal values.

These results which demonstrate that hyaluronidase is an ineffective spreading agent in the absence of local increase of skin interstitial pressure and volume, indicate that, under normal physiological conditions, hyaluronidase diffuses through skin at a slow rate. Under these circumstances, hyaluronidase can destroy the hyaluronic acid component of the dermal barrier to fluid diffusion only to the limited extent that it attacks the barrier in its immediate vicinity.

When, however, the slow hyaluronidase diffusion is accelerated by the pressure and volume of injection, the enzyme is enabled to approach and remove the dermal hyaluronic acid at sites adjacent to and distant from the point of injection and the typical spreading response is obtained.

These considerations serve to explain the sigmoid dosage-response curve of hyaluronidase. Since the administered fluid containing hyaluronidase can only spread to a particular maximal area (controlled by the volume of administered fluid) the amount of hyaluronic acid substrate in skin is limited to a finite value. Using a constant injection volume, as increasing concentrations of hyaluronidase are administered intradermally, the rate of spreading to the maximal area determined by the volume of injected fluid will also increase and then level off. At this point presumably the hyaluronic acid substrate in skin is saturated with enzyme, and further increase of hyaluronidase has no further effect.

The rapid spreading in skin induced by injection of hyaluronidase has been shown not to be influenced by profound alterations in skin blood flow. This indicates that hyaluronidase is not rapidly eliminated from the injected area by circulatory absorption; and further, that circulating plasma antihyaluronidases do not significantly affect the spreading activity of injected hyaluronidase. The hyaluronidase spreading reaction likewise does not appear to be influenced by adsorption of enzyme on a constituent in skin, or by substances present in extracts of normal skin or dermis.

The view that hyaluronidase exhibits spreading activity only in the presence of localized increase in interstitial pressure and volume of skin is supported by an early finding of McClean (15) which has been overlooked in considering S. F. action. McClean observed that intradermal administration of vaccine virus into an area previously injected with testis S. F. led to marked facilitation of virus spread. When, however, the virus was rubbed into freshly shaven areas of rabbit skin which had been injected with S. F., no enhancement of virus spread was noted. Since papules developed in all areas wherein virus had been applied to the skin surface, it was clear that the latter result was not due to the inability of the virus to enter the skin. McClean's results now appear to be explained by the fact that the virus could be spread by hyaluronidase only when the interstitial pressure and volume of the skin were increased by fluid administration, as was the case when virus was injected.

This view of hyaluronidase action in skin has been obtained with a purified preparation which did not alter capillary permeability or produce inflammation. However, hyaluronidase activity as it occurs in nature (*i.e.*, in bacteria, poisonous venoms, etc.) is regularly associated with factors which damage capillaries and produce inflammatory edema (1). Consequently, it was of interest to determine whether the spreading reaction of naturally occurring toxic products containing hyaluronidase would be explicable in terms of the concept

that rapid hyaluronidase diffusion is controlled by local skin interstitial pressure and volume. It has been shown that the continued secondary spreading produced by venom but not by purified S. F. is associated with the inflammatory edema these agents produce. Thus (*a*) snake venom injected into either ischemic skin of living animals or into skin of dead animals produces no secondary spreading, and (*b*) moccasin snake venom, whose S. F. activity has been destroyed by alkali treatment, retains the power to augment the spreading produced by maximal concentrations of purified testis hyaluronidase in the skin of living, but not of dead, animals.

Thus, the observations on snake venoms also are in agreement with the view that hyaluronidase spreading activity is controlled by mechanical forces. When purified hyaluronidase is injected intradermally, the solution spreads until the pressure of the injected bleb approaches the normal interstitial pressure of the skin. When hyaluronidase is associated with inflammatory agents, spreading does not level off but continues because the interstitial pressure and volume of the bleb initially dependent upon the volume of fluid injected is maintained by the volume of edema fluid which enters the injected area consequent to capillary damage.

It would appear that the results of this study may be of importance with reference to the rôle of hyaluronidase in bacterial invasion in skin and to the circulatory absorption of poisonous venoms.

Bacteria often penetrate the skin through abrasions with only minimal amounts of fluid accompanying the organism. If the invading organism produces hyaluronidase, this would presumably not of itself promote bacterial spreading in the skin, for hyaluronidase appears to require the presence of a local increase of the interstitial skin pressure and volume before it can exhibit its spreading activity. This localized increase of interstitial pressure and volume necessary for the bacterial spreading activity could result, however, from edema formation secondary to toxic effects of bacteria upon skin capillaries. Indeed it may be that the spread of bacteria through the intercellular spaces of skin in man depends as much upon edema-inducing "leukotaxin-like" substances (16) as it does upon the hyaluronidase that accompanies, or is produced by, the invading organism.

While inflammatory edema facilitates the spread of snake venoms in skin, it may be predicted that the edema factor is of less importance in snake bites than in natural bacterial invasion. In the latter instance the fluid volume and pressure necessary for the promotion of spreading by hyaluronidase may largely be the result of inflammatory edema. In snake bites, however, sizable volumes of fluid venom are injected by snakes. Although the exact volume of venom ejected by a snake when it bites is difficult to estimate (and is undoubtedly influenced by a multiplicity of factors), it has been asserted that cotton mouth moccasins eject on a single bite about 20 cc. venom, and diamond-

back rattlesnakes approximately 7.5 cc. venom (17). It would, therefore, appear that venoms could spread rapidly independent of the effect of inflammatory edema upon spreading, by virtue of the volume of fluid introduced.

The significance of these results concerning S. F. action for the assay of S. F. may be briefly mentioned. As has already been pointed out differences in the effects of various concentrations of hyaluronidase upon the rate of spreading are best evident during the initial phases of the reaction and tend to become smaller as the reaction proceeds. It would therefore appear that the practice of determining the rate of spread in the first minute after injection instead of the 20 minute time interval utilized in the methods of Bacharach *et al.* (2) and Humphrey (5) might facilitate the assay of S. F. activity. In addition, utilization of this short time interval minimizes the possibility that inflammatory edema produced by the material under investigation may complicate the results by leading to an overestimation of S. F. activity.

SUMMARY

The spreading response induced by intradermal administration of hyaluronidase is influenced not only by enzyme concentration, but also by the volume and pressure of the injection. These pressure-volume factors influence the rate of spreading and determine the final area of spreading. When a constant volume is injected, the rate of spreading to the area determined by the volume of injection is a function of the enzyme concentration. These and other findings have led to the conclusion that hyaluronidase is an effective spreading agent only when the slow diffusion of enzyme in skin is accelerated by a localized increase of interstitial pressure and volume. These considerations of hyaluronidase action in skin have been utilized to explain the shape of the dosage-response curve of hyaluronidase spreading activity, and the differences in the spreading reactions between crude snake venoms and purified hyaluronidase. The significance of the findings as related to the rôle of hyaluronidase in bacterial invasiveness, and in the assay of S. F. are briefly discussed.

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BIBLIOGRAPHY

1. Duran-Reynals, F., *Bact. Rev.*, 1942, **6,** 197.
2. Bacharach, A. L., Chance, M. R. A., and Middleton, T. R., *Biochem. J.*, 1940, **34,** 1464.
3. Evans, M. G., and Madinaveitia, J., *Biochem. J.*, 1940, **34,** 613.
4. McClean, D., *J. Path. and Bact.*, 1942, **54**, 284.
5. Humphrey, J. H., *Biochem. J.*, 1943, **37,** 177.
6. Leonard, S. L., and Kurzrock, R., *Endocrinology*, 1945, **37,** 171.
7. Haas, E., *J. Biol. Chem.*, 1946, **163**, 63.

8. Welker, W. H., and Williamson, C. S., *J. Biol. Chem.*, 1920, **41,** 75.
9. Madinaveitia, J., *Biochem. J.*, 1938, **32,** 1806.
10. Madinaveitia, J., *Biochem. J.*, 1941, **35,** 447.
11. Hoffman, D. C., and Duran-Reynals, F., *J. Exp. Med.*, 1931, **53,** 387.
12. Rigdon, R. H., *Arch. Surg.*, 1940, **41,** 96.
13. Rocha e Silva, M., and Dragstedt, C. A., *J. Pharmacol. and Exp. Therap.*, 1941, **73,** 405.
14. Madinaveitia, J., *Biochem. J.*, 1939, **33,** 1470.
15. McClean, D., *J. Path. and Bact.*, 1930, **33,** 1045.
16. Menkin, V., Dynamics of Inflammation, New York, The Macmillan Co., 1940.
17. Allen, R., personal communication.