STUDIES ON SPREADING FACTORS

II. The Effect of Serum upon Hyaluronidase Spreading Activity*

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INTRODUCTION

The fact that normal serum from non-immunized animals contains a factor which inhibits certain activities of hyaluronidase now seems well established (1–3). Although the biological significance of this antihyaluronidase factor is obscure, it has been recently suggested that the serum factor is the fundamental body defense mechanism against invasive processes catalyzed by hyaluronidase (3). This view, however, is based on an extrapolation of *in vitro* findings to invasion in skin. It should be noted that there is little or no information concerning the effect of serum upon hyaluronidase spreading activity in skin and that the principal evidence for the existence of the serum antihyaluronidase factor is derived from *in vitro* studies on decapsulation of streptococci (1), removal of follicle cells from mammalian ova (2), and viscometric studies using hyaluronic acid preparations (3).

Since the spreading effect of hyaluronidase in skin is the activity of hyaluronidase which has been implicated in bacterial, and other, invasive processes, the need for an understanding of the effect of the serum factor upon hyaluronidase action in skin is clear, particularly since it is known that the activity of hyaluronidase in skin is markedly influenced by non-enzymatic factors (4). To date, the only information available concerning the effect of the serum factor upon hyaluronidase spreading activity is: (*a*) an isolated statement by Humphery (5) that serum inhibits the spreading activity of testicular hyaluronidase, with no details of this work presented, and (*b*) the illustration of a similar inhibitory effect of rabbit serum in spreading of vaccine virus by Duran-Reynals (6) although this observation was not mentioned in the text of the paper.

Accordingly, studies were undertaken to determine the effect of serum upon hyaluronidase spreading activity. In this paper the results of these studies will be described and the significance of the serum antihyaluronidase factor as a body defense mechanism against invasive processes potentiated by hyaluronidase will be discussed.

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Methods

The plan of these experiments has been to study the reaction between serum and hyaluronidase, under both ***in vitro*** and ***in vivo*** conditions. Hyaluronidase was measured using an assay method based on spreading activity which will be described later in this section of the paper. It should be emphasized immediately that although the spreading method of hyaluronidase assay is highly sensitive, the quantitative evaluation of hyaluronidase activity with this method is much less satisfactory than with simple ***in vitro*** tests using the viscosimeter (3), mucin clot prevention (7), or turbidimetric (8) methods of assay. Consequently, for a study of the ***in vitro*** reaction between serum and hyaluronidase, one of the latter procedures would be the method of choice. In this study, however, we are interested in the reaction between serum and hyaluronidase under environmental conditions in skin, and thus the spreading method is most informative for this purpose.

Hyaluronidase spreading activity was measured using hemoglobin or methemoglobin as an indicator in shaved rabbit abdominal skin. The measurement of areas of spread, preparation of indicator solutions, and of the solutions for intradermal injection have been described in a previous paper (4). The hyaluronidase preparation used in these studies was a partially purified bovine testis preparation (Schering). Rabbit and pig serum was used in these studies. We have found no significant differences in antihyaluronidase activity between normal serum and the serum derived from defibrinated blood. The samples of the pig sera used contained high ***in vitro*** antihyaluronidase activity as determined by the viscometric technic of Haas (3). Thus, 0.1, 0.2, and 0.3 cc. of these sera, incubated for 10 minutes with 50 μg. of hyaluronidase in a total volume of 3 cc. inhibited hyaluronidase viscosity—reducing activity an average of 75, 85, and 90 per cent respectively. Rabbit serum, while not tested simultaneously as regards its inhibitory activity in the spreading reaction and in the viscosimeter, contains an approximately equivalent amount of antihyaluronidase activity as measured in the latter test (9).

Evaluation of the effect of ***in vitro*** incubation of serum with hyaluronidase was obtained by assay of hyaluronidase spreading activity using the following procedure: To eliminate the factor of animal variation, all tests of spreading activity in a given experiment were made in the same animal. Initially, varying concentrations of enzyme in a constant volume and indicator concentration were injected into duplicate sites and the area of spread 1, 2, 5, and 10 minutes after injection determined. After the dosage-response curves of hyaluronidase had been obtained, concentrations of enzymes were selected for ***in vitro*** incubation at 25°C. with serum. At varying intervals after the initiation of the ***in vitro*** reaction, a sample was removed and the spreading activity was determined in the same animal. To check the results obtained in the single animal, all tests for results were repeated at least three times with three different rabbits.

The quantitative aspects of the method require some mention. Twofold differences in enzyme concentration can be detected *only on the linear portion of the sigmoid dosage-response curve.* This method therefore has quantitative significance only in certain dosage ranges, and at best is not capable of detecting slight differences in hyaluronidase concentration.

In order to assess the ***in vivo*** inhibitory activity of serum uncomplicated by factors involving the rate of diffusion of antihyaluronidase through the capillary wall, etc., the effect of incubating serum with hyaluronidase in skin was studied. In these experiments, hyaluronidase and serum were mixed and immediately thereafter injected intradermally in a small volume (0.1 cc.) of fluid. After varying intervals of time, the residual spreading activity of the bleb was tested by reinjecting the initial site with 1.0 cc. of indicator solution. The basis for this assay depends on the fact that the final area of spread of intradermally injected hyaluronidase is directly related to the volume of injection; and that administered hyaluronidase in the absence of the pressure of injection, diffuses only slowly through skin (4). As a consequence of this, a portion of the injected hyaluronidase may be expected to remain in the area of injection. This hyaluronidase is available for further spreading activity if a second large injection

of fluid without enzyme is made into the area initially treated with enzyme. If, serum is introduced with the spreading enzyme and inactivates the hyaluronidase in the skin, the rate of spread of the second injection of a large volume of fluid should be correspondingly decreased. In these experiments, on *in vivo* incubation of serum and hyaluronidase the large areas of spread following the administration of the second injection limited the number of injections

TABLE I

*The Effect of in Vitro Incubation of Rabbit Serum with Varying Concentrations of Hyaluronidase (Serum Concentration Constant)**

Final concentration before injection		Time after injection, *min.*‡						Average I
		1		2		5		
Hyaluronidase	Serum	A§	I‖	A	I	A	I	
μg./cc.	*cc./cc.*	*cm.²*	*per cent*	*cm.²*	*per cent*	*cm.²*	*per cent*	*per cent*
0	0	1.70	—	2.04	—	2.36	—	—
0	0.3	1.74	—	1.94	—	2.42	—	—
0.1	0	1.70	—	2.08	—	2.70	—	—
1.0	0	1.97	—	2.53	—	3.28	—	—
2.0	0	2.80	—	3.60	—	4.34	—	—
	0.3	1.84	85	2.48	50	3.08	65	67
5.0	0	3.65	—	3.87	—	4.53	—	—
	0.3	1.69	98	2.76	78	3.46	78	84
10.0	0	3.72	—	4.47	—	5.01	—	
	0.3	2.12	89	2.51	90	3.46	90	90
100.0	0	3.79	—	4.30	—	4.85	—	—
	0.3	4.02	0	4.50	0	4.78	0	0

* Incubated at 25°C. for 10 minutes.

‡ All solutions were injected intradermally in a volume of 0.20 cc.

§ A, area of spread in $cm.^2$

‖ I, Inhibition produced by *in vitro* incubation, in per cent, as evaluated from the dosage-response curves.

that could be made into the abdominal skin; thus it was not possible to make duplicate injections as in the case of those experiments involving *in vitro* incubation of serum with hyaluronidase. As in the previous case, the results of the single experiment were checked by repeating the test in three different rabbits.

EXPERIMENTAL

Effect of in Vitro Incubation of Serum with Hyaluronidase.—The effect of incubating varying concentrations of hyaluronidase with a constant amount of

rabbit serum was studied in seven experiments (four using pig serum and three with rabbit serum). Essentially similar results were obtained with both types of serum and the results of a single experiment with rabbit serum are shown in Table I.

In this experiment, 0.3 cc. of rabbit serum was incubated with 0.2 cc. of solutions containing 10, 25, 50, or 500 μg. hyaluronidase per cc. for 10 minutes at 25°C.; 0.5 cc. of the hemoglobin indicator was then added so that the final concentrations were equivalent to 2, 5, 10, and 100 μg. per cc. of hyaluronidase. A 0.2 cc. sample of the incubation mixture was then injected intradermally and the rate of spreading determined. The degree of inhibition produced by serum was determined by comparing the spreading activity of the incubated samples with the dosage-response curve of hyaluronidase alone.

It will be seen from Table I that the spreading activity of hyaluronidase is inhibited by 10 minutes *in vitro* incubation with serum. Thus, 2, 5, and 10 μg. hyaluronidase incubated with 0.3 cc. serum in a volume of 0.5 cc. is inactivated approximately 67, 84, and 90 per cent respectively as regards spreading activity. The finding that 100 μg. of hyaluronidase is not significantly inhibited under these conditions, does not mean that some degree of hyaluronidase inactivation did not occur. It should be pointed out that in order to demonstrate an inhibition with this concentration of hyaluronidase, almost 100 per cent of the spreading activity would have had to be removed.

In the next experiments, the effect of varying the serum concentration, using a constant amount of hyaluronidase, upon inhibition of spreading activity was studied. The results of four experiments of this type (three with rabbit serum, one with pig serum) were essentially similar, and the results of a single representative experiment are shown in Table II.

After determination of the dosage-response curve of hyaluronidase in a rabbit (*cf.* Table II) using a constant injection volume of 0.2 cc. a concentration of hyaluronidase was selected for incubation with varying amounts of rabbit serum so that the final concentration of hyaluronidase was 2.5 μg. per cc. Then, 0.25 cc. of a 10 μg per cc. solution of hyaluronidase was mixed with 0.05, 0.125, or 0.25 cc. of rabbit serum, saline was added to make a total volume of 0.5 cc., and the mixture was incubated for varying intervals of time at 25°C. At the completion of the incubation period, 0.5 cc. of indicator solution was added, the solution mixed, and spreading activity measured by intradermal injection of 0.2 cc. of the mixture. The degree of inhibition produced by serum was obtained by comparing the response with the previously determined dosage-response curve.

It will be seen from Table II that the inhibitory action of serum incubated *in vitro* with hyaluronidase is dependent both upon the serum concentration and the period of incubation. Thus, for a 10 minute incubation period, the undiluted serum (present in a final concentration of 0.25 cc. serum per cc. of the injected mixture) inhibited 2.5 μg. of hyaluronidase to the extent of about 70 per cent; serum diluted 1:2 and 1:5 with saline, under similar circumstances had no inhibitory influence as measured by spreading activity. If, however,

the incubation period of hyaluronidase with the 1:2 and 1:5 dilutions of serum was increased to 20 and 50 minutes respectively, serum inhibitory activity (approximately 75 per cent) is demonstrable.

Since it had been shown that *in vitro* incubation of undiluted serum with hyaluronidase for 10 minutes produced inactivation of spreading activity, it was of interest to determine whether serum introduced simultaneously with hyaluronidase into the skin without prior *in vitro* incubation, would influence spreading activity. A typical experiment of this type is illustrated in Table III.

TABLE II

The Effect of in Vitro Incubation of Hyaluronidase with Varying Amounts of Rabbit Serum (Hyaluronidase Concentration Constant)

Final concentration before injection		Time of *in vitro* incubation*	Time after injection,‡ *min.*				Average I
			2		5		
Hyaluronidase	Serum		A§	I‖	A	I	
μg./cc.	*cc./cc.*	*min*	*cm.²*	*per cent*	*cm.²*	*per cent*	*per cent*
0	0	—	1.64	—	1.84	—	
0.1	0	—	1.79	—	2.01	—	
0.5	0	—	1.89	—	2.35	—	
1.0	0	—	2.67	—	2.94	—	
2.5	0	—	2.99	—	3.70	—	
5.0	0	—	3.43	—	3.76	—	
	0.25	10	2.11	76	2.80	64	70
	0.125	10	3.00	0	3.74	0	0
2.5	0.05	10	2.94	4	3.72	0	2
	0.125	20	2.11	76	2.44	76	76
	0.05	50	1.88	80	2.39	79	80

* Incubated at 25°C.

‡ All solutions were injected intradermally in a volume of 0.20 cc.

§ Area of spread in cm.².

‖ Inhibition produced by *in vitro* incubation, in per cent, as evaluated from dosage-response curves.

In this experiment 2, 5, or 10 μg. of hyaluronidase in a volume of 0.2 cc. was added to 0.5 cc. of indicator solution; 0.3 cc. of rabbit serum was then added, the solution mixed, and immediately thereafter 0.2 cc. of the final solution injected. Although the serum was in contact with the hyaluronidase for perhaps as long as 1 minute before the mixture was injected (time required to mix the solutions, load the syringe, etc.) the time of *in vitro* incubation has been taken as zero. For comparative purposes, results obtained by incubating similar concentrations of serum and hyaluronidase for 15 minutes *in vitro*, prior to injection, are likewise shown in Table III. The results of the above experiments were compared with those obtained by injecting hyaluronidase in the absence of serum.

Table III illustrates the inability of serum to affect hyaluronidase spreading activity when both materials are injected almost immediately after mixing, in

contrast to the significant inhibition produced by *in vitro* incubation prior to injection. This result is not due to a possible effect of the indicator affecting the serum inhibition for it was observed that the *in vitro* reaction between serum and hyaluronidase is not significantly influenced by the presence or absence of the hemoglobin indicator during the course of the reaction. The finding that serum does not affect spreading activity when injected together with hyaluronidase, under conditions of minimal prior incubation could be explained in either of two ways: (*a*) the rate of reaction of the serum factor upon hyaluronidase is slow as compared to the rate of hyaluronidase action upon the hyaluronic acid component of the dermal barrier, or (*b*) the reaction between serum and hyaluronidase does not take place *in vivo* in skin. The former view is supported

TABLE III

The Effect of the Simultaneous Injection of Rabbit Serum and Hyaluronidase without in Vitro Incubation

Final concentration before injection		Time of *in vitro* incubation*	Time after injection, *min.*			Average I
Hyaluronidase	Serum		1	2	5	
			I‡	I	I	
μg./cc.	*cc./cc.*	*min.*	*per cent*	*per cent*	*per cent*	*per cent*
2.0	0.3	0	2	0	0	1
		15	90	78	85	84
5.0	0.3	0	0	0	0	0
		15	95	88	88	90
10.0	0.3	0	—	0	0	0
		15	—	90	94	92

* Incubated at 25°C.

‡ Inhibition produced by addition of serum to hyaluronidase, in per cent, as evaluated by dosage-response curves determined in the same animal.

by recent viscometric work of Hadidian (9) who has found that the addition of hyaluronic acid (prepared from human umbilical cord) to a system containing testis hyaluronidase and serum, completely prevents the serum inhibition of hyaluronidase viscosity-reduction activity usually obtained during the first two minutes of the reaction and significantly decreases the inhibitory activity of the serum upon hyaluronidase ten minutes after initiation of the reaction. These viscometric results obtained by Hadidian, taken in conjunction with the fact that the spreading reaction produced by hyaluronidase is 75 to 80 per cent complete within two minutes (4) could explain the ineffectiveness of the serum antihyaluronidase to influence the spreading reaction of hyaluronidase when both are injected together with a minimal period of prior *in vitro* incubation.

It is also possible that the reaction between serum and hyaluronidase under *in vivo* conditions does not lead to significant inhibition of spreading activity. This latter possibility was tested in the next section of this paper.

Effect of in Vivo Incubation of Serum with Hyaluronidase.—The ability of the serum factor to react with hyaluronidase *in vivo* was tested by incubating hyalu-

TABLE IV

The Ineffectiveness of Serum to Inhibit Hyaluronidase Spreading Activity under Conditions of in Vivo Incubation in Skin

Initial injection (0.1 cc.) contains		Period of *in vitro* Incubation*	Interval between 1st and 2nd injections	Time after injection of 1.0 cc. indicator, *min.*								Average H
				1		2		5		10		
Hyaluronidase	Serum			A‡	H§	A	H	A	H	A	H	
μg.	*cc.*	*min.*	*min.*	*cm.²*	*μg.*	*cm.²*	*μg.*	*cm.²*	*μg.*	*cm.²*	*μg.*	*μg.*
0	0	—	3	3.18	—	4.12	—	4.65	—	5.25	—	—
0.1	0	—	3	4.13	—	5.34	—	5.97	—	7.52	—	—
0.5	0	—	3	5.00	—	6.10	—	8.60	—	9.65	—	—
1.0	0	—	3	6.85	—	8.90	—	12.32	—	12.70	—	—
2.5	0	—	3	6.53	—	8.70	—	12.00	—	12.83	—	—
1.0	0.09	15	3	3.66	0.1	4.48	0.1	6.32	0.1	7.65	0.1	0.10
1.0	0.09	0	30	6.02	0.7	7.23	0.6	8.00	0.4	10.12	0.6	0.57
	0	0		5.90	0.7	6.77	0.5	7.88	0.4	9.85	0.6	0.55
1.0	0.09	0	65	6.55	0.9	7.35	0.6	8.55	0.5	10.10	0.6	0.65
	0	0		5.60	0.6	7.27	0.6	7.88	0.4	9.90	0.6	0.55
1.0	0.09	0	120	6.85	1.0	7.35	0.6	8.73	0.5	9.80	0.6	0.67
	0	0		6.45	0.9	7.00	0.6	8.57	0.5	9.80	0.6	0.65

* Incubated at 25°C.

‡ A, area of spread in cm.² of the indicator.

§ H, is the hyaluronidase equivalent, to the closest 0.1 μg., as obtained from the dosage-response curves.

ronidase with serum in skin for varying intervals of time and then measuring residual hyaluronidase spreading activity. Table IV shows the results of a typical experiment of this series.

Concentrations of hyaluronidase ranging from 1 to 25 μg. per cc. were injected intradermally without indicator in a volume of 0.1 cc. The needle hole and initial bleb were marked with india ink, and 3 minutes later, 1.0 cc. of methemoglobin indicator solution was injected directly

into the previously treated area. The area of spread of the indicator 1, 2, 5, and 10 minutes after injection was measured. On the basis of these results, an amount of hyaluronidase equivalent to 1 μg. in 0.1 cc. was selected for *in vivo* incubation with serum. Thus, 0.1 cc. of a 100 μg. per cc. solution of hyaluronidase was mixed with 0.9 cc. of rabbit serum, and immediately thereafter, 0.1 cc. volumes of this mixture were injected intradermally. After time intervals ranging from 30 to 120 minutes, these areas were reinjected with 1.0 cc. of indicator solution and the rate of spreading determined in the usual manner. The spreading reactions observed in the areas previously treated with hyaluronidase plus serum, were compared with those obtained by injecting areas which had been treated with 0.1 cc. containing 1 μg. of hyaluronidase alone 30 to 120 minutes previously. The *in vitro* antihyaluronidase activity of the serum used in these experiments was checked in the following manner: 0.1 cc. of hyaluronidase (containing 10 μg.) was incubated for 15 minutes at 25°C. with 0.9 cc. serum; 0.1 cc. of this mixture was then injected into the skin, and 3 minutes later this area was reinjected with 1.0 cc. of indicator solution.

It will be seen from Table IV that serum incubated *in vivo* with hyaluronidase for periods ranging from 30 to 120 minutes had no inhibitory activity; but that the same concentrations of serum and hyaluronidase incubated *in vitro* for only 15 minutes produced marked inhibition of spreading activity (approximately 90 per cent). It should be emphasized that in three other experiments with rabbit serum the ineffectiveness of the serum factor to inhibit hyaluronidase on *in vivo* incubation was consistently observed, despite the fact that the period of *in vivo* incubation in skin in some experiments was as long as 5 hours. These results indicate that in the *in vivo* environment of skin, the reaction between serum antihyaluronidase and hyaluronidase, either does not occur, or if a reaction does take place, it is readily reversible.

DISCUSSION

The results obtained in this study demonstrate that the *in vitro* reaction between normal rabbit or pig serum and hyaluronidase is profoundly modified by the *in vivo* conditions that exist in rabbit skin. Serum incubated *in vivo* with hyaluronidase in skin for as long as 5 hours, fails to produce a significant inhibitory effect, although marked inhibition of hyaluronidase activity is demonstrable when similar concentrations of serum and hyaluronidase are incubated *in vitro* for 10 to 15 minutes. This disparity between the *in vivo* and *in vitro* effectiveness of serum to inhibit hyaluronidase spreading activity, indicates that environmental conditions in the skin are unfavorable for the reaction between the serum inhibitor and hyaluronidase which regularly occurs *in vitro*.

While it is possible to explain these results on the basis that the serum factor is inactivated in skin or eliminated, an alternative explanation seems equally likely. Recent studies by Hadidian and Pirie (10) using the viscometric method, clearly demonstrate that the inhibition of hyaluronidase by serum is not an enzymic reaction, but is due to a reversible binding of the enzyme activity by the serum factor. Alterations of various conditions (salt and phos-

phate concentration, etc.) modify the rate of the inhibitory binding reaction as well as the rate of the release of hyaluronidase from its inactive form. It therefore seems possible that factors in skin could either inhibit the serum binding of hyaluronidase, or increase the dissociation of the hyaluronidase bound by serum. One factor operative *in vivo*, which could function in the former manner, is dermal hyaluronic acid; for it has been shown using the viscometric technic that hyaluronic acid significantly decreases the rate of reaction of the serum factor upon hyaluronidase, presumably by competitive inhibition (9).

Although it is not possible at this time to completely explain the ineffectiveness of the serum factor under *in vivo* conditions in skin, the results of this study would nevertheless appear to have significance concerning the rôle of the serum inhibitor factor in those invasive processes catalyzed by hyaluronidase. Thus the results of this study cast considerable doubt upon the biological significance of the enzymatic theory for the mechanism of invasion recently proposed by Haas (3). By extrapolation of viscometric data to bacterial invasiveness *in vivo*, Haas has developed the concept that the serum inhibitory factor ("antinvasin I") represents the body defense mechanism against bacterial, and other, invasions facilitated by hyaluronidase. According to his view, "proinvasin I," a factor found in bacteria and snake venoms, destroys "antinvasin I" and thus facilitates the invasive process. Other enzymes, "antinvasin II," and possibly "proinvasin II" and "antinvasin III" participate in the manner indicated by their names (3). In substance this theory views the process of invasion in skin, as the resultant of enzymatic activities present in the invading agent and in the body defense mechanism. Without considering the question as to whether the activities described by Haas, are, in fact, enzymatic, our results which demonstrate that hyaluronidase inactivation by "antinvasin I" does not occur *in vivo* would seem to remove "antinvasin I" from the position of representing the body defense mechanism against invasions facilitated by hyaluronidase. It is of interest to mention that having removed the "antinvasin I" factor from the Haas theory of invasion, the physiological significance of the subsequent factors in the scheme ("proinvasins I and II" and "antinvasin II and III") disappears because all of these are believed indirectly to affect hyaluronidase *via* "antinvasin I."

The results of these studies throw no light on the *in vivo* function of the antihyaluronidase factor of serum and further work will be necessary to elucidate the biological rôle of this factor.

SUMMARY

The reaction between normal serum and hyaluronidase has been studied *in vitro* and under *in vivo* conditions in skin. Using *in vitro* conditions of incubation, serum exhibits antihyaluronidase activity as measured by assay of hyaluronidase spreading activity in skin. This confirms the work of others, who

have previously described the serum inhibitory factor using other tests of hyaluronidase activity. When, however, hyaluronidase and serum are allowed to incubate in skin under *in vivo* conditions, no inhibitory influence of serum upon hyaluronidase spreading activity is evident. This latter finding has been taken to indicate that the environmental conditions in skin are unfavorable for the inhibitory reaction of serum upon hyaluronidase. The disparity between the *in vivo* and *in vitro* effectiveness of serum, and the significance of the serum factor as a defense mechanism against invasive processes, have been briefly discussed.

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