# THE RELATIONSHIP BETWEEN SPREADING FACTOR AND HYALURONIDASE*

By GLADYS L. HOBBY, Ph.D., MARTIN H. DAWSON, M.D., KARL MEYER, M.D., and ELEANOR CHAFFEE

*(From the Departments of Medicine and Ophthalmology, College of Physicians and Surgeons, Columbia University, the Edward Daniels Faulkner Arthritis Clinic, and the Institute of Ophthalmology, Presbyterian Hospital, New York)*

(Received for publication, September 24, 1940)

The term spreading factor was originally employed to describe a property present in testicular tissue and sperm which is active in enhancing bacterial and virus infections. Subsequently the use of the term was extended to describe the same or a similar property present in various tissues, bacteria, and other material, which is responsible for the spreading of solutions of dyes and toxins in the skin of experimental animals.

Hyaluronidase is the term which has been given to the enzyme responsible for the hydrolysis of hyaluronic acid, an acid polysaccharide which has been isolated from a variety of human and animal sources.

*Spreading Factor.*—In 1928 Duran-Reynals (1) demonstrated that normal testicular tissue increases the infective power of neurovaccine. In the following year he reported (2) that the infectivity of neuro-, testicular, and cow strains of vaccine virus is enhanced to an extraordinary degree by contact with sensitive tissues of susceptible animals. He further reported that this effect was due to the presence of an extractable factor which exerted a similar influence on many viruses and organisms. Later Hoffmann and Duran-Reynals (3) demonstrated that spreading factor induces a spreading of solutions of dyes and toxins injected intracutaneously into experimental animals. They attributed the phenomenon to the capacity of the injected material to increase tissue permeability (3, 4).

In the following years the phenomenon of spreading and the properties of spreading factor were studied by Duran-Reynals and a large number of other investigators. Spreading factor has been demonstrated in extracts of invasive bacteria;—*Staphylococcus aureus, Streptococcus hemolyticus, Clostridium welchii, Cl. chauvei, Cl. oedematis-maligni,* pneumococcus, and other bacteria (5); in certain malignant tumors (6); in snake and other animal venom (7); and in leech extracts (8). Likewise, Claude has shown that certain azoproteins possess a similar property (9). The mechanism of spreading has also been studied by several investigators. McClean (10) has shown that the phenomenon occurs when injections are made in the isolated skin of a rabbit. Favilli (11), as well as Duran-Reynals (12), has further shown that the same phenomenon can be demon-

* This work has been supported in part by a grant from the John and Mary Markle Foundation.

strated in muscles, tendons, and in the walls of stomach and intestine. Finally Duran-Reynals has shown (4) that the permeability of the vascular system can be locally or generally increased by means of preparations rich in spreading factor.

*Hyaluronidase.*—Hyaluronic acid is an acid polysaccharide of high molecular weight which is composed of acetyl-glucosamine and glucuronic acid in equimolar concentrations. It has been isolated from the following sources: vitreous humor (13), umbilical cord (14), the mucoid phase of group A hemolytic streptococci (15), synovial fluid (16), fowl sarcoma (17), and the pleural fluid of a patient with a mesothelioma (18). Hyaluronic acid forms viscous solutions and the mucinous character of the material from which it has been isolated is due to the presence of this constituent.

An enzyme or enzymes which decrease the viscosity of solutions containing hyaluronic acid, with the liberation of reducing substances, has been prepared from the following sources: two strains of pneumococcus, one strain of group A hemolytic streptococcus, one strain of *Cl. welchii*, and from splenic tissue (19). Such enzyme preparations specifically hydrolyze isolated hyaluronic acid, and the enzyme has been therefore designated as hyaluronidase.

In a recent paper Chain and Duthie (20) reported that testicular extracts decrease the viscosity of synovial fluid and vitreous humor, with the liberation of reducing substances. Since such extracts also induce the phenomenon of spreading these investigators suggested that spreading factor is probably identical with the "mucinase" which hydrolyzes the polysaccharide in these fluids. Meyer and Chaffee (21) confirmed the observations of Chain and Duthie and, in addition, demonstrated that testicular extracts hydrolyze isolated hyaluronic acid. Claude (22) in a study of leech extracts also contributed evidence in support of the theory proposed by Chain and Duthie. He showed that such material, which is rich in spreading factor, contains a "mucolytic" enzyme which reduces the viscosity of Chicken Tumor I extracts and of a "mucoprotein" isolated from skin. He concluded that the strength of the "mucolytic' enzyme paralleled the strength of spreading factor and suggested that the two agents might be identical. The present study was undertaken to determine more precisely the relationship between spreading factor and hyaluronidase. Some of the results have already been published in a preliminary communication (23).

## EXPERIMENTAL

The presence of hyaluronidase and spreading factor was tested in preparations from the following sources: pneumococci (Type I, virulent and avirulent strains; Type II, avirulent strain), group A hemolytic streptococci (virulent and avirulent strains in the mucoid, matt, and rough phases (24)), *Cl. welchii*, testis, pigskin, leech extract, commercial spleen extract, commercial hirudine, and certain chemical substances.

## *Methods*

*Spreading Factor.*—The spreading capacity of the various preparations was tested by the intradermal method using the dye T.1824. The substances to be tested were dissolved in saline and 0.125 cc. of the test solution was mixed with an equal volume of a 2 per cent aqueous solution of the dye. The resultant mixture was injected intradermally into male albino rabbits weighing 4.5 to 5.5 pounds. The area of diffusion was measured at 3 hours and 20 hours after injection. Four injections were made on each rabbit, using only the area between the fore and hind limbs on each side. A mixture of 0.125 cc. of saline and an equal volume of dye was used as a control in each rabbit. Individual animals were found to vary considerably in their skin permeability. In order to eliminate error due to this variation, an index of diffusion was determined, according to the method of Claude (8), by dividing the area of spread produced by the test solution by the area of spread produced by the saline control. Only an index of 1.4 or more was accepted as an indication of spread. An index of 1.1 to 1.3 was considered doubtful and 1.0 or less negative.

*Hyaluronidase.*—Hyaluronidase preparations were made according to the procedure described in an earlier paper (19). The action of hyaluronidase was tested (*a*) by the hydrolysis of isolated hyaluronic acid and (*b*) by determining changes in the viscosities of various fluids known to contain hyaluronic acid.

Hydrolysis of the polysaccharide was carried out as follows: 0.1 mg. of the powdered enzyme preparation was incubated at 37°C. with 5 mg. of the polysaccharide in the presence of toluene at pH 5.8. Loss of the viscosity of the solution was observed almost immediately. Reducing values were determined after 2 hours, 20 hours, and 44 hours. Reducing sugar was determined by the ceric sulfate method (25). The theoretical reducing value of a given preparation was calculated from the acetyl-glucosamine and glucuronic acid content as determined by analysis and expressed as equivalents of glucose.

Change in viscosity was determined by means of the Ostwald viscosimeter. Viscous pleural fluid obtained from a patient with a mesothelioma of the pleura was used as a test material. It had previously been shown that the viscosity of the fluid was due to the presence of hyaluronic acid (18). 5 cc. of the pleural fluid were incubated at 37°C. with 1 mg. of enzyme preparation dissolved in 0.1 cc. of water. Readings were made at varying intervals and the results interpolated graphically. A control experiment with 5 cc. of the pleural fluid plus 0.1 cc. of 0.9 per cent sodium chloride served as control. The recorded values were expressed as per cent reduction of viscosity relative to the viscosity of 0.9 per cent sodium chloride.

The relation between the two reactions, hydrolysis and decrease in viscosity, is discussed in detail elsewhere (26). It is sufficient to state at this point that although the two reactions apparently are catalyzed by the same agents, it has not been proven conclusively that they are due to the same enzyme.

Experimental observations suggesting a relationship between spreading factor and hyaluronidase will first be presented. Following this certain findings and observations suggesting dissimilarities between the two will be reported.

## *I. Similarities between Spreading Factor and Hyaluronidase*

*1. The Presence of Spreading Factor in All Preparations Containing Hyaluronidase.*—Preparations of hyaluronidase were made from the following sources: pneumococcus (Type I virulent, strain 1/230; Type I avirulent, strain 1/192/R); group A *Streptococcus hemolyticus* (type 4, strain H 44);[1]

TABLE I

*Presence of Spreading Factor in Preparations Containing Active Hyaluronidase*

| Source of preparation | Spread | | Hydrolysis | | |
|---|---|---|---|---|---|
| | Amount injected | Average index of spread | Enzyme concentration | 2 hrs. | 20 hrs. |
| | *mg.* | | *per cent* | *per cent* | *per cent* |
| Hemolytic streptococcus | | | | | |
| Strain H 44, preparation 1 | 3.1 | 2.6 | 0.5 | 7 | 12 |
| " " " 2 | 2.5 | 2.4 | " | 5 | 8 |
| " " " 3 | 5.0 | 2.8 | " | 6 | 39 |
| Pneumococcus | | | | | |
| Strain 1/230 | 3.2 | 4.3 | 0.05* | 28 | 59 |
| " 1/192/R | 3.0 | 3.3 | " * | 26 | 60 |
| " D/39/R, preparation 1 | 5.0 | 6.0 | " | 68 | 92 |
| " 2 | 3.0 | 6.3 | " * | 51 | 62 |
| " 3 | 2.5 | 3.6 | " | 11 | 54 |
| " 4 | 1.3 | 10.0 | " | 23 | 55 |
| *Cl. welchii* | | | | | |
| Strain Lister A | 2.3 | 3.4 | 0.25 | 44 | 74 |
| Testis | | | | | |
| Preparation 1 | 0.7 | 3.1 | 0.5 | 42 | 47 |
| " 2 | 3.8 | 1.4 | " | 20 | 38 |
| Leech (dried) | 1.25 | 2.5 | 0.25 | 37 | 67 |

* These preparations of enzyme also produced a reduction of viscosity of approximately 90 per cent within 10 minutes.

*Cl. welchii* (Lister A strain); crude leech extract;[2] and testicular extracts prepared according to the methods of Morgan and McClean (27) and of Duran-Reynals (4). When tested by the methods previously described all the foregoing preparations were found to possess the capacity of hy-

[1] Attempts to obtain hyaluronidase from other strains of group A hemolytic streptococci have so far proved unsuccessful. The strain employed (H 44) produces only matt colonies and it seems possible that its failure to produce mucoid colonies may be due to the presence of the enzyme.

[2] We are indebted to Dr. Claude for a generous supply of leech extract.

drolyzing hyaluronic acid. Similarly when tested for the presence of spreading factor all preparations were found to produce marked spreading. However, no parallelism was observed between the strength of the spreading factor and of the enzyme from the same preparations. The results are summarized in Table I.

TABLE II

*Effect of Heat on Spreading Factor*

| Source of preparation | Amount injected | Average index of spread | |
|---|---|---|---|
| | | Unheated | Heated at 65°C. for 30 min. |
| | *mg.* | | |
| Pneumococcus (strain D/39/R) | 5.0 | 6.0 | 3.6 |
| Preparation 1 | 3.0 | 6.3 | 3.4 |
| | 2.5 | 5.6 | 2.2 |
| | 0.25 | 3.0 | 1.1 |
| | 0.06 | 2.3 | 1.2 |
| | | Unheated | Heated at 100°C. for 30 min. |
| Preparation 2 | 2.5 | 4.9 | 2.0 |
| | | Unheated | Heated at 65°C. for 30 min. |
| Hemolytic streptococcus | 8.6 | 6.5 | 4.3 |
| (strain H 44) | 5.0 | 2.8 | 2.6 |
| | 3.1 | 2.6 | 1.8 |
| | 2.5 | 2.4 | 1.6 |
| | 0.5 | 2.4 | 1.4 |
| | 0.25 | 2.0 | <1.0 |
| | 0.06 | 1.7 | <1.0 |
| | | Unheated | Heated at 100°C. for 30 min. |
| | 2.5 | 2.3 | 1.4 |

*2. Effect of Heat on Spreading Factor and Hyaluronidase.*—Preparations made from pneumococcus and *Streptococcus hemolyticus* were dissolved in physiological saline and heated at 65°C. for 30 minutes and at 100°C. for the same period. The activity of the heated material was then compared with that of the unheated.

After heating at 65°C. for 30 minutes the activity of the spreading factor was diminished but not completely destroyed. Heating at 100°C. for 30 minutes produced a similar effect (Table II). The effect of heat on hyaluronidase is shown in Table III. In one experiment the activity of

the enzyme was markedly weakened by heating at 65°C. for 30 minutes and in the second experiment it was destroyed entirely. Similarly heating at 100°C. for 30 minutes completely inactivated the enzyme.

TABLE III

*Effect of Heat on Hyaluronidase*

| Source of preparation | Enzyme concentration | Hydrolysis, *per cent* | | | |
|---|---|---|---|---|---|
| | | Unheated | | Heated at 65°C. for 30 min. | |
| | *per cent* | 2 hrs. | 20 hrs. | 2 hrs. | 20 hrs. |
| Pneumococcus (strain D/39/R) Preparation 1 | 0.05 | 40 | 62 | 5 | 37 |
| | | Unheated | | Heated at 100°C. for 30 min. | |
| | | 2 hrs. | 20 hrs. | 2 hrs. | 20 hrs. |
| Preparation 2 | 0.1 | 22 | 59 | 0 | 0 |
| | | Unheated | | Heated at 65°C. for 30 min. | |
| | | 2 hrs. | 20 hrs. | 2 hrs. | 20 hrs. |
| Hemolytic streptococcus (strain H 44) | 0.5 | 6 | 21 | 0 | 2 |

TABLE IV

*Effect of Iodine on Spreading Factor and Hyaluronidase**

| Source of preparation | Average index of spread | | Inhibition of hydrolysis, *per cent* | |
|---|---|---|---|---|
| | Before treatment with $I_2$ | After treatment with $I_2$ | Before treatment with $I_2$ | After treatment with $I_2$ |
| Pneumococcus, strain D/39/R | | | | |
| Preparation 1 | 2.0 | 1.7 | 0 | 100 |
| " 2 | 4.0 | 1.7 | 0 | 100 |

* In these experiments 0.25 cc. N/10 iodine was used. Under the experimental conditions neither spreading factor nor hyaluronidase was reactivated by N/10 sodium sulfite. In later experiments it was found that by using lower concentrations of $I_2$ the inactivation of hyaluronidase could be reversed by $As_2O_3$.

*3. Inactivation of Spreading Factor and Hyaluronidase by Iodine.*—A preparation of hyaluronidase from pneumococcus was made up in a concentration of 20 mg. per cc. To 0.5 cc. of this solution was added 0.25 cc. of N/10 iodine and, after 10 minutes, 0.25 cc. of N/10 sodium sulfite. As a control, 0.5 cc. of the enzyme solution was diluted with 0.5 cc. of physi-

ological saline. The resultant mixtures were tested for hyaluronidase[3] and for spreading power. As indicated in Table IV the activity of spreading factor was greatly reduced by iodine while the activity of the enzyme was completely destroyed. Under the conditions of the experiment, neither was reactivated by sodium sulfite.[4]

*4. Presence of Hyaluronic Acid in Skin.*—The theory that spreading factor is identical with hyaluronidase postulates the presence in skin of either hyaluronic acid itself, or a related substrate, upon which hyaluronidase may act. Recently in this laboratory an acid polysaccharide which exhibits all the properties of hyaluronic acid has been isolated from pigskin. The method of preparation and the analytical data pertaining to this polysaccharide are being presented in detail elsewhere (28). This skin polysaccharide has the same optical rotation and is hydrolyzed by hyaluronidase at the same rate as hyaluronic acid obtained from other sources. The demonstration of hyaluronic acid in skin is consistent with the theory that the spreading phenomenon is due to the enzymatic hydrolysis of this polysaccharide.

### *II. Dissimilarities between Spreading Factor and Hyaluronidase*

*1. The Presence of Spreading Factor in Preparations in Which Hyaluronidase Has Not Been Demonstrated.*—It has already been reported that a number of preparations made from strain H 44 of group A hemolytic streptococcus possessed both spreading factor and the ability to hydrolyze hyaluronic acid. Certain other preparations made from strain H 44 as well as from other strains of group A hemolytic streptococci failed to hydrolyze hyaluronic acid although they did possess spreading factor. The results of nine experiments are summarized in Table V. Marked spreading was obtained with eight of the nine preparations yet none hydrolyzed hyaluronic acid. So, also, a preparation from strain 6163 of *Cl. welchii*, received from Dr. Fritz Schiff, contained no demonstrable hyaluronidase but did produce marked spreading.

Other substances tested for the presence of spreading factor and hyaluronidase were as follows: commercial hirudine (Eimer and Amend Company); a commercial preparation of beef-spleen (Armour spleen liquid 40 per cent) which had been concentrated 10 times by drying *in vacuo*; a prep-

[3] Due to difficulties encountered with the ceric sulfate method in the presence of iodine and the reducing agents, the liberated reducing groups in this case were measured by the Folin-Wu method.

[4] In subsequent experiments it was found that by using lower concentrations of iodine the inactivation of hyaluronidase could be reversed by arsenious oxide.

aration from pigskin; two azoprotein preparations; arsenious oxide in 0.025 and 0.0025 molar concentrations; and, finally, isolated hyaluronic acid itself. As indicated in Table V none of these preparations hydrolyzed hyaluronic acid but all were capable of inducing spreading phenomena.

In interpreting the spreading phenomena induced by these various agents consideration must be given both to the rate and to the type of reaction. Madinaveitia (29) has

TABLE V

*Presence of Spreading Factor in Inactive Hyaluronidase Preparations and in Other Material Which Does Not Contain Hyaluronidase*

| Material injected | Amount injected | Average index of spread | Hydrolysis |
|---|---|---|---|
| **A. Inactive hyaluronidase preparations** | | | |
| | *mg.* | | *per cent* |
| Hemolytic streptococcus | | | |
| Strain H 44, preparation 1 | 3.0 | 2.4 | 0 |
| " " " 3 | 1.0 | 3.1 | 0 |
| " C203MV " 1 | 3.5 | 2.4 | 0 |
| " " " 2 | 1.0 | 3.0 | 0 |
| " " " 3 | 3.2 | 6.5 | 0 |
| " C203R | 3.2 | 5.5 | 0 |
| " Render " 1 | 2.2 | 4.5 | 0 |
| " " " 2 | 3.4 | 2.4 | 0 |
| *Cl. welchii*, strain 6163 | 0.5 | 2.0 | 0 |
| **B. Other material which does not contain hyaluronidase** | | | |
| Azoprotein, preparation 1 | 0.25 | 1.4 | 0 |
| " 2 | 1.2 | 15.5 | 0 |
| Hyaluronic acid | 2.8 | 1.9 | 0 |
| Hirudine | 2.6 | 2.2 | 0 |
| Pigskin | 3.3 | 1.5 | 0 |
| | *cc.* | | |
| Spleen extract (10 × conc. soln.) | 0.125 | 2.1 | 0 |
| Arsenious oxide 0.05 M | 0.125 | 3.3 | 0 |
| " " 0.005 M | 0.125 | 1.5 | 0 |

called attention to the difference in the rate of spread produced by kallikrein compared with that induced by testicular extracts and other preparations. While all preparations were effective in causing spread he found that the rate of the reaction was much slower with kallikrein than with the other agents tested. He therefore inferred that the mechanism of the reaction was not the same in both instances. Claude (30) has called attention to a difference in the type of spreading reaction produced by various agents. According to him there is more thickening and edema of the skin in the area of the spread with arsenious oxide than with leech extract. The type of reaction produced by azoproteins is intermediate between these two.

The observations of Madinaveitia and of Claude indicate that great care must be taken in interpreting spreading phenomena in general. The test is purely a biological one and subject to many variations. Except in the experiments with arsenious oxide we were unable to detect any significant differences in either the rate or the character of the reaction. However, readings were only made at the end of 3 and 20 hour periods and no histological studies of the skin were made in the tested areas. It is therefore possible that future studies will show that spreading is not a single phenomenon but a group of reactions depending upon a variety of mechanisms, one of which may be the hydrolysis of hyaluronic acid.

*2. Effect of Antisera to Hyaluronidase Preparations on Hyaluronidase and on Spreading Factor.*—The following experiments were undertaken in order to determine whether hyaluronidase preparations were antigenic, and if so, whether the antisera so prepared would exert the same effect on the hyaluronidase and on the spreading factor known to be present in these preparations.

*(a) Precipitation of Hyaluronidase Preparations by Antisera.*—

*Preparation of Antisera.*—50 mg. of a sodium flavianate preparation of pneumococcus hyaluronidase were dissolved in 24 cc. of physiological saline and 1 cc. of alum was added. Four rabbits were injected 3 times a week for a period of 4 weeks. Each animal received a total of 110 mg. of enzyme preparation. The rabbits were bled on the 6th day following the last injection.

Antisera so prepared were set up against the homologous hyaluronidase preparations as well as against hyaluronidase preparations made from *Streptococcus hemolyticus* and *Cl. welchii*. In the case of the homologous enzyme preparations definite precipitates were obtained with amounts of 1.0 and 0.1 mg. per cc. Weaker reactions were obtained with the preparations from *Streptococcus hemolyticus* and no reaction with preparations from *Cl. welchii*. The results are summarized in Table VI.

The results of these experiments show that the hyaluronidase preparations made from pneumococcus possess antigenic properties. However, the fact that weak or no precipitation was obtained with heterologous enzyme preparations suggests that the precipitation obtained with homologous preparations might well have been due to extraneous antigenic material present in the pneumococcal hyaluronidase preparation.

*(b) Effect of Antisera to Hyaluronidase Preparations on Hyaluronidase Activity.*—

The capacity of antisera to inhibit the activity of hyaluronidase preparations was tested as follows:—

Samples of each of the antisera above described were precipitated by half saturation with sodium sulfate at 35°C. and the precipitate dissolved in water to the original volume. 0.1 cc. of each antiserum fraction was mixed with 0.1 mg. of enzyme preparation and 5 mg. of hyaluronic acid. The mixture was incubated at 37°C. at pH 5.8 and the amount of hydrolysis determined after 2 and 20 hours. Controls were set up in which the antiserum fraction was replaced by normal rabbit and normal human sera.

TABLE VI

*Precipitin Reactions with Various Enzyme Preparations and Antisera against Pneumococcal Hyaluronidase*

| Antiserum | | | Enzyme preparation | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Pneumococcus | | | | | Hemolytic streptococcus | | | *Cl. welchii* | | | |
| No. | Dilution | Control | 1 mg./cc. | 0.1 mg./cc. | 0.01 mg./cc. | 0.001 mg./cc. | 0.0001 mg./cc. | 1 mg./cc. | 0.1 mg./cc. | 0.01 mg./cc. | 1 mg./cc. | 0.1 mg./cc. | 0.01 mg./cc. |
| 1 | 0 | − | Ring ++± | ++ | ± | − | − | Cloud ++ | + | − | ± | − | − |
| 2 | 0 | − | Ring ++± | +± | ± | − | − | Cloud + | ± | − | ± | − | − |
| 3 | 0 | − | Ring ++± | +± | ± | − | − | Ring ++± | + | − | ± | − | − |
| 4 | 0 | − | Ring ++± | +± | ± | ± | − | Ring ++± | + | − | ± | − | − |
| Control | | | − | − | − | − | − | − | − | − | − | − | − |

The tests were read after 30 minutes at room temperature and the presence of rings or clouding noted. The tubes were then shaken, incubated at 37°C. for 1½ hours, and stored in the refrigerator overnight. Final readings were made after refrigeration.

The results in Table VII show that three of the four antisera completely inhibited the activity of the homologous hyaluronidase preparations while the fourth caused over 80 per cent inhibition. On the other hand, only 25 to 50 per cent inhibition was observed when the same antisera were tested against heterologous hyaluronidase preparations made from *Streptococcus hemolyticus* and *Cl. welchii*. Control experiments showed that normal human and normal rabbit serum exerted approximately the same degree of inhibition. It is therefore concluded that antiserum inhibits the activity of the homologous hyaluronidase preparation but exerts little or no effect on heterologous preparations.

In interpreting the results of this experiment attention is directed to two complicating factors. In the first place the inhibition of the activity of the hyaluronidase prepara-

tion exhibited by the homologous antiserum may be due, in part at least, to the adsorption of the active material on an extraneous precipitate, rather than an actual antibody inhibition. Secondly the inhibition produced by normal human and normal rabbit serum may be due to the salt formation which takes place between the serum protein and hyaluronic acid at the pH of the experiment.

TABLE VII

*Effect of Hyaluronidase Antisera on Hyaluronidase Activity*

| Antiserum to hyaluronidase (pneumococcus) | Hyaluronidase activity—hydrolysis, *per cent* | | | | | |
|---|---|---|---|---|---|---|
| | Pneumococcus | | Streptococcus | | *Cl. welchii* | |
| | 2 hrs. | 20 hrs. | 2 hrs. | 20 hrs. | 2 hrs. | 20 hrs. |
| No serum | 26 | 86 | 7 | 42 | 10 | 61 |
| Normal rabbit serum | 14 | 69 | 8 | 31 | 4 | 27 |
| Antiserum 1 | 3 | 14 | 12 | 39 | 1 | 30 |
| " 2 | 0 | 2 | | | | |
| " 3 | 0 | 2 | | | | |
| " 4 | 1 | 1 | 7 | 33 | 6 | 33 |

TABLE VIII

*Effect of Hyaluronidase Antisera on Spreading Factor*

| Antiserum to hyaluronidase (pneumococcus) | Source of spreading factor | Average index of spread |
|---|---|---|
| No serum | Pneumococcus | 1.5 |
| Antiserum | " | 1.8 |
| No serum | Streptococcus | 5.6 |
| Antiserum | " | 5.6 |

(*c*) *The Effect of Antisera to Hyaluronidase Preparations on Spreading Factor.*—

The effect on spreading factor of antisera made against pneumococcal and streptococcal hyaluronidase preparations was tested as follows:—

0.25 cc. of each of two enzyme preparations containing 10 to 12 mg. per cc. was mixed with equal volumes of antiserum. The resultant mixture was injected immediately into the skin of rabbits and again after the material had been allowed to stand for 2 hours at room temperature. Serum mixed with an equal volume of water was used as a control.

The results presented in Table VIII show that antisera to hyaluronidase preparations did not affect the spreading property of either the pneumococcal or the streptococcal preparations. These results differ from those reported in the preceding section in which it was shown that the same antisera

did inhibit the activity of homologous hyaluronidase preparations *in vitro*. Therefore, as far as the capacity of antisera to inhibit their respective effects is concerned, there appears to be a distinction between spreading factor and hyaluronidase. Whether this difference is real or only apparent is considered in the general discussion.

## DISCUSSION

Since spreading factor is not as yet a precisely defined agent it is obviously impossible to express any final opinion as to its identity or non-identity with hyaluronidase. In the present study an attempt has been made merely to determine a possible relationship between the phenomenon of spreading and hyaluronidase activity. A comparison of the properties of the two reactions has resulted in the demonstration of many points of similarity and, at the same time, certain apparent points of difference.

It has been shown that all preparations containing hyaluronidase also contain spreading factor. It has likewise been shown that spreading factor and hyaluronidase possess certain properties in common. Both are weakened by heating at 65°C. for 30 minutes and their activity is almost entirely destroyed by heating at 100°C. for the same period. Both are partially inactivated by iodine, and, under the conditions of the experiment, neither is reactivated by sodium sulfite. An even more significant fact in establishing a possible relationship between the two has been the demonstration of the presence of hyaluronic acid in skin. The occurrence of this substrate in skin offers a plausible explanation for the mechanism of the spreading phenomenon.

On the other hand certain evidence has been presented indicating that the phenomenon of spreading is not identical with hyaluronidase activity. Many preparations which possessed no hyaluronidase activity were shown to exhibit marked spreading properties. So also an apparent difference was demonstrated in the inhibition of the activity of the two agents by antisera. Antisera made against pneumococcal hyaluronidase preparations specifically and completely inhibited the activity of the homologous enzyme but did not inhibit the spreading property of these preparations.

In considering the apparent differences between spreading and hyaluronidase activity certain facts should be borne in mind:

1. It is possible that spreading may be due to several agents of which hyaluronidase may be one. That more than one spreading factor may exist has been implied by several investigators. Duran-Reynals and Stewart (6) observed a lack of parallelism between the enhancement of

experimental vaccinia infections and the spread of dyes; Medinaveitia (29) called attention to the difference in the rate of spreading produced by different types of agents; Claude (30) pointed out that the nature of the spreading reaction was not the same in all instances; finally, in the present study, it has been shown that hyaluronic acid itself acts as a spreading agent. It would therefore appear probable that spreading is not a single phenomenon but a group of reactions depending upon a variety of mechanisms, one of which may be hyaluronidase activity.

2. The observed differences in the effect of antisera on spreading factor and hyaluronidase also require some comment. It is possible that the apparent difference may be due to the fact that the demonstration of hyaluronidase activity is carried out *in vitro* while that of spreading is shown *in vivo*. In the former case the antiserum may combine with hyaluronidase to form a loose inactive complex which dissociates to an active form *in vivo*.

The theory that spreading factor is adsorbed onto an extraneous precipitate has been advanced by Duran-Reynals (31) to account for the inactivation of spreading factor by antisera to testicular extracts. The evidence available indicates that hyaluronidase is not inactivated by such adsorption. Its inhibition by antisera to homologous enzyme preparations, therefore, probably cannot be explained on this basis.

3. The apparent differences between the two agents may be explained by the greater sensitivity of the test for spreading factor. It is possible that the intradermal test may detect the presence of an agent in amounts too small to be demonstrated by the hydrolysis and viscosity experiments. Although this possibility cannot be denied, the lack of parallelism in the activity of many preparations makes such an explanation improbable.

Another finding which complicates the interpretation of spreading phenomenon is the demonstration that extracts of skin itself contain spreading factor. This observation, which was made by Duran-Reynals, has been confirmed in the present study. There is thus always the possibility that the spreading effect may be due to the release of this factor by the injected material, whatever it may be. Such an explanation may account for the spreading produced by simple chemical substances such as arsenious oxide.

In conclusion certain evidence suggests that hyaluronidase may play an important rôle in the spreading reaction. It is apparent, however, that the phenomenon is a complex one and that the entire mechanism cannot be explained on the basis of a simple chemical reaction.

## SUMMARY AND CONCLUSIONS

A comparative study of spreading factor and hyaluronidase in preparations from various sources revealed the following points of similarity and dissimilarity in the two reactions.

1. Similarities: (*a*) All preparations containing hyaluronidase also produced spreading. (*b*) Heating at 65° and 100°C. for 30 minutes produced a comparable effect on both reactions. (*c*) The demonstration of the presence of hyaluronic acid in skin offers a plausible explanation for the mechanism of spreading on the basis of hyaluronidase activity.

2. Dissimilarities: (*a*) No parallelism was observed in the degree of activity of spreading factor and hyaluronidase in the same preparations. (*b*) All preparations which produced spreading did not contain hyaluronidase. (*c*) Antisera to hyaluronidase preparations specifically and completely inhibited the activity of the homologous enzyme but did not inhibit the spreading factor in the same preparations.

The significance of the similarities and dissimilarities between the two reactions is discussed. It is concluded that while hyaluronidase may play a rôle in the spreading reaction the phenomenon is a complex one and cannot be explained on the basis of a simple chemical reaction.

## BIBLIOGRAPHY

1. Duran-Reynals, F., *Compt. rend. Soc. biol.*, 1928, **99,** 6.
2. Duran-Reynals, F., *J. Exp. Med.*, 1929, **50,** 327.
3. Hoffmann, D. C., and Duran-Reynals, F., *Science*, 1930, **72,** 508; *J. Exp. Med.*, 1931, **53,** 387.
4. Duran-Reynals, F., *Yale J. Biol. and Med.*, 1939, **11,** 601.
5. Pijoan, M., *J. Exp. Med.*, 1931, **53,** 37. Duran-Reynals, F., *J. Exp. Med.*, 1933, **58,** 161. McClean, D., *J. Path. and Bact.*, 1936, **42,** 477. Pradham, M. G., *Brit. J. Exp. Path.*, 1937, **18,** 90. Wilson, H., *Australian J. Exp. Biol. and Med. Sc.*, 1939, **17,** 393.
6. Duran-Reynals, F., and Stewart, F. W., *Am. J. Cancer*, 1931, **15,** 2790. Boyland, E., and McClean, D., *J. Path. and Bact.*, 1935, **41,** 553.
7. Duran-Reynals, F., *J. Exp. Med.*, 1933, **58,** 161. McClean, D., *J. Path. and Bact.*, 1936, **42,** 477.
8. Claude, A., *J. Exp. Med.*, 1937, **66,** 353.
9. Claude, A., *J. Exp. Med.*, 1935, **62,** 229; 1939, **69,** 641.
10. McClean, D., *J. Path. and Bact.*, 1931, **34,** 459.
11. Favilli, G., *Sperimentale*, 1935, **89,** 724.
12. Duran-Reynals, F., *Ann. Inst. Pasteur*, 1936, **57,** 597.
13. Meyer, K., and Palmer, J. W., *J. Biol. Chem.*, 1934, **107,** 629.
14. Meyer, K., and Palmer, J. W., *J. Biol. Chem.*, 1936, **114,** 689.
15. Kendall, F. E., Heidelberger, M., and Dawson, M. H., *J. Biol. Chem.*, 1937, **118,** 61.
16. Meyer, K., Smyth, E. M., and Dawson, M. H., *J. Biol. Chem.*, 1939, **128,** 319.

17. Kabat, E. A., *J. Biol. Chem.*, 1939, **130,** 143.
18. Meyer, K., and Chaffee, E., *J. Biol. Chem.*, 1940, **133,** 83.
19. Meyer, K., Hobby, G. L., Chaffee, E., and Dawson, M. H., *J. Exp. Med.*, 1940, **71,** 137.
20. Chain, E., and Duthie, E. S., *Nature*, 1939, **144,** 977.
21. Meyer, K., and Chaffee, E., *Proc. Soc. Exp. Biol. and Med.*, 1940, **43,** 487.
22. Claude, A., *Proc. Soc. Exp. Biol. and Med.*, 1940, **43,** 684.
23. Meyer, K., Hobby, G. L., Chaffee, E., and Dawson, M. H., *Proc. Soc. Exp. Biol. and Med.*, 1940, **44,** 294.
24. Dawson, M. H., and Hobby, G. L., *J. Infect. Dis.*, 1938, **62,** 138.
25. Miller, B. F., and Van Slyke, D.D., *J. Biol. Chem.*, 1936, **114,** 583.
26. Meyer, K., Chaffee, E., Hobby, G. L., and Dawson, M. H., *J. Exp. Med.*, 1941, **73,** in press.
27. Morgan, W. T. J., and McClean, D., *J. Soc. Chem. Ind.*, 1932, **51,** 44, 912.
28. Meyer, K., and Chaffee, E., unpublished.
29. Madinaveitia, J., *Biochem. J.*, 1939, **33,** 1470.
30. Claude, A., personal communication.
31. Duran-Reynals, F., *J. Exp. Med.*, 1932, **55,** 703.