due to Dr. T. W. Stallybrass, County Medical Officer of Health for Dorset, and Dr. T. V. Cooper, Pathologist to the County Laboratory, Dorchester, who kindly allowed the investigation to be continued at the County Laboratory.

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IDENTITY OF HYALURONIDASE AND SPREADING FACTOR.

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IN a preliminary communication (Chain and Duthie, 1939) we have presented suggestive evidence for the identity of the so-called spreading factor of Duran-Reynals (1928, 1929) and McClean (1930, 1931) with an enzyme acting on a mucin-like substance present in synovial fluid and vitreous humour. Since the appearance of our preliminary report we have been engaged in collecting more conclusive evidence for the identity of the two substances which we present in the following paper. The evidence is based on the facts that (1) the enzyme has been demonstrated to be present in all sources of spreading factor investigated, and an immediate spread in the skin has never been observed in solutions not containing the enzyme; (2) there exists a satisfactory quantitative correlation between enzyme activity and spreading activity; (3) a mucin-like substance has been isolated from skin with properties similar to those of vitreous humour and synovial fluid, and is acted upon by solutions containing spreading factor in the same way as the substances in vitreous humour and synovial fluid.

While this work was in progress a number of publications arising from our preliminary report have appeared, partly confirming our results and partly anticipating some of the work on which we were actually engaged (Favilli, 1940; McClean and Hale, 1940; Meyer *et al.*, 1940*b*, *c*; Claude, 1940; Madinaveitia, 1940; Madinaveitia *et al.*, 1940). With the exception of the last-mentioned all the investigators agree with our view of the existence of a close relationship between spreading and enzyme activity.

Meyer and Palmer (1936) have isolated from vitreous humour and umbilical cord a protein-free polysaccharide composed of glucuronic acid and N-acetylglucosamine in equimolecular proportions. This substance was termed hyaluronic acid. In a later communication Meyer, Smyth and Dawson (1939) were able to show that a substance with identical chemical properties, but

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with a much higher viscosity, was present in synovial fluid and was, in fact, responsible for the two main characteristic properties of this fluid, its high viscosity and string formation after the addition of acetic acid.

We have prepared purified hyaluronic acid according to the prescriptions of Meyer, and his collaborators, both from vitreous humour and synovial fluid. We have found that the prepared substances were attacked by the " mucolytic " enzyme present in spreading factor solutions of different origin and, in fact, were the only substances in vitreous humour and synovial fluid acted upon by the enzyme. It is therefore certain that hyaluronic acid is the substrate for the " mucolytic " enzyme in spreading factor solution which accordingly is termed " hyaluronidase."

The purified polysaccharide acid from synovial fluid gave very viscous solutions, and the effect of the mucolytic enzyme on it was the same as on the original unpurified synovial fluid, consisting of an immediate fall of viscosity, followed later by a liberation of glucuronic acid and N-acetylglucosamine. The purified polysaccharide acid from vitreous humour gave much less viscous solutions than the polysaccharide acid from synovial fluid. Meyer (1938*a*) has suggested that the differences in viscosity between the polysaccharide acids may be due to differences in their degree of polymerization, the vitreous humour hyaluronic acid being partially depolymerized by a hyaluronidase present in the ciliary body. Though we have not been able to confirm the presence of the enzyme in the ciliary body of ox and sheep eyes, it is possible that the depolymerization of vitreous humour by hyaluronic acid may have been caused by some other, perhaps non-enzymatic substance (see Robertson *et al.*, 1939). The depolymerization of hyaluronic acid, as it occurs in vitreous humour, is, however, only partial, and it is acted upon, like synovial fluid hyaluronic acid, by all spreading factor containing solutions, with complete depolymerization, as demonstrated by the rapid and complete loss of its original moderate viscosity, followed by the liberation of glucuronic acid and N-acetylglucosamine.

METHODS.

Tests for Hyaluronidase Activity.

Hyaluronidase activities can be studied by three methods :

(1) By measuring the rate of decrease in viscosity of hyaluronic acid solutions when acted upon by the enzyme.

(2) By measuring the increase in reducing substances produced from hyaluronic acid by the action of the enzyme. This method can be used as a specific test only with protein-free hyaluronic acid preparations, since proteolysis leads to the liberation of reducing substances (Hewitt, 1938).

(3) By measuring the increase in N-acetylglucosamine from hyaluronic acid by the action of the enzyme.

The rate of liberation of reducing substances and N-acetylglucosamine is too small to be measured accurately ; hence methods (2) and (3) are not suitable for accurate quantitative estimations of the enzyme. It was found, however, that the rate of decrease of viscosity of hyaluronic acid solutions

from synovial fluid when acted upon by the enzyme under certain conditions was proportional to the concentration of the enzyme within a certain concentration range; and it was sufficiently rapid and reproducible for a quantitative assay of the enzyme.

Viscometric Determination of the Enzyme.

Enzyme activity was measured with an error of about $\pm$ 10 per cent. in Ostwald viscometers using synovial fluid from the tibio-astragaloid joint of cattle as substrate. The substrate used was prepared in two ways. In some cases freshly aspirated fluid was preserved by the addition of acriflavine to a concentration of 1 : 10,000, and after filtering through pleated filter paper, was adjusted before use to a viscosity 3 to 3·5 times that of water, and to a slightly acid pH by the addition of KH_2PO_4 and saline. It was then used directly, or was stored frozen. If unfrozen it underwent a rapid loss in viscosity after some days, presumably because of infection. In most cases the substrate used was a dried product prepared as follows: The fresh synovial fluid was shaken with chloroform and n-butyl alcohol (Seastone, 1939) and precipitated with two volumes of alcohol. The powder was dried with alcohol and ether, and was kept in a desiccator in the cold. This is essential, since the powder is hygroscopic and therefore becomes easily infected. Yield 2 g. per 100 c.c. 1 g. of the powder was dissolved by grinding and subsequent shaking at 37° C. for a few minutes in 2 c.c. $N/2$ NaOH, and about 70 c.c. of distilled water. The solution was made approximately neutral with $M/5$ KH_2PO_4 and acriflavine to 1/10,000 was added, after which it was filtered through pleated filter paper in the cold. A sample sufficient for one to two days was adjusted to a viscosity about 3·5 times that of water, and to a final molarity of about $M/15$ salt by the addition of freshly filtered phosphate buffer pH 7·0, the remainder being stored at 4° C. as a stock solution. This stock solution undergoes a slow loss in viscosity at 4° C., a solution of a viscosity three times that of water losing some 10 per cent. in 24 hours at 4° C. In carrying out tests 1·5 c.c. of the diluted substrate was measured into four or five test tubes 5·5 cm. by 1·0 cm., using a 1 c.c. record tuberculin syringe and a small hypodermic needle, the tubes being then placed to stand for some time in the bath at 26° C., containing the viscometers. 0·4 c.c. of enzyme in a concentration as judged by preliminary tests sufficient to cause a fall in viscosity between 10 per cent. and 35 per cent. in 30 minutes (1 to 4 units per c.c.) was added, two tubes receiving a standard preparation of known potency. The tubes were corked and rotated vertically on a turntable for five minutes, 1·7 c.c. of the contents being then transferred to the viscometers by a syringe. These were of the Ostwald type, having a bulb capacity of about 0·8 c.c. and capillary 7·5 cm. and 0·05 mm. internal bore. They were made from the same piece of capillary tube, and gave almost identical viscosity values over the range used. As many readings as were possible were taken over a 40 to 60-minute period, and were plotted as multiples of the water time for the viscometer against time from the beginning of mixing. Under these conditions it was found (Fig. 1) that the time required to reach any definite multiple of the

water time was inversely proportional to the concentration of enzyme present, and comparison with the standard preparation was therefore possible.

Several points should be observed. Specimens of substrate which undergo a rapid loss in viscosity (more than 1 second in 5 minutes) should be rejected. As recommended by Madinaveitia (1940), it is better to blow the liquid into the bulb of the viscometer than to suck. Dust particles must be eliminated from all solutions used, by repeated filtering and by taking care during the manipulations, and bubbles should be excluded from the viscometers. Water times should be determined daily, using a water volume equivalent to that of the enzyme-substrate mixture measured. The viscometers should not be heated above the temperature of the bath.

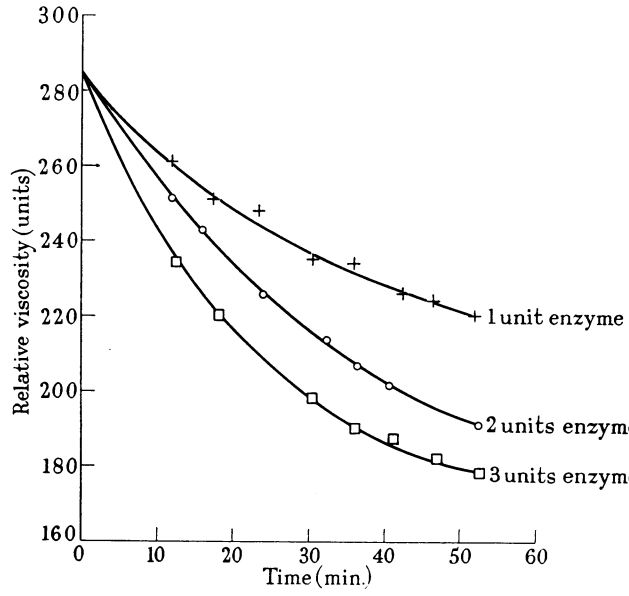

FIG. 1.—Rates of decrease of viscosity of hyaluronic acid caused by different amounts of hyaluronidase.

Choice of Unit.

In all comparisons the standard enzyme solution used was a sterile (filtered) extract of dried testis powder in $M/15$ acetate buffer pH 4·6 containing 1500 units per c.c. In the rabbit skin 0·1 unit in 0·25 c.c. haemoglobin solution gave an area about 1·25 to 1·5 times that of a control haemoglobin solution. 0·05 unit was barely detectable. Viscometrically it was found convenient to carry out determinations using solutions of between 1 and 2 units per c.c., i.e. a final concentration of between approximately 0·2 and 0·4 units per c.c. in the substrate.

Measurement of Diffusing Activity.

White albino rabbits, selected for thin skin and absence of moulting, were clipped on both sides of the vertebral column. Solutions injected were as

follows: 0·25 unit and 0·1 unit of standard testis preparation, 0·1 unit of two or three unknown solutions (first estimated viscometrically) and a control of haemoglobin alone, all in 0·25 c.c. of homologous haemoglobin. Each solution was injected in duplicate with a No. 20 needle. Twenty minutes after each injection the long and short diameters were measured, holding the rabbit skin gently stretched, and the results expressed as the ratio of the products of the diameters to those of the control injections. Similar measurements after 12 and 24 hours were not substantially different, and are not recorded.

Reference to Table I shows that in a series of nine animals there was in every case a clearly recognizable difference between the area of spread produced by 0·25 and 0·1 units of the standard testis preparation.

TABLE I.—*Comparison of Area of Spread Produced by* 0·25 *and* 0·1 *Units of Standard Testis Preparation.*

Animal number.	Spread with 0·25 unit.	Spread with 0·1 unit.	Spread given by 0·25 unit. / Spread given by 0·1 unit.
1	1·30	1·20	1·08
2	1·57	1·37	1·12
3	1·60	1·39	1·15
4	1·65	1·39	1·18
5	1·69	1·23	1·38
6	1·74	1·26	1·38
7	1·82	1·55	1·17
8	1·93	1·49	1·30
9	2·11	1·78	1·18

The figures are multiples of control areas (control area = 1·0), each value representing the mean of two determinations, measured twenty minutes after injection.

Determination of N-acetylglucosamine.

Hyaluronidase action can be detected by the colorimetric determination of N-acetylglucosamine, liberated from hyaluronic acid by the action of the enzyme. Crude hyaluronic acid preparations, still containing proteins, can be used as substrates; in this work hyaluronic acid preparations from vitreous humour, prepared according to the method of Seastone (1939) have been used. Usually, substrate solutions, containing 1–2 mg. of crude hyaluronic acid per c.c., were incubated for 16 hours with the enzyme at pH5 at 37° C., and the liberated N-acetylglucosamine was then determined in 1 c.c. samples. Controls with boiled enzyme were set up as a routine, but invariably negative results were obtained. The determinations were done according to the method of Morgan and Elson (1934). The colour intensities were measured by a Spekker photo-electric cell which was calibrated with solutions of N-acetylglucosamine of known strength.

Material Investigated for the Presence of Hyaluronidase and Spreading Activity.

Organ extracts were prepared by grinding fresh tissues in the homogenizer of Elvehjem and Potter (1936) or in a pestle with sand with one, two or three volumes of $M/10$ acetate buffer pH 4·6. They were subsequently centrifuged and the supernatant filtered and tested. The leeches used were chopped into pieces and ground in a pestle and mortar with sand, buffer being added. Venoms used were dried products. The specimen of bee sting was obtained through the courtesy of Messrs. Antibody Products Ltd., and represented twelve bee stings per c.c. The organisms tested were 24 to 48-hour cultures grown aerobically or anaerobically in broth, centrifuged at 7000 r.p.m., and preserved in the cold by the addition of acriflavine to 1/10,000. The specimens of *Cl. welchii* toxin tested were obtained from the Wellcome Physiological Laboratories through the kindness of Dr. J. W. Trevan.

Comparison of Viscometric Determinations and Diffusing Activity.

In order to establish the identity of hyaluronidase and diffusing factor we have examined extracts from as many as possible of those sources in which the presence of diffusing factor has been reported by various workers. In all cases qualitative comparisons were made between the amount of enzyme as detected by the viscometric method and diffusing effect. In a number of representative cases summarized in Table II, exact quantitative comparisons were carried out between the spread given in 20 minutes by 0·1 unit of extract determined viscometrically and that given by 0·1 unit and 0·25 unit of the

TABLE II.—*Comparison of Viscometric Measurements with Diffusing Activity of Hyaluronidase Preparations from Various Sources.*

Source.	Enzyme content (units/mg.).	Area of spread of— Solutions containing 0·1 unit hyaluronidase.	0·1 unit standard testis extract.	0·25 standard testis extract.
Dialysed testis extract	245	1·63	1·56	2·12
Dialysed testis extract heated 60 min. at 54° C.	60	1·39	1·26	1·74
Testis extract after $(NH_4)_2SO_4$ precipitation and dialysis	415	1·26	1·23	1·69
Leech extract dialysed	200	1·52	1·56	2·12
Copperhead venom	27 (units/c.c.).	1·32	1·23	1·69
Black tiger venom	111	1·54	1·23	1·69
Bee sting	24	1·26	1·26	1·74
Cl. welchii toxin	220	1·76	1·49	1·93
Pneumococcus culture	150	1·70	1·49	1·93

The figures are multiples of control areas (control area = 1), each value representing the mean of two determinations.

standard testis preparation. In addition to the viscometric measurements, in the majority of cases determinations of N-acetylglucosamine were carried out.

DISTRIBUTION OF HYALURONIDASE AND SPREADING FACTOR ACTIVITY.

Tissue Extracts.

Testis was the only mammalian organ showing either hyaluronidase activity or immediate spreading in the dermis. In the cases of certain organ extracts such as sheep spleen, ox ciliary body and rabbit pancreas, slight activity was sometimes observed both in the skin and viscometrically. Repeated experiments using freshly prepared tissues were entirely negative, and we are obliged to conclude that the few positive results were due to bacterial contamination. The following were the organs examined in the rabbit—lung, liver, kidney, brain, spleen, pancreas, muscle and preparations of polymorphonuclear leucocytes and lymphocytes. In the ox we examined ciliary body, pituitary lymph glands and thymus as well as those organs just listed for the rabbit. In the sheep we examined spleen and ciliary body. Tissue from the Jensen rat sarcoma was also investigated. As will be seen from Table II purification of testis extract by dialysis and precipitation with $(NH_4)_2SO_4$ as described below, or partial destruction by heat, did not alter the relationship between hyaluronidase content and diffusing activity. These results contradict those recently published by Madinaveitia *et al.* (1940). In their short preliminary note insufficient experimental details are given to enable us to give an explanation of the discrepancy. The partial destruction of hyaluronidase activity was achieved by incubating a dialysed testis extract at 54° C. for 60 min.

No liberation of N-acetylglucosamine from hyaluronic acid was found with any tissue extract except testis. (For pH activity of testicular enzyme see Table IV.) In these experiments 2 c.c. of tissue extracts, prepared by grinding the tissue with one volume of water in the homogenizer of Elvehjem and Potter (1936), were incubated at pH 5 or pH 6 with 5 c.c. of hyaluronic acid, containing 2 mg./c.c.

Bacterial Filtrates.

Of the bacteria examined only *Staph. aureus*, pneumococcus, *Cl. welchii* Types A and B and *Cl. septique* contained any appreciable quantity of hyaluronidase or spreading factor, the most potent being given by *Cl. welchii* Type A. In all cases examined the diffusing activity was definitely greater than would be expected from the enzyme content, corresponding approximately to 0·2 unit instead of the 0·1 injected. Slight enzyme activity (less than 0·2 unit per c.c.) was found in 48 hours' broth cultures of anaerobic streptococci, but as would be expected, there was no diffusing effect at that dilution. No enzyme or diffusing activity could be detected in at least 25 strains of *Strept. haemolyticus*, the majority of which were virulent. In several strains of *Strept. viridans*, meningococcus, *Bact. coli* and *Cl. welchii* Type C and D, neither enzyme activity nor diffusing effect was found.*

* Many of these determinations of hyaluronidase and spreading activity in bacterial filtrates were carried out by Dr. J. M. Barnes, to whom we are indebted for placing his results at our disposal.

After this work was completed papers by Robertson, Ropes and Bauer (1940) and Meyer *et al.* (1940*a*) dealing with hyaluronidase activity in bacterial cultures have appeared. Their results agree substantially with ours except for the fact that we have never been able to find any hyaluronidase activity whatever in any of the streptococcal cultures tested. The liberation of N-acetylglucosamine from vitreous humour hyaluronic acid was always found to go parallel with the viscometric determinations. The enzyme solutions were added to 5 c.c. of the substrate solutions (2 mg. hyaluronic acid per c.c.) in amounts of 0·1–2 c.c. depending on the strength of the enzyme.

Leech Extracts and Venoms.

Leech extract contained enormous amounts of hyaluronidase and gave a good correlation with diffusing activity. Equally good agreement between hyaluronidase and diffusing activity was obtained with bee venoms. In the case of snake venoms examined the diffusing activity was somewhat stronger than would be expected from their hyaluronidase content. With both bacterial filtrates and venoms the skin injected was definitely thicker (more oedematous) than that which had received testis preparations, and it is possible that the increased permeability of the vessels in the injected area caused by toxic substances may account for the greater area of spread. Leech extract and snake venoms liberated N-acetylglucosamine from hyaluronic acid. Leech extract was added in amounts of 0·1 c.c. to each 5 c.c. of the substrate solutions (2 mg. hyaluronic acid per c.c.), the snake venoms in amounts of 1–2 mg. to each 5 c.c. of the substrate solutions.

Azoproteins.

Azoproteins have been shown to have a spreading effect on the skin similar to that of the testicular spreading factor (Claude, 1935). The spread caused by azoproteins differs, however, from the spread caused by testis spreading factor in that it is very much slower, and is accompanied by oedema. We have diazotized sulphanilic acid and coupled it with ox serum following the prescription of Claude (1935). One volume of the resulting azoprotein solution was diluted with three volumes of distilled water. 0·4 c.c. of this solution were added to 1·5 c.c. of synovial fluid. The synovial fluid used, when diluted with a corresponding amount of 0·9 per cent. saline, had a viscosity of 3·25 times that of water. The azoprotein solution produced a loss in viscosity of 14 per cent. in 30 minutes. Three units of testicular hyaluronidase produced a loss in viscosity of 18 per cent. over the same period. The azoprotein caused spreading in the skin of rabbit, but quantitative comparisons of its spreading and viscosity decreasing power were not carried out, since the mode of spreading of azoproteins differs from that of hyaluronidase and therefore no strict comparisons are possible.

PROPERTIES OF HYALURONIDASE.

Stability.

Contrary to the statements of Madinaveitia the testicular hyaluronidase is stable over a fairly wide pH range. Sterile solutions containing 15 units

per c.c. either in 0·85 per cent. NaCl containing $M/50$ buffer or in $M/50$ buffer alone were incubated twelve hours at 37° C., and when tested viscometrically showed no loss of activity between pH 4·6 and 9. At pH 4 and 9·6 there was some 30 per cent. loss, and the destruction was much greater on either side of this range (90 per cent. at pH 3). Using unsterilized solutions, results similar to those of Madinaveitia's were obtained, and it is likely that the destruction obtained by him was due to bacterial action.

The enzyme is rapidly destroyed by gentle shaking, being most rapidly destroyed in dilute salt solutions, and especially in distilled water. Stability was greatest when shaken in 0·85 per cent. NaCl buffered to 4·6 with $M/50$ acetate as shown in Table III. For these reasons all solutions tested by us were shaken as little as possible and were stored in the cold.

TABLE III.—*Inactivation of Testicular Hyaluronidase by Shaking.*

Hyaluronidase solution contained 1·5 units per c.c. Temperature 25° C.

Diluting medium.	Percentage activity after 3 hours. Shaken.	Unshaken.
0·15 M NaCl + 0·02 M acetate buffer pH 4·6	50	100
0·15 M NaCl + 0·02 M phosphate pH 6·0	0	100
0·15 M NaCl + 0·02 M phosphate pH 7·0	0	100
0·02 M acetate buffer pH 4·6	0	100

Effect of Antiseptics.

Hyaluronidase is not affected by being left in contact with toluene without shaking, but is markedly inhibited by chloroform under these conditions.

The amount of enzyme destroyed by shaking is greater in the presence of chloroform or toluene, as shown in the following experiment. 2 c.c. of testicular enzyme solution, containing 15 units per c.c., showed no loss over a period of one hour at 37° C. in the presence of chloroform or toluene, if unshaken ; the same solution shaken without either antiseptic showed a 70 per cent. loss of activity ; if toluene or chloroform were added, the loss of activity was approximately 100 per cent. The amount of antiseptic added was 0·1 c.c. to 2 c.c. of enzyme ; the tubes were revolved in a vertical plane at 80 r.p.m. In view of the sensitivity of the enzyme to even mild shaking, care was taken to shake the solutions as little as possible. Merthiolate, phenyl mercuric nitrate and acriflavine do not affect the activity of the enzyme, and these antiseptics were used for the protection of the enzyme solutions from bacteria.

pH Activity and Salt Effect.

Table IV shows the activity of testis and *Cl. welchii* hyaluronidase at different pH values. Vitreous humour hyaluronic acid was used, prepared according to the method of Seastone (1939). The substrate was used in solutions containing 2 mg./c.c. of buffer. To each c.c. of the substrate solutions were added 0·1 mg. (32 units) of purified testis hyaluronidase dissolved in 0·1 c.c. of water, respectively 0·1 c.c. of a filtrate of *Cl. welchii*, containing 220 units per c.c. The buffers were $M/10$ citrate buffers for pH 2–4, $M/10$ acetate buffer for pH 5, $M/10$ phosphate buffer for pH 6 and pH 7, $M/10$ borate buffer for pH 8 and pH 9. The substrate solutions were incubated with

the enzyme for 16 hours at 37° C. and the liberated N-acetylglucosamine was determined colorimetrically.

TABLE IV.—*Effect of pH on Hyaluronidase Activity.*

Hyaluronidase from	γ/c.c. N-acetylglucosamine liberated at pH							
	2.	3.	4.	5.	6.	7.	8.	9.
Testis . . .	0	50	100	270	150	120	65	0
Cl. welchii . .	0	0	265	280	487	532	235	55

Both testicular and bacterial hyaluronidase are active over a fairly wide range of pH. The optima of the two enzymes are slightly different, pH 5 for the testicular and pH 6 for the bacterial enzyme.

The activity of the testicular hyaluronidase at different pH values is influenced by the salt concentration, as shown in Fig. 2. *M*/5, *M*/15 and *M*/90 buffers were used, and the amounts of liberated N-acetylglucosamine were determined after 16 hours' incubation of the substrate with testis hyaluronidase. Concentrations of enzyme and substrate and the types of buffers used were the same as in the experiments recorded in Table IV. The enzyme in diluted buffers is much more active at lower pH and much less active at higher pH than the enzyme in stronger buffers.

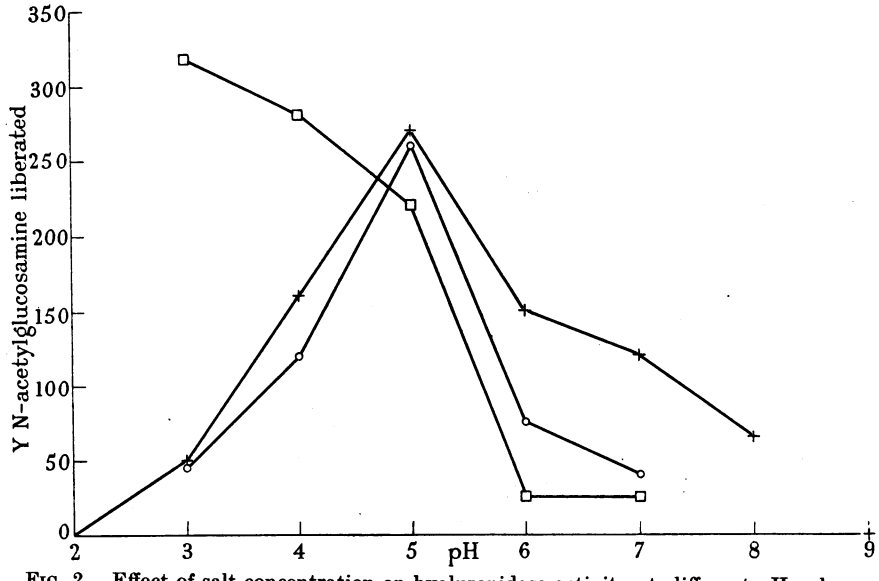

FIG. 2.—Effect of salt concentration on hyaluronidase activity at different pH values: — □ — = *M*/90 buffers. — + — = *M*/15 buffers. — 0 — = *M*/5 buffers.

Effect of Inhibitors.

The possible inhibitory action on hyaluronidase of a number of substances was tested as follows: *M*/100 solutions of sodium fluoride, potassium cyanide, sodium arsenite, iodine and sodium iodoacetate were prepared containing 1·5 units per c.c. of standard testis preparation and added to substrate (0·4 c.c.

enzyme to 15 c.c. substrate) and tested immediately in the viscometer for activity. At this final dilution of $M/500$ inhibitor there was complete inhibition only by iodine, the enzyme activity not being restored by the addition of sodium bisulphite, and a 50 per cent. loss of enzyme activity in the case of iodoacetate. Fluoride, arsenite and cyanide had no effect on enzyme activity. On the other hand both $(NH_4)_2SO_4$ and Na_2SO_4 produced considerable destruction in comparatively short periods. The addition of two volumes of a saturated solution of Na_2SO_4 or one volume of a saturated solution of $(NH_4)_2SO_4$ produced some 50 per cent. loss in 12 hours in strong solutions containing 3000 units per c.c. The activity was not restored on dialysis.

Partial Purification of Enzyme.

Because of the instability of the enzyme in the presence of the usual protein precipitates it has not been possible to carry its purification very far. A preparation having an activity of about 400 units per mg. equal to that prepared by the lead hydroxide method of Morgan and McClean (1932) was prepared as follows :

Dried bull testicle powder (obtained from the Instituto Biologico Argentino, Buenos Aires) was extracted three times, twice with six volumes, and once with three volumes of $M/50$ acetate buffer pH 4·6. The combined extracts (14 per cent. dry weight) had an activity of about 2500 units per c.c. The extract was evaporated to one-fifth of its volume in a large dish by passing a stream of warm air over the surface at 37° C., and was centrifuged. It was then dialysed with the minimum amount of shaking against tap water containing $M/50$ buffer pH 4·6, and, after centrifuging, precipitated with an equal volume of a saturated solution of $(NH_4)_2SO_4$. The precipitate was centrifuged off immediately, and the supernatant dialysed against distilled water in the cold. The solution containing $M/50$ acetate buffer pH 4·6 was dried from the frozen state *in vacuo*.

Isolation of a Substance closely Resembling Hyaluronic Acid from the Skin of Rabbits.

Five rabbit skins were clipped and then depilated with barium sulphide. They were thoroughly washed with running tap-water, minced through an ordinary mincer, treated twice with acetone (24 hours each time) and once with ether, dried first on filter paper and finally over P_2O_5. Portions of 100 g. of the dried skin powder were extracted twice with $N/100$ NaOH (600 c.c. and 750 c.c.) at room temperature for eight hours. The extracts were brought to pH 7·4, concentrated to 65 c.c. and precipitated with 600 c.c. of acetone. The precipitate, after drying in a desiccator, was taken up in portions of 2·7 g. in 20 c.c. of water and shaken for several hours. The insoluble residue was centrifuged and extracted with 10 c.c. water. The very viscous solution was dialysed against distilled water for 27 hours. The dialysed solution was concentrated to 30 c.c. and precipitated with 300 c.c. of acetone. The precipitate, after washing with acetone and ether, was dried over P_2O_5. The material dissolved in water to a 2 per cent. solution with a viscosity of 4 (water = 1). The viscosity was decreased to that of water by the addition of 320 units of testis hyaluronidase solution to 5 c.c. of a 2 per cent. solution

of the skin substance in phosphate buffer pH 6 within 30 minutes at room temperature.

After incubation at 37° for 16 hours 150 γ/c.c. of N-acetylglucosamine were set free.

Effect of Testis Hyaluronidase on Sexual Skin Mucin.

Ogston *et al.* (1940) have investigated some properties of a mucin-like substance accumulating in the sexual organs of female monkeys during the oestrous phase (Aykroyd and Zuckermann, 1938). This mucin resembled the mucin from synovial fluid, giving extremely viscous, string-forming solutions, precipitable by acetic acid in the same typical way as synovial mucin. We therefore decided to test the effect of testis hyaluronidase on a sample of this mucin, kindly given to us by Dr. Zuckermann. The result of the addition of testis hyaluronidase was an immediate fall in viscosity to that of water, loss of precipitability with acetic acid, liberation of reducing substances, consisting of N-acetylglucosamine and glucuronic acid. The mucin of sexual skin must therefore be hyaluronic acid or a substance closely related to it.

Specificity of Testis Hyaluronidase.

As was to be expected the testis hyaluronidase is strictly specific, acting only on hyaluronic acid. Other mucins, e.g. salivary mucin, gastric mucin, duodenal mucin, the substrate for lysozyme, are not acted upon. Salivary mucin was prepared from cow salivary gland by extraction with water; gastric and duodenal mucins were obtained from fistulae of the cat and pig (kindly given to us by Mrs. M. A. Jennings and Prof. H. W. Florey); the substrate for lysozyme was prepared by Mr. L. A. Epstein, in this Department, from *M. lysodeikticus* (Epstein and Chain, 1940).

Kurzrok and Miller (1928) made the observation that semen contained an enzyme capable of dissolving the cervical plug mucin and thus enabling the spermatozoa to penetrate into the uterus. We have tested whether hyaluronidase was identical with this enzyme. 0·2 c.c. of mucin obtained from the uterine cervix of a cow (given to us through the kindness of Dr. Walton of the Institute of Animal Pathology, Cambridge) were incubated with 1 c.c. testicular hyaluronidase (containing 4000 units) at 37° C. for one hour without any effect. Similar results were obtained with specimens of human cervical plug, very kindly collected for us by Prof. J. C. Moir, of the Nuffield Department of Gynaecology and Obstetrics. Kurzrok and Miller themselves have tested extracts from bulls' testicles for the presence of the enzyme, with negative results.

DISCUSSION.

In extensive comparisons between the hyaluronidase activity (determined viscometrically) and spreading activity in the skin of a large number of animal organs, numerous bacterial filtrates, snake venoms, leech extracts and azoproteins, we have noticed in all cases a strict qualitative correlation between the two. There was always spreading in the skin when hyaluronidase was present, and in no case was spreading activity noticed in the absence of hyaluronidase activity.

Quantitatively good agreement was found when spreading activity and hyaluronidase activity of testis extracts of different enzyme strength, leech extracts and bee sting venom were compared. In the cases of some of the bacterial filtrates, especially with *Cl. welchii* and the pneumococcus, and some snake venoms a somewhat bigger spread in the skin was observed than would have been expected from the hyaluronidase activity of the preparations, solutions containing 0·1 unit of hyaluronidase activity giving rise to a spread corresponding to a figure between 0·1 and 0·25 unit when injected into the skin. This moderate inconsistency is easily accounted for by the fact that injection of snake venoms and bacterial filtrates containing toxins was always followed by considerable oedema, due to capillary damage. We have observed that many substances, such as proteolytic enzymes, nucleic acid, arsenious acid, histamine and the permeability increasing polypeptide (Duthie and Chain, 1939), when injected under the skin, produce oedema and some spread, which, however, appears much later than the typical spread caused by testis extracts or bacterial filtrates, and does not show the immediate flattening of the injected bleb, characteristic of solutions containing spreading factor. This non-specific spread may have been confused in the past with the true spreading effect, giving rise to false positive results. This must have been the case with animal organs and Jensen rat sarcoma tissue, which in our experience were found to be entirely devoid of both hyaluronidase and spreading activity.

The supposition that hyaluronidase and spreading factor are identical postulates the existence of a substance, closely related to synovial hyaluronic acid, in the skin of rabbits. The assumption of the existence of such a substance was proved correct by the isolation of a highly viscous substance from the skin of rabbits which is acted upon by hyaluronidase in the same way as hyaluronic acid from vitreous humour and synovial fluid, with an immediate loss of its viscosity and the liberation of N-acetylglucosamine.

A bleb of fluid injected into the skin retains its shape because it is walled off by jelly-like connective-tissue substance, and is submitted to the tensile force of the stretched fibres (Bensley, 1934). We consider that the removal of a highly viscous compound from this tissue must necessarily result in the passage of fluid through the wall of the bleb, bringing about its flattening and the spread of contained fluid through the adjacent tissues. These are two typical manifestations of spreading factor, and we conclude therefore that spreading factor and hyaluronidase are identical.

However, since the spread in the skin caused by hyaluronidase can be influenced by non-specific irritants, it cannot possibly be represented correctly by the viscometric determinations of hyaluronidase alone.

For the understanding of the physiological function of hyaluronidase it seems to us to be important and significant that the enzyme occurs exclusively in testis. It is not identical with the mucolytic enzyme in semen described by Kurzrok and Miller, which dissolves the mucinous plug in the uterine cervix and thus facilitates the penetration of the spermatozoa into the uterus. No trace of hyaluronidase has been found in any other mammalian organ when prepared freshly, and it seems probable that the contrary findings of Meyer *et al.* (1938*b*, 1940*a*), who found hyaluronidase activity in ciliary body and spleen, were due to bacterial contamination.

We would point out that hyaluronidase cannot be regarded as one of the causes either for the rapid growth or for the invasiveness of tumours since the Jensen rat sarcoma, like all other mammalian tissues, is devoid of it.

Skin hyaluronic acid has an important function with regard to permeability of the skin. It is therefore possible that certain states of increased skin permeability, occurring in infectious diseases such as can be demonstrated by the test of Aldrich and McClure (1924), may be due to a decrease of the hyaluronic acid content of the skin. This may be caused either by the action of bacterial hyaluronidases or by a decreased hyaluronic acid synthesis. It seems that the amount of hyaluronic acid present in the skin is subject to the influence of hormones. This is especially pronounced in the sexual skin of monkeys during the oestrous period. In this connection Sprunt *et al.* (1938*a*, *b*) found a decrease of the spread of ink in the skin of rabbits injected with oestrogenic hormone. The thyroid gland may also be of importance for the regulation of the hyaluronic acid content of skin since there seems to be an accumulation of hyaluronic acid in the skin in myxoedema.

SUMMARY.

1. Evidence for the identity of the spreading factor of Duran-Reynals and McClean with the enzyme hyaluronidase is presented. This enzyme acts on the polysaccharide acid hyaluronic acid present in synovial fluid and vitreous humour and responsible for the mucinous character of these liquids. The enzyme hydrolyses hyaluronic acid with the liberation of N-acetylglucosamine and glucuronic acid. The high viscosity of hyaluronic acid is quickly reduced to that of water.

2. A viscometric method for the determination of the enzyme has been worked out. It is accurate to $\pm$ 10 per cent.

3. Hyaluronidase has been found in all sources of spreading factor. No spreading activity was encountered in the absence of hyaluronidase. Testis was the only mammalian organ in which the enzyme occurred.

4. In quantitative comparisons between hyaluronidase activity determined viscometrically and spreading activity in the skin of rabbits, good agreement was obtained with testis hyaluronidase of different strengths, and with leech and bee venom hyaluronidase. With bacterial and snake venom hyaluronidase somewhat bigger spreads were obtained than were expected from their hyaluronidase content. The reason for this is most probably the oedema caused by these fluids.

5. A substance closely resembling hyaluronic acid was isolated from the skin of rabbits. It was acted upon by testis hyaluronidase in the same way as is hyaluronic acid.

6. The mucinous substance which accumulates in the sexual skin of monkeys during the oestrous phase is acted upon by hyaluronidase and is therefore closely related to or identical with hyaluronic acid. Salivary, gastric and duodenal mucin and mucin of the uterine cervix are not attacked by hyaluronidase.

7. Stability and pH activity of hyaluronidase have been investigated.

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