17. STUDIES ON DIFFUSING FACTORS THE HYALURONIDASE ACTIVITY OF TESTICULAR EXTRACTS, BACTERIAL CULTURE FILTRATES AND OTHER AGENTS THAT INCREASE TISSUE PERMEABILITY

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AQUEOUS extracts of mammalian testicle contain a factor that dramatically increases the permeability of the tissues to injected fluids [McClean, 1930; 1931; Hoffman & Duran-Reynals, 1931]. This can be demonstrated by the rapid disappearance of the bleb following intracutaneous injection of these extracts and by the rapid spread through a large area of skin of any suitable coloured indicator that may be injected together with them. This factor is associated with the germinal epithelium and can be extracted from spermatozoa [McClean, 1931]. Factors with similar diffusing properties have been obtained from the most diverse sources, for example, from filtrates and extracts of staphylococcus and streptococcus [Duran-Reynals, 1933], from organisms of the gas-gangrene group and virulent pneumococci [McClean, 1936], from extracts of malignant tissues [Duran-Reynals & Stewart, 1931; Boyland & McClean, 1935], from snake and spider venoms [Duran-Reynals, 1939] and from leeches [Claude, 1937].

Various methods for the partial purification of testicular extracts and of bacterial culture filtrates of the gas-gangrene group have been described [Morgan & McClean, 1932; Favilli, 1936; Claude & Duran-Reynals, 1937; McClean, 1936; Madinaveitia, 1938; 1939, 1, 2; Mannozi-Torini, 1939], but no naturally occurring diffusing factor has been isolated in the pure state. Nobody had succeeded in elucidating the mechanism whereby this diffusion effect is produced until Chain & Duthie [1939] reported that purified preparations of testicular diffusing factor exhibit a remarkable mucolytic activity characterized by a rapid fall in the viscosity of mucoprotein preparations and the liberation therefrom of reducing substances. This observation suggested that the mucolytic and diffusing activities might be due to the same factor and that the spread in the tissues might be due to the action of the enzyme on the mucin-like interfibrillar substance in the collagen of the dermis.

It was obviously important to confirm the observations of Chain & Duthie, to determine whether diffusing factors from other sources exhibit a similar mucolytic activity and to make a comparative study of these enzymes. Madinaveitia & Quibell [1940] have already described a viscosimetric method whereby testicular mucinase may be accurately estimated and they have investigated many of the characters of the reaction. During the progress of our own observations Meyer *et al.* [1940, 1] reported the hydrolysis of hyaluronic acid by bacterial enzymes; it had previously been shown by Meyer & Palmer [1936] that the mucoproteins of vitreous humour and umbilical cord contain a mucopolysaccharide which they had designated by the term hyaluronic acid. More recently

Robertson *et al.* [1940] described an enzyme derived from culture filtrates of *Cl. welchii* and other organisms which hydrolyses synovial fluid; neither these authors nor Meyer and his co-workers had at that time connected the mucolytic activity of their preparations with the action on the permeability of the tissues. Subsequently, however, Meyer & Chaffee [1940] published a preliminary account of the composition of a polysaccharide obtained from the cornea and a comparison of its properties with the polysaccharides obtained from vitreous humour and synovial fluid which they consider to be identical with it and which are hydrolysed by the same enzyme preparations. They suggested that the substrate in the skin upon which the enzyme acts to produce the spreading effect may not be hyaluronic acid itself but its sulphuric acid ester. Meyer *et al.* [1940, 2] have since published a note on the relationship between 'spreading factor' and 'hyaluronidase'. They found that all preparations containing hyaluronidase also exhibit spreading properties and that both factors possess attributes in common. They have succeeded in isolating a polysaccharide from the skin which is hydrolysed by hyaluronidase. On the other hand, these authors state that a number of preparations which possess marked spreading action do not hydrolyse hyaluronic acid or reduce the viscosity of solutions containing the polysaccharide. They also reported that antisera prepared against pneumococcal hyaluronidase, which completely neutralize the hydrolytic activity, fail to inhibit the spreading activity of the pneumococcal preparations. This result is not in agreement with ours obtained with antisera prepared against the purified diffusing and hyaluronidase factors from *Cl. welchii* and *Vibrio septique*; our results will be described in the course of this paper, but it may be suggested here that the failure of Meyer and his co-workers to neutralize the spreading activity might be due to failure to balance the dose of the factor against that of the antiserum; one of us [McClean, 1936] has shown that the most potent antisera then obtainable would only neutralize a limited number of minimal diffusing doses of the spreading factor.

Favilli [1940] reported that several snake venoms which possess high diffusing activity also show a marked mucolytic effect and that this effect is inhibited by appropriate antisera. This author also reported that the diffusing extracts from leeches exhibit mucolytic activity. Claude [1937] had reported the diffusing activity in the skin of leech head extracts and he [1940] confirmed the mucolytic activity of these extracts and noted a parallelism between the mucolytic and spreading powers of various extracts. He also prepared a mucoprotein from normal rabbit skin, the viscosity of which is rapidly reduced by leech extracts, and concluded that the spreading action in the skin was due, at least in part, to the power of these extracts to cause hydrolysis or depolymerization of the same compound *in vivo*. Claude [1935] had previously reported that azoproteins diffused in the skin in a manner similar to, though with less rapidity than testicular extracts; Favilli showed that these diazotized compounds cause a marked fall in the viscosity of mucoprotein preparations, but he was unable to decide whether reducing substances were liberated as a result of this action.

Our own observations have been designed to study the characteristics of the hyaluronidase activities of extracts from testis, from various bacteria and from other sources in order to determine how far the enzymes are identical in their properties. We have also attempted to correlate the hyaluronidase and spreading activities of these preparations in order to determine whether diffusion in the tissues may be due to the action of hyaluronidase. Our observations have led us to investigate the relationship between activities of hyaluronidase and of other

enzymes such as phosphatase and amylase present in the bacterial filtrates. We have also made some preliminary observations on the part played by hyaluronidase in the metabolism of certain bacteria with a view to elucidating the activity of this enzyme in the tissues during infection. Some of the observations to be described in this paper have already been briefly reported in preliminary communications [McClean & Hale, 1940, 1, 2].

Methods

Preparation of substrate. In preliminary experiments vitreous humour was used as substrate, but, in view of its spontaneous loss of viscosity on storage, to which reference will be made later, this substance was replaced by preparations of mucoprotein from umbilical cords. The cords were stored in acetone and minced as required. 100 g. of the dried powder were extracted with 1 l. of distilled water for 24 hr. in the refrigerator and this extraction was repeated once. The fluid was squeezed through twill and both supernatants combined. The aqueous solution was purified by the method described by Meyer & Palmer [1936] which depends upon precipitation with ice-cold alcohol. We avoided the use of glacial acetic acid in this method in order to preserve the viscosity of the mucoprotein; flocculation of the precipitate was accelerated by the addition of a few ml. of saturated alcoholic sodium acetate solution. The final precipitate was washed with acetone and dried over P_2O_5 *in vacuo*. About 3 g. of a white fluffy powder can be obtained from 100 g. of dried umbilical cord. This powder contains about 7% total N and a 0.25% solution of it in water has a relative viscosity of about 400 compared with water as 100. Aqueous solutions were preserved by the addition of a few drops of chloroform and were kept in the refrigerator. A very slow loss of viscosity occurs. This preparation was used for most of our experiments.

For some observations, it was desired to use a purified salt of hyaluronic acid as substrate. For this purpose the preparation described above was further purified by the method of Robertson *et al.* [1940], which depends upon tryptic digestion of the protein, precipitation of this with trichloroacetic acid and final precipitation of the polysaccharide with alcohol as a potassium salt. About 350 mg. of this powder are obtained from 1 g. of the mucoprotein. 0.2% aqueous solutions of the polysaccharide have a relative viscosity of about 350. The powder contains about 3% total N and 0.5–1.0% P.

Preparation and purification of enzymes. Samples of testicular enzyme purified by methods described by Morgan & McClean [1932] and by Madinaveitia [1938; 1939, 1, 2] for the purification of diffusing factor were used. Unpurified bacterial culture filtrates were employed as well as samples from *Cl. welchii* filtrates purified by the method described by McClean [1936], which depends upon precipitation of the active fraction with basic lead acetate. Filtrates from *Cl. oedematis maligni* (*Vibrio septique*) were purified with alumina C_7 by the method used by Madinaveitia [1939, 2] for *Cl. welchii* filtrates and testicular extracts. In our experience it is impossible to purify *Vibrio septique* filtrates by the lead precipitation method successfully employed with *Cl. welchii*. Our observations with Russell's viper venom were made with the dried venom in the form of 'Stypven' which was kindly supplied by Dr Parish of the Wellcome Physiological Research Laboratories.

Viscosimetric technique. Madinaveitia & Quibell [1940] have shown that, under certain defined experimental conditions, the time taken by any sample of enzyme to reduce the viscosity of the substrate to a level half-way between its original level and that of the solvent is inversely proportional to the concentration of the enzyme. They have also shown that the reaction time, that is the time taken to reach half viscosity level, is independent of the concentration of the substrate and of its original viscosity. Both these facts have been confirmed by us and, for the purpose of this work, we have provisionally defined a viscosity-reducing unit (v.r.u.) as that concentration of enzyme which will reduce the viscosity of a standard substrate preparation to a level half-way between its original figure and that of the solvent employed in 20 min. The selection of this period of time is determined by experimental conditions that will be described. Madinaveitia & Quibell employed a final concentration of 0.66 M NaCl in their system. It appeared that the $M/6$ citrate buffer supplied sufficient electrolyte and, since we did not wish to work in a salt concentration so widely different from that present under biological conditions, we omitted the addition of NaCl in

our earlier experiments. Later, for reasons that will appear in the text, we have added NaCl to a final concentration of 0.1 *M*.

Four vol. of 0.25 % mucoprotein or 0.2 % potassium hyaluronate in distilled water are mixed with 1 vol. of *M* sodium citrate buffer at pH 4.6. The buffer is prepared by mixing 1 vol. of *M* citric acid with 2 vol. of *M* NaOH. The mixture of substrate and buffer is placed in the water bath at 34°. 0.5 ml. of the appropriate dilution of the enzyme is also placed in the water bath in a separate tube. A similar dilution of the enzyme previously heated for 30 min. in boiling water serves as control. Two Ostwald viscosimeters with the same or closely similar flow times are placed in the bath; these and the sample of enzyme and substrate are left to warm for at least 10 min. After this interval 2.5 ml. of the buffer substrate mixture are run into each of the tubes containing heated and unheated enzyme. A stopwatch which is started at this moment will record the reaction time (*R*). The substrate, buffer and enzyme mixtures are mixed with warm pipettes and 1.5 ml. of each are run into the viscosimeters so that one viscosimeter contains the test mixture and the other the

Fig. 1. Typical curve of enzyme containing one viscosity-reducing unit.

control. The fluid is blown (not sucked) into the bulb of the viscosimeter in order to avoid the formation of bubbles. The first reading is taken on another stopwatch 1 min. after mixing and thereafter at frequent intervals until the flow time of the unheated enzyme system is less than half the sum of that of the heated enzyme system and that of the solvent employed. The flow time readings on either side of the half viscosity level are plotted against time and the point at which the line crosses the half viscosity level is taken as the reaction time of the sample of enzyme under test. A correction is made for the fall in viscosity that occurs during a reading of the flow time; half the flow time is added to the time at the beginning of the run of the fluid through the viscosimeter. In order to facilitate calculation of relative viscosities, half viscosity levels and enzyme strength, all the flow times are considered to be relative to a flow time of 100 for *M*/6 sodium citrate buffer. Thus

$$\frac{\text{Flow time of substrate}}{\text{Flow time of buffer}} \times \frac{100}{1} = \text{relative viscosity.}$$

Viscosimeters were closely matched and there were not more than 1 or 2 sec. difference in the time of flow of the buffer solution. The viscosimeters used gave a flow time of about 50 sec. for 1.5 ml. of this fluid. In order to compare the potencies of different enzyme samples accurately it is necessary to use dilutions which give reaction times falling between 15 and 45 min. If the experimental conditions described are carefully observed assays of the viscosity-reducing activity of enzymes can be made with an error of $\pm 10\%$. A typical calculation of the activity of a sample of enzyme is as follows:

Reaction time to half viscosity level (R) = 30 min.

Amount of dilution of enzyme sample (D) = 60 (dilution of 1 : 60).

$$\begin{aligned}\text{Then viscosity-reducing units (already defined)} &= \frac{D \times 20}{R} \\ &= \frac{60 \times 20}{30} \\ &= 40.\end{aligned}$$

A typical viscosity reduction curve for a sample containing 1 unit of activity is shown in Fig. 1.

Estimation of reducing substances. *N*-Acetylhexosamine was estimated by the method of Morgan & Elson [1934]. The colour produced in the test solutions was matched against standard solutions in an optical colorimeter. The method gave most accurate results when the concentrations of acetylhexosamine were between 20 and 200 $\mu\text{g./ml.}$ Total reducing substances were estimated by the method of Hagedorn & Jensen.

Diffusion in the dermis. Three methods of assaying diffusing activity in the skin are available. The original method described by one of us [McClean, 1930; 1931] depends upon the determination of the highest dilution of a sample that will produce a significant (20%) increase in the area of spread in the skin. Madinaveitia [1938] has described a method which depends upon the comparison of the rate of spread of the sample under test with that of a standard preparation of diffusing factor. He did not claim that it was possible to differentiate between less than tenfold dilutions of the diffusing factor by this method. Bacharach *et al.* [1940] have recently described a method for estimating testicular diffusing factor depending upon a comparison of the areas of spread after 20 min. of two dilutions of a sample solution with those of two dilutions of a standard preparation. The injections are so arranged on the shaved backs of four rabbits as to allow for corrections for variation in animal susceptibility to the diffusing factor. These authors claim to be able to distinguish between doses in the ratio of 2 to 1. An investigation is being made into the correspondence between determinations of diffusing activity made by the method of Bacharach *et al.*, and by the original method of determining the minimal diffusing dose; observations are also in hand to determine whether both methods are equally applicable to testicular, bacterial and other diffusing substances. Pending the result of this investigation it has seemed advisable to adhere to assay by the determination of the minimal diffusing dose for the purpose of the work to be reported in this paper. Notwithstanding the fact that we cannot claim to be able to distinguish between less than tenfold dilutions, we have noticed that the dose response curve does become steeper as the limiting dilution of the diffusing factor is being reached. There is therefore less liability to confusion between the areas of spread of two succeeding dilutions than when more concentrated solutions are employed. The unsatisfactory nature of this animal test from a quantitative point of view must be admitted, but it does suffice for the broad comparison of diffusing and hyaluronidase activities attempted in this work.

Tenfold dilutions of the sample to be tested were made in isotonic haemoglobin solution. 0.4 ml. samples of these dilutions were injected into the backs of clipped or depilated white-skinned rabbits. Two control injections of 0.4 ml. of haemoglobin solution alone were made into each rabbit. The length and breadth of the areas stained with haemoglobin were measured 20 min. after injection and the areas calculated on the assumption that they were ellipses according to the formula $\pi Dd/4$ when D = the long axis and d = the short one. The assumption that the area is elliptical is valid for the higher dilutions of the spreading factor only. Estimation of the area of spread of haemoglobin is facilitated if the animal's back is smeared with liquid paraffin before the measurements are made. Our results were expressed as percentage increase in area compared with that of the control injections of haemoglobin solution alone.

EXPERIMENTAL

Association between the hyaluronidase activities of various extracts and their diffusion in the dermis

The mucolytic activity of testis extract reported by Chain & Duthie was confirmed with mucoprotein from either vitreous humour or umbilical cord as substrate. Preparations of this diffusing factor purified either by the method of Morgan & McClean or by those of Madinaveitia showed similar activities in the reduction of the viscosity of the substrate and the liberation of reducing substances. Our preliminary experiments were designed to demonstrate a qualitative association between the diffusing and hyaluronidase activities of enzymes from various naturally occurring sources; no quantitative comparisons of the two activities were made at this stage.

Purified preparations of the diffusing factor from filtrates of *Cl. welchii* and *Cl. oedematis maligni* (*Vibrio septique*), which in high dilution cause rapid diffusion in the skin, also show marked hyaluronidase activity as demonstrated by viscosity reduction and the liberation of *N*-acetylhexosamine. These organisms are both members of the group of gas-gangrene-producing anaerobic organisms. Filtrates from certain strains of staphylococcus, streptococcus and from a virulent type 1 pneumococcus are known to diffuse actively in the skin; as will be seen in Table 1, these filtrates also showed marked hyaluronidase activity. On the other hand filtrates from a virulent strain of *S. typhi*, from *Cl. tetani* and from *C. diphtheriae* which produce no diffusion also contain no hyaluronidase.

Table 1. *The qualitative association between diffusion in the dermis and hyaluronidase activity*

Source of enzyme	Diffusion in 20 min.		Hyaluronidase units Undiluted sample	Liberation of <i>N</i> -acetyl- hexosamine
	Dose ml.	Increase in area %		
Testis extract (bulls)	4×10^{-5}	84	90	+
Russell's viper-venom (‘Stypven’)	0.016	38	0.5	+
<i>Cl. welchii</i> filtrate (strain 107)	4×10^{-6}	35	70	+
<i>Cl. oedematis maligni</i> filtrate (<i>V. septique</i> KF 5)	4×10^{-5}	82	22	+
Staphylococcus filtrate (strain Wood 46)	4×10^{-2}	150	60	+
Pneumococcus filtrate (type 1 virulent)	4×10^{-2}	115	6	+
Streptococcus filtrate (5 strains)	4×10^{-2}	50–100	0.3	+
Streptococcus filtrate (3 strains)	4×10^{-2}	Nil	Nil	–
<i>C. diphtheriae</i> filtrate (strain 110)	0.2	Nil	Nil	–
<i>Cl. tetani</i> filtrate	0.2	Nil	Nil	–
<i>S. typhi</i> filtrate	0.2	Nil	Nil	–

Note. *Diffusion tests*: 0.4 ml. of a dilution of the sample to be tested was injected intracutaneously. The area of spread of these single injections is not an accurate expression of the relative diffusing activities of the different samples and no quantitative correlation between diffusing and hyaluronidase activities was attempted in this experiment.

The activity of the filtrate from virulent type 1 pneumococci is of interest, since one of us [McClean, 1936] has reported that the same strain rendered avirulent by cultivation in type-specific immune serum loses its diffusing power; on the other hand Meyer *et al.* obtained their hyaluronidase from the autolysate of a 'non-encapsulated non-type-specific variant' of a type 2 strain and drew a distinction between the enzyme obtained from pneumococci which is bound to the cell structure and that from *Cl. welchii* which is free in the fluid medium. It is clear from our observation that encapsulated virulent pneumococci excrete the enzyme into the medium in a manner similar to that of members of the gas-gangrene group.

It has already been mentioned in the introduction that several snake and spider venoms diffuse in the skin and that Favilli had reported that such snake venoms also showed a marked mucolytic activity. Through the courtesy of Dr Parish of the Wellcome Physiological Research Laboratories we were able to examine these properties in dried Russell's viper venom ('Stypven'). The results are summarized in Table 1. It will be seen that there is again an obvious association between diffusing and hyaluronidase activities. Further observations on the activity of this venom will be described later when the general properties of these enzymes are considered.

Certain artificially prepared compounds such as azoproteins and ascorbic acid and its oxidation products were found to diffuse in the skin and to reduce the viscosity of mucoprotein. Since, however, the characters of the diffusion and of the action on mucoprotein differ in many respects from those of the naturally occurring enzymes consideration of the behaviour of these substances will be postponed until that of the enzymes has been described.

Characters of the hyaluronidase activity of enzymes from various sources

Influence of pH. We have confirmed the result reported by Madinaveitia & Quibell that the action of the testicular enzyme on the viscosity of the substrate is greatest in the region of pH 4.6. The rate of the reaction decreases sharply on the acid side of this and more gradually as the pH approaches neutrality. Similar observations on enzymes obtained from culture filtrates of *Cl. welchii*, *Vibrio septique* and from Russell's viper venom indicate that they are all most active in the region of pH 4.6. All these enzymes show the same sharp decline in activity on the acid side of this pH, but whereas the enzyme from viper venom is completely inactive on the alkaline side of pH 6.5 those from *Cl. welchii* and *Vibrio septique* still cause a slow fall of viscosity at pH 8.5.

Liberation of N-acetylhexosamine by these enzymes. Preliminary observations on the rate of liberation of N-acetylhexosamine from mucoprotein by these enzymes indicated that, whereas in the presence of a sufficient concentration of enzyme the fall in viscosity may be so rapid that half viscosity level is reached in a few minutes, no acetylhexosamine can be detected for some hours. An experiment was performed in which purified potassium hyaluronate was exposed to the action of the purified *welchii* enzyme with the result shown in Table 2. It will be seen that although half viscosity level was reached in between 1 and 10 min. no measurable amount of acetylhexosamine could be detected for 50 min.; the maximum liberation was not attained until considerably after 2 hr. had elapsed. Similar results were obtained with the testicular enzyme.

This result indicated that two separate mechanisms might be involved and, furthermore, our early experiments suggested that enzymes from different sources might differ qualitatively as well as quantitatively in their capacities to liberate reducing substances. Thus it appeared that the bacterial enzyme

Table 2. *Time relation of viscosity reduction and liberation of N-acetylhexosamine*

Substrate: Potassium hyaluronate 2 mg./ml.

Enzyme: Purified from *Cl. welchii* filtrate.

Time		Relative viscosity	N-acetylhexosamine
Hr.	Min.		
0	0	350	0
0	1	250	0
.....			
0	10	162	0
0	20	138	Trace
0	30	127	Trace
0	50	120	<0.1 mg./ml.
1	10	115	<0.1 "
2	0	108	0.1 "
18	0	102	1.0 "

Viscosity of buffer solution + enzyme = 100. The dotted line indicates the half viscosity level and the approximate time taken to reach it.

liberated more reducing substances than that from testis and that no reducing substance could be detected after the action of viper venom. When, however, the concentrations of the enzymes from different sources were adjusted so that their reaction times for the reduction of viscosity were comparable, it was found that the amount of enzyme necessary to liberate measurable quantities of reducing substances was approximately the same whatever the source of the enzyme. The result of this experiment is shown in Table 3.

Table 3. *Relation between N-acetylhexosamine liberation and viscosity-reducing activity of enzymes from various sources*

Substrate: Potassium hyaluronate 1.5 mg./ml.

Source of enzyme	Dilution containing 1 viscosity-reducing unit (v.r.u.)	Amount of N-acetylhexosamine (mg./ml.) liberated in 18 hr. by		
		60 v.r.u.	6 v.r.u.	3 v.r.u.
<i>Cl. welchii</i> (strain 107)	1:66	1.0	0.1	Trace
<i>Staphylococcus</i> (Wood 46)	1:60	0.8	0.1	Trace
<i>Pneumococcus</i> (type 1 virulent)	1:6.6	—	Trace	Trace
Russell's viper venom ('Stypven' 2 mg./ml.)	1:10	—	0.1	0.1
Testis (Madinaveitia standard prep. 10 mg./ml.)	1:5	—	0.4	0.2

Later observations indicated that there is not complete correlation between viscosity reduction and liberation of reducing substances by different samples of enzyme from the same source. Thus, in the experiment illustrated in Table 3, the testicular enzyme liberated at least four times as much acetylhexosamine as comparable concentrations of enzymes derived from bacteria and venom; in another experiment, with a sample of testicular enzyme purified by a different method, it was found to liberate less than half the amount of acetylhexosamine liberated by a *welchii* enzyme of the same viscosity-reducing activity. The time lag in the liberation of reducing substances, coupled with the lack of complete correlation between this activity and the reduction of viscosity, suggest that two separate mechanisms may be involved; if the enzymes are not identical, they

must be very closely associated since they are not separated by any existing method of purification and, as will appear, they are similarly influenced by variations in the environment of the reaction.

Neutralization of enzyme activity by antisera. One of us [McClean, 1936] has already reported that antisera can be prepared which inhibit the spread of diffusing factors of bacterial origin in the dermis; it was also reported that the animals actively immunized against the purified diffusing factor in order to produce these antisera were themselves resistant to the diffusing activity of the homologous factor. Experiments were made to determine whether these sera

Fig. 2. The neutralization of hyaluronidases by anti-diffusing sera.

Table 4. *The influence of anti-diffusing sera on the liberation of N-acetylhexosamine*

Substrate: Vitreous humour.

Enzyme: Prepared from culture filtrate of *Cl. oedematis maligni* (*Vibrio septique*).

Serum	Liberation of <i>N</i> -acetylhexosamine (mg./ml.) in 18 hr. by falling dilutions of enzyme from <i>Vibrio septique</i>		
	Undiluted	1 : 10	1 : 100
<i>Vibrio septique</i> anti-diffusing serum	Nil	Nil	Nil
<i>Cl. welchii</i> anti-diffusing serum	1.0	0.1	Trace
Normal rabbit serum	1.0	0.1	Trace
Saline	1.0	0.1	Trace
Heated enzyme + saline + vitreous humour	Nil	—	—

inhibited the viscosity-reducing activity of the bacterial enzymes and the liberation of reducing substances by them. The influence on viscosity reduction is shown in Fig. 2 and on liberation of reducing substances in Table 4. Similar experiments on the inhibition of the activity of the testicular enzyme could not be undertaken since the purified enzyme from this source has never been obtained in an antigenic form.

Equal volumes of undiluted serum and the enzyme preparations were mixed and left at room temperature for 30 min. before addition to the substrate. In the observation on viscosity-reducing activity both *Cl. welchii* and *Vibrion septique* enzymes were set up with the homologous and heterologous antisera using a substrate of mucoprotein derived from umbilical cord. In the observation on the liberation of acetylhexosamine the enzyme from *Vibrion septique* was the only one used; it was set up against the homologous antiserum, against a *welchii* antiserum, normal rabbit serum and saline. The amount of acetylhexosamine liberated was estimated after 18 hr. incubation at 37°. It will be seen that in both experiments the inhibition of enzyme activity was complete and strictly specific; the heterologous antisera and normal serum exerted no effect. The diffusing activity of these enzymes in the skin occurs at a much higher dilution than that at which any *in vitro* viscosity-reducing activity can be demonstrated. It has already been shown [McClean, 1936] that these antisera can only completely inhibit a limited number of minimal diffusing doses of diffusing factor in the skin and this probably explains the failure of Meyer *et al.* [1940, 2] to demonstrate inhibition of diffusion with pneumococcal antiserum.

Influence of electrolytes on the reaction. Madinaveitia & Quibell [1940] employed a final concentration of 0.66 *M* NaCl and 0.166 *M* sodium citrate in their viscosimetric tests. For reasons given in the section describing our methods, we omitted the NaCl from our system, but retained the 0.166 *M* sodium citrate buffer. A comparison of estimations of the activities of several samples of both testicular and bacterial enzymes revealed a gross disparity between us and led to the investigation of the influence of electrolytes on this reaction.

The apparent viscosity-reducing activities of a purified testicular enzyme and of purified enzymes derived from *Cl. welchii* and *Vibrion septique* were compared at concentrations of NaCl varying from nil up to *M*. The concentration of sodium citrate at 0.166 *M* was kept constant throughout the experiment. The influence of varying concentration on the initial viscosity of the substrate was controlled in all titrations of enzyme activity. The result of this experiment is shown in Fig. 3 in which the apparent strength of the enzyme is expressed in viscosity-reducing units plotted against NaCl concentration.

It will be seen that there is a marked retarding effect on enzyme activity as the salt concentration is increased above 0.3 *M*, but that below this level there is an apparent difference between the behaviours of testicular and bacterial enzymes; the testicular enzyme shows a rapid apparent fall in activity as the salt concentration is reduced to nil, whereas the activity of the bacterial enzymes appears to be maintained at a high level. Madinaveitia (personal communication) has shown that in salt concentrations below 0.1 *M* the direct relationship between enzyme concentration and reaction time on viscosity breaks down and that this lack of correlation is more marked for weak enzyme preparations. Since the method of estimating enzyme activity depends upon the employment of a dilution of the enzyme which will give a reaction time of 15–45 min. it is possible that considerable errors may develop in the assay of activity at very low salt concentrations. Thus it appeared that the difference between the behaviours of testicular and bacterial enzymes might be more apparent than real. Further-

Fig. 3. The influence of NaCl concentration on the viscosity-reducing activity of testicular and bacterial enzymes.

Fig. 4. The influence of NaCl concentration on the rate of liberation of N-acetylhexosamine by testicular and bacterial enzymes.

more, the purified bacterial enzymes were derived from culture filtrates and might contain small amounts of salts or other impurities sufficient to maintain a high level of activity. It should be stated, however, that both the testicular and bacterial enzymes had been exposed to prolonged dialysis during purification, were of considerable potency and were therefore used in dilutions which should have sufficed to eliminate the influence of impurities.

In order to obtain confirmatory evidence of the influence of salt concentration on the rate of the reaction, an experiment was performed to compare the rates of liberation of acetylhexosamine by testicular and *welchii* enzymes in the presence of NaCl concentrations ranging from nil to 0.5 *M*. The amount of acetylhexosamine was estimated after 2, 4 and 20 hr. incubation at 37°. The result is shown in Fig. 4.

In this experiment ninety viscosity-reducing units of each enzyme were used; the enzymes were therefore much more concentrated than in the foregoing experiment and there is no question of direct proportionality between reaction time and enzyme concentration. It will be seen that after 2 and 4 hr. the maximum liberation of acetylhexosamine by the *welchii* enzyme has occurred at the lowest NaCl concentrations and that there is a steady fall as the salt is increased. The testicular enzyme, however, shows an increase in activity up to 0.2 or 0.3 *M* NaCl, similar to that which appears in the experiments on viscosity reduction; there is a fall in the yield as the salt concentration is further increased. There is no significant difference in the amount of acetylhexosamine liberated by either enzyme at any salt concentration after 24 hr.; the reaction is then presumably complete. Both activities appear to be similarly affected by variations in salt concentration and the difference between the viscosity-reducing activities of testicular and bacterial enzymes is confirmed in respect of the liberation of acetylhexosamine. These experiments show that the potency assigned to enzyme preparations will be profoundly affected by the NaCl concentration of the system and this has some bearing on the correlation between diffusing activity in the skin, which presumably occurs in isotonic salt, and *in vitro* hydrolysis of the substrate; this point will be considered later.

The part played by the different ions in the system was investigated. In order to determine the part played by the Na ion the concentration of sodium citrate at pH 4.6 was increased by amounts calculated to give a weight of Na equivalent to the various amounts of Na present as NaCl plus the constant amount of sodium citrate employed in the previous test. The activities of both testicular and bacterial enzymes at various sodium levels were estimated viscosimetrically in the normal way, allowance being made for the influences of sodium citrate concentrations on the initial viscosity of the system. The results showed that increase in Na concentration exaggerated the depressing effect on enzyme activity and almost eliminated the initial rise in the apparent activity of testicular enzyme which occurs in the presence of small concentrations of NaCl.

In order to vary the concentrations of the Cl ion in the presence of a constant amount of Na as citrate an experiment was set up in which the usual concentration of *M*/6 buffer was used in the presence of increasing concentrations of NH_4Cl . The result with both enzymes was in every way comparable with the action of Cl as NaCl.

A further experiment was performed on the influence of increasing concentrations of Na_2SO_4 in the presence of the constant amount of citrate buffer. The result obtained was the same as that with increasing amounts of sodium citrate.

These observations indicate that the accelerating action on both enzymes at low salt concentration is due to the Cl ion and that the Na ion exerts a depressing

effect at similar concentrations. As the amount of salt is increased beyond a certain point both ions exert depressant influences. A preliminary account of these investigations together with those made by Madinaveitia has been published [Madinaveitia & McClean, 1940], and he has shown that the intensity of the apparent accelerating effect is in the order $\text{Cl} > \text{Br} > \text{I} > \text{SO}_4$ and the retarding effect at higher concentrations the opposite.

Relation of viscosity to concentration of substrate and the influence on enzymic activity. Madinaveitia & Quibell [1940] reported that the reaction constant and the half-life time of the viscous substrate are constant for a fixed amount of enzyme regardless of the concentration of the substrate. Robertson *et al.* [1940], on the other hand, found that the time taken for a fixed amount of enzyme to break down increasing amounts of substrate was proportional to the concentration of the latter. We have found that the reaction time of the enzyme is constant in concentrations of substrate varying from 0.66 to 6.6 mg./ml., thus confirming the findings of Madinaveitia and Quibell over a much greater range of viscous substrate than that of their experiment. In order to clear up this discrepancy we were led to investigate the relation of the viscosity to concentration of the substrate and the influence of NaCl on this relationship; we also wished to find out whether it is justifiable to use viscosity as a direct measurement of the concentration of mucous material or to assume that the half-viscosity level is that at which half of the substrate has been rendered non-viscous.

The viscosity of mucoprotein at concentrations increasing from 0.66 to 6.6 mg./ml., in the whole system as set up for enzyme titrations, was determined both in the absence of salt and in the presence of 0.66 *M* NaCl. The results are shown in Fig. 5.

It will be seen that the viscosity increases at a much greater rate than the concentration of the substrate and that this effect is more marked as the concentration increases. Therefore the reduction of viscosity by half does not indicate that half the substrate has been rendered non-viscous. If we assume that when half-viscosity level is reached the substrate consists of a mixture of completely viscous and completely non-viscous material, the following conclusions can be drawn from these curves: as the concentration of the substrate is raised a gradually increasing amount but decreasing proportion of the total weight of substrate is rendered non-viscous when half-viscosity level is reached. Whether NaCl is present or not, the proportion of substrate rendered non-viscous at this level is approximately the same; therefore we think it is justifiable to assume that the half-viscosity level at different NaCl concentrations does represent a comparable decrease in the concentration of viscous material, but it is not true to say that the percentage decrease in the concentration is independent of the original concentration of mucoprotein. As already confirmed by us, however, it is an experimental fact that the reaction time of a sample of enzyme remains constant and is completely independent of the concentration of the substrate. Since the proportion of substrate destroyed by the enzyme is not constant in different concentrations of substrate this raises interesting questions as to how the constant reaction time is attained. It may be that a balance is established between increased rapidity in the formation of enzyme-substrate complex as the concentration is raised and slowing of molecular movement due to the increased viscosity of the system.

These observations on the relation between viscosity and concentration of substrate provide a possible explanation for the apparent discrepancy between Madinaveitia & Quibell's findings that the reaction time is constant regardless of the concentration of substrate and the report by Robertson *et al.*, that the rate of

reaction is proportional to the concentration. The latter workers took their reactions to an end-point when either the viscosity was completely destroyed or the precipitability of the mucoprotein was abolished. From the curves in Fig. 5 it can be deduced that if the original substrate contains 6.6 mg./ml. then, when half-viscosity level is reached, only 1.45 mg./ml. will have been destroyed and 5.15 mg./ml. will remain. If, however, the original substrate only contains

Fig. 5. The relation of viscosity to concentration of mucoprotein.

0.66 mg./ml., then at half-viscosity level 0.30 mg./ml. will have been destroyed and only 0.32 mg./ml. remain. Therefore the latter reaction, at the lower initial concentration, will probably be carried to completion more quickly, although the time taken to reach half-viscosity level is the same with both samples of substrate.

*Relation of hyaluronidase activity to amylase and phosphatase,
and to the action on other polysaccharides*

Robertson *et al.* [1940] stated that mucinase causes a loss of viscosity of starch paste and that in some respects mucolytic and amylolytic activities are similar. They also stated that the first stage in the breakdown of mucin is catalysed by serum phosphatase. It seemed desirable, therefore, to investigate the amylolytic and phosphatase activities of our testicular and bacterial hyaluronidase preparations in order to determine whether there is any correlation between these activities.

Pastes were prepared from rice, maize, potato and wheat starches according to the method described by Waldschmidt-Leitz & Mayer [1935] except that the concentration of starch employed was slightly less than half that used by these authors. In the experiments with testicular and bacterial enzymes the proportions of enzyme and of sodium citrate buffer to the starch substrate were the same as those used in the determination of hyaluronidase activity with mucoprotein substrate.

Preliminary observations indicated that testicular enzyme did not reduce the viscosity of any of these starch pastes and that purified enzymes derived from *Cl. welchii* and *Vibrio septique* reduced the viscosity of both potato and wheat starch, but that the rate of the reaction bore no relation to their hyaluronidase activities.

An experiment, the results of which are shown in Table 5, was performed to compare the amylase and hyaluronidase activities of several bacterial and testicular preparations and to determine whether an increase in NaCl concentration exerts an effect on the amylase activity similar to that on the hyaluronidase.

Table 5. *A comparison of the amylase and hyaluronidase activities of various enzyme preparations*

Source of enzyme	NaCl concentration	Amylase activity. Viscosity reduction	Hyaluronidase activity. Viscosity-reducing units
<i>V. septique</i> A	Nil	30 min. to reach H.V.L.	13
" B	Nil	35 min. to reach H.V.L.	3.5
" B	0.1 M	2 hr. 14 min. to reach H.V.L.	—
" B	0.2 M	2 hr. 50 min. to reach H.V.L.	—
" C	Nil	No activity	10
<i>Cl. welchii</i> A	Nil	392-334 in 1 hr. 14 min. (H.V.L. = 246)	55
" B	Nil	354-340 in 1 hr. 35 min. (H.V.L. = 227)	120
		H.V.L. not reached in 21 hr.	
Testis	Nil	No activity	2.5
"	0.3 M	No activity	91

H.V.L. = Half-viscosity level.

Viscosity figures refer to relative viscosity. Buffer + heated enzyme = 100.

It will be seen that there is no correlation between the hyaluronidase and amylase activities of these preparations and that the addition of NaCl up to a final concentration of 0.3 M does not produce any amylolytic activity in the testicular enzyme, in striking contrast to its marked enhancing action on the hyaluronidase activity of the same specimen. The addition of NaCl produces a retarding effect on amylolytic activity of the *Vibrio septique* preparation similar to that produced on the hyaluronidase activity of bacterial enzymes.

When the starch pastes used were hydrolysed with $N/10$ H_2SO_4 a positive Benedict reaction was produced after boiling for 15 min. No Benedict reaction could be obtained even after 24 hr. incubation with the bacterial enzymes notwithstanding the substantial fall in viscosity that had occurred; the iodine reaction also remained positive. Takadiastase, which contains no hyaluronidase and does not diffuse in the skin, causes a rapid fall in the viscosity of starch paste with the liberation of reducing sugars.

We are indebted to Dr King of the Post-Graduate Medical School, London, for the phosphatase estimations on samples of hyaluronidase from various sources. The method employed has been described by King [1939]; disodium phenyl phosphate was employed as substrate for the phosphatase estimations and diphenyl phosphate for the estimations of phosphodiesterase.

Table 6. *A comparison between the hyaluronidase, phosphatase and phosphodiesterase activities of various enzyme preparations*

Source of enzyme	Hyaluronidase activity. Viscosity-reducing units	Primary phosphatase units		Phosphodiesterase units	
		pH 4.5	pH 10	pH 4.5	pH 10
Testis	10	3.0	1.8	Nil	3
<i>Cl. welchii</i> A	118	4.2	Nil	Nil	1
" B	54	2.1	—	Nil	—
<i>V. septique</i> A	8	—	Nil	—	—
" B	20	3.4	—	Nil	—
Viper venom A	10	—	6.4	—	Nil
" B	3	2.1	—	Nil	—
<i>S. typhi</i>	Nil	25	Nil	Nil	Nil
Takadiastase (1%)	Nil	68	—	Nil	—

It will be seen that two samples of enzyme from *Cl. welchii* purified by different methods and of widely different hyaluronidase activities are included in the list and that samples of viper venom were tested in addition to the testicular and bacterial enzymes. A filtrate from a culture of *S. typhi* was included as an example of a bacterial filtrate containing no hyaluronidase and a solution of takadiastase as a control substance known to contain a phosphatase active at pH 4.6. These estimations indicate that, when the above-mentioned phosphoric esters are employed, there is no evidence of any correlation between the phosphatase or phosphodiesterase and the hyaluronidase contents of any of these samples; the phosphatase activities of all the samples were very slight. It appears that amylase and phosphatase play no part in the hyaluronidase activity of any of these enzyme preparations.

Neither the testicular nor bacterial enzymes exerted any action on the viscosity of gum acacia or that of the bacterial polysaccharide isolated from *Bact. dysenteriae* (Shiga) by Morgan & Partridge [1940].

Robertson *et al.* [1940] state that mucinase derived from *Cl. welchii* does not affect gastric mucin (human or swine), but Meyer *et al.* [1940, 1] report that whereas the enzymes from pneumococcus and streptococcus have no action on the neutral polysaccharide or mucoitin-sulphuric acid from pig gastric mucosa, the enzyme from *Cl. welchii* does hydrolyse these substrates. We tested the viscosity-reducing activity of hyaluronidase from testis, streptococci, *Vibrio septique* and *Cl. welchii* on a 1% solution of commercial gastric mucin (granular mucin, type 1701 W. Wilson Laboratories), with the results presented in Table 7. In addition we performed an experiment similar to that to be described in the section on the part played by hyaluronidase in bacterial metabolism; *Cl. welchii* was cultivated in a basal medium alone and with the addition of glucose, potassium hyaluronate or gastric mucin. As will be seen in the second part of Table 7, the addition of glucose causes a slight increase and the addition of hyaluronate a considerable increase in the production of hyaluronidase. The inclusion of gastric mucin results in the production of less hyaluronidase than in the culture containing glucose, but the action of this filtrate on gastric mucin shows considerable enhancement.

Our results show no correlation between hyaluronidase activity and the action on gastric mucin. Whereas testicular and streptococcal enzymes of considerable hyaluronidase potency have no action on gastric mucin, a weak hyaluronidase from *Vibrio septique* caused a slight fall in the viscosity of gastric

Table 7. *The relationship between hyaluronidase and an enzyme which hydrolyses mucin from pig gastric mucosa*

Source of enzymes	Hyaluronidase. Viscosity- reducing units. pH 4.6	Fall in viscosity of gastric mucin in 2 hr. (undiluted sample of enzyme). pH 7.0
I. Testis extract (no NaCl)	2.5	No activity
Testis extract (in 0.3 M NaCl)	90	No activity
Streptococcus filtrate	330	No activity
<i>Vibrio septique</i> filtrate	10	292-272 (7%)
II. <i>Cl. welchii</i> filtrate. Medium:		
A. 3.5% Evans peptone alone	15	268-256 (7%)
B. 3.5% Evans peptone + 0.5% glucose	180	268-237 (18%)
C. 3.5% Evans peptone + 0.5% potassium hyaluronate	590	268-246 (13%)
D. 3.5% Evans peptone + 0.5% gastric mucin	77	268-213 (33%)

Note. No action on gastric mucin by any of these enzymes could be detected at pH 4.6.

mucin. *Cl. welchii* enzymes of widely different hyaluronidase activities possessed slight actions on gastric mucin which were not proportional to their activities on umbilical mucoprotein. The action of *Cl. welchii* on gastric mucin is, however, exalted by cultivation in the presence of this substance. Culture filtrates from *Vibrio septique* and *Cl. welchii* both liberate reducing substances from gastric mucin.

Relation of hyaluronidase in viper venom to its coagulant action on blood

Russell's viper venom which diffuses in the dermis also causes a rapid fall in the viscosity of mucoprotein with the liberation of reducing substances. This venom promotes the coagulation of haemophilic blood, but its mode of action has not been completely explained. Since the chemical constitution of heparin is closely allied to that of hyaluronic acid, it was thought that the hyaluronidase in the venom might destroy the heparin present in the circulating blood and thus accelerate coagulation. It was, therefore, of interest to determine whether this venom and the hyaluronidase from testis and *Cl. welchii* exert any destructive action on heparin and whether they accelerate the coagulation of blood.

Several experiments were performed in which plasma, to which sufficient heparin had been added to delay coagulation at 37° for 1-2 hr., was mixed with varying amounts of viper venom and with testicular and bacterial enzyme preparations. These observations were made with heparinized plasma alone and in the presence of 0.1% lecithin which itself reduces the anticoagulant action of heparin. Control tests were made with the addition of varying amounts of brain pulp as a source of thrombokinase to the heparinized plasma; this pulp induced rapid coagulation. No evidence was obtained from many experiments that viper venom, testicular or *Cl. welchii* enzymes reduce the anticoagulant action of heparin or that they act in a manner comparable with thrombokinase.

A further experiment was performed to determine whether viper venom, testicular or bacterial enzymes exert any direct destructive action on heparin in the absence of plasma. Heated and unheated samples of these enzymes were mixed with constant volumes of a heparin solution of known strength and the mixtures were incubated for 18 hr. at 37°. After incubation the enzymes were inactivated by boiling for 30 min. and, after cooling, a constant volume of blood was added to each tube. There was no reduction of clotting time in those tubes in

which the heparin had been exposed to the various enzyme preparations; it can therefore be concluded that the heparin molecule was still intact and these observations confirm and extend the statement by Meyer *et al.* [1940, 1] that the enzymes obtained by them from pneumococci and *Cl. welchii* do not hydrolyse heparin. In view of the uniformly negative results of all our experiments they are not presented in detail, but we wish to record our thanks to Dr R. G. MacFarlane of the Wellcome Physiological Research Laboratories for supplies of lecithin emulsion and for much invaluable advice on the arrangement and interpretation of these experiments.

*Artificial diffusing factors; influence on the viscosity of
mucoprotein and diffusion in the dermis*

Claude [1935] reported that azoproteins obtained by coupling diazobenzenesulphonic acid with serum, albumin, casein or gelatin diffuse in the skin in a manner comparable with testicular extract. Favilli [1940] reported that these azoproteins, diazobenzenesulphonic acid and its sodium salt reduce the viscosity of synovial fluid, but he encountered difficulties in deciding whether reducing substances are liberated as a result of this action. We have confirmed Favilli's observation that diazotized compounds reduce the viscosity of mucoprotein and that the fall in viscosity is slow compared with that produced by naturally occurring enzymes. The relative slowness of this reaction is of interest, since it is comparable with the rate of diffusion of these compounds in the skin; the greatest diffusion of naturally occurring enzymes takes place in the first 20 min. after injection, while that produced by diazotized compounds develops slowly, is not obvious for an hour or more and attains its maximum after 9 hr.

We have overcome the difficulties which arise in determining whether reducing substances are liberated. These difficulties are due to the coloured nature of the diazotized compounds, their own reducing power and the difficulty of removing soluble protein. After the azoprotein had been incubated with the mucoprotein substrate for 18 hr., attempts were made to remove the protein by precipitation with ammonium sulphate or with neutral lead acetate. Neither of these methods was satisfactory; treatment with ammonium sulphate resulted in a heavy precipitation when Ehrlich's reagent was added in the estimation of acetylhexosamine and, after treatment with neutral lead acetate it was impossible to remove the lead with H_2S in the usual manner. It was found, however, that if basic lead acetate (B.D.H.) is added to the mixture of azoprotein and substrate, to which sufficient NaOH has already been added to produce a deep red colour, the protein is precipitated and the residual lead can be removed with H_2S ; the latter gas is removed by bubbling air through the mixture. The efficacy of this method was tested on samples to which a known quantity of acetylglucosamine had been added and also on mixtures of substrate with natural enzyme in which reducing substances had been liberated. Our observations were also controlled by tests on mixtures of the substrate with normal serum and with water and on the substrate alone. When acetylglucosamine is estimated in the usual manner by Morgan & Elson's method a yellow colour develops on the addition of Ehrlich's reagent which alters the final colour slightly, but this can be compensated for by blending with the contents of known positive and negative tubes. Total reducing sugars can be estimated by the method of Hagedorn & Jensen after this lead treatment. Our observations indicate that after the viscosity of mucoprotein has been reduced by the action of diazotized compounds no increase of reducing substances can be detected; there is no evidence that diazotized compounds destroy reducing substances already present in the mixture.

As already explained in the section describing our methods, vitreous humour was replaced by mucoprotein from umbilical cord as substrate in our observations because of the spontaneous loss of viscosity by the former substance. Robertson *et al.* [1940] suggested that this loss of viscosity is due to traces of ascorbic acid and stated that the first stage in the breakdown of mucin is catalysed rapidly by 'ascorbic acid-peroxide'. It was of interest to investigate the nature of this reaction and to determine whether ascorbic acid and its oxidation products show any diffusing action in the skin.

Ascorbic acid was dissolved in freshly boiled distilled water at $M/5$ and $M/50$ concentrations. Samples of each concentration were taken, the first being treated with one molecular equivalent of H_2O_2 , the second with two molecular equivalents and the third not treated. These specimens were tested for their action on the viscosity of the usual mucoprotein substrate buffered at pH 4.6. The fall in viscosity caused by all three specimens at $M/50$ concentration was extremely rapid, but it appeared that the sample containing one equivalent of H_2O_2 was slightly more active than the other two. The test was repeated with $M/500$ solutions, giving a final concentration of $M/3000$ in the system, and the result is shown in Fig. 6. It will be seen that the ascorbic acid and its oxidation

Fig. 6. The reduction of viscosity by ascorbic acid derivatives. A =control, water only. B = $M/3000$ ascorbic acid + two equivalents of H_2O_2 . C = $M/3000$ ascorbic acid alone. D = $M/3000$ ascorbic acid + one equivalent of H_2O_2 .

products all cause a rapid fall in viscosity, but that the specimen with one equivalent is slightly more active than the other two, half-viscosity level being reached in less than 15 min.

In order to test the diffusing activity of these preparations in the dermis it was necessary to buffer them at pH 7.0 before injection. The results of these diffusion tests are shown in Fig. 7. The diffusion of all three preparations in the skin is marked but develops slowly, attaining a maximum after 3 hr., and is of the same type as the spread due to diazotized compounds; a curve illustrating

the typical rapid diffusion of testicular enzyme is included in the figure for comparison. The preparation with one equivalent of H_2O_2 spread over a greater area than either of the others.

Fig. 7. The diffusion of ascorbic acid derivatives compared with that of testis diffusing factor.

It was thought that the action of ascorbic acid and its products on the viscosity of mucoprotein might be reversible. Samples of the substrate were taken after 40 min. and 18 hr. exposure to the dehydroascorbic acid, together with controls of substrate with water, and these were dialysed for 48 hr. The viscosity of the dialysed material was measured, but no evidence was obtained of any recovery of viscosity.

Notwithstanding the extremely rapid fall in viscosity of the substrate, no detectable acetylhexosamine is liberated by ascorbic acid or its derivatives; in this respect the action resembles that of the diazotized compounds. The addition of enzyme, obtained from *Cl. welchii* to substrate which has already been exposed to ascorbic acid oxidation products results in the liberation of the normal amount of acetylhexosamine.

The characteristics of the action of these preparations on mucoprotein and their diffusion in the skin differ in several respects from that of naturally occurring hyaluronidase. The failure to liberate acetylhexosamine and the slow diffusion in the skin has already been noted. In addition we observed that the reaction time measured viscosimetrically is not proportional to the concentration of ascorbic acid or its products that are present. The increase in reaction time as the dilution is increased from $M/5$ to $M/500$ is very small. The reaction time is not independent of the concentration of substrate as it is with the naturally occurring enzymes; increase in this concentration causes a marked lengthening of the reaction time. The dermal diffusion of naturally occurring enzymes is evident at a much higher dilution than that at which reduction of viscosity of mucoprotein can be detected; on the other hand, the viscosity-

reducing activity of ascorbic acid and its derivatives can be detected in dilutions much greater than those necessary to demonstrate dermal diffusion.

An accidental observation in the course of some control tests revealed the fact that 0.0066 *M* thiolacetic acid reduces the viscosity of mucoprotein and that this reduction passes the half-viscosity level during 18 hr. incubation. This led us to investigate whether other reducing substances, such as H_2S , quinol, pyrogallol, glucose, sodium sulphite and 'metol', had similar actions and whether they showed any diffusing action in the skin. Tests were made to exclude the possibility that these substances activated an enzyme already present in the substrate and to determine whether reducing substances were liberated as a result of the action on viscosity. All the above substances, with the exception of glucose, cause a similar reduction of viscosity of the substrate and none of them liberate reducing substances; the viscosity of heated substrate is reduced, which excludes the possibility that an enzyme already present is activated by these agents. Several of these substances produce definite diffusion in the skin which is of the slow type similar to that caused by diazotized compounds and ascorbic acid. This activity and its relation to the reduction potential of the various substances is being investigated and the results will be described in a later communication; it is hoped that it may help to elucidate the mode of action of diazotized compounds and ascorbic acid.

The influence of hyaluronidase on bacterial metabolism

Preliminary experiments have been made to determine whether bacteria which produce hyaluronidase can use hyaluronic acid as a metabolite if it is included in the culture medium and what influence the presence of this substance has upon the production of the enzyme by the organism.

A rapidly growing 2 hr. culture of *Cl. welchii* was washed in saline to remove any hyaluronidase present in the culture fluid; the washed suspension was used as inoculum for tubes containing the following four media:

- (a) A basal medium of 3.5 % Evans peptone alone.
- (b) 3.5 % Evans peptone + 0.5 % glucose.
- (c) 3.5 % Evans peptone + 0.5 % potassium hyaluronate.
- (d) The same composition as (c) with the addition of 1 unit of hyaluronidase to start the enzymic action on the hyaluronate.

Anaerobiosis was maintained in these tubes by the addition of 1 % by vol. of a 2 *M* thiolacetic acid in *NHCl*; the *pH* was corrected by neutralization with *NaOH*. The cultures were incubated for 18 hr.; the amount of bacterial multiplication was estimated by comparison with standard opacity tubes; the hyaluronidase and spreading activities of the culture fluids were titrated in the usual way. The result of this experiment is recorded in Table 8.

It will be seen that the addition of glucose to the medium results in an eight-fold increase in bacterial multiplication and an approximately fivefold increase in hyaluronidase production. The substitution of potassium hyaluronate for glucose does not significantly increase multiplication of the organism, but the concentration of hyaluronidase shows a dramatic ninefold increase as compared with the medium containing glucose; this is accompanied by a considerable increase of spreading activity in the skin. The medium containing 1 unit of hyaluronidase shows a further slight increase of hyaluronidase which may be significant. This experiment, which has been repeated with consistent results, shows that *Cl. welchii* can use a salt of hyaluronic acid as a metabolite and that the inclusion of the specific substrate in the medium induces a greatly enhanced production of hyaluronidase.

Table 8. *Cultivation of Cl. welchii in various media*

Medium	Bacterial multiplication. Opacity tube number	Hyaluronidase. Viscosity-reducing units	Dermal diffusion. Highest dilution producing diffusion
3.5 % Evans peptone alone	1	13	10 ⁻⁶
3.5 % Evans peptone + 0.5 % glucose	8	70	10 ⁻⁷
3.5 % Evans peptone + 0.5 % potassium hyaluronate	8	1340	10 ⁻⁹
3.5 % Evans peptone + 0.5 % potassium hyaluronate + 1 unit <i>welchii</i> hyaluronidase	8	1660	10 ⁻⁹

Infections by *Cl. welchii* are characterized by extremely rapid spread through the tissues and marked generalized toxæmia. The rapid adaptation of this organism in the presence of a salt of hyaluronic acid in the culture medium is of considerable interest in this connexion. It is known that there is a mucinous interfibrillar structure in the tissues, and recently Claude [1940] and Chain & Duthie [1940] have described the extraction from the skin of a substance indistinguishable from hyaluronic acid which is destroyed by hyaluronidase. We have also found that reducing substances are liberated by the action of this enzyme on crude extracts from the skin. Therefore, it appears likely that as soon as *Cl. welchii* start to proliferate in the tissues the hyaluronic acid there is attacked by means of this enzyme. Thus the organism is able to use this metabolite and, at the same time, by destroying the viscosity of this material to increase the permeability of the tissues and render more of the specific substrate accessible. In addition, the presence of hyaluronic acid causes the organism to produce greatly increased amounts of the enzyme. In this way a vicious circle is set up that promotes the rapid extension of the infection.

Further investigations on this adaptive enzyme effect with other strains of *Cl. welchii* are in progress and similar observations on strains of streptococcus and staphylococcus which do and those which do not produce the diffusing factor are being made. An attempt is being made to correlate this phenomenon with the relative virulence or local invasiveness of different strains of these organisms, but results of these observations will be reported in detail elsewhere.

Neither sulphanilamide nor sulphapyridine (M and B 693) inhibit the *in vitro* production of hyaluronidase by *Cl. welchii* when these substances are introduced into the culture medium. Neither of the substances have any direct action on the viscosity of the mucoprotein substrate, nor do they inhibit the *in vitro* activity of the enzyme.

Correlation between hyaluronidase activity and diffusion in the dermis

The close association between hyaluronidase activity and diffusion in the dermis that is obtained with tissue extracts, various bacterial extracts and snake venoms presents a strong *prima facie* case for assuming that the same enzyme is responsible for both phenomena. This assumption is further supported by the isolation of a substance from the skin [Claude, 1940; Chain & Duthie, 1940] which is indistinguishable from hyaluronic acid, and the viscosity of which is reduced by hyaluronidase. We have ourselves observed that enzyme preparations cause the liberation of reducing substances from a crude extract of skin. The fact that diazotized compounds, ascorbic acid and its oxidation products and other artificially prepared substances which reduce the viscosity of the

substrate also diffuse in the skin, though in a slower manner than enzymes, adds support to the belief that both diffusion and viscosity reduction have an identical cause. Recently, however, Madinaveitia *et al.* [1940] have published a warning that it is not justifiable to assay skin-diffusing factors by merely measuring their hyaluronidase activity. Using the method of Bacharach *et al.* [1940], these authors failed to obtain parallel figures for the titrations of diffusing factor potency and hyaluronidase activity measured viscosimetrically and they suggest that it may be possible to separate diffusing factors from hyaluronidase. While the validity and relative accuracy of various methods of assaying diffusing factors is under investigation it may be suggested that conclusions based upon the lack of correspondence between titrations of diffusing factor and of hyaluronidase should be treated with caution. The environmental conditions of the test in the skin and that in the viscosimeter differ profoundly and may exert differential effects upon samples of enzymes from different sources or those from the same source purified by different methods. Viscosimetric tests were carried out by Madinaveitia & Quibell [1940] in 0.66 *M* NaCl and at pH 4.6, whereas skin diffusion presumably takes place in isotonic salt at or about neutrality. The marked influence of salt concentration and pH on the *in vitro* activity of the enzymes has already been demonstrated; it has also been shown that testicular and bacterial enzymes differ in their responses to changing salt concentration. There is also evidence that reducing substances affect the viscosity of the substrate and the permeability of the tissues; little is known at present about the oxidation-reduction potential of the *in vitro* enzyme system or of the tissues into which these factors are injected. Investigations on this point are in progress. In addition, there are many possible factors concerned in the response of an animal to these substances which it is not possible to control adequately at present. In the existing state of knowledge it is certainly unjustifiable to assay skin-diffusing factors in terms of hyaluronidase activity but, in view of the considerations set out above, it does not follow that the two activities are due to separate agents.

Diffusion in the skin is caused by enzyme preparations that are too weak to be titrated viscosimetrically and those of moderate hyaluronidase content produce significant diffusion at high dilution. The skin test is therefore very much more delicate though much less accurate than viscosimetric assay. In the section describing our methods, we have explained why, for the purposes of this work, we preferred to continue testing for diffusing activity by determining the minimal diffusing dose rather than by the methods devised by Madinaveitia or by Bacharach *et al.* When the limiting diffusing dilution is determined it is not claimed that differences of less than tenfold dilutions can be distinguished, but it is possible to determine whether samples assayed by this method fall into the same order of activity as they do when titrated viscosimetrically. Table 9 contains the result of such a comparison.

It will be seen that, broadly speaking, the enzyme samples fall into the same order of activity by both tests. There is slight overlapping of those samples showing limiting diffusion at dilutions of 10^{-4} and 10^{-5} , but otherwise there is as good agreement as could be expected when the limitations of the animal test are taken into consideration. Further work is required to determine how far the diffusion test of Bacharach *et al.* will give results in agreement with our test, and these comparative observations are in progress. In the meantime the results presented in this table support the belief that diffusion in the skin may be due to hyaluronidase activity. This conclusion is further supported by the work of Chain & Duthie [1940] which has just appeared; these authors find good quanti-

tative agreement between hyaluronidase activity determined viscosimetrically and spreading activity in the skin with various testicular extracts and with leech and bee venoms.

Table 9. *Correlation between diffusion in the dermis and viscosity-reducing activity*

Source of enzyme	Highest dilution producing diffusion in the dermis	Viscosity- reducing units (in 0.146 M NaCl)
<i>Cl. welchii</i> filtrate 1a	10^{-8}	6.5
<i>Cl. welchii</i> filtrate 1	10^{-4}	13
<i>Cl. welchii</i> (purified by method of Morgan & McClean [1932])	10^{-4}	25
<i>Cl. welchii</i> (purified by method of Robertson <i>et al.</i> [1940])	10^{-5}	18
<i>Cl. welchii</i> (purified by method of Morgan & McClean)	10^{-5}	25
<i>Cl. welchii</i> filtrate 2a	10^{-5}	29
Testis, Glaxo standard, 1 mg./ml.	10^{-5}	36
Testis (purified by method of Morgan & McClean)	10^{-5}	70
<i>Cl. welchii</i> filtrate 2	10^{-6}	70
<i>Cl. welchii</i> filtrate 3a	10^{-7}	920
<i>Cl. welchii</i> filtrate 3	10^{-8}	1340
<i>Cl. welchii</i> filtrate 4	10^{-8}	1660

SUMMARY

1. The association between hyaluronidase activity and diffusion in the tissues of testicular extracts, certain bacterial filtrates and snake venom is described.

2. The influence of pH on the hyaluronidase activity of these substances has been investigated.

3. The liberation of reducing substances by the enzymes has been studied. Quantitative comparisons of viscosity-reducing activity and the time relation of both reactions suggest that two separate mechanisms may be involved.

4. The reduction of viscosity and liberation of reducing substances by bacterial enzymes can be completely neutralized by appropriate antisera which also inhibit diffusion in the skin. This neutralization is strictly specific.

5. A study of the influence of the concentration of electrolytes on the reduction of viscosity and liberation of reducing substances by testicular and bacterial enzymes reveals that the relative potency assigned to enzyme samples from different sources is profoundly modified by the salt concentration of the system. Testicular enzymes appear to differ in their behaviour from bacterial enzymes as the salt concentration is reduced.

6. The relation between viscosity and concentration of substrate has been investigated. The viscosity is not proportional to the concentration. The bearing of this observation on estimation of enzyme activity is discussed.

7. There is no correlation between the hyaluronidase activities of enzymes from various sources and their amylase or phosphatase contents, nor with their actions on gastric mucin or several other polysaccharides.

8. The hyaluronidase of Russell's viper venom ('Stypven') is not associated with any action on heparin. Neither this venom nor testicular or bacterial enzymes have any direct action on heparin, nor do they promote the coagulation of heparinized plasma.

9. Diazotized compounds are known to diffuse in the dermis; the observation that they also reduce the viscosity of mucoprotein has been confirmed. These compounds do not liberate reducing substances.

10. Ascorbic acid and its oxidation products cause a rapid fall in the viscosity of mucoprotein without the liberation of reducing substances. They also diffuse in the skin in a manner similar to diazotized compounds.

11. Various reducing agents also cause a fall in viscosity of the substrate and diffuse in the skin. The action of these artificially prepared compounds is compared with that of naturally occurring enzymes. Further investigations on the activity of artificial compounds are in progress.

12. Preliminary experiments on the influence of the inclusion of potassium hyaluronate in the culture medium of *Cl. welchii* are reported. A large increase in the yield of hyaluronidase results. The significance of this increase in relation to the proliferation of these organisms in the tissues is discussed. Further observations with this and other organisms that produce hyaluronidase are in progress.

13. Sulphanilamide and sulphapyridine do not inhibit production of this enzyme by *Cl. welchii* nor do they inhibit the *in vitro* activity of the enzyme.

14. The question whether diffusion in the tissues is due to hyaluronidase is discussed. The correlation between tests for the two activities is considered. Our experimental results support the belief that diffusion in the skin is due to hyaluronidase activity.

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