The Complexity of the Hyaluronidases Produced by Micro-organisms

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Immunologically distinct hyaluronidases are formed by a variety of micro-organisms when grown in ordinary culture media and are present in large amounts in mammalian testes, leech heads and certain venoms. Because of the complexity and size of the hyaluronate molecule various methods have been used to measure the degree of hydrolysis brought about by these enzymes. Those most frequently employed have utilized viscosity changes or the appearance of reducing sugars in such solutions. Hahn (1945 *a*, *b*; 1946 *a*, *b*), however, has suggested that there are two enzymes present in preparations of testicular hyaluronidase. One of these degrades hyaluronate to a disaccharide and is responsible for lowering the viscosity and for the liberation of about one half of the theoretical amount of reducing sugars; the second hydrolyzes the resulting *N*-acetylhexosamine glucuronide to the constituent monosaccharides. If the ratio of the amounts of these two enzymes varies from preparation to preparation, determinations of hyaluronidase activity by viscosity techniques need not always correlate with those using the liberation of reducing sugars. Other work (Rogers, 1946 *a*, *b*) suggests that the hyaluronidase enzyme system may be even more complex, since, when purified hyaluronic acid is partially hydrolyzed by enzymes prepared from three different sources so that *c.* 50% of the theoretical amount of reducing sugar has been liberated, the size of the units remaining depends upon the source from which the enzyme has been obtained. This observation is best explained (Rogers, 1946 *a*) by assuming that several enzymes successively degrade the polysaccharide to smaller and smaller units; the optimal conditions of pH and salt concentration for the individual enzymes in these systems may differ according to the source of the preparation. Thus, when the hyaluronidases are acting upon the polysaccharide at a single pH and salt concentration, some of the components active in preparations from one source might be inactive in those from another.

If, as McClean (1941*a*) and Lythgoe & Madinaveitia (1943) suggest, the hyaluronidases supply organisms with available carbohydrate by hydrolyzing the hyaluronic acid of the mesodermal tissues, the complexity of the bacterial hyaluronidases has implications for the *in vivo* function of the enzyme, since the organisms will be as dependent upon their ability to form enzymes which liberate fermentable reducing sugars from hyaluronate under physiological conditions as upon their ability to increase skin permeability.

In the present work, the potency of preparations of hyaluronidase obtained from three different bacterial sources has been compared using two different methods of estimation. The first method measures the relatively slight degradation of the polysaccharide (i.e. reduction of the viscosity) and the second, upon a more extensive hydrolysis (i.e. the liberation of reducing sugars). No attempt has been made to separate completely the various enzymes present in any one of the preparations, and no claim is made that the work designates the precise number of enzymes involved in the complete hydrolysis of hyaluronate to monosaccharides. It is thought that claims for specific enzymes, based on the ability of enzyme preparations to hydrolyze *ad hoc* fractions obtained from hyaluronate, such as disaccharides, may be misleading until more knowledge is available of the structure of the complex, undegraded polysaccharide.

METHODS

Viscosity experiments. The technique used was that described by McClean & Hale (1941) as modified by McClean (1943). The final concentration of the phosphate-citrate buffer was M/60 and of NaCl, 0·1 M. In the determination of pH optimum the pH of the final buffer-hyaluronate mixture was always measured because the hyaluronate itself had a considerable buffer action; the glass electrode was used. As in all the previous work, the enzyme potencies (recorded as viscosity reducing units, V.R.U.) are measured by comparison with a dried testicular hyaluronidase preparation of known strength.

Enzyme preparations. The partially purified preparations of streptococcal and staphylococcal hyaluronidase were made by growing a strain of *Streptococcus haemolyticus*, group C (Lancefield) type 7 (Griffiths) (called *Strep.* C7) and *Staphylococcus aureus*, strain Humphrey (called *Staph.* Humphrey) in the casein hydrolysate media already described (Rogers, 1945). The enzymes were partially purified from these cultures by the method appearing under 'partially purified streptococcal hyaluronidase' (Rogers, 1946*a*). Since this latter work appeared it has been found

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that the value for the ratio of V.R.U./mg. N (i.e. 4550 in Table 1, Rogers, 1946*a*) is too low for the majority of enzymes prepared by this method. When the preparations have been exhaustively dialyzed against a buffer-saline mixture this ratio rises to as high as 66,000 in some instances, and to an average value of 15,000–20,000. It has been found that aqueous solutions of the enzymes are not stable at 0–4° unless protected by the presence of glycerol. Consequently the preparations were dialyzed for about 6 hr. at room temperature against undiluted glycerol and stored at −10°. Under these conditions only a 10–20% loss of enzyme potency has occurred in 9 months.

Table 1. *The effect of dilution upon a concentrated preparation of* Clostridium welchii *hyaluronidase using various diluents and various periods between diluting and testing*

(The pH of the diluents was always 7·0, temp. 18–20°. The results are expressed as viscosity-reducing units.)

Diluent	Time (min.)	Apparent potency (V.R.U.)
Distilled water	1	227,000
Distilled water	30	116,000
Distilled water	60	100,000
Gelatin (0·2%)	15	650,000
Peptone* (1%)	15	865,000
Gum arabic (0·5%)	2	1,560,000
Gum arabic (0·5%)	30	1,600,000
Gum arabic (0·5%)	60	1,520,000

* As supplied by Evans Sons, Lescher and Webb.

The dilutions made for determination of enzymic activity were in 0·5% (w/v) gum arabic solution. This precaution was necessary since the partially purified enzymes are rapidly inactivated by dilution in water. Table 1, for example, shows the influence of diluting even a relatively crude preparation of *Clostridium welchii* hyaluronidase in a number of diluents. This enzyme preparation was kindly supplied by Dr W. E. van Heyningen, of the Wellcome Physiological Research Laboratories. It was prepared by growing strain S 107 of the organism upon a papain digest medium, and concentrating the enzyme by precipitation with $(NH_4)_2SO_4$ from the culture filtrate. Reducing sugars were estimated by the method described by Somogyi (1937).

Method for estimating the potency of hyaluronidase preparations by the amount of reducing sugar liberated from hyaluronate. No satisfactory test, in which the amount of reducing sugars liberated from potassium hyaluronate is strictly proportional to the concentration of enzyme used, has so far been described. Preliminary tests showed that at pH 7·0 and in the presence of 0·1 M-NaCl the rate at which reducing sugars were liberated by streptococcal hyaluronidase (1–5 V.R.U./ml.) acting at 37° was approximately constant for 30 min. Fig. 1 shows that the enzyme was saturated with substrate under these conditions at a concentration of 0·3 g./100 ml. The sample of hyaluronate used was not pure, and according to the glucosamine determinations on acid hydrolysates, the true concentration was estimated as 0·16 g./100 ml. The method of testing finally adopted was as follows: A solution of crude K hyaluronate (3·3 ml. of 0·5% solution) is mixed with the appropriate (see later) 0·1 M-phosphate buffer containing 0·6 M-NaCl (0·83 ml.) and water (0·37 ml.), and the mixture is warmed at 37° for 1 hr. before the diluted hyaluronidase (0·5 ml. of potency *c.* 10 V.R.U.) is added by carefully running it down the side of the tube to avoid mixing the substrate and enzyme. The tube is corked and the contents mixed at zero time by two inversions. After 22 min. the tube is rapidly cooled in ice water and left for 1 hr. Two samples (each 1·5 ml.) are then taken into a mixture of the Somogyi (1937) $CuSO_4$ reagent (5·0 ml.) and water (3·5 ml.). The reducing sugar is estimated in the usual manner. It is important to boil the tubes to be used in this part of the estimation in chromic acid a few days before use, otherwise the two duplicate estimations do not always agree satisfactorily. The final titrations of the liberated I_2 using 0·005 N-$Na_2S_2O_3$ should agree to within 0·05 ml. for duplicate samples, and usually to within 0·02 ml.

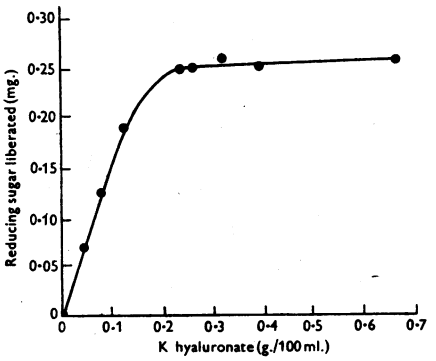

Fig. 1. Influence of substrate concentration on the amount of reducing sugar liberated from potassium hyaluronate by streptococcal hyaluronidase.

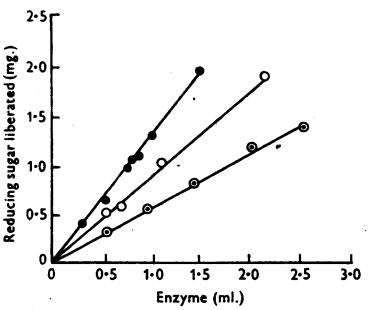

Fig. 2. The influence of enzyme concentration upon the liberation of reducing sugars from hyaluronate by hyaluronidase from *Streptococcus* C7 —●—, *Staphylococcus* Humphrey —○— and *Cl. welchii* —⊙—.

The unit of enzymic activity adopted (R.S.U.) has been arbitrarily defined as the amount of enzyme which will release 1 mg. of reducing sugar, estimated as an equimolar mixture of *N*-acetylglucosamine and glucurone, in 5·0 ml. of the above hydrolysate mixture. Each batch of the $CuSO_4$ reagent must be standardized with an equimolar solution of the *N*-acetylglucosamine and glucurone before use. The same batch of hyaluronate has been used as substrate for all the estimations of enzymic activity by both viscosity reduction and sugar liberation.

Fig. 2 shows that under these conditions the amount of reducing sugar liberated from hyaluronate by hyaluronidases from three different bacterial sources is strictly proportional to the amount of enzyme added.

RESULTS

The pH *optimum of hyaluronidases*

As measured by viscosity reduction. The optimal pH values for the viscosity-reducing activities of testicular and *Cl. welchii* hyaluronidases are 7·0 and 6·0 respectively (McClean, 1943). On the basis of these observations, and because conditions approaching those in the skin of animals were required, McClean (1943) chose pH 7·0 for the standard viscosity reduction assay. Later Hale (1944) showed that streptococcal hyaluronidase could also be estimated viscosimetrically at pH 7·0, thus overcoming the difficulty met by McClean (1941 *b*), who found that streptococcal hyaluronidase could not be estimated by the viscosimetric technique of Madinaveitia & Quibell (1940). This failure was due, as Hale (1944) showed, to the rapid inactivation of the enzyme at pH 4·6 which was that originally employed by Madinaveitia & Quibell (1940) and subsequently used by McClean (1941 *b*).

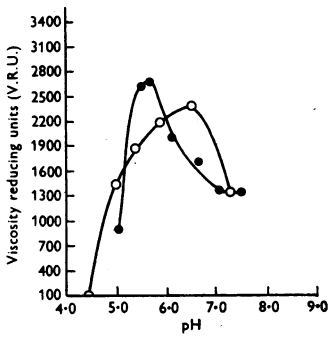

Fig. 3. The influence of pH on the viscosity-reducing activity of hyaluronidase from *Streptococcus* C7 —●— and *Staphylococcus* Humphrey —○—.

Fig. 3 shows the pH optimum of the viscosity-reducing activity of streptococcal (*Strep.* C7) and staphylococcal (*Staph.* Humphrey) hyaluronidase. Both enzymes show a single sharp optimum. The result for the streptococcal enzyme is somewhat unexpected since it shows optimal activity at a pH value of about 5·5, less than 1 unit above that at which inactivation is too rapid for determinations to be made. In this connexion it may be mentioned that the inactivation of this hyaluronidase at pH 4·6 in the presence of substrate is almost completely reversible. After 10 min. at pH 4·6 and 37°, only 4·4% of the original enzymic activity could be detected in a mixture of streptococcal hyaluronidase and substrate. After a further 30 min. the residual value had fallen to 1·0%. At this time the pH was readjusted to 7·0 with 0·5N-NaOH, and an enzyme estimation made immediately showed a 10% recovery of activity, which increased on 3 hr. standing at pH 7·0 and room temperature to 89%. Inactivation of enzyme at pH 4·6 in the absence of substrate could not be reversed in this way.

The staphylococcal enzyme had an optimal pH of about 6·6; this value lies between those for the *Cl. welchii* and testicular enzymes. These curves were constructed using the same buffer system at constant ionic strength. Other types of buffers and other ionic concentrations would probably have altered both the pH optima and the shape of the pH activity curves (cf. McClean, 1943; Madinaveitia & Quibell, 1940; Myrbäck, 1926; Sherman, Caldwell & Adams, 1928).

As measured by release of reducing sugar. Meyer, Hobby, Chaffee & Dawson (1940) and Meyer, Chaffee, Hobby & Dawson (1941) have measured the relations between activity and pH for pneumococcal, *Cl. welchii* and testicular hyaluronidases using the liberation of reducing sugars. Evidence in their paper shows that the amount of reducing sugars, liberated under their conditions, was not proportional to the amount of enzyme used, and consequently their pH optima cannot be accepted without further examination.

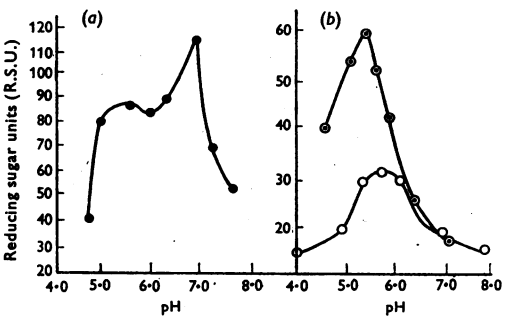

Fig. 4. The influence of pH upon the amount of reducing sugar liberated from potassium hyaluronate by hyaluronidase from (*a*) *Streptococcus* C7; (*b*) *Staphylococcus* Humphrey —○— and *Cl. welchii* S107 —⊙—.

The potencies of preparations of hyaluronidase obtained from *Strep.* C7, *Staph.* Humphrey and *Cl. welchii* strain, S107, were determined at various pH values (glass electrode) by the technique outlined above.

The results are shown in Fig. 4. Noteworthy is the double optimum obtained with the streptococcal enzyme (Fig. 4*a*). Whilst several estimations made with the same preparation were in agreement, the relative values for the maxima were different when a second preparation was examined. This showed a more marked minimum, and the optimum at pH 5·6 represented a higher enzymic activity than that at pH 7·1.

The above result for the streptococcal hyaluronidase suggests that more than one enzyme is involved in the liberation of reducing sugar. It may be significant that one of these optima agrees with the optimum at pH 5·7 obtained by viscosity reduction. A comparison of the pH optima of the other two bacterial enzymes, as measured by the liberation of reducing sugar and by the viscosity reduction technique, shows that only with the *Cl. welchii* preparation is there a close similarity. The optimum pH for reducing-sugar liberation by this enzyme is 5·6, and McClean (1943) obtained the value of 6·0 by viscosity-reduction test. The difference between the optima for the staphylococcal preparation is a whole pH unit.

The ratio of V.R.U./R.S.U. *for different preparations of the same enzymes and for fractions obtained from them*

At this stage in the work a number of glycerinated preparations of staphylococcal and streptococcal hyaluronidase had been accumulated. Some of these had been made from separate batches of enzyme using different seed inocula, whilst others were fractions separated from the same batch during elutions from $Fe(OH)_3$ (Rogers, 1946*a*). The enzymic activities of these preparations were measured by the two techniques at the optimal pH determined above; e.g. the activity of the staphylococcal enzyme was measured at pH 6·6 by the viscosity-reduction technique and at pH 5·5 by the reducing sugar-liberation technique. Table 2 summarizes the results of these tests. There is a large variation between the ratios of the activities estimated by the two techniques, except for those between the two samples of *Cl. welchii* hyaluronidase. The greatest error involved in assessing V.R.U./R.S.U. ratios is ± 15–20%, being the sum of the maximum experimental errors of the two methods. The difference between the values for the two *Cl. welchii* preparations (i.e. about ± 7%) is well within the experimental error, and is therefore not significant. These results indicate that the reduction in viscosity, and the liberation of reducing sugars from solutions of hyaluronate, are not brought about by the same enzyme or enzymes in the preparations of streptococcal and staphylococcal hyaluronidase.

A further separation of the several enzymes was then attempted by chromatography. This method had already given promising results in the purification of *Cl. welchii* hyaluronidase (Rogers, 1946*a*).

Two adsorbents were used: (1) a mixture of equal weights of $Ca_3(PO_4)_2$ and acid-washed powdered glass (pyrex). (2) Al_2O_3 (Savory and Moore Ltd., London). The glycerinated enzyme preparations (5·0 ml.) were dialyzed for 4 hr. against 0·01 M-phosphate-citrate buffer (2 l.) at pH 5·6 and room temperature. It was still necessary to adjust the pH of the dialysates to 5·6 with 0·1 M-citric acid after this period of dialysis. The volumes and the enzyme potencies were then measured, and the dialysates (10 ml.) were passed down the columns of the adsorbents without suction. The sizes of the columns were: $Ca_3(PO_4)_2$-glass powder, 3 × 2 cm.; Al_2O_3, 9 × 2 cm. The columns were washed with water (10 ml.) and then with 0·2 M-Na_2HPO_4 solution. Successive fractions (3–5 ml.) were removed and called E_1, E_2, E_3, etc. These samples were immediately adjusted to pH 7·0 by the careful addition of 0·1 M-citric acid, diluted 1 : 5 in gum arabic (0·5 g./100 ml.) and were stored at −10° until tested. The potencies of the samples were measured by both the viscosity-reduction and reducing sugar-liberation techniques at the appropriate pH.

Table 2. *Activities of various preparations of bacterial hyaluronidase as measured by the viscosity reduction technique and by the liberation of reducing sugar*

(Both estimations were made at the predetermined optimum pH and with final concentrations of M/60 phosphate-citrate buffer and 0·1 M-NaCl.)

Code number of preparation	Strain of micro-organism	Enzyme potency V.R.U.	Enzyme potency R.S.U.	V.R.U./R.S.U.
C7IVE_1	*Streptococcus haemolyticus* Strain C7	2,150	17·4*	123
C7IVE_2		1,710	10·2*	169
C7IIIE_2		2,800	91·0*	30·6
H I	*Staphylococcus aureus* Strain Humphrey	2,620	12·9	203
H Ib		860	6·9	125
H IIE_2		2,260	16·2	139
H IIE_3		1,610	16·0	100
Pf21	*Cl. welchii* Strain S 107	3,740	19·8	189
Pf21 P_1		15,340	75·9	202

* The liberation of reducing sugar by these samples was measured at pH 7·2, corresponding to the second optimum shown in Fig. 4*a*.

Again the ratios of the activities measured by the two methods show very considerable variation (Table 3), amounting in some cases to fourfold differences. In almost all cases the variation is at least twice the maximum total experimental error of ± 15–20%. Similar chromatographic runs were also made with the *Cl. welchii* preparations; there

was no significant difference, however, with these enzymes, between the values for the V.R.U./R.S.U. ratios. These results again confirm those obtained for the similar pH optima of the *Cl. welchii* preparations. In Table 3, the differences between the ratios for the various fractions separating from the column of Al_2O_3 are greater than the values for those from the $Ca_3(PO_4)_2$ column, due, presumably, to the more effective separation of the bands of enzyme.

Table 3. *The* V.R.U./R.S.U. *ratio for bacterial hyaluronidase fractions prepared by chromatography*

	Adsorbent $Ca_3(PO_4)_2$ + glass powder		Adsorbent Al_2O_3	
Fraction no.	*Strep.* prep. (V.R.U./R.S.U.)	*Staph.* prep. (V.R.U./R.S.U.)	*Strep.* prep. (V.R.U./R.S.U.)	*Staph.* prep. (V.R.U./R.S.U.)
E_2	50·8*	—	—	—
E_3	79·9*	220	—	—
E_4	91·1*	199	—	—
E_5	—	144	—	—
E_6	—	104	—	—
E_7	—	—	31·0*	89·0
E_8	—	—	133*	59·2
E_9	—	—	67·2*	24·5
E_{10}	—	—	—	—
Original dialysate	93·8	—	80·1	112

* Reducing-sugar liberation by these samples was measured at pH 7·2 corresponding to the second optimum of the curve shown in Fig. 4*a*.

Variation in the V.R.U./R.S.U. *ratio with age of the culture*

On examining the values for the V.R.U./R.S.U. ratio of the various preparations of streptococcal hyaluronidase, it was noticed that the largest differences occurred between batches of enzyme for which different seed innocula had been used rather than between different fractions obtained from the same batch. This suggests that the organisms, in different phases of growth and by inherent variation, might produce different amounts of the various enzymes. It is known, for example, that the production of the viscosity-reducing enzyme is adaptively increased by the inclusion of whole hyaluronate (McClean, 1943; Rogers, 1945), or of hyaluronate degraded as far as is possible by testicular hyaluronidase (Rogers, 1945, 1946*a*), but not by the products remaining after it has been hydrolyzed by streptococcal enzyme. Thus it was supposed that, during the growth of streptococci in a medium containing hyaluronate, the level of production of the viscosity-reducing hyaluronidase would be high only so long as there remained an adequate supply of sufficiently large units of hyaluronic acid in the medium to stimulate the organisms. If the formation of enzymes attacking smaller units of the polysaccharide should also be adaptive, then there would be a supply of units of the necessary size in the medium for a somewhat longer period, the large units being progressively degraded by the hyaluronidase formed by the growing organisms. If, on the other hand, the production of these latter types of enzyme were not adaptive, the organism might be expected to go on producing them at a steady level whilst the production of the viscosity-reducing enzyme falls. These considerations suggest that, if there are several enzymes responsible for various stages in the hydrolysis of hyaluronate, there should be considerable variations in the V.R.U./R.S.U. ratios during the course of growth of the organisms.

Two aspects of the problem were investigated: (1) by measuring the V.R.U./R.S.U. ratio in ordinary cultures of organisms at various stages of growth in casein hydrolysate media, both supplemented with hyaluronate (0·5 g./100 ml.) and unsupplemented (20 ml. quantities in pyrex glass tubes incubated at 37°); (2) by measuring the V.R.U./R.S.U. ratio of cultures growing in supplemented medium in cellophan dialysis sacs surrounded by medium containing no polysaccharide. Under these latter conditions it was hoped to prolong considerably the logarithmic phase of growth. The flasks containing the sterile medium and cellophan sacs, mounted on glass tubes in bungs which fitted tightly in the necks of the flasks, were shaken at 37° on an eccentric shaker designed by Duthie (unpublished communication). The shaking helped to stir the contents of the flask and sacs.

In both types of experiment, samples were taken at the intervals indicated in Tables 4 and 5, and centrifuged immediately on an angle centrifuge. The supernatant liquids were drawn off, a drop of toluene added to each, and stored at 0–4° until the end of the experiment. All the samples were then placed in small cellophan sacs and dialyzed for 24–48 hr. at 0–4° against 4–5 l. of 0·01 M-citrate-phosphate buffer, pH 5·8. This dialysis was necessary to remove traces of unfermented glucose and other reducing substances before testing the samples. The viscosity-reduction tests were also made on the dialyzed samples, so that any inactivation of enzymes, which had taken place during dialysis, would be unlikely to upset the evaluation of the V.R.U./R.S.U. ratio.

Table 4 shows the results of two experiments carried out by the first method, in which the V.R.U./R.S.U. ratios were determined after different periods of growth of the *Streptococcus* C7 in the ordinary tube cultures. The figures recorded are the mean of two cultures for Exp. 1 and of three cultures for Exp. 2.

The figures in Table 4 are among the most convincing evidence for the occurrence of differences

between the values of potency of the enzyme preparations when measured by the two methods; the V.R.U./R.S.U. values show an extreme difference of 12·6–192, giving very satisfactory proof of the complexity of the enzymes hydrolyzing hyaluronate. The hypothetical sequence of events in the formation of enzymes by cultures, as elaborated above, is, however, probably inadequate. An added complication seems to be that some enzyme or enzymes, estimated by the sugar-liberation technique, are sometimes destroyed during the prolonged incubation of the culture.

Table 4. *Values for the ratio* V.R.U./R.S.U. *in the dialyzed supernatant fluids from cultures of* Streptococcus C7

(The organisms were grown for the indicated periods in the casein hydrolysate medium A (Rogers, 1945) supplemented with 0·5 g./100 ml. of a crude sample of potassium hyaluronate, and in the unsupplemented medium. Exps. 1 and 2 were inoculated with different seed cultures of the organism and the results for Exp. 1 are the mean of two duplicate cultures, whilst those for Exp. 2 are the mean of three cultures.)

Time of incubation (hr.)	V.R.U./R.S.U. +Hyaluronate Exps. 1	2	No hyaluronate Exp. 2
10	12·6	—	—
24	11·3	21·2	56·2
48	—	60·5	125
96	68·2	94·7	192

Table 5. *The amounts of hyaluronidase formed by* Streptococcus C7 *in tubes and dialysis sacs*

(The organisms were grown at 37° in 25 ml. of the casein hydrolysate medium A (Rogers, 1945) supplemented by 0·5 g./100 ml. of a crude sample of potassium hyaluronate (1) in 'pyrex' glass tubes, and (2) in cellophan dialysis sacs surrounded by 1 l. of unsupplemented medium. The values given are the means for two duplicate cultures.)

Time of incubation (hr.)	V.R.U. D*	V.R.U. T†	R.S.U. D*	R.S.U. T†	V.R.U./R.S.U. D*	V.R.U./R.S.U. T†
10	112	62·4	4·13	4·9	27·2	12·0
24	670	221	18·7	19·0	39·4	13·2
48	1250	247	26·2	14·3	47·5	17·2
96	2850	410	26·7	4·7	107	87·0

* D = dialysis sac cultures. † T = tube cultures.

It was apparent from the results that the adaptive increase of the enzymes, involved in the liberation of reducing sugars from hyaluronate, was at least as great as that of the enzymes estimated in the viscosity-reduction test. It seemed, therefore, of interest to proceed with the experiments in which the organisms were grown in a dialysis sac. In such cultures the smaller saccharide units, formed by the action on the hyaluronate of the hyaluronidase produced by the organisms growing inside the sac, would also be removed. Thus it might be expected that the V.R.U./R.S.U. ratios for the sac cultures would be higher than those for the tube cultures if it is the smaller saccharide units which stimulate the production of enzymes estimated by the R.S.U. technique. The results in Table 5 support this suggestion. The ratios of the V.R.U./R.S.U. values for the sac/tube cultures are 2·3/1, 3·0/1, 2·8/1 and 1·2/1 for the various times at which the cultures were sampled. Apart from the last pair of values, these differences are well removed from the maximum experimental error of ± 20%. A further point of interest emerged from this series of experiments. The V.R.U. titre increased after a period of 96 hr. growth in the dialysis sacs to 2850. Yet, since the formation of hyaluronidase, as assessed by the viscosity-reduction technique in cultures grown in tubes, is strictly proportional to the amount of hyaluronate present in the medium, up to a concentration of 0·6 g./100 ml. (Rogers, 1945), it might be supposed that the concentration of hyaluronate in the medium would be the factor limiting enzyme formation. Apparently, however, when essential metabolites are added and toxic metabolic products removed by dialysis, enzyme production proceeds up to a level of 2850 V.R.U., compared with 250 in the tube cultures. The ratio of V.R.U./mg. bacterial N in the dialysis sac and tube cultures was identical at the end of the experiment. Hence it seems that, once they have begun to produce hyaluronidase at the adapted level, the organisms can continue to do so in the absence of the polysaccharide, providing fresh growth substances are added and toxic catabolites removed. Organisms from dialysis-sac cultures, re-inoculated into fresh medium without hyaluronate, form enzyme only at the unadapted level when the logarithmic phase of growth is resumed.

DISCUSSION

Measurements of streptococcal and staphylococcal hyaluronidases by the methods which depend upon the rate of liberation of reducing sugars do not agree with those which use viscosity-reduction estimations. The differences amount to more than one hundred times the experimental error, and it seems legitimate to conclude that the two methods do not measure the activity of one and the same enzyme. The conditions of buffer and NaCl concentrations were the same in both methods of estimation; therefore the objections raised by McClean & Hale (1941) and by McClean (1943) to the basis for the conclusions

reached by Madinaveitia, Todd, Bacharach & Chance (1940) cannot be applied to the present work. The last-named authors thought that the hyaluronidase and diffusing activities of testicular extracts were probably due to the presence of different entities, because of large discrepancies between the potency of the preparations assayed by the viscosity-reducing and skin-diffusion techniques. McClean & Hale (1941) and McClean (1943), however, pointed out that Madinaveitia *et al.* (1940) were measuring hyaluronidase activity by viscosity reduction at pH 4·6 and in M/6 NaCl, conditions far removed from those of the *in vivo* tests in the skin, and showed that the discrepancies were likely to be due to this difference. This is now rather easier to understand, since under the conditions used by Madinaveitia *et al.* (1940) enzymes may be active in reducing viscosity, which would be inactive in the skin of injected animals and in the hyaluronidase assay used by McClean (1943). If this is the true explanation, it might also apply to the results of Humphrey (1946) who found that viscosity reduction and sugar liberation by testicular and *Cl. welchii* hyaluronidase preparations ran parallel, when tests were conducted at a low pH and high ionic concentration.

It is necessary here to consider further the meaning of the variations in the V.R.U./R.S.U. ratios which have been observed in the present paper. The methods of measurement were not designed to determine the precise number of enzymes involved in completely hydrolyzing the complex hyaluronate molecule. First, the extent of the hydrolysis accomplished by the enzyme or enzymes measured in the viscosity reduction technique is unknown. Using a physical measurement such as that of viscosity, hydrolysis can only be followed so long as the molecules are large enough to exert their effect. Consequently we do not know the V.R.U./R.S.U. value characteristic of this stage of hydrolysis, which will presumably be the upper limit for its variation. The highest value which has been obtained in the present work is about 280. Secondly, in a series of enzymes acting successively in the hydrolysis of a high molecular weight substrate, such as is suggested here for the hydrolysis by hyaluronidase of hyaluronic acid, the individual enzymes are not independent in their functioning. Alteration in the concentration of enzymes acting at an early stage in the scheme will alter the rate of action of those coming later. The importance of this type of interrelationship in influencing V.R.U./R.S.U. ratios will depend upon such factors as the number of enzymes involved and their kinetics.

In order to decide how many enzymes are present in hyaluronidase preparations we must know more of the structure of hyaluronate. The most important single contribution would be towards an understanding of the chemical changes underlying the loss of viscosity of solutions of the polysaccharide.

There is evidence that, when viscous solutions of hyaluronate are allowed to react with simple chemicals, such as reducing reagents, hydrolysis stops long before the mono- or disaccharide stage is reached (Hale, 1944). (The results obtained by Skanse & Sundblatt (1943), indicating the formation of disaccharides in this type of degradation, could not be confirmed in these laboratories (Hale, unpublished results).) If the chemical nature of the oligosaccharide resulting from this relatively slight hydrolysis of the molecule were known, it might be of considerable help in the selection of suitable substrates for an examination of the precise degree of complexity of the hyaluronidase preparations. Until such knowledge is available, it is unprofitable to draw dogmatic conclusions from studies in which some particular fraction obtained from the polysaccharide is used as a substrate. The purpose of using the two methods chosen in the present study has merely been to indicate in a qualitative manner the complexity of the bacterial hyaluronidases. Further work on the polysaccharide may shed more light on the understanding of the results, as, for example, the recognition of the amylose and amylopectin fractions of starch has helped in studies of the amylases.

SUMMARY

1. An accurate method has been devised for the assay of bacterial hyaluronidase activity with respect to the liberation of reducing sugars from potassium hyaluronate.

2. The pH optima of streptococcal, staphylococcal and *Cl. welchii* hyaluronidases have been measured using this method. The results obtained for the first two enzymes do not agree with those obtained using a viscosity-reduction technique.

3. The relative potencies of samples of streptococcal and staphylococcal hyaluronidase, measured by the liberation of reducing sugars from hyaluronate, do not agree with measurements made by the viscosity-reducing technique.

4. It is deduced that preparations of streptococcal and staphylococcal hyaluronidase contain several enzymes responsible for various stages in the degradation of hyaluronate.

5. Highly potent hyaluronidase can be produced by growing streptococci inside dialysis sacs surrounded by medium containing no hyaluronate.

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