The Effect of Amino-Acids and Other Substances on the Activity of Streptococcal Hyaluronidase

With a Note on the Activity of Fractions obtained during the Isolation and Purification of Components of Streptococcal Hyaluronidase

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SUMMARY. The titre of streptococcal polysaccharidase was increased by incubation with β -alanine, serine, tyrosine, cystine, cysteine or methionine. Dextrinogenic amylase and hyaluronidase measured by a turbidity end-point were both activated by cysteine, cystine, serine or methionine. Polysaccharidase activity also increased after incubation with hyaluronic acid, starch, pectin or dextran. Pantothenic acid suppressed activity of SH-activated enzymes, and the rise of titre observed during incubation with substrate was decreased twofold or fourfold by addition of valine. The activation of streptococcal proteinase by cysteine was also prevented or retarded by pantothenic acid or valine. Pantothenyl alcohol was more active than either pantothenate (DL-calcium salt) or DL-valine.

Non-specific depolymerizing activity by hyaluronidase and amylase of Group A streptococci was reported by Crowley (1951), who also found that pro-enzyme in inactive cultures was activated by thiol groups, partially activated by metallic ions, and that pantothenic acid caused a transient rise of titre of inactive cell free filtrates. Hyaluronidase was decreased twofold in the presence of high proteinase concentration but there was no evidence to explain the proteinase effect. The present paper reports experiments made with partially purified polysaccharidase, with reference to amino-acids and other substances which activate polysaccharidase and proteinase.

MATERIAL AND METHODS

Strains. The strains of Group A streptococci were described in previous papers (Crowley, 1950, 1951). The serological classification and polysaccharidase activity are shown in Table 1.

Enzymic material was prepared from cell free filtrates of 2-day broth cultures. A note on the method of isolation and the activity of fractions obtained during purification is appended to this paper.

Activity. Amylase. Hydrolysis of 0.2 % soluble starch was tested by the method of Crowley (1951). **Proteinase.** The clotting time of milk + thiolacetate was tested by the method of Elliott & Dole (1947).

Hyaluronidase was tested by the M.C.P. method of McClean (1942), but human synovial fluid was used as substrate. Synovial fluid was obtained from knee joints at autopsy by first injecting 2 ml. sodium β -glycerophosphate (0.126 M) and withdrawing mucin + buffer which was collected in a sterile bottle and immediately layered over with toluene. Volumes of fluid obtained varied from 3 to 5 ml., therefore all samples were pooled. The first precipitate which

developed while standing under toluene, together with cell debris, was removed by centrifuging. The 'clean' pool was maintained at a volume not less than 150 ml., and 25–30 ml. removed at any one time to make the test substrate. Standing under toluene at -4° or at bench temperature the viscosity remained very constant, and the pH value was in the range 6.8–7.2. A sample from the original pool contained 1 % hyaluronic acid (this estimation was kindly made by Dr W. T. Morgan). A dilution of synovial fluid in distilled water containing approximately 0.2 % hyaluronic acid clotted with 2N acetic acid without additional protein. The hyaluronidase titre of a Type 4 strain (H713) was estimated using a cell free filtrate which was concentrated by dialysis against glycerol and stored at -10° . Subsequently, this 'standard' enzyme was used to estimate the strength of fresh batches of substrate. Usually 1 vol. of synovial fluid was diluted with 5 vol. of distilled water, and diluted by further half volumes as required. Enzyme samples in buffer + saline (Na β -glycerophosphate 0.063M + NaCl 0.1M) were diluted in distilled water at 20 % differences. The M.C.P. test was carried out at pH 7.0.

Method of reading results. The M.C.P. end-point was recorded in the usual way, but a record was also made of the greatest dilution of enzyme which was associated with an *increased* turbidity on acidification compared with two control tubes containing substrate + water or + buffer saline. (This end-point differs from the end-point observed in the usual turbidity reduction tests.) After standing overnight at bench temperature further readings were taken on lines similar to the ACRA test (Burnet, 1948). Coarse flocculation was seen in dilutions sometimes $\times 4$ higher than the M.C.P. end-point, and increased turbidity was seen just above as well as several tubes below the M.C.P. end-point in some conditions. Meyer (1947), using testicular hyaluronidase, has suggested that the M.C.P. and turbidimetric methods may not measure the same enzyme. After streptococcal hyaluronidase had been considerably purified turbidity changes were seen in synovial mucin substrate which were not apparent using purified umbilical hyaluronate + horse serum, or + crystalline albumin (horse or human). In this connexion changes in starch, pectin and dextran substrates were also best observed with purified enzymes, and moreover differences between enzymes from different strains became clearer with increasing purification. It was convenient to designate the primary depolymerizing activity as the β -activity or liquefying component, and the turbidity changes as α -activity (see p. 916). It was not clear whether two enzymes or two aspects of the same enzyme were measured.

Units. The end-point tube contained one unit of enzyme in 0.5 ml.; therefore, 1 ml. of undiluted samples contained $2 \times$ reciprocal of endpoint dilution.

Polysaccharide substrates. Dextran (*Leuconostoc mesenteroides*) was supplied in 6 % solution prepared for intravenous use by the East Anglia Chemical Co. The solution was boiled for 60 min. before use. Citrus pectin (Messrs Reckitt and Colman Ltd., Carrow Works, Norwich) was twice precipitated from aqueous solution with 1.5 vol. of pure acetone. Five samples of umbilical hyaluronate were used at different times, of which three were commercially prepared. Crystalline albumin was given by Dr R. A. Kekwick.

EXPERIMENTAL

The effect of pantothenic acid and amino-acids on activity of hyaluronidase isolated from cell free filtrates

Enzymic material was prepared from culture filtrates of ten strains of Group A streptococci (Table 1).

Table 1. *Strains of Group A streptococci used as source of extracellular enzymes*

Group A streptococci. Strains	Serological type	Polysaccharidase activity		
		Hyaluronidase	Amylase	Depolymerase
BB	Type 4 (M)	++	++	++
H713	Type 4 (M)	++	++	++
765/E	{ T antigen group 4, 24, 26, 28, 29, 46 }	++	++	++
P315		+	±	+
R1823	Type 24 (M)	—	+	+
6794	Type 28 (M)	—	+	+
63T	Type 22 (T)	++	—	+
P674	Type 22 (T)	++	—	+
4226	Type 2 (M)	—	++	+
1182	Untypable	—	+	+

(M, T = antigen identified; ++, +, ± = degrees of activity; —, enzyme component absent or inactive.)

Enzyme samples in buffer + saline were mixed with equal volumes of polysaccharide substrate (0.2 % aqueous solution), namely hyaluronate, starch, pectin and dextran. Enzyme was also distributed in samples (a) without substrate, (b) with test substance before addition of substrate, (c) with test substance after preliminary incubation with substrate. Test samples were removed at hourly or daily intervals during incubation at 37° and frozen until the end of the experiment. Changes in enzyme titre were measured in terms of hyaluronidase or amylase. Pantothenic acid (DL-calcium salt), pantothenyl alcohol, or DL-amino-acids were added in 0.002M concentration in the first instance, but tests were also made with 0.004, 0.02 and 0.04M concentrations.

The effect of incubation with substrate. Hyaluronidase titre increased after incubation with starch, pectin or dextran for 2–4 days. Amylase titre of enzymes from three hyaluronidase positive strains showed equivalent increase during incubation with hyaluronate, pectin and dextran. Enzyme from each strain showed greatest activity with one particular substrate suggesting some specificity (Table 2) which agreed with the previous estimations of predominant activity (Table 1). No bacterial growth occurred during incubation. These results suggested either that an inactive precursor was present in the partially purified material or that inactivated enzyme protein was reactivated. The possibility of adaptation in the sense that substrate protected enzyme from inactivation during incubation had also to be considered.

The effect of pantothenic acid and β-alanine. Fig. 1 shows the hyaluronidase titre of enzyme from a Type 4 strain (P315) during incubation with pantothenyl alcohol. Similar results were recorded for five enzymes from hyaluroni-

Table 2. *Increased hyaluronidase activity after incubation with four substrates: relative specificity of components*

Purified enzyme from filtrate	Relative rise of titre after 4 days, 37° with substrates (0.2 % aq. dilution)			
	Hyaluronic acid	Starch	Pectin	Dextran
H713	+++	+++	+	+
765/E	+++	+++	+	+
BB	++	++	+	+
P315	+++	+	+	+
R1823	+	++	+	+
6794	+	++	+	+/-
63T	+++	+	+++	+
P674	+++	+	++	+
4226	+	++++	+++	+/-
1182	+	++	+	+

(+ = twofold rise of titre, each additional + = $\times 2$ increase; +/- = less than $\times 2$ increase after 4 days.)

Enzymes were diluted in buffer + saline. M.C.P. and starch hydrolysis tests were made on samples taken at daily intervals. The final estimations were made after dialysis of enzyme + substrate mixture against water and buffer + saline to remove the products of the reaction.

dase positive strains. By comparison with control solutions the turbidity titre was decreased in the presence of pantothenic acid. While this effect was maintained by pantothenyl alcohol during prolonged incubation (10 days at 37°), pantothenate was less active. The results of incubation with β -alanine are shown in Fig. 2, using another Type 4 enzyme (BB). Amylase activity was used as a screening test comparing enzymes from ten strains. Pantothenic acid also suppressed dextrinogenic amylase; starch solution, however, showed loss of viscosity and flocculation though remaining blue with iodine. When hyaluronic acid, starch, pectin or dextran were added during incubation of enzyme with pantothenic acid, the rise of hyaluronidase or amylase titre was greater than with substrate alone. Only the β -activities were increased immediately; α -activities were suppressed for as long as 10 days in the presence of pantothenyl alcohol.

The effect of DL-cysteine. Figs. 1 and 2 also show the results of incubation with cysteine on the hyaluronidase activity of the same enzymes. Cysteine activated hyaluronidase, the turbidity titre showing relatively greater increase than the M.C.P. titre. Amylase was also activated, the rise of titre following the pattern of the turbidity curve. Ravel & Shive (1946) reported that biological antagonism between pantothenic acid and cysteic acid was neutralized by β -alanine.

The effect of DL-amino-acids. Thoai, Roche & Roger (1947) reported the reactivation of inactive alkaline phosphatase by alanine. Edlbacher & Wiss (1946) reported that histidine was the most efficient activator of D-amino oxidase. The effect of certain DL-amino-acids was tested using a Type 4 and a Type 22 enzyme. Hyaluronidase titre increased after incubation with serine, tyrosine or cystine, but neither alanine nor histidine affected activity.

Fig. 1. Activity of streptococcal hyaluronidase during incubation with pantothenyl alcohol and cysteine. ---, M.C.P. titre; x—x, primary depolymerase (β -activity); ●—●, increased turbidity of synovial mucin (α -activity). Purified enzyme from Type 4 streptococcus (P815) incubated for 7 days at 37° in buffer + saline, pantothenyl alcohol (0.04 % v/v) or cysteine (0.002M) added after 1 day.

Fig. 2. The effect of β -alanine on hyaluronidase activity compared with that of cysteine. ---, M.C.P. titre; x—x, depolymerase; ●—●, turbidity. Amino-acids (0.002M) added to enzyme from Type 4 strain BB. Experimental conditions as in Fig. 1.

Table 3. *The activation of purified streptococcal hyaluronidase by DL-amino-acids; the effect on enzymes isolated from six strains*

DL-Amino-acids	Concentration (M)	Effect on titre of hyaluronidase components	
		Liquefying-depolymerizing activity Stages I and II	Increased turbidity of depolymerized substrate Stage III
Alanine	0.02	—	—
β -Alanine	0.02	+	+/-
Serine	0.002	++	++
Tyrosine	0.02	+	+/-
Histidine	0.02	—	—
Cysteine	0.002	.	+++
Cystine	0.002	.	+++
Methionine	0.002	.	+++
Valine	0.02	+/-	Decrease
Arginine	0.02	—	—
Proline	0.02	+/-	Decrease

(+/- = variable; — = no change; + = twofold rise of titre, each additional + sign = further twofold rise.)

The results of tests with a number of DL-amino-acids are shown in Table 3. With the exception of methionine, only certain alanine derivatives promoted polysaccharidase activity, but valine and proline decreased hyaluronidase titre of four different enzymes. Threonine, glycine and hydroxyproline reduced the titre of one enzyme but this finding has not been confirmed. At present, constituents of proteins found in animal tissues (Rose, Haines & Johnson, 1942) and bacterial phosphoprotein have been tested.

The effect of DL-valine. Twofold or fourfold reduction of hyaluronidase titre occurred after addition of valine (Fig. 3). The effect was neutralized by equimolar concentrations of cysteine, cystine or methionine, which also neutralized the effect of pantothenic acid. After a few hours the reaction was spontaneously reversed. The required concentration of thiol or potential thiol groups to reverse the effect of either pantothenic acid or valine varied for enzymes from different strains. The action of methionine was more prolonged than equivalent concentration of cysteine or cystine. The rise of turbidity titre coincided with a fall of pH value to 6.2–6.4, and with a fall of OR potential (Methylene blue indicator). In systems containing pantothenic acid, spontaneous reversal of the effect of thiol amino-acids coincided with re-oxidation of methylene blue.

Anti-proteinase activity. Proteinase was separated from polysaccharidase by precipitation with 25 % ethanol at pH 5.6. Purified polysaccharidase showed no proteinase activity, and two of the ten strains were proteinase negative. After cysteine was used to activate hyaluronidase or amylase, proteinase was detected in some of the purified enzymes, including the two preparations from proteinase negative strains. This suggested that the material was contaminated with inactive proteinase precursor (Elliott & Dole, 1947) and it was used to

study the proteinase effect on hyaluronidase activity. It was found that high concentration of proteinase in culture filtrates was correlated with twofold reduction of hyaluronidase titre (Crowley, 1951). The inhibiting concentration was constant for the strain but showed considerable strain variation. There was no evidence to explain the effect.

Fig. 3. Decrease of M.C.P. after addition of valine, compared with the effect of β -alanine or serine. A, M.C.P. titre before addition of amino-acids, M.C.P. titre after addition of valine, $\circ$ — $\circ$; β -alanine, $\bullet$ — $\bullet$; serine, $\times$ — $\times$ (0.002M). B, reversal of valine effect by serine. C, reversal of serine effect by valine. Purified enzyme from Type 4 strain (H713). Experimental conditions as for Figs. 1 and 2.

The concentration of proteinase in purified polysaccharidases varied widely between different preparations. The appearance of proteinase coincided with a fall of pH value to pH 6.0 or lower, and increasing proteinase activity was associated with decreasing hyaluronidase activity.

Adjustment of the pH to 7.4 with Na glycerophosphate partially neutralized the effect of increasing proteinase activity. The addition of hyaluronic acid also delayed the usual fall of hyaluronidase titre. These results suggested that proteinase inactivated but did not destroy hyaluronidase.

Not unexpectedly the activation of precursor by cysteine was prevented by pantothenic acid and by valine in the experimental conditions described. Pantothenyl alcohol suppressed proteinase activity indefinitely when the autocatalytic conversion of precursor would normally be expected. This result may perhaps be explained by a change in OR potential in the presence of pantothenic acid. Using methylene blue as a rough indicator 'turbidity' hyaluronidase and dextrinogenic amylase were activated at a higher OR potential than proteinase.

The effect of amino-acids on material isolated from inactive cultures

Hyaluronidase activity was reduced in cultures grown at 23° in filtered broth, and in broth containing proteose peptone or bicarbonate buffer (Crowley, 1951). Inactive cell-free filtrates were treated with $(\text{NH}_4)_2\text{SO}_4$ to give 25, 50

Fig. 4. The M.C.P. titre of material prepared from inactive culture after incubation with amino acids. M.C.P. titre of control, ----; M.C.P. titre with ●—●, serine; x—x, methionine; —, cysteine; ○—○, β-alanine. Amino-acids in 0.002M concentration, added to solution of fraction precipitated by $(\text{NH}_4)_2\text{SO}_4$, 0.5 sat., from inactive culture filtrate. Two-day culture of Type 4 strain BB, grown at 23° proteose peptone filtered broth. The volume of solute was approximately one-tenth of the original volume of filtrate.

and 75 % saturation. Weakly active material was precipitated at all concentrations. This material was incubated with test amino-acids in the same way as the active enzymes.

The effect of amino-acids. Fig. 4 shows the results of M.C.P. tests on material from inactive cultures of a Type 4 strain (BB) before and after incubation with β-alanine, serine, cysteine and methionine.

The rise of hyaluronidase titre followed the pattern usually seen when this

strain was grown at 37° in culture medium favourable for hyaluronidase production.

The final yield of active enzyme was greatest from cultures grown at 23° in proteose peptone filtered broth buffered with sodium β -glycerophosphate. This medium also contained the greatest number of viable organisms. Two- or three-day cultures were treated with 3 vol. saturated $(\text{NH}_4)_2\text{SO}_4$ solution. An aqueous suspension of the precipitate was treated with a 75–80 % saturated solution of $(\text{NH}_4)_2\text{SO}_4$, resuspended and twice reprecipitated. After dialysis against running water until the dialysate gave no colour with Nessler reagent, and against buffer + saline, no viable organisms were detected. Any insoluble material was removed by filtration through paper, and the solution was filtered through a Seitz pad. After incubation with serine + methionine, hyaluronidase titre showed 12-fold increase. It seemed possible that products in the immediate environment of the organism, or intracellular constituents, provided a rich source of pro-enzymic material.

DISCUSSION

The possibility of an inactive hyaluronidase precursor analogous to streptococcal proteinase precursor (Elliott & Dole, 1947) was previously postulated on the basis of the similarity of environmental conditions affecting production of both enzymes (Crowley, 1951). The experiments described suggest that a component of a complex hyaluronidase or polysaccharidase may be derived from an inactive pro-enzyme and be activated by SH groups at a higher pH value and OR potential than proteinase. There is no evidence that the increased turbidity of synovial fluid substrate seen with purified streptococcal enzymes + potential thiol groups was in fact due to a hyaluronidase. All the material was contaminated with proteinase precursor, and the possibility of an artefact seems worth consideration. It might be possible that in some circumstances proteinase could attack the protein moiety of a protein-polysaccharide complex. In this connexion increased turbidity of synovial mucin has frequently been seen with crude culture filtrates of Group A streptococci of various serological types when hyaluronidase activity could not be demonstrated by any other method (unpublished data). These strains had one feature in common, namely, proteinase activity.

As a third possibility the amino-acids may have reversed inhibitions by factors at present unknown. Meyer & Rapport (1951) have shown that SH compounds including cysteine reversed the inhibition of testicular hyaluronidase by heavy metals. Weil-Malherbe (1948) suggests that apparent activation of enzymes by amino-acids is a non-specific protective affect against the hazards of purification *in vitro*. The inactive material prepared from cultures and cell free filtrates which subsequently yielded active enzymes did not merit the description 'purified'. The same groups of amino-acids appeared to activate hypothetical components in both crude and considerably purified material. There is insufficient evidence at present to support a theory of specific activity by amino-acids or by the pantoyl residue in relation to these streptococcal enzymes.

I wish to thank Dr Alexander Lawson and Dr J. H. Humphrey who gave me valuable help and advice.

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A NOTE ON THE ACTIVITY OF FRACTIONS OBTAINED DURING THE ISOLATION AND PURIFICATION OF COMPONENTS OF STREPTOCOCCAL HYALURONIDASE

The enzymes used above were isolated during attempts to purify and separate components of streptococcal polysaccharidase. Finally there was insufficient material for the usual criteria of purity to be applied.

Isolation of material

Filtrates of 2-day broth cultures (37°) were treated with $(\text{NH}_4)_2\text{SO}_4$ to bring to 70 % saturation, and left standing at bench temperature for 3 days. The precipitate was suspended in distilled water, filtered through paper, and the paper washed with phosphate buffer (pH 7.0). The solution was treated with $(\text{NH}_4)_2\text{SO}_4$ at 25, 50 and 75 % saturation successively (Table 1). The bulk of active material was precipitated at 50 % saturation, and this fraction contained streptococcal proteinase. The fraction was dissolved in water and reprecipitated by $(\text{NH}_4)_2\text{SO}_4$ at a lower pH value, when better separation was obtained. No progress was made until two types of polysaccharidase activity were distinguished: (1) depolymerizing activity, and (2) dextrinogenic amylase activity, which was associated with hyaluronidase measured by turbidity, as

described on p. 907 of this *Journal*. These activities were designated β and α and further purification was attempted following the method of Wittler & Pillemer (1949) using methanol or ethanol at varied concentration and pH value in the cold.

Separation of polysaccharidase components by solubility in alcohol + water mixtures

The material isolated by salting out with $(\text{NH}_4)_2\text{SO}_4$ was dialysed against running water until the dialysate was Nessler negative, and against phosphate buffer until the bulk of material was in solution. The protein concentration

Table 1. *Activity of polysaccharidase components obtained during isolation and purification with $(\text{NH}_4)_2\text{SO}_4$*

(Precipitated at bench temperature, pH value adjusted by $\text{Na}_2\text{HPO}_4 + \text{NaH}_2\text{PO}_4$ (M/15 conc.). Precipitates dissolved in distilled water.)

$(\text{NH}_4)_2\text{SO}_4$ (% saturation)	pH	Amylase	Hyaluronidase	Proteinase
25	7.0	+	+	+
50	7.0	++++	++++	++++
75	7.0	+/-	++	+/-
50 % fraction,				
$(\text{NH}_4)_2\text{SO}_4$				
50	6.0	++	++	+
.	6.4	++++	++++	+
70	7.0	+/-	++	-
> 70	7.0	+/-	+	-
Polysaccharidase activity				
50 % pH 6.4 fraction		Components activated by thiol groups α	Depolymerases liquefying components β	
$(\text{NH}_4)_2\text{SO}_4$ (%)	pH value			
50	6.0-6.4	++++	+	
70	7.0	+	+++	
> 70	7.0-7.4	+	++	

++++ to +/- = relative activity.

of the soluble material from ten strains varied between 10.9 and 18.9 %. The results of fractional precipitation by methanol or ethanol are shown in Tables 2 and 3. Fraction I showed dextrinogenic amylase and 'turbidity' hyaluronidase activity. Precipitates were formed with 26 % Na_2SO_4 (at 37°), with 50 % ZnSO_4 and 3 % sulphosalicylic acid. The protein-copper test was positive, and the material was precipitated from solution by dialysis against water. Non-specific depolymerase was associated with Fraction II, which showed activity against hyaluronic acid, starch, pectin and dextran as substrates. Deoxyribonuclease tested by the method of McCarty (1948) was present in Fraction II of all ten strains. Precipitates formed with tannic acid, trichloroacetic acid and lead salts, but not with 26 % Na_2SO_4 at 37°. The protein-copper test was negative. After addition of cysteine, streptococcal proteinase was detected in this fraction, suggesting that inactive precursor was present. Elliott (1950) reported that inactive precursor was

Table 2. *Solubility of polysaccharidase components in methanol or ethanol at various pH values*

(Material was precipitated at -10° ; precipitates were dissolved in phosphate buffer. Protein concentration in solutions 3–5 %.)

Methanol (%)	pH value	Polysaccharidase activity	
		β	α
25	6.4	++	++++
40	6.0	++++	+
	5.6	+	+
	4.8	+	+
	4.2	+	+
70	5.6	+	+
	7.0	++	+

Ethanol (%)	pH value	Polysaccharidase activity	
		β	α
18	5.2	+	+++
25	6.4	+	++++
40	5.6	+	+/-
	4.8	++++	+/-
	4.2	+++	+/-
70	5.6	++	+
	6.8	++	+

+ = activity based on unit/mg. PN_2 , each additional + = twofold increase;
+/- = weak activity.

Table 3. *Activity of polysaccharidase components after further purification with methanol + water mixtures*

Fraction I, 25 % methanol, pH 6.4			
Methanol (%)	pH value	Polysaccharidase components	
		α	β
25	6.4	++++	+
40	6.0–4.8	No precipitate	
50	4.2	Faint precipitate	
> 50 + CaCl_2 , 0.0025 M	4.2–6.8	+/-	++

Fraction II, 40 % methanol, pH 6.0			
Methanol (%)	pH value	Polysaccharidase components	
		α	β
25	6.4	No precipitate	
40	4.8	+/-	++++
70	5.6	+	++

Symbols as Table 2.

more soluble than active proteinase. Inactive α -components in Fraction II were demonstrated by activation with cysteine or methionine. It is not clear whether the enzymes were inactivated during purification or whether inactive precursor was present in the material.

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(Received 1 February 1951)