The Influence of Hydrolysates of Hyaluronate upon Hyaluronidase Production by Micro-organisms

By H. J. ROGERS (Beit Memorial Research Fellow)
The Lister Institute of Preventive Medicine Elstree, Herts

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It has been shown (McClean & Hale, 1941; McClean, 1941; Rogers, 1945) that hyaluronidase production by certain streptococci and *Cl. welchii* is increased adaptively by the inclusion of hyaluronate in growth media for these organisms. Enzyme production by streptococci is similarly increased by including the products formed from hyaluronate by the action of testicular hyaluronidase at pH 7·0, but not by the addition of substances resulting from the hydrolysis of the polysaccharide by hyaluronidase derived from streptococci (Rogers, 1945).

The present paper extends these observations to a study of the influence of other hydrolysates of hyaluronate upon hyaluronidase production by *Strep. haemolyticus* Group C (Lancefield) type 7 (Griffiths), in the sequel called the streptococcus C7, and by a strain of *Cl. welchii*. The relation between the amount by which enzymic hydrolysates stimulate hyaluronidase formation and the degree to which they inhibit the viscosity reducing activity of the enzymes has also been studied. A direct relation between these two values might be expected if Yudkin's (1938) mass action theory for the mechanism of adaptive enzyme formation is applicable to the production of hyaluronidase by micro-organisms.

METHODS

The medium and technique for growing the organisms used, together with the methods of estimating growth and the amount of enzyme produced, were as previously described (Rogers, 1945). Reducing sugars were estimated by Somogyi's (1937) reagent standardized with an equimolar mixture of *N*-acetylglucosamine and glucurone. Glucosamine was estimated by Elson & Morgan's (1933) method, *N*-acetylglucosamine by Morgan & Elson's (1934) method, and nitrogen by the micro-Kjeldahl method.

Preparation of the enzymes used for hydrolysis

(1) *Testicular hyaluronidase*

(*a*) Crude preparation. Fresh bulls' testes from the abattoir were ground with five times their weight of distilled water. The pulp obtained was filtered through glass-wool and the filtrate frozen at $-10°$. Measured samples of this extract were thawed and spun at 2–3000 r.p.m. for 15 min. on an angle centrifuge immediately before use. The enzyme titre of the supernatants was 290 viscosity reducing units (V.R.U.; McClean & Hale, 1941; McClean, 1943).

(*b*) Partially purified preparations were made by the method of Madinaveitia (1941) or by that of Morgan & McClean (1932).

(2) *Streptococcal hyaluronidase*

(*a*) Crude preparation. A filtrate from a culture of the streptococcus C7 grown in a peptone medium was saturated to 70% with $(NH_4)_2SO_4$. The precipitate obtained was collected and dialyzed in the presence of toluol against running tap water until free of $(NH_4)_2SO_4$. This solution was used as the crude hyaluronidase preparation.

(*b*) Partially purified preparation. The streptococcus C7 was grown for 24 hr. in the casein hydrolysate medium B (Rogers, 1945) containing 0·2 g./100 ml. crude potassium hyaluronate. The organisms were removed by mixing the culture with kieselguhr and filtering through fluted papers. The filtrate was then dialyzed against running tap water for 24 hr. in the presence of toluol. The dialysate was adjusted to pH 5·6 with 2N-acetic acid and 50 ml./l. of a solution of dialyzed iron (British Drug Houses Ltd.) added. The precipitate of $Fe(OH)_3$ was allowed to remain in contact with the dialysate for 15–20 min. after which it was removed by centrifugation through a Sharples super-centrifuge. The enzyme was eluted from the $Fe(OH)_3$ with 0·2M-Na_2CO_3. It was sometimes necessary to perform as many as five elutions in order to recover the maximum amount of enzyme. The yield from this process varied from 20 to 50%; further attempts are being made to improve the method.

(3) Cl. welchii *hyaluronidase*

(*a*) Crude preparation. This was made from the filtrate from a culture of *Cl. welchii* type A, strain 107, by the same technique as that used for the crude preparation of hyaluronidase from the streptococcus C7.

(*b*) Partially purified preparation. A concentrated filtrate from cultures of *Cl. welchii*, strains 107, which had already been fractionated by $(NH_4)_2SO_4$ precipitation was kindly provided by Dr W. E. van Heyningen (The Wellcome Physiological Research Laboratories). This concentrate had an enzyme titre of 122,000 V.R.U. and contained 37·8 mg. N/ml.; 10 ml. were mixed with buffer (12 ml. of McIlvaine buffer at pH 4·6) and water (20 ml.) and passed through a column 8 cm. long × 2 cm. diameter of Al_2O_3 (Savory and Moore Ltd. London), using very gentle suction. The column was washed with 50 ml. of water, and phosphate buffer (0·1M at pH 7·0 or 0·1M-Na_2HPO_4) then passed down the column. Successive fractions of 1–3 ml. of the effluent were taken and tested by the mucin clot prevention test (McClean, 1943); the most active fractions were checked by the more exact V.R.U. method. The passage of buffer was continued until no further enzyme could be detected in the effluent. Using this procedure it was found that about 50%

of the enzyme applied to the column was obtained in three of the fractions with a total volume of 3–4 ml. These fractions contained a total of only 4% of the original N in the preparation. Similarly promising results were obtained with four other crude preparations of *Cl. welchii* hyaluronidase. One preparation, however, failed to give a sharp band, and the method in its present form is not applicable to the purification of streptococcal hyaluronidase. Further studies of the chromatographic method for the purification of this group of enzymes are now to be undertaken.

EXPERIMENTAL

The hydrolysis of hyaluronate by hyaluronidase of different degrees of purity

The streptococcal enzyme used in the previous work (Rogers, 1945) was a relatively crude preparation, whereas the testicular hyaluronidase used here had been partially purified by Morgan & McClean's (1932) method. It was previously suggested (Rogers, 1945) that the different behaviour of the hydrolysates produced by these two enzyme preparations might be explained by this difference in degree of purification. To test this hypothesis, the following series of hydrolysates was made:

Pairs of enzyme preparations of differing purity (as indicated by the ratio of enzyme activity to their content of total N) were used to hydrolyze solutions of purified potassium hyaluronate under the following conditions:

The hyaluronate solution (10 ml. of 0·5 g./100 ml. solution) was mixed with 2·5 ml. of McIlvaine buffer (0·1 M, pH 7·0 and containing 0·6 M-NaCl) and incubated at 37° with the hyaluronidase preparations indicated in Table 1, until no further reducing sugar was liberated; chloroform was present throughout to prevent bacterial growth. From 600 to 1200 V.R.U. of hyaluronidase were used for the 12·5 ml. of the substrate buffer mixture.

The hydrolysates were sterilized and the enzymes they contained inactivated by heating in a boiling water-bath for 15 min.; the pH of the solutions was first lowered to 6·5. This process produced very much less caramelization than did autoclaving (Rogers, 1945) and the enzymes were always completely inactivated. Sterility was tested by adding 1 ml. of the treated hydrolysates to 4 ml. of a glucose-peptone broth and incubating this mixture for 48 hr. at 37°. An occasional hydrolysate contained resistant organisms and was autoclaved for 15 min. at 115°. The technique for testing the influence of the hydrolysates upon hyaluronidase production by the streptococcus C7 was as previously described (Rogers, 1945). Tubes of medium were also supplemented by buffer and inactivated enzyme as controls.

All the hydrolysates listed in Table 1 were not tested in the same experiment. For this reason both the absolute stimulation (Rogers, 1945), which varies somewhat with different seed cultures, and the ratio of this to the stimulation caused by an equivalent concentration of unhydrolyzed hyaluronate, are given. This ratio has been found to be constant for any one hydrolysate from experiment to experiment. Table 1 shows the influence upon hyaluronidase production by the streptococcus C7 of adding the various enzymic hydrolysates of hyaluronate to the casein hydrolysate medium B (Rogers, 1945).

Irrespective of the purity of the enzyme preparations used, the hydrolysis products formed by testicular hyaluronidase cause the same degree of stimulation, within the experimental error of the method, as an equivalent concentration of unhydrolyzed hyaluronate. The streptococcal and *Cl. welchii* type A strain 107 hyaluronidase preparations all left hydrolysis products which had little influence upon hyaluronidase production by the streptococcus C7. Hence the different behaviour of the hydrolysates produced using testicular hyaluronidase and these two bacterial enzymes cannot be explained on the basis of the purity of the enzyme preparations.

Table 1. *The influence of various enzymic hydrolysates of hyaluronate upon hyaluronidase production by the streptococcus C7*

Source of enzyme used for hydrolysis	Preparation	Purity of enzyme (V.R.U./mg. N)	Hydrolysis* of substrate (%)	Stimulation (*S*)	*S/W*†
Bull testes	A‡	—	57	3·12	0·80
	B§	120–320	54·2–78·0	2·1–4·7	1·03–1·09
Cl. welchii	A‡	3470	65	0	0
	B§	58,100	54·2	0·11	0·04
Streptococcus C7	A‡	520	83	0·30	0·26
	B§	4550	45·8	0·41	0·17

* Measured by the release of reducing sugars from the polysaccharide.

† $S/W = \dfrac{\text{Stimulation by hydrolysate}}{\text{Stimulation by an equivalent amount of unhydrolyzed hyaluronate}}$.

‡ Crude preparation. § Partially purified preparation.

Hydrolysis of hyaluronate by mild treatment with acids

Hydrolysis of hyaluronate by strong acids such as 2–4N-HCl yields glucosamine, acetic acid and various degradation products of glucuronic acid. Thus such treatment not only hydrolyzes the glycosidic bonds of the polysaccharides, but disrupts the constituent sugar derivatives. The mixtures obtained were not suitable for an investigation of the influence of acid hydrolysates of hyaluronate upon hyaluronidase production. Hydrolysis of hyaluronate at the temperature of a boiling water-bath by N-acetic acid yields *N*-acetylglucosamine as determined by the Morgan & Elson (1934) test (Rogers, 1946). After 30 hr. treatment, 100% of the *N*-acetylglucosamine predicted to be present in the polysaccharide from its hexosamine content had been liberated. Some destruction of glucuronic acid does take place during this treatment. Experiments, however, using more dilute acid, did not result in less destruction of glucuronic acid for an equivalent release of *N*-acetylglucosamine.

The addition of N-acetate solution to the test medium somewhat suppressed hyaluronidase production by the streptococcus C 7 and acetic acid was thus an undesirable hydrolytic agent. An oxalic acid-sodium oxalate mixture at pH 2·3 was found to be satisfactory and the oxalate could be removed as the calcium salt before the hydrolysates were added to the test medium. The conditions of hydrolysis were as follows:

Purified hyaluronate (0·2 g.) was dissolved in 40 ml. of distilled water. Oxalic acid (2·522 g.) was then added, dissolved, and the pH of the mixture adjusted to 2·5 with N-NaOH, using a glass electrode. A precipitate of sodium oxalate formed and was removed by filtration; the volume of the filtrate was adjusted to 50 ml. with washings from the precipitate. The solution was then heated under reflux in a boiling water-bath and samples (12 ml.) were withdrawn at appropriate time intervals. These were cooled and approximately 0·5 g. of $CaCO_3$ added in small portions with mechanical stirring. The calcium oxalate and unchanged carbonate were then removed by centrifuging and the small amounts of oxalate remaining in the supernatant fluid were precipitated by the addition of $CaCl_2$ solution. Glucosamine was estimated before and after removal of the oxalate to check that no adsorption of the hydrolytic products by the precipitated Ca salts had taken place; *N*-acetylglucosamine was determined in the final supernatant liquids. The hydrolysates were neutralized to pH 7·0 and sterilized by autoclaving at 115° for 15 min. during which no caramelization took place.

The influence of the hydrolysates upon hyaluronidase production by streptococcus C 7 was tested in the usual manner. Table 2 shows that the ability of hyaluronate to stimulate hyaluronidase production by the streptococcus C 7 was progressively destroyed during hydrolysis. It will be seen from Table 2 that when 70% of the calculated amount of *N*-acetylglucosamine had been released, the hydrolysis products had no influence on enzyme formation. This hydrolysis by acids resembles that by streptococcal and *Cl. welchii* hyaluronidase. It may be noted that the degree of stimulation caused by the acid hydrolysates is consistently less than that to be expected from the amount of *N*-acetylglucosamine calculated to be still in unhydrolyzed units (i.e. not reacting to give colour with the *p*-dimethylaminobenzaldehyde reagent under the conditions used by Morgan & Elson, 1934).

Table 2. *The influence of hydrolysis of hyaluronate by an oxalic acid-sodium oxalate mixture at pH 2·5 upon the ability of the hydrolysis products to influence hyaluronidase formation by the streptococcus C 7*

Hydrolysis* (%)	Stimulation (Observed)	(Calculated)†
0	4·30	—
16·7	2·90	3·50
43·7	1·46	2·60
70	0	1·29

* Measured by the release of *N*-acetylglucosamine.

† Calculated from the amount of bound *N*-acetylglucosamine still present in the hydrolysate.

The influence of enzymically hydrolyzed hyaluronate on hyaluronidase production by Cl. welchii *type A strain A 118 d*

Gale & van Heyningen (1942) found that maximum amounts of hyaluronidase were produced by a strain of *Cl. welchii* type A after 6–7 hr. growth in a peptone medium. They stated, however, that more hyaluronidase was sometimes found in 24 hr. cultures. Hahn (1945) working with a peptone medium, and Byers, Tytell & Logan (1945) using a meat digest medium containing hyaluronate, both found that the amounts of enzyme in filtrates from *Cl. welchii* cultures were greater when the incubation period was increased from 24 hr. to 5 days.

In the present work it was found, with strain A 118*d* of *Cl. welchii* and the casein hydrolysate Medium A (Rogers, 1945), that considerable variation occurred in the rate of production of hyaluronidase. Whereas in some experiments maximum amounts of this enzyme were produced after only 18 hr. incubation, in others hyaluronidase formation continued up to 4–5 days' incubation. This slow production of the enzyme was very much more pronounced in media supplemented by hyaluronate. Enzyme formation by the organisms growing in the unsupplemented media was usually maximal after 18–40 hr. incubation. These observations made necessary considerable changes in the technique

hitherto found satisfactory for examining the influence of the composition of the growth medium upon hyaluronidase production by organisms. The new technique was as follows:

Hyaluronidase production in all the cultures in an experiment was measured at 24 hr. intervals until no further increase in enzyme formation could be detected. The mucin clot prevention test (McClean, 1943) was used in the preliminary work. The cultures at the end of this period were centrifuged and the amount of hyaluronidase was estimated in the supernatants by the more accurate V.R.U. method. When hyaluronidase production had ceased, the bacterial cells had very largely autolyzed. It was for this reason not possible to estimate the mass of bacterial growth which had occurred. Consequently the stimulation caused by both hyaluronate and the preparations of enzymically hydrolyzed hyaluronate was calculated simply from the amount of enzyme in the culture supernatants without correction for variations in growth.

The influence of the concentration of hyaluronate upon hyaluronidase production by Cl. welchii *strain A* 118*d*

The stimulation of hyaluronidase production by the streptococcus C7 was directly proportional to the concentration of hyaluronate present in the medium, up to 0·6 g./100 ml. (Rogers, 1945). The influence of the polysaccharide content of the medium upon hyaluronidase production by *Cl. welchii* strain A118*d* was investigated as follows:

Volumes of a sterile solution of crude potassium hyaluronate (0·5 g./100 ml.) were added to 5 ml. of double-strength test medium A (Rogers, 1945) to give final concentrations of 0·03, 0·06, 0·09, 0·125, and 0·180 g./100 ml. of the polysaccharide. Sufficient sterile distilled water was then added to adjust the volumes in the tubes to 10 ml. The media all contained glucose (0·25 g./100 ml.) and each tube was inoculated with one drop of an 18 hr. meat culture of the organism. Two tubes of each test medium were inoculated.

This experiment was repeated several times. There was considerable variation in the degree of stimulation caused by the same amount of hyaluronate from experiment to experiment. Fig. 1 shows the final amounts of enzyme in the cultures from two such experiments; the variation was probably due to differences in the seed culture rather than to uncontrolled variation of the medium since the same batch of medium and of hyaluronate solution was used for two experiments quoted and results for the duplicate pairs of cultures in different experiments agreed within 10%.

In the next series of experiments enzymically hydrolyzed hyaluronate was added to the culture medium. The technique for the preparation of the hydrolysates was as already described when investigating their action upon the streptococcus C7. Partially purified enzyme preparations were used for hydrolysis. Each 10 ml. of casein hydrolysate growth medium contained 2·0 ml. of the hydrolysates. The technique for examining hyaluronidase production by *Cl. welchii* strain A118*d* was as already described.

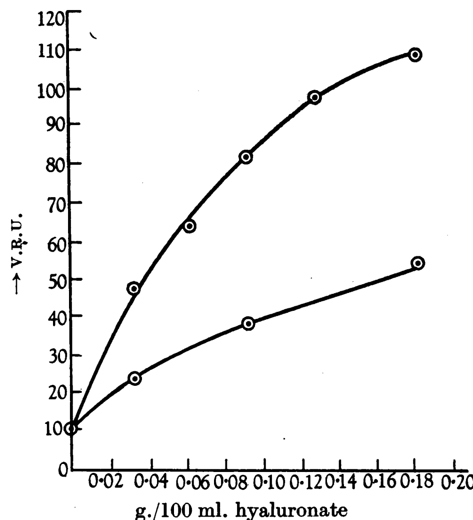

Fig. 1. The influence of the concentration of hyaluronate (g./100 ml.) present in the casein hydrolysate medium on hyaluronidase production measured in V.R.U. (McClean & Hale, 1941) by *Cl. welchii* type A strain A118*d*. The results of two separate experiments are plotted in the figure; different seed cultures were used for these two experiments.

The products formed by testicular enzyme stimulated hyaluronidase production. Table 3 shows that hydrolysates prepared using purified streptococcal and *Cl. welchii* enzymes also increased enzyme production. The stimulation in the medium containing hyaluronate hydrolyzed with streptococcal hyaluronidase is sufficiently high to be significant. The result in the medium supplemented

Table 3. *The influence of various enzymic hydrolysates of hyaluronate upon hyaluronidase production of* Cl. welchii *strain A* 118*d*

Source of enzyme used for hydrolysis	Hydrolysis* of substrate (%)	Stimulation	*S/W*†
Bull testes	30·5	3·49	1·16
Cl. welchii	54·2	1·35	0·44
Streptococcus C7	45·8	2·44	0·81
0·06 g./100 ml. unhydrolyzed hyaluronate		1·94	—
0·12 g./100 ml. unhydrolyzed hyaluronate		3·62	—

* Measured by the release of reducing sugars from the polysaccharide.

† Stimulation observed/stimulation by an equivalent concentration of unhydrolyzed hyaluronate.

with the products formed by the *Cl. welchii* enzyme is less definite but is probably significant. Owing to the less accurate nature of the test that had to be used for this organism, however, it is not possible to be quite certain of the significance of this latter result.

The inhibition of streptococcal and Cl. welchii *hyaluronidase*

Yudkin (1938) suggested that the adaptive formation of enzymes by micro-organisms was a mass-action phenomenon. In this theory the small amounts of enzyme initially formed by the organism combine with the substrate leading to the production of more enzyme. As emphasized by Stephenson (1939) substances which competitively inhibit an enzyme should also stimulate its microbiological production if Yudkin's hypothesis is correct. McClean (1942) showed that heparin and chondroitin sulphuric acid both inhibited the action of purified testicular hyaluronidase upon hyaluronic acid and upon the capsules of Lancefield group A streptococci. These capsules are known to consist of hyaluronate.

It has been confirmed by viscosimetric test that both heparin and chondroitin sulphuric ester inhibit the action of a partially purified preparation of streptococcal hyaluronidase upon hyaluronic acid, e.g. 0·21 mg./ml. of heparin (Roche Products Ltd.) in the final solution used for the viscosity reduction test (McClean & Hale, 1941; McClean, 1943) reduced the titre of the enzyme from 256 to 126 V.R.U. It was, however, previously reported (Rogers, 1945) that neither of these polysaccharides increases hyaluronidase production by the streptococcus C7.

The influence of the heated enzymic hydrolysates of hyaluronate upon the action of streptococcal and *Cl. welchii* hyaluronidase has now been examined as follows:

Mixtures containing increasing ratios of amounts of enzyme to hydrolysate were prepared and then diluted so that 0·5 ml. of the mixture would reduce the viscosity of the batch of hyaluronate used for viscosimetric determinations to half its initial value in approximately 20 min. under the usual conditions of the test. The apparent strength of the enzyme in the mixture was calculated in the usual manner (McClean & Hale, 1941).

Fig. 2 shows the results obtained when two partially purified enzyme preparations were used in these tests. The actual degree of inhibition varied according to the nature of the enzyme preparation. For example, there was very little inhibition by either of the hydrolysates when an unmodified filtrate from a streptococcus C7 culture was used as a source of enzyme. The somewhat greater inhibition of streptococcal hyaluronidase caused by the hydrolytic products formed by testicular enzyme appeared significant. When, however, the degree of inhibition of a streptococcal enzyme preparation by the dialysate and diffusate from this hydrolysate was tested it was found that 85% of the inhibiting substance was present in the diffusate. It was previously found (Rogers, 1945) that all the substance stimulating hyaluronidase production by the streptococcus C7 was present in the dialysate, the diffusate having no influence upon enzyme formation. Attempts were made to determine whether the inhibition of enzyme activity caused by the hydrolysate and diffusate were competitive. The viscosity reduction test, however, does not lend itself easily to such experiments since increased substrate concentration leads to very long times of flow of the substrate-enzyme mixture through the viscometer and great inaccuracies in determining enzyme titres. The results, however, did indicate a somewhat decreased degree of inhibition when the substrate concentration was raised from 0·12 to 0·5 g./100 ml. From these experiments it does not seem that Yudkin's (1938) hypothesis can be applied to the microbiological production of hyaluronidase.

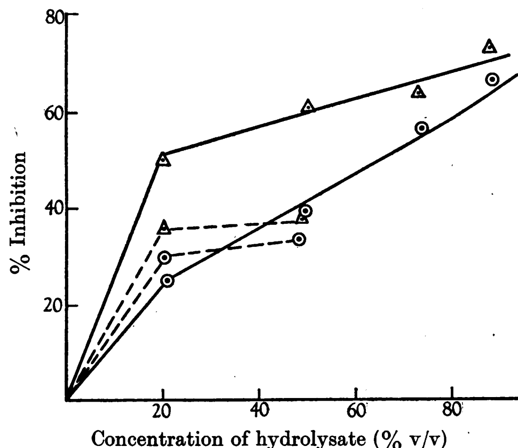

Fig. 2. The influence of increasing concentration (% v/v in the mixture diluted for test) of hyaluronate hydrolyzed by different enzyme preparations on the activity of hyaluronidase produced by the streptococcus C7 and by *Cl. welchii* strain S107. (1) Hydrolysis by testicular hyaluronidase: effect upon (*a*) streptococcal enzyme △—△, (*b*) *Cl. welchii* enzyme △---△. (2) Hydrolysis by streptococcal hyaluronidase: effect upon (*a*) streptococcal enzyme ⊙—⊙, (*b*) *Cl. welchii* enzyme ⊙---⊙.

DISCUSSION

The influence of enzymic hydrolysates of hyaluronate upon the production of hyaluronidase by those organisms which form this enzyme adaptively appears to be a delicate qualitative method for

distinguishing between the hydrolysates. The substances obtained when the polysaccharide is broken down by testicular hyaluronidase at pH 7·0 are still as active in stimulating enzyme production by both multiplying streptococci and by *Cl. welchii*, as is the undegraded hyaluronate. Chemically these hydrolysates are distinguished (Rogers, 1946) from those obtained by using streptococcal or *Cl. welchii* hyaluronidase, by the presence of oligosaccharides not diffusible through cellophan, but which show very considerable reducing power towards the alkaline copper reagent of Somogyi (1937). The results suggest that the smaller but still relatively complex molecules of oligosaccharide, such as are present in these hydrolysates, are as effective as the molecules of undegraded polysaccharide in stimulating the organisms. If the hyaluronate molecules are attacked by the enzymes in the testicular preparations primarily at their ends, then the oligosaccharides remaining would be in the same molecular concentration as the original polysaccharide. This might explain the quantitatively equivalent response of the organisms to the hydrolyzed and unhydrolyzed hyaluronate. It has also been shown (Rogers, 1946) that after hydrolysis of hyaluronate at pH 7·0 by streptococcal hyaluronidase, complex but smaller, diffusible molecules remain. These hydrolysates have little or no influence upon hyaluronidase production by streptococci. Thus it would seem that relatively large molecules are necessary to stimulate these organisms. The smaller molecules are, however, probably effective for *Cl. welchii*. This result would appear to indicate an interesting difference in the mechanism of adaptive enzyme formation by the two organisms.

The comparative rates of production of hyaluronidase by *Cl. welchii* and streptococci are particularly interesting in light of the statement by Meyer, Hobby, Chaffee & Dawson (1940) that no hyaluronidase can be extracted from the cells of *Cl. welchii* grown on a medium not containing hyaluronate, but that, as confirmed by general experience, the culture filtrates are a potent source of enzyme. They found that the reverse situation was true for pneumococcal cultures. It appears from the results in the present paper, however, that in some cultures most of the enzyme production by *Cl. welchii* takes place while the organisms are autolyzing. Casein hydrolysate media are the only ones that have been used but the results of Hahn (1945) and Byers *et al.* (1945) indicate that enzyme must also appear in culture-filtrates during autolysis of organisms grown on a peptone medium; but again Gale & van Heyningen's (1942) results suggest that this may only occasionally be true. It would be of interest to know more of the conditions which determine whether the enzyme shall be secreted freely during the growth phase of the organisms or only released on autolysis of the cells. With the strain of streptococcus used here (C 7), and grown on the casein hydrolysate growth medium, hyaluronidase was always produced during the active growth of the organisms, although Meyer, Chaffee, Hobby & Dawson (1941) found that hyaluronidase was present in both the cells and the cell-free supernatant fluid from cultures of streptococci.

SUMMARY

1. Potassium hyaluronate, hydrolyzed by crude or purified testicular hyaluronidase, can stimulate hyaluronidase formation by the streptococcus C 7 or *Cl. welchii* strain A 118*d* equally as well as the unhydrolyzed polysaccharide.

2. Potassium hyaluronate hydrolyzed by streptococcal or *Cl. welchii* enzymes does not stimulate hyaluronidase production by the streptococcus C 7. The streptococcal hydrolysate does, however, stimulate hyaluronidase production by *Cl. welchii* A 118*d*, whilst the *Cl. welchii* hydrolysate has a small but probably significant influence on this organism.

3. Hydrolysis of hyaluronate by mild treatment with acids destroys the ability of the polysaccharide to enhance hyaluronidase production by the streptococcus C 7.

4. The ability of substances to inhibit competitively the activity of hyaluronidase is not correlated with their ability to stimulate microbiological formation of the enzyme.

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