The Conditions Controlling the Production of Hyaluronidase by Micro-organisms Grown in Simplified Media

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The purpose of the present work was to design protein- and peptone-free media (to be called 'simplified media') in which various micro-organisms would grow and synthesize hyaluronidase. The antigenically specific enzymes thus produced could then be purified from the culture filtrates and examined. In this work it has been found that various groups of organisms have different nutritional requirements for the production of hyaluronidase. A study of the response of organisms to the inclusion of hyaluronate and its breakdown products in the medium has revealed that hyaluronidase preparations from various sources differ in the degree to which they hydrolyze hyaluronate. Brief preliminary reports of this work have already appeared (Rogers, 1944*b, c*).

METHODS

The amounts of hyaluronidase in the supernatant fluids from 16–18 hr. centrifuged cultures were determined by the mucin clot-prevention (M.C.P.) method (McClean, 1943) using twofold dilutions, and by the viscosity reduction method (McClean & Hale, 1941) as modified by McClean (1943) (this modification allows the accurate determination of streptococcal enzyme; Hale, 1944). A single batch of potassium hyaluronate was used as substrate for all the enzyme estimations. The enzyme titres of the samples, as assayed by either test, are recorded in the tables as comparisons with a dried preparation of testicular hyaluronidase of known potency. The mucin clot-prevention results are recorded (under M.C.P.) as reciprocals of the enzyme titres; viscosity reduction results are recorded as viscosity reduction units (under V.R.U.) (cf. McClean & Hale, 1941; McClean, 1943).

Bacterial-N was estimated by the micro-Kjeldahl method upon twice-washed deposits, centrifuged from 10 ml. of culture. Both the quantity of the enzyme produced and the amount of bacterial-N were determined in duplicate cultures for each modification of the medium. The results given in the protocols represent the mean of these two determinations. The amount of enzyme synthesized by unit mass of bacterial cells was calculated from the ratio: Mean V.R.U./Mean value for mg. bacterial-N per 10 ml. of culture. When enzyme synthesis by an organism was to be compared in a series of different media all the tubes were inoculated from the same seed culture.

Glucosamine estimations were made by the method of Elson & Morgan (1933) upon the samples of K hyaluronate hydrolyzed by treatment for 4 hr. with 4*N*-HCl in sealed ampoules immersed in a boiling water-bath. Reducing sugars were estimated upon the enzymatically hydrolyzed

K hyaluronate by Somogyi's (1933) modification of the Hartman-Shaffer alkaline copper reagent. Equimolecular mixtures of *N*-acetylglucosamine and glucurone were used as standards in the latter estimation, since neither of these substances alone brings about the degree of reduction of the reagent to be expected from an equivalent quantity of glucose.

Preparation of purified hyaluronate

Considerable degradation of the polysaccharide occurs when methods such as that described by Madinaveitia & Stacey (1944) are used; for this reason the method of purification employed in this work is described. The work of Meyer & Palmer (1936), Morgan & King (1943), and more recently of Blix & Snellman (1944, 1945) has revealed the necessity for using gentle methods of treatment if the physical properties of polysaccharides are to be preserved. This is emphasized by the results recorded in Fig. 1, which shows the deleterious influence of

Fig. 1. The influence of heating upon the relative viscosity of solutions of crude potassium hyaluronate (0.5 g./100 ml.) adjusted to pH 7.0 ○—○ and 8.0 ●—● and maintained at the temperature of a boiling water-bath.

maintaining, at the temperature of a boiling water-bath, sealed ampoules of crude potassium hyaluronate solution adjusted to pH 7.0 or 8.0. The method to be described yields a highly viscous product.

(1) $1\frac{1}{2}$ vol. of ethanol saturated with potassium acetate and cooled to -10° were added to an aqueous solution at 0° of crude potassium hyaluronate (prepared by Glaxo Laboratories Ltd.) containing 0.5 g./100 ml. and adjusted to pH 9.0 (McClellan, 1943). The fibrous clot which formed was collected on a stirring rod, transferred to a flask, washed thrice with ethanol, twice with ether and dried *in vacuo* at 90° . The dry product was dissolved and reprecipitated, once.

(2) The twice-precipitated material was ground in a mortar with 2 vol. of 90% (w/v) phenol solution (Morgan & Partridge, 1941), each equal to one-tenth of the volume of the original polysaccharide solution. The insoluble hyaluronate was then washed free of phenol with ethanol followed by ether and dried as before.

(3) The material from (2) was dissolved in sufficient water to give a 0.5 g./100 ml. solution. 10% by volume of a 50:50 mixture of *n*-butanol and chloroform was added to the solution, which was then shaken for 6 hr. The emulsion formed was separated by centrifuging for 2 hr. at 2000–3000 r.p.m.

(4) The supernatant from (3) was diluted with an equal volume of water, adjusted to contain 7 g./100 ml. NaCl to decrease its viscosity reversibly (Madinaveitia & Quibell, 1940) and shaken for 5 min. with kaolin; the latter was then removed by gravity filtration through fluted no. 2 Whatman papers over a period of 48 hr. The concentration of chloroform was maintained throughout to prevent bacterial growth.

(5) A few drops of toluene were added to the filtrate from (4) which was then dialyzed for 48 hr. against running tap water, when the viscosity of the solution became restored. The dialysate was adjusted to pH 9.0 and the hyaluronate precipitated by the addition of 2 vol. of ethanol and a trace of potassium acetate. The precipitate, removed by centrifuging and dried with absolute ethanol and ether, was equivalent to 20–30% of the initial crude product.

The final product gave solutions which varied from crystal clarity to slight opalescence. Biuret tests and the usual protein precipitants all gave negative results with solutions of the purified material. These tests are, however, comparatively crude for the examination of polysaccharides and no claim is made that the described method of preparation yields hyaluronate completely free from protein or polypeptide contaminants. Table 1 gives analytical figures for two batches of the purified material and for the crude samples from which they were prepared. The relative viscosity of a 0.1 g./100 ml. solution of batch E in distilled water was 3.70 at 34° as compared with 2.6 for a solution in buffer (2.3 g./100 ml.) of the better of the two preparations of Madinaveitia & Stacey (1944). The nitrogen content of the preparations was about 3.0% of their dry matter, compared with 5.0% for those of Madinaveitia & Stacey (1944); Meyer & Palmer's (1936) ash-free preparations of hyaluronic acid contained about 3.3% N. Thus it may be said that the preparations used here were more likely to be free from protein, or from amino-acid-containing contaminants, than those of Madinaveitia & Stacey (1944), yet they were not grossly degraded.

Table 1. *Analyses of crude and purified samples of potassium hyaluronate*

Sample	Glucos-amine* (%)	N* † (%)	Ash† (%)	Moisture (%)	Relative viscosity of 0.1% solution in water (water = 1.00)
A	29.2	4.89	20.9	15.4	—
A purified	36.0	3.28	13.1	10.1	—
E	20.9	4.13	27.6	15.4	4.94
E purified	37.2	2.90	18.0	14.0	3.72
Calculated§	41.7	3.21	19.8	—	—

* Corrected for moisture but not for ash content.

† By the micro-Kjeldahl method.

‡ As sulphate.

§ Calculated from the disaccharide consisting of *N*-acetylglucosamine and potassium glucuronate.

The simplified growth media

The simplified medium which seemed most likely to be suitable for the production of hyaluronidase by various species of bacteria was that designed by Bernheimer, Gillman, Hottle & Pappenheimer (1942) for the production of erythrogenic toxin from Lancefield Group A streptococci. Sufficient of a commercial dry preparation of casein acid hydrolysate ('Casamino acids', Difco Laboratories Inc.) was substituted for the laboratory-prepared casein hydrolysate to give the medium the same N concentration as that used by the above authors. Sterile solutions of all the growth factors were added to 9 ml. of the autoclaved amino-acid-salt solution, which contained casein hydrolysate, cystine, tryptophan and salts, to give the concentrations indicated by Bernheimer *et al.* (1942). The solutions of pyridoxin, nicotinic acid, adenine, biotin, thiolacetic acid, magnesium chloride and glucose (the latter acidified with one drop of *N*-HCl/100 ml.) were sterilized by autoclaving at 115° for 15 min.; those of riboflavin, aneurin and glutamine were Seitz-filtered. The final glucose concentration in the media, except where otherwise stated, was 0.25 g./100 ml. Good growth of strains of Lancefield Group A streptococci and *Cl. welchii* type A, and moderate growth of strains of *Cl. septicum*, was obtained in this medium, which will be called Medium A. In Medium B, for the cultivation of Lancefield Group C streptococci, the uracil concentration was maintained at 10 mg./l., and biotin and adenine were omitted (Rogers, 1944a). For staphylococci all the growth factors except aneurin and nicotinic acid (Knight, 1937a, b) were omitted (Medium C). The sodium bicarbonate recommended by Bernheimer *et al.* (1942) was not included in any of the media.

RESULTS

Hyaluronidase production by a streptococcus of Group C (Lancefield), Type 7 (Griffiths); called C7

The amount of hyaluronidase produced by the streptococcus C7 was very low when the organism was grown in medium B, despite the luxuriant growth. McClellan & Hale (1941) showed that the addition of hyaluronate to a peptone medium increased hyaluronidase production by *Cl. welchii*,

and McClean (1941) obtained similar results for various groups of streptococci. The influence of the inclusion of hyaluronate in the simplified medium was examined as follows:

Three sets of tubes of medium B were inoculated, each tube receiving two drops from the same glucose broth culture grown for 18 hr. at 37°. One set of tubes was supplemented by hyaluronate (0.1 g./100 ml.) added to the amino-acid-salt mixture before autoclaving, and by glucose (0.25 g./100 ml.) added before inoculation; the second set contained no glucose but hyaluronate (0.1 g./100 ml.); the third set contained only glucose (0.25 g./100 ml.).

Table 2 shows the results obtained in this experiment. The addition of glucose and hyaluronate together completely suppressed enzyme formation, whereas hyaluronate, when present as the sole carbohydrate, stimulated hyaluronidase formation.

Table 2. *The influence of the addition of crude potassium hyaluronate on hyaluronidase production by the streptococcus C7*

(Hyaluronate added to medium B. M.C.P. = 1/enzyme titre in mucin clot-prevention assay (McClean, 1943).)

Glucose concentration (g./100 ml.)	Hyaluronate concentration (g./100 ml.)	M.C.P.	Final pH* of culture
0.25	0	10-50	6.3
0	0.1	1000	6.8
0.25	0.1	<10	5.6

* As determined by indicators.

The relatively low pH induced by the organisms in the medium which contained both glucose and hyaluronate (Table 2) provides a possible explanation for the suppression of hyaluronidase formation in this medium. To test this the following experiment was done:

Three sets of six tubes of medium B containing phenol red (2 mg./100 ml.) were prepared. Two sets contained 0.25 and 0.5 g./100 ml. of glucose and were called media I and II respectively, the third contained potassium hyaluronate

(0.1 g./100 ml.) and glucose (0.25 g./100 ml.) (medium III). All the tubes were inoculated with streptococcus C7 in the usual manner. Two tubes of each set (cultures *e* and *f*) were buffered with Na- β -glycerophosphate (2.5 g./100 ml.) (Rogers, 1944*b*) and two cultures (*c* and *d*) in each set of the three media were maintained at pH 7.4 during growth by the frequent addition of sterile N-NaOH. The third pair of tubes in each set served as unneutralized control cultures (cultures *a* and *b*).

From Table 3 it will be seen that enzyme production in the presence of hyaluronate is the same when growth takes place both in the buffered and the frequently neutralized cultures (*c* and *d*, *e* and *f*); the presence of hyaluronate causes a great increase in the quantity of enzyme produced under these conditions. Results have been obtained which indicate that the pH of the culture is equally important for hyaluronidase production by the other organisms studied. Therefore Na- β -glycerophosphate (2.5 g./100 ml.) was added to all the simplified media (A, B and C) as a routine.

The response of Streptococcus C7 to potassium hyaluronate

Although streptococcus C7 was selected here for particular study, results have been obtained which indicate that, in part, they can be extended to other organisms, such as group A streptococci and *Cl. welchii*.

Different amounts of a sample of crude potassium hyaluronate were added to tubes of the amino-acid + salts solution before they were autoclaved. After this the necessary growth factors described for medium B were added. Three tubes of medium were used for each concentration level of the polysaccharide. The method of inoculation, of estimation of enzyme yield, and of growth were as already described. The bacterial-N value was corrected for the N contained in the precipitate formed when the hyaluronate + amino-acid + salt solutions were autoclaved.

Fig. 2 shows that enzyme synthesis per unit wt. of bacterial-N is directly proportional to the amount of crude hyaluronate added to the growth medium. This result may be due to an adaptive response of

Table 3. *The influence of glucose and hyaluronate on hyaluronidase production by the streptococcus C7*

(Simplified medium B with additions of glucose and hyaluronate; frequently neutralized (cultures *c* and *d*) and in medium containing Na- β -glycerophosphate (2.5 g./100 ml.) (cultures *e* and *f*).

M.C.P. = 1/enzyme titre in mucin clot-prevention assay (McClean, 1943).)

Growth conditions	M.C.P. with		
	hyaluronate absent		hyaluronate present (0.1 g./100 ml.)
	Medium I (glucose 0.25 g./100 ml.)	Medium II (glucose 0.5 g./100 ml.)	Medium III (glucose 0.25 g./100 ml.)
Control (cultures <i>a</i> and <i>b</i>)	150	80	<10, 640*
Frequently neutralized (cultures <i>c</i> and <i>d</i>)	330	640	10,000
Buffered with glycerophosphate (cultures <i>e</i> and <i>f</i>)	300	640	10,000

* The enzyme titres for cultures *a* and *b* in medium III are recorded individually and not as a mean because of the large difference between the duplicates.

the streptococci to the presence of hyaluronate. The polysaccharide used, however, was not pure and the increased enzyme formation might have been due to impurities. The ratio: (stimulation of enzyme production (defined in Table 4) caused by a sample

Fig. 2. The influence upon hyaluronidase production by streptococcus C7 of the concentration of crude potassium hyaluronate in the simplified growth medium. Each point on the curve represents the mean value for three separate cultures. All the points were determined for media inoculated from the same 16 hr. seed culture in glucose broth.

Table 4. *The influence of different samples of potassium hyaluronate, added to simplified medium B, on hyaluronidase production by the streptococcus C7*

(v.r.u. = viscosity reduction units. Different 16 hr. seed cultures of the organism grown in glucose broth were used for inoculation in Exps. I and II.)

Sample of hyaluronate	Analysis of samples		Concentration of hyaluronate (g./100 ml. medium)	Exp. no.	v.r.u.	v.r.u./growth*	Stimulation†	s/w‡
	Glucosamine (%)	N (%)						
A (crude)	29.2	4.89	0.5	I	652	1350	14.2	0.97
B (crude)	24.7	3.32	0.5	I	331	980	10.1	0.83
C (crude)	31.9	2.60	0.5	I	530	1500	16.0	1.01
E (crude)	20.9	4.13	0.25	II	136	408	3.5	0.68
E (purified)	37.2	2.90	0.25	II	163	600	5.7	0.62
None added	—	—	—	I	30.1	88	—	—
None added	—	—	—	II	48.6	131	—	—

* v.r.u./growth = Mean ratio of v.r.u. to mg. bacterial-N/10 ml. culture.

† Stimulation = $\frac{\text{v.r.u./growth on supplemented medium} - \text{v.r.u./growth for unsupplemented medium}}{\text{v.r.u./growth for unsupplemented medium}}$

‡ Stimulation/wt. hyaluronate as measured by the glucosamine released by acid hydrolysis from the hyaluronate sample.

of polysaccharide)/(its content of hyaluronate) should be constant if the streptococci are showing a true adaptive response. The hyaluronate content of the polysaccharide samples was estimated from the amount of glucosamine present after acid hydrolysis. Table 4 shows the influence of six samples of hyaluronate upon hyaluronidase production by the streptococcus C7.

The results for the 'stimulation ratio' (s/w) described above are recorded in column 9 of Table 4. They vary from the mean value of 0.82 by $\pm 18.8\%$. This variation does not differ significantly from $\pm 12.9\%$, the mean value for the difference between the ratio enzyme produced/growth, for the individuals of 25 pairs of duplicate cultures. Thus it is unlikely that the streptococci are stimulated by impurities in the hyaluronate preparations. Moreover, the addition of heparin or of chondroitin sulphuric acid in a concentration of 0.5 g./100 ml. to the simplified medium had no influence upon the enzyme formation. The differences between the glucosamine content of the samples of polysaccharide, however (see Table 4), are of a relatively low order, the greatest being between 20.9 and 37.2% for sample E before and after purification. It was desirable, therefore, to seek additional evidence on the nature of the increase in enzyme production caused by hyaluronate.

Influence of enzymatically hydrolyzed hyaluronate

If the streptococci are responding to hyaluronate and not to concomitant impurities, hydrolysis by hyaluronidase should decrease or destroy altogether the stimulating properties of the polysaccharides. It was already known that an equimolar mixture of *N*-acetylglucosamine and glucuronic acid had no influence upon enzyme production by the streptococcus C7 when added to medium B.

Two samples of hyaluronidase were used for hydrolyzing the polysaccharides; one was a purified

preparation prepared from testis (bull) by the method of Morgan & McClean (1932). The other enzyme preparation was made from a culture of streptococcus C7 grown upon a peptone medium and concentrated by precipitation of the culture filtrate with $(\text{NH}_4)_2\text{SO}_4$, 53 g. being added to 100 ml. of culture filtrate, and subsequent dialysis of the precipitate until free from sulphate ions.

Samples (10 ml. of 0.5 g./100 ml.) of solutions of the K hyaluronate (sample E, purified, Table 1) were mixed with 2.5 ml. of the buffer (pH 7.0) used in the routine viscosimetric assays. Volumes of the enzyme solutions equivalent to 1000 v.r.u. of streptococcal enzyme and 2000 v.r.u. of the testicular preparation were added to the polysaccharide solutions; a few drops of chloroform were added to prevent bacterial growth and the mixtures incubated at 37°. Reducing-sugar determinations were made after 24, 48 and 96 hr.

It was found that, when the streptococcal enzyme was used, hydrolysis ended after about 80 % of the theoretical amount of reducing sugars had been released from the polysaccharide; when the testicular enzyme was used, hydrolysis was only about 66 % complete. The theoretical amount of reducing sugar was calculated on the assumption that hyaluronic acid consists of equimolar proportions of *N*-acetylglucosamine and glucuronic acid (Meyer & Palmer, 1936).

In view of the results obtained when the influence of these hydrolysates upon enzyme production was examined, it seemed important to obtain a more complete breakdown of the hyaluronate by the testicular enzyme. When more of this enzyme was added and the incubation period lengthened by a further 3 days, the degree of hydrolysis was increased to 78 %. Attempts to obtain a further increase, by removing the products of hydrolysis by dialysis or by the addition of more enzyme, failed. The hydrolysates were sterilized and the enzymes inactivated by autoclaving at 115° for 15 min. During this process the hyaluronate solution hydrolyzed by streptococcal enzyme, but not that treated with the testicular enzyme, showed a slight degree of caramelization. It was, however, imperative to inactivate the enzymes used for hydrolysis, since the

addition of active hyaluronidase to the test medium would have unduly complicated determinations of enzyme production by the organisms. Hence filtration sterilization could not be used and this small amount of caramelization of hydrolysates prepared with the streptococcal enzymes had to be ignored.

In order to test the influence of the autoclaved hydrolysates upon enzyme synthesis they were mixed with an equal volume of the simplified medium B, prepared at twice its usual concentration.

The medium was also supplemented with an equal volume of undegraded hyaluronate solution (0.5 g./100 ml.) mixed with buffer and sterilized by autoclaving, to serve as a positive control. These media were inoculated in the usual manner with streptococcus C7 and after 18 hr. the cultures were examined for the quantity of enzyme produced and for mass of growth.

It will be seen (Table 5) that hyaluronate hydrolyzed by the streptococcal enzyme possessed only 25 % of the stimulating properties of the undegraded polysaccharide, whereas the testicular hydrolysates showed undiminished activity.

Since hydrolysis of the hyaluronate by neither of the enzymes was complete, and since the exact nature of the products of enzymic hydrolysis of hyaluronate are unknown, it seemed possible that the testicular enzyme had degraded the polysaccharide predominantly to disaccharide units. If this were so, the remaining linkage between the *N*-acetylglucosamine and the glucuronic acid might be sufficient to stimulate enzyme formation, in which case the materials diffusible through cellophan should also stimulate.

Ten ml. of each of the hydrolysates were dialyzed for 96 hr. against two changes of 50 ml. of distilled water. The two diffusates were combined and concentrated to 10 ml. *in vacuo* (25°); the dialysate was also concentrated to 10 ml. It was found that 80 % of the reducing sugars present in the original hydrolysate had passed through the cellophan. The diffusates and dialysates were sterilized by autoclaving at 115° for 15 min. and tested for their influence upon hyaluronidase formation by the streptococcus C7 in the usual manner. Table 6 shows that the material which stimulates enzyme formation does not diffuse,

Table 5. *The influence of the products of enzymic hydrolysis of K hyaluronate on hyaluronidase production by the streptococcus C7*

(The basal growth medium was medium B.)

Exp. no.	Enzyme used for hydrolysis of hyaluronate	Degree of hydrolysis of hyaluronate (%)	Stimulation*	(Stimulation by hydrolysate) (Stimulation by the unhydrolyzed sample)
1†	Crude streptococcal hyaluronidase	83	0.3	0.26
1a†	Purified testicular hyaluronidase	78	3.3	1.03
2	Purified testicular hyaluronidase	65	2.1	1.09
3	Purified testicular hyaluronidase	61	4.7	1.07

* See Table 4.

† Results recorded opposite Exps. 1 and 1a were taken from one experiment inoculated from the same seed culture. Therefore, the results under the column headed 'Stimulation' for Exps. 1 and 1a can be directly compared.

the diffusates having no influence upon enzyme synthesis.

Table 6. *The influence of fractions from enzymically hydrolyzed hyaluronate on the production of hyaluronidase by streptococcus C7*

The enzyme used in both experiments was purified testicular hyaluronidase. Basal medium B supplemented by dialysates or diffusates.

Fraction	Stimulation* by whole hydrolysate	Stimulation* by fraction	Stimulation* by maximum amount of undegraded hyaluronate possibly remaining in fraction†
Dialysate	2.1	2.2	0.53
Diffusate		0	—
Dialysate	3.3	1.9	0.21
Diffusate		0	—

* See Table 4.

† The maximum hyaluronate content was calculated by assuming that all the glucosamine released by acid hydrolysis of the dialysates was originally bound in them as undegraded hyaluronate.

The production of hyaluronidase by other organisms

Table 7 shows the quantities of hyaluronidase produced by organisms other than streptococcus C7, when grown for 16–18 hr. upon the buffered simplified media.

Table 7. *The production of hyaluronidase by various organisms grown for 16 hr. on the simplified medium in the presence and absence of crude hyaluronate*

(M.C.P. = 1/enzyme titre in mucin clot-prevention assay (McClean, 1943); V.R.U. = viscosity reduction units.)

Organisms	Medium	Simplified medium unsupplemented		Simplified medium + K hyaluronate (0.2 g./100 ml.)	
		M.C.P.	V.R.U.	M.C.P.	V.R.U.
<i>Cl. welchii</i> S 107	A	600	83.0	3,200	350
<i>Cl. welchii</i> A 118		800	49.8	16,000	496
<i>Streptococcus</i> A 4		600	5.5	6,400	159
<i>Streptococcus</i> C 7	B	1600	8.5	24,000	142
<i>Staph. aureus</i> (Wood) 46	C	200	—	100	—
<i>Staph. aureus</i> H		1500	115	1,500	133
<i>Cl. septicum</i> K.F.	A	15	2.5	30	4.0
<i>Cl. septicum</i> V.S. 54		<5	—	<5	—

The seed cultures of the streptococci and staphylococci were 16 hr. cultures in glucose broth, those for the *Cl. welchii* and *Cl. septicum* strains were 16 hr. cultures in Robertson's heart-meat medium. One drop of the seed culture was used to inoculate 10 ml. of the simplified medium.

Two points of interest will be noted: (i) the absence of response to added hyaluronate by the staphylococci and the strains of *Cl. septicum*; (ii) the very small amount of enzyme formed by the strains of *Cl. septicum*.

Hyaluronidase production by staphylococci

The lack of response to hyaluronate by the staphylococci may be due to a difference between the mechanism of hyaluronidase synthesis by staphylococci and that used by streptococci and *Cl. welchii*. Or it may be due to the absence from the simplified medium C of factors which limit enzyme formation and thus mask any response to added hyaluronate. The simplified medium C contained only aneurin and nicotinic acid as growth factors. Hyaluronidase production was examined in medium A, which contains a greater range of growth factors, and in the two following empirical media:

(i) Evans's peptone (Evans Sons, Lescher and Webb Ltd.) (3.5 g./100 ml.) sterilized by autoclaving for 15 min. at 115°.

(ii) A laboratory-prepared, Seitz-filtered, papain digest of horse muscle. This medium, when suitably supplemented, was found by Dr W. E. van Heyningen (private communication) to yield culture filtrates from *Cl. welchii* strain S107 containing a high yield of α -toxin.

Both of these media contained Na- β -glycerophosphate (2.5 g./100 ml.) and glucose (0.25 g./100 ml.). Two sets of each medium were prepared, one of which contained crude K hyaluronate (0.2 g./100 ml.), added to the Evans's peptone medium before sterilization. The viscosity of the papain-digest medium when containing hyaluronate was too great to permit Seitz filtration. Consequently, an autoclaved solution containing hyaluronate (0.5 g./100 ml.) was

added aseptically to this medium prepared so that the final N concentration, after dilution with the polysaccharide solution, was 6.1 mg./ml. The media were inoculated in the usual manner.

Table 8 shows that hyaluronidase formation after 16 hr. growth upon the empirical media which contained hyaluronate was no higher than upon media not containing hyaluronate. At the same time the increased enzyme production by the organisms grown on the papain-digest medium is to be noted.

Table 8. *The production of hyaluronidase by staphylococci*

(Growth for 16 hr. upon simplified and empirical media without and with potassium hyaluronate (0.2 g./100 ml.). All three media contain Na- β -glycerophosphate (2.5 g./100 ml.) and glucose (0.25 g./100 ml.).

V.R.U. = viscosity reduction units.)

Strain	Medium	No hyaluronate		K hyaluronate (0.2 g./100 ml.)	
		V.R.U.	V.R.U./growth*	V.R.U.	V.R.U./growth*
Humphrey	Simplified C	138	242	138	216
Farrance	"	9.7	11.9	10.3	14.7
Wood 46	"	<5	<25	<5	<25
Humphrey	Evans's peptone	124	165	100	185
Farrance	"	8.6	15.9	10.3	16.9
Wood 46	"	12	36	13	—
Humphrey	Papain digest	600	740	510	620
Wood 46	"	65	170	58.7	133

* V.R.U./growth: see Table 4.

Hyaluronidase production upon simplified medium A was the same as that upon medium B with or without hyaluronate.

Hyaluronidase production by Cl. septicum

Hyaluronidase production by two strains of *Cl. septicum* upon the simplified medium A (Table 4) was very low; it was already known that enzyme production by the strain K.F. was significantly higher upon Evans's peptone medium (30–50 V.R.U.). For this reason the influence upon hyaluronidase formation of the addition of a diffusate prepared from Evans's peptone to medium A was investigated.

The diffusate was prepared by dialyzing a solution of the peptone (60 g./100 ml.), against three changes of distilled water at 0–4°. A biuret test on the third diffusate was only weakly positive. The combined diffusates were evaporated to dryness *in vacuo* at 25°, and the resulting material dissolved in 120 ml. distilled water. This solution contained 22 mg. N/ml.; 60% of the total-N in the peptone had dialyzed through the cellophan. The concentrated diffusate (E.P.D.) was sterilized by autoclaving at 115° for 15 min.

Table 9 shows that the addition of E.P.D. to the medium significantly increased hyaluronidase pro-

Table 9. *The production of hyaluronidase by Cl. septicum, strain K.F.*

Growth in simplified media and peptone media containing glucose (0.25 g./100 ml.) and Na- β -glycerophosphate (2.5 g./100 ml.).

Medium	V.R.U.	V.R.U./growth
Simplified A	2.5	9.5
Simplified A + 0.5 g./100 ml. crude K hyaluronate	4.0	6.1
3.5% Evans's peptone	8.7	28.2
3.5% Evans's peptone + 0.5 g./100 ml. crude K hyaluronate	21.2	36.2
Simplified A	3.2	10.3
Simplified A + 1 ml./100 ml. E.P.D.*	8.8	17.2
Simplified A + 20 ml./100 ml. E.P.D.*	14.6	23.9

* E.P.D. = Evans's peptone diffusate.

duction by *Cl. septicum*, strain K.F. Enzyme formation was not increased by the inclusion of hyaluronate in either the simplified or the peptone media.

DISCUSSION

The one property common to all the organisms so far examined is that optimal formation of hyaluronidase occurs only in well-buffered media; enzyme synthesis apparently ceases when the pH of the culture falls below 6.0. This result may be compared with that obtained by Gale & van Heyningen (1942) with *Cl. welchii* cultivated in a peptone medium, in which hyaluronidase formation per unit volume of culture filtrate was greatest when the initial pH of the culture was 7.5. Larger amounts of hyaluronidase were produced per unit weight of organisms at pH 5.5 than at pH 7.5, however. From the results recorded here the organisms would be unlikely to synthesize appreciable amounts of hyaluronidase in casein hydrolysate media adjusted to pH values as low as 5.5; cultures maintained at a pH other than 7.4 have not been examined.

Comparing the conditions necessary for the formation of hyaluronidase by strains of streptococci, *Cl. welchii* type A, *Cl. septicum* and staphylococci, we can distinguish two groups of organisms: (i) those which form increased amounts of hyaluronidase when hyaluronate is included in the medium; (ii) those which show no such enhanced enzyme production but which require so far unrecognized factors for optimum enzyme formation. Schwabacher, Cunliffe, Williams & Harper (1945) have confirmed the lack of response of staphylococci to hyaluronate added to a broth medium, when a series of freshly-isolated strains is used. Thus, just as different organisms have different growth requirements, so also may the requirements (conditions of growth or need for specific substances) for enzyme formation differ with different organisms. *Cl. welchii*, for example, forms no lecithinase (α -toxin) upon simplified media which yield high titres

of hyaluronidase; the presence of other 'toxigenic factors' appears to be necessary.

It appears probable that the enhancement of hyaluronidase production by streptococci and *Cl. welchii* in the presence of hyaluronate is a true adaptation to hyaluronate, and is not due to impurities in the polysaccharide preparations used. The evidence is: (a) the ratio of the increase in enzyme production to the amount of pure hyaluronate contained in the crude polysaccharide is constant, within experimental error; (b) purification of crude hyaluronate does not decrease its stimulating properties; (c) the addition of heparin and chondroitin sulphuric acid to the test medium, had no influence upon hyaluronidase formation.

Dubos (1932) found that an enzyme hydrolyzing the *Pneumococcus* type III polysaccharide was also produced in response to the inclusion of the enzyme substrate in the growth medium used for his soil organism. The only other substance which also stimulated enzyme formation was the aldobionic acid, 4- β -glucosidoglucuronide, formed by acid hydrolysis of the specific polysaccharide. However, the substance responsible for enhancing hyaluronidase production by the streptococcus C7, which is formed when hyaluronate is hydrolyzed by testicular enzyme, will not diffuse through cellophan. Hence it is likely to be of higher molecular weight than a disaccharide. This substance cannot be residual undegraded hyaluronate, since, when a dialysate is used, the final polysaccharide concentration in the test medium cannot be greater than 0.01-0.02 g./100 ml., while the stimulation consistently obtained is that to be expected from four to eight times this concentration. Two explanations of these observations may be suggested. The samples of polysaccharide used may, in fact, be mixtures of two substances of similar composition. If so, the one present in lower concentration, and not hydrolyzed by testicular hyaluronidase, must be responsible for the increased enzyme production. Both of these substances, on this hypothesis, must be considerably hydrolyzed by the streptococcal enzyme. The second possibility is that the difference lies in the degree of hydrolysis brought about by the two enzyme preparations. Very recently Hahne (1945) has stated that crude extracts from swine testis contain two enzymes, only one of which can hydrolyze hyaluronic acid to monosaccharides. It there-

fore seems possible that hyaluronidase purified from bull testis by the method of Morgan & McClean (1932) consists almost entirely of an enzyme responsible for partial breakdown of the polysaccharide. The streptococcal enzyme preparation, on the other hand, may accomplish a more complete breakdown of hyaluronate. Preliminary observations on the inhibition of streptococcal hyaluronidase by hydrolysates, which were made before the publication of Hahne's (1945) work, may provide tentative evidence for a difference in the degree of breakdown caused by the testicular and streptococcal enzyme preparations. If this hypothesis is substantiated by further work, the stimulation of enzyme formation by the products of hyaluronate hydrolysis by testicular enzyme may be compared with other instances in which the products of partial hydrolysis of a substrate have been shown to stimulate the formation of the corresponding enzyme (Yudkin, 1938).

SUMMARY

1. Peptone-free and protein-free media have been devised in which strains of streptococci, *Cl. welchii* type A, staphylococci and *Cl. septicum* will produce large amounts of hyaluronidase.

2. The conditions controlling enzyme synthesis by these organisms have been studied.

3. All the organisms require an adequately buffered medium.

4. Streptococci and *Cl. welchii* produce hyaluronidase in proportion to the amount of hyaluronate added to the buffered growth medium. This is an adaptive response and is not due to the presence of impurities in the polysaccharide.

5. Hydrolysis of hyaluronate by streptococcal hyaluronidase destroys its stimulating properties, whereas hydrolysis by testicular hyaluronidase does not.

6. Staphylococci and *Cl. septicum* do not respond to the inclusion of hyaluronate either in the simplified or the more complex growth media.

7. *Cl. septicum* and staphylococci require factors present in peptone or digest media for optimal hyaluronidase production. Streptococci and *Cl. welchii* do not require such factors.

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The Linkage of Glutamine in Proteins

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The excess of free amino-groups over the number of lysine molecules in different proteins has been attributed by Chibnall (1942) to the existence of several smaller peptide units within the protein molecule, each of them containing a free terminal α -amino-group. In lactoglobulin these peptide chains are supposed to be held together by non-peptide linkages involving carboxyl and not amino-groups; three possibilities were discussed: (1) ester linkages with the hydroxyamino acids; (2) imide linkages $R.CO.NH.CO.R'$ formed with the amide groups of asparagine or glutamine, and (3) thiol-ester linkages formed with cysteine.

The problem of chain-branching in the protein molecule being under investigation in our laboratory for some years, we have examined the possibility of imide linkages of the type suggested by Chibnall (1942) in formula (1):

Several proteins were deaminized and then hydrolyzed by boiling with acids. It was expected that the action of nitrous acid on the residue (1) would lead to the formation of α -hydroxyglutaramide residues (2), which after complete hydrolysis should yield free α -hydroxyglutaric acid (3):

The same acid would be formed if residues of glutamic acid were bound in the peptide chain by their two carboxyl groups, thus leaving the α -amino-group free (formula (4)):

The hydrolysate of deaminized proteins was tested for α -hydroxyglutaric acid by extraction with ether and oxidation of the ether-soluble fraction with permanganate. Under these conditions a considerable part of hydroxyglutaric acid is oxidized

to succinic acid, which can be determined manometrically with succinic dehydrogenase (Krebs, 1937).

If the excess of free amino-groups in the protein molecule were due to mono-amino-acids other than glutamic acid, the corresponding α -hydroxyacids would be formed by the action of nitrous acid, and these would also pass into the ether extract of the hydrolysate.

EXPERIMENTAL

Estimation of α -hydroxyglutaric acid in protein hydrolysates

Pure α -hydroxyglutaric acid was prepared according to Fischer & Moreschi (1912). 2 g. of casein with varying amounts of α -hydroxyglutaric acid, as indicated in Table 1, were boiled with 20 ml. of 23% (w/w) HCl or 8*N*-sulphuric acid. The hydrolysate was evaporated under reduced pressure, the residue dissolved in 10 ml. of water and continuously extracted with ether for 8 hr. The ether was removed by distillation, the residue dissolved in 3 ml. of water and filtered. The filtrate and washings, after evaporation to dryness, furnished much less than the expected amount of ether-soluble substances (Table 1), and prolonged extraction with ether did not give higher yields. Hence, it follows that a part of the hydroxyglutaric acid is lost during the boiling with acid. The ether-soluble residue containing α -hydroxyglutaric acid or its lactone (butyrolactone carbonic acid) was dissolved in 5 ml. of 0.1*N*-NaOH and the lactone hydrolyzed by keeping the solution in a boiling water-bath for 30 min. The solution was cooled to 25°, acidified with 2 ml. of 2*N*-sulphuric acid, mixed with 3 ml. of 0.1*N*-permanganate and heated slowly to 60°. Precipitated oxides of manganese were dissolved by the addition of sodium sulphite; then potassium sulphate was added and succinic acid was extracted as described by Goepfert (1940). The ether extract was evaporated, dried, weighed, dissolved in small amounts of water and neutralized by NaOH. Succinic acid was determined in the neutral solution by treatment with succinic dehydrogenase prepared from pigeon's breast muscle (Krebs, 1937). These experiments, as well as those in which α -hydroxyglutaric acid was treated