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Studies on Diffusing Factors

5. THE INFLUENCE OF SOME ENVIRONMENTAL CONDITIONS
ON THE ACTIVITY OF HYALURONIDASEBy C. W. HALE, *The Lister Institute of Preventive Medicine, Elstree, Herts*

(Received 19 February 1944)

In the course of other work (Hale, 1944), observations were made to determine whether reducing agents or other substances which affect the viscosity of hyaluronic acid also exert any influence on the activity of hyaluronidase.

METHODS

The methods used were those described previously (McClean & Hale, 1941; McClean, 1943; Hale, 1944).

RESULTS

Influence of oxygen on hyaluronidase activity. Experiments were carried out in the viscosimeter apparatus described by Hale (1944). There was no significant difference in the activity of the enzyme under any of the conditions shown in Table 1. It is concluded that the enzyme does not need molecular oxygen for its activity, nor does the enzyme during its action on the substrate at pH 7.0 produce conditions which will either oxidize reduced rosinduline or reduce indophenol. The activity of the enzyme is not readily affected by exposure to oxygen.

Influence of Cu and Na diethyldithiocarbamate on hyaluronidase. Since Cu increases the rate of reaction in a reductant + oxygen + hyaluronate mixture it was thought that it might have a similar action upon hyaluronidase + hyaluronate. Copper (0.1 mg.

Table 1. *The influence of oxygen on hyaluronidase activity*

(The enzyme + substrate mixture had the following composition:

2.0 ml. K-hyaluronate (0.25%) } previously boiled
0.5 ml. McIlvaine's buffer (pH 7.0) } and cooled.
0.5 ml. crude *Cl. welchii* hyaluronidase (1:100 in boiled and cooled water).

Temperature of thermostat 30°.

Rosinduline, exactly reduced with $\text{Na}_2\text{S}_2\text{O}_4$, included as a reducing agent and as an indicator of oxidation.

Indophenol added as an indicator of reduction.)

Conditions	v.r.u.* activity	Remarks
In nitrogen + trace of reduced rosinduline	125	Rosinduline not re-oxidized
"	105	"
In nitrogen + trace of indophenol	100	Indophenol not reduced
In air; no dye	108	—
"	96	—
In oxygen; enzyme and substrate saturated with oxygen	95	—

* v.r.u. = viscosity reducing units (McClean, 1943).

as sulphate) was added to 2 ml. hyaluronate (0.25%) + 0.5 ml. of 0.1M-McIlvaine's buffer (pH 7.0) + 0.5 ml. *Cl. welchii* hyaluronidase dilution,

10 min. after the viscosity fall had begun. No change in the rate of fall of viscosity was observed after adding the copper. In another experiment Na diethyldithiocarbamate (1 mg.) was added to the usual enzyme + substrate mixture. Again no change in rate of fall of viscosity was observed. It is concluded that copper plays no part in the activity of hyaluronidase.

Influence of oxidizing agents on hyaluronidase activity. Meyer, Dubos & Smyth (1937) and, in confirmation, Meyer, Chaffee, Hobby & Dawson (1941), reported that hyaluronidase prepared from an autolysate of a type I *Pneumococcus* was inactivated by iodine and could be reactivated by arsenite or sulphite. Robertson, Ropes & Bauer (1940) found that hyaluronidase from a culture of *Cl. welchii* was also inactivated by iodine, but were unable to reverse the inactivation. In view of these contradictory reports, and of the relative insusceptibility of *Cl. welchii* hyaluronidase to oxygen, the matter was investigated by means of the viscosimetric assay method. It was found that crude hyaluronidase from *Cl. welchii* and hyaluronidase from testis purified by the method of Madinaveitia (1941) were both partially inactivated by KMnO_4 and by iodine (0.001–0.0001 M). It was found that the testis enzyme (dry wt. 5.55 mg./ml.; activity 180 v.r. units/mg.) (McClean, 1943) was not completely inactivated when 0.001 M iodine was used. This was an excess of iodine for, after 30 min. exposure, free iodine could still be detected. Each enzyme preparation, partially inactivated by either iodine or KMnO_4 , was not reactivated by $\text{Na}_2\text{S}_2\text{O}_4$.

Influence of pH on streptococcal hyaluronidase. The anomalous behaviour of hyaluronidase derived from haemolytic streptococci when examined viscosimetrically has been reported by Meyer *et al.* (1941), and McClean (1941). This behaviour of these enzyme preparations was of importance, since it prevented their assay by the viscosimetric method then in use. Madinaveitia & Quibell (1940) stated that, under defined conditions of viscosimetric assay, samples of hyaluronidase derived from mammalian testis decrease the viscosity of hyaluronic acid at a rate directly proportional to the enzyme concentration. The enzymes from *Cl. welchii*, *Cl. septicum* and *Staph. aureus* behave similarly (McClean & Hale, 1941). The composition of the mixture which was used by McClean & Hale (1941) was as follows: 4 ml. of a 0.25% hyaluronate solution, 1 ml. M-Na citrate (pH 4.6) containing 0.6 M-NaCl and 1 ml. of enzyme dilution in distilled water. Under these conditions, hyaluronidase from group A and C streptococci did not behave in the same quantitative way as the enzymes from other sources. An example of the effect observed is illustrated in Fig. 1. Whereas a 1:10 dilution of the enzyme reduced the viscosity of the system to half in 10 min., a 1:20 dilution did

not cause this reduction in 50 min., and a 1:40 dilution barely caused any decrease in viscosity. The enzyme appeared to be destroyed or inhibited during its action. It has been found (McClean, 1943) that testicular and bacterial enzymes show

Fig. 1. The activity of three concentrations of group A streptococcal hyaluronidase in 0.16 M-Na citrate at pH 4.6.

their optimum activity in a system containing 1 vol. of McIlvaine's 0.1 M-citric acid-phosphate buffer (pH 7.0) instead of the 1 vol. of M-Na citrate (pH 4.6) previously used. For this reason the technique for viscosimetric assay was modified so that the reaction

Fig. 2. The activity of three concentrations of group A streptococcal hyaluronidase in 0.016 M-McIlvaine buffer at pH 7.0.

of the system was now pH 7.0, instead of pH 4.6, and the salt content decreased. When streptococcal hyaluronidase was tested in this way it caused a normal viscosity fall (Fig. 2). The enzyme sample used in this experiment was the same as that used to obtain the values of Fig. 1. Several experiments were performed using Na-citrate buffer (pH 4.6) in concentrations varying from 0.16 to 0.016 M. Exposure of streptococcal hyaluronidase to this buffer

before it is mixed with the substrate results in complete inactivation of the enzyme. Similar results are obtained if citric acid-phosphate buffer is used at pH 4.6; if this pH is employed in the viscosimetric assay of streptococcal hyaluronidase, abnormal curves are always obtained and no quantitative assays can be made. It appears that hyaluronidase from either group A or group C streptococci differs from the enzymes obtained from testis, *Cl. welchii* and staphylococci in that it is rapidly destroyed at pH 4.6, but is relatively stable at pH 7.0.

SUMMARY

1. Hyaluronidase from *Cl. welchii* is not readily inactivated by molecular oxygen, does not require

oxygen for its activity and does not produce conditions which will either oxidize reduced rosinduline or reduce indophenol during its activity on the hyaluronate at pH 7.0.

2. The presence or absence of Cu has no influence on the activity of hyaluronidase from *Cl. welchii*.

3. Hyaluronidase derived either from *Cl. welchii* or testis is partially inactivated by KMnO_4 and by iodine; this inactivation is not reversed by $\text{Na}_2\text{S}_2\text{O}_4$.

4. Hyaluronidase derived either from group A or group C streptococci is rapidly inactivated at pH 4.6, but is relatively stable at pH 7.0. This inactivation explains previous reports of the anomalous viscosimetric behaviour of streptococcal hyaluronidase.

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The Antibacterial Action of some Diphenyl Derivatives

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(Received 15 August 1944)

The observations recorded in this paper are a continuation of those previously reported; similar methods have been used (Faulkner, 1943, 1944).

EXPERIMENTAL

The anti-bacterial test. In the standard experiment organisms are incubated at 37° in broth containing the substance being investigated, and subcultured on to solid media after 24 and 48 hr. to test survival. To test bacteriostatic action the organisms are sown on to agar-medium in which the bactericide is incorporated in a concentration 1:2500.

Organisms used. All the organisms used were freshly isolated from material submitted for bacteriological examination, and were as follows: *Staph. aureus*, three strains; haemolytic *Streptococcus* Group A, three strains; *C. diphtheriae*, four strains all virulent to guinea-pigs; *Diplococcus pneumoniae*, two strains; *Streptococcus faecalis*, three strains; Gram-negative diplococci, two strains; *Klebsiella pneumoniae* (Friedländer's bacillus); and seven strains of Gram-negative intestinal bacilli: *Esch. coli*, *Shigella paradysenteriae*, *Shigella sonnei* (*B. dysenteriae* Sonne), *Salmonella typhimurium*

(*B. aertrycke*), *Salmonella* sp. *oranienburg* type, *Salmonella schottmuelleri* (*Bact. paratyphosum* B) and *Eberthella typhosa*.

Compounds used. The starting-point of this series of experiments was diethylstilboestrol, i.e. 4-OH.C₆H₄CEt:CEt.C₆H₄.OH-4'. Most of the substances tested contain two benzene rings; a few compounds were included containing three benzene rings, or only one, but none of these showed any antibacterial activity. Many of the compounds are not soluble in the concentrations used. In these cases they were used as a fine suspension obtained by adding an ethanol solution to broth.

RESULTS

Table 1 summarizes the results of experiments with the most active compounds. Such a compilation does not give a complete picture, as it does not take into account the number of different organisms against which the substance is active, or the variations in the relative susceptibilities of the organisms to the compounds. In general the order of decreasing susceptibility is: *C. diphtheriae*; haemolytic *Streptococcus* and *Diplococcus pneumoniae* approxi-