The Effects of Serum and of Hyaluronic Acid Derivatives on the Action of Hyaluronidase

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In the preceding paper (Hadidian & Pirie, 1948) the viscosity of hyaluronic acid preparations in a defined environment was used as a criterion in selecting standard preparations. In this paper the loss of viscosity by such a preparation has been used to follow the course of hyaluronidase action. This method has the primary advantage that loss of viscosity appears to be the first change that can be recognized during the enzymic decomposition of hyaluronic acid. Factors influencing this *in vitro* change are more likely, therefore, to affect the action of hyaluronidase *in vivo* than those influencing the later stages of the decomposition of the molecule. It is clear that several enzymes are involved in the hydrolysis of hyaluronic acid to reducing sugars. Thus Hahn (1945) has effected a partial separation of the enzyme responsible for oligosaccharide production from that responsible for monosaccharide production; and Rogers (1946*b*) finds differences between the products of the reaction catalyzed by enzymes from different sources. He interprets this as evidence that the enzymes studied by Hahn are present in different ratios in these sources and points out that it is improbable that either enzyme brings about the reduction in viscosity.

Several inhibitors are known for the action of hyaluronidase on hyaluronic acid, whether this is measured by fall in viscosity, mucin-clot prevention or appearance of acetylglucosamine. McClean & Hale (1941) and Hobby, Dawson, Meyer & Chaffee (1941) found that the sera of animals, that had been injected with hyaluronidase from different sources, inhibited the action of the enzyme used for immunization but did not inhibit the action of other hyaluronidases. Later, however, McClean (1942) mentioned briefly an inhibitor present in normal serum, and this substance has also been investigated by Haas (1946). Heparin, gastric mucin, chondroitin sulphuric acid and partly depolymerized hyaluronic acid also inhibit the enzyme. McClean (1942) suggests that these glucosamine-containing substances act competitively because of their structural similarity to hyaluronic acid. Working on this suggestion we have made some inhibitors by modifying hyaluronic acid. We also record some observations on the inhibitor present in serum.

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EXPERIMENTAL

General

Viscosity measurements were made by the technique used in the preceding paper (Hadidian & Pirie, 1948). The time taken for the viscosity to fall half way from the initial to the presumed final value has been taken as a measure of the rate of enzyme action. This measurement of 'half-time' is conventional in studies on this enzyme and has been adopted for convenience, although it is clear that it has no particular merit. It does not, for example, correspond to the loss of viscosity by half of the hyaluronic acid present but to a smaller proportion than this; and to a proportion that varies inversely with the original viscosity of the hyaluronic acid. This is due to the non-linearity of the relation between the concentration and the viscosity of a hyaluronic acid solution, and to the non-linearity of the relation between the axial ratios of elongated particles and the viscosity of their solutions. The enzyme action we are studying can be pictured in two extreme ways. The enzyme may attack a particle of hyaluronic acid and proceed to break linkages in that particle until the products have little or no effect on the viscosity of the solution; the half-time would correspond to a state where less than half the particles have been affected because of the first non-linearity. On the other hand, it may break all the particles to some extent before any one particle has been so far reduced as to have little effect on the viscosity of the solution; because of the second non-linearity the half-time would correspond to the breaking of less than half the relevant linkages.

Some confusion is also introduced into the use of half-time measurements in studying inhibition reactions by the viscosity of the inhibitors. In many cases the viscosity of the inhibitor is unaffected by the enzyme; the final value for the end of the action can therefore be taken as the viscosity of the inhibitor solution in the salt mixture used. In some cases, however, the sum of the increments in relative viscosity, due to hyaluronic acid and inhibitor separately, is less than the observed increments when they are present together. This effect, when it occurs, is small and as long as no significance is attached to differences in half-time less than 30%, it is unlikely to alter the conclusions.

Measurements of the rate of loss of viscosity have been made on an extensive range of hyaluronic acid preparations under identical conditions and with the same enzyme preparation. The half-times have varied by a factor of ten. Part of this variation is due to differences in the initial viscosities of the preparations. If differences in viscosity are due to differences in the average length of the particles in a preparation, the half-time corresponds to the breaking of a smaller number of linkages when the particles are long than when they are short. The generalization that the more viscous the preparation, the shorter the half-time, is not,

however, found to be invariably true. This has suggested to us that umbilical cord may contain substances that activate or inhibit the action of hyaluronidase on hyaluronic acid, and that these may have been removed to varying degrees during the purification. The fact that viscous preparations are the most uniform in their half-time is in favour of this interpretation. We have tested many of the products discarded during the purification and have found, in agreement with other workers, that fractions of very low viscosity are inhibitors. No fraction giving activation has been consistently preparable.

Preparation of inhibitors

Nitration of hyaluronic acid and substances related to it. Dry hyaluronic acid, with the loose, open structure it has after drying by sublimation, is cooled in a freezing mixture to about $-10°$. Sixty parts of fuming HNO_3 (sp.gr. 1·59), also cooled to the same temperature, are added and the mixture is maintained at $-10°$ for 15 min. with occasional stirring. All the hyaluronic acid dissolves in a few minutes. Ice (300–400 parts) is now added with assiduous stirring. The temperature falls still further and a white curd separates from a brilliant blue fluid. As soon as most of the ice has melted and all pockets of undiluted HNO_3 in the curd have been broken up with a rod, the mixture is centrifuged for a few minutes and the solid is washed 3–4 times on the centrifuge with ice water. It is kneaded carefully each time to get rid of as much HNO_3 as possible, and the washing is continued until the wash water can be neutralized by a drop or two of 0·1 N-NaOH. The solid is now suspended in distilled water and brought into solution by adding sufficient NaOH to bring the pH to 6–7. This pale yellow, viscous fluid will be referred to as acid-insoluble nitrate.

All the supernatant fluids are mixed and kept cold. They are neutralized with 5 N-NaOH with vigorous stirring and cooling so that the temperature never rises above 20° during the neutralization. The fluid, at pH 6, is concentrated on a hot plate in a draught of air until $NaNO_3$ begins to crystallize out. It is then dialyzed for 10–20 hr. to remove most of the nitrate, concentrated again, and redialyzed. At this stage the solution from 100 mg. of hyaluronic acid has a volume of 2–3 ml. The solution remaining after thorough dialysis will be referred to as acid-soluble nitrate.

The ratio of acid-soluble to acid-insoluble nitrate varies from preparation to preparation. When hyaluronic acid of low viscosity (e.g. with a relative viscosity of 1·4 for a 1 g./l. solution measured in the presence of salt) is used as the starting material there is little acid-insoluble product. When even less viscous fractions are used there is no acid-insoluble material at all. It seemed possible, therefore, that all the acid-soluble material was derived from the less viscous components of an initially non-homogeneous hyaluronic acid. It is unlikely that this is the sole origin; for if the acid-insoluble nitrate is dried by sublimation and nitrated again by the method described, a further quantity of soluble nitrate is formed. Some degradation of the molecule during nitration is to be expected, and this explanation of the origin of some of the soluble nitrate is borne out by the increased proportion of product in that form if the nitration is continued for longer than 15 min. The sum of the weights of nitrate in both forms is about equal to the starting weight of hyaluronic acid and, when highly viscous hyaluronic acid is used, 85% is in the acid-insoluble form.

Nitration always leads to some fall in the viscosity of hyaluronic acid. The extent of this fall is variable, but in general the viscosity increment of the acid-insoluble nitrate is about half that of the parent hyaluronic acid. The acid-soluble nitrate is much less viscous. Nitrated hyaluronic acid, of both types, can be salted out from neutral solution by $(NH_4)_2SO_4$. With the acid-insoluble product, precipitation begins at about one tenth of saturation and is complete at one third of saturation. The more readily precipitated fractions are the more viscous, and they are precipitated fully by lower concentrations of $(NH_4)_2SO_4$ than the less readily precipitated fractions. It is clear, therefore, that the nitrate is a non-homogeneous mixture, but it is not certain whether this reflects an original non-homogeneity of the parent hyaluronic acid or is due to destruction during the nitration. Precipitation of the acid-soluble nitrates and of nitrates derived from hyaluronic acid preparations of very low initial viscosity does not start until one third saturation by $(NH_4)_2SO_4$. Salting out does not therefore seem to be a useful alternative to neutralization, evaporation and dialysis in the preparation of acid-soluble nitrates because, although acid-insoluble nitrate is precipitated by saturated $NaNO_3$, the soluble nitrates are not, and the high nitrate concentration confuses $(NH_4)_2SO_4$ precipitation.

Hyaluronic acid is not salted out by $(NH_4)_2SO_4$ from solutions containing no other component, but, as one of the methods suggested for the purification shows, it can be salted out if pyridine or some forms of protein are also present. The nitrates behave similarly, for, although esterification of the hydroxyl groups has made the nitrates sufficiently hydrophobic to be salted out, they can be salted out at lower concentrations of $(NH_4)_2SO_4$ in the presence of pyridine.

There is no reason to doubt that the hyaluronic acid has nitrated as other polysaccharides do (cf. Speiser & Eddy, 1946); for the dry product burns in a flash and the nitrogen content (Dumas) has risen from 3–4 to 8·6%. No estimate other than the measurement of nitrogen content has been made of the number of nitrate groups introduced. If hyaluronic acid has the structure generally assigned to it, a chain of alternate glucuronic acid and acetylglucosamine residues connected by glucoside linkages, there are four free hydroxyl groups for each repeating disaccharide unit. The sodium salt of the tetranitrate that could be made from such a substance would contain 12% of nitrogen and that of the dinitrate 8·55%. Esterification of the hydroxyl groups in this way does not apparently affect the *N*-acetyl group, for the acetyl content falls from 10–11 to 7–8%. The value to be expected for the dinitrate is 8·7%. Control experiments show that neither nitrous nor nitric acids, in amounts comparable to those present in a hydrolysate, are estimated as volatile acid after hydrolysis and distillation by the procedures used on these samples. On the other hand direct estimation, by the Elson & Morgan (1933) method, on the nitrated material shows an apparent disappearance of the glucosamine. This result is due to the action of the HNO_3 liberated during hydrolysis. The same disappearance can be brought about by hydrolyzing normal hyaluronic acid in the presence of a quarter of its weight of $NaNO_3$, an amount corresponding to four nitrate groups. Furthermore, de-esterification, by the method to be described later, restores the apparent glucosamine content, and hydrolysis in the presence of a reducing agent such as HI protects the glucosamine to a great extent from destruction. Fuming HNO_3 probably acts to some extent as an oxidizing as well as a nitrating agent, but it is clear that no oxidation involving

the glucosamine is going on to an extent at all comparable to that found by Skanse & Sandblad (1943) to accompany peroxide oxidation.

Acetylation of hyaluronic acid

Like some other acid polysaccharides (e.g. alginic acid, pectin and agar) hyaluronic acid does not acetylate readily. Acetic anhydride in neutral or alkaline aqueous solution, or in the presence of pyridine under anhydrous conditions, failed to bring about significant acetylation in a few hours at room temperature or on slight warming. Two recent methods are, however, effective; in one the acid is swollen with acetic acid and H_2SO_4 is used as catalyst (Chamberlain, Cunningham & Speakman, 1946), and in the other the acid is swollen with formamide and acetylated with acetic anhydride and pyridine (Carson & Maclay, 1946). The products of these two acetylations differ somewhat in their properties.

(1) Hyaluronic acid is swollen with acetic acid by adding glacial acetic acid in a thin stream to a 3–10 g./l. solution. Mixing is complete until about 2 vol. have been added, after which precipitation begins and is substantially complete with 4 or 5 vol. The mixture is centrifuged and the solid washed with acetic acid and used immediately. This precipitation does not lead to any significant fractionation of the hyaluronic acid, for only about 3% of the starting material is found in the solution when this is evaporated to dryness. This soluble fraction has the normal nitrogen and glucosamine content, but its viscosity is low. The precipitate, on the other hand, if dialyzed immediately, gives a solution with the original viscosity. We do not, therefore, agree with the statement made by many workers on hyaluronic acid that precipitation with acetic acid leads to a fall in viscosity; but our observations are in agreement with those of Meyer, Smyth & Dawson (1939) who prepared a relatively viscous product from synovial fluid by acetic acid precipitation.

The fibrous clot of hyaluronic acid is pressed as free from acetic acid as possible and added to 200 times its weight of a mixture of 1 part of conc. H_2SO_4, 90 parts of acetic anhydride and 120 parts of benzene. There is little or no solution of the hyaluronic acid, and, after a suitable interval, the mixture is centrifuged and the solid suspended in water and dialyzed. If acetylation is allowed to proceed for a few minutes only, the product has an acetyl content of 16–20%; after 40 min. at 22° this rises to 25–33% and is not increased by longer acetylation at that temperature. By following the method of Chamberlain *et al.* (1946) and raising the temperature to 60° for 15 min., after acetylation has proceeded for 1 hr. at 25°, the acetyl content can be raised to 36%. Acetyl determinations were made by the method used in the preceding paper (Hadidian & Pirie, 1948) and include both *O*-acetyl and *N*-acetyl. The theoretical value for the tri-*O*-acetyl derivative of a polysaccharide built up from equal quantities of glucuronic acid and *N*-acetyl glucosamine is 34% and for the tetra-*O*-acetyl derivative 39·4%. It would therefore appear that, as in the case of nitration, esterification remains incomplete. Products that have been acetylated for a few hours only are still soluble in water over the whole pH range, but those that have been acetylated at room temperature for many hours, or have been heated to 60° during acetylation, are not soluble on the acid side of neutrality. There is a progressive fall in viscosity during acetylation. Products acetylated for a few minutes only retain 70–80% of the original viscosity increment, whereas the tri-*O*-acetates retain only 10% or less.

(2) Hyaluronic acid that has been dried by sublimation does not change its appearance when left for a few hours at 20° in formamide, but at 40° it swells somewhat and at 50° turns in 1 hr. to a viscous dough when suspended in ten times its weight of formamide. This dough can be mixed with an equal volume of pyridine by stirring for a few minutes. The plastic, rather than fluid, mixture is now mixed with half its volume of acetic anhydride, which causes an initial shrinkage, but is incorporated into the mixture in a few minutes. As in the other method of acetylation the properties of the final product depend on the duration of acetylation and the temperature at which it proceeds. The mixture is stirred with three times its weight of ice; when it is brought to pH 2 with 5N-HCl a soft curd separates. The fluid is dialyzed. If acetylation has proceeded for 1 hr. at 20° this acid-soluble product has an acetyl content of 14–16%, while after 3 hr. at 30° it rises to 30%. In neither case has it any significant viscosity. The curd is not soluble at neutrality in the absence of salt and is only partly soluble in the presence of salt, but it swells slowly to give a suspension of gelatinous particles, which dissolve at pH 9–10. The solutions have only one fifth of the viscosity of the parent hyaluronic acid. All these acetylated fractions coagulate with strong acid and, like the nitrates, can be precipitated with $(NH_4)_2SO_4$.

De-esterification. The nitrates and acetates of hyaluronic acid can be partly de-esterified without extensive degradation by exposure to alkali in the presence of ethanol. Solutions of either ester containing 3–10 g./l. are mixed with 10 vol. of ethanol. With the esters, unlike with hyaluronic acid itself, no precipitate separates even on the addition of a salt such as potassium acetate. The addition of ethanolic KOH at 22° leads to the appearance of an opalescence, and later, after a time interval depending on the amount of alkali added, of a precipitate. For example, an ethanolic solution containing 0·8 g./l. of a moderately viscous nitrate (rel. visc. 2·3 at 1 g./l. in salt) gave an immediate precipitate with 7 g./l. KOH; but with 2·5 g./l. precipitation took 2 hr., and with 0·7 g./l. precipitation occurred only on the addition of some salt. Similarly with the acetate there is immediate precipitation with 1–2 g./l. KOH, but with less than this amount precipitation is delayed. Under these conditions de-esterification remains incomplete as determined from the nitrogen and acetyl content of these products. The immediate purpose of the work, the making of substances that inhibit hyaluronidase is, however, satisfied; and conditions that would lead to complete de-esterification have not been investigated.

Factors affecting the hyaluronidase-hyaluronic acid reaction

Effect of salt concentration on the rate of reaction. In studying the effect of possible inhibitors and activators on the hyaluronidase-hyaluronic acid reaction, it would first be necessary to establish conditions of salt concentration under which such work can be carried out with the least possible interference from salt effects. Results of Robertson, Ropes & Bauer (1940), McClean & Hale (1941), and

Madinaveitia & Quibell (1941) show that there is an optimum salt concentration for the reduction of the viscosity of hyaluronic acid by hyaluronidase, but the optima they find vary with their different substrate and enzyme preparations. We have tried the effect on this reaction of several salts, previously studied for their viscosity-reducing activity, with results shown in Fig. 1. These reactions were carried

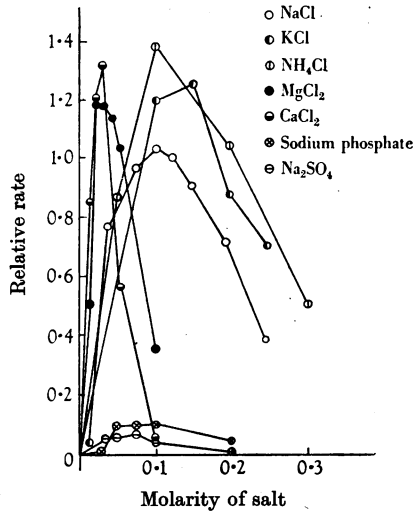

Fig. 1. Variations in the rate of hydrolysis of hyaluronic acid by hyaluronidase with varying concentrations of different salts. Temp. 25°; pH 6·9±0·10; 12·5 mg./l. bull-testis enzyme; 0·3 g./l. solution of a medium viscous hyaluronic acid preparation. In the experiments with NaCl, KCl, NH_4Cl and Na_2SO_4 the pH was maintained with phosphate buffer, and in those with $MgCl_2$ and $CaCl_2$ with borate buffer. In all cases the molar ratio of salt to buffer was 100. In expressing the relative rates, the reciprocal of the half-time in 0·125 M-NaCl is taken as unity.

out at pH 7·0, using salt solutions buffered with 1% of phosphate or borate buffer. Of necessity, pH control in low salt concentrations was not quite adequate, but frequent checks showed that variation was not more than 0·2 unit; this may produce as much as 20% variation in the rate of reaction. From the results shown and from others on different substrates, three groups are distinguishable: $CaCl_2$ and $MgCl_2$, effective in very low concentrations; NaCl, KCl and NH_4Cl, just as effective but in higher concentrations; Na_2SO_4 and Na_2HPO_4, relatively ineffective. Sodium acetate is intermediate between the chloride and phosphate. Lineweaver & Ballou (1945), in their study of the effect of salt concentration on the activity of pectin esterase, find similar variations in the effectiveness of various salts in activating the enzymic reaction, but with that enzyme there is the same maximum activation at the optimum concentration of each salt. We agree with McClean & Hale (1941) that the chloride ion is an important activating agent. However, the difference in the effectiveness of the chlorides cannot be attributed to Cl^- alone. Expressed as normality of Cl^-, assuming complete ionization, the optima with Mg^{++}, Ca^{++}, Na^+, NH^+ and K^+ are at 0·05, 0·06, 0·1 and 0·15 respectively. Furthermore, in the presence of 0·05 M-phosphate, maximum activation is obtained with 0·05 M-NaCl. Two factors seem to be involved; general salt concentration and the presence of an activating ion, such as Cl^-. The pH optimum in the presence of 0·05 M-NaCl and 0·05 M-phosphate is at 6·3; this value is intermediate between the optima of 6·8 and 5·0 found by McClean (1943) in 0·017 M- and 0·167 M-NaCl respectively. Our experiments have been carried out at pH 7 as a compromise between the pH optimum and the pH at which the enzyme and inhibitors are used in *in vivo* tests that will be described elsewhere.

Estimation of inhibitory activity. Since no method exists for direct estimation of hyaluronidase, the course of the reaction between it and an inhibitor has to be followed by the indirect method of determining the amount of hyaluronidase that is made unavailable for the hyaluronic acid-hyaluronidase reaction in the presence of the inhibitor or after the hyaluronidase has been exposed to the action of the inhibitor for a given period of time. If, then, one works in a region where there is a linear relationship between hyaluronidase concentration and the rate of loss of viscosity by hyaluronic acid, one can justifiably consider the rate as a direct measure of the amount of active hyaluronidase in the system. The range over which such a relation exists varies with different preparations of hyaluronic acid tested at the same concentration. With highly viscous preparations and with the partially purified enzyme preparation that we use, deviation from linearity is slight up to an enzyme concentration of 8 mg./l. With less viscous preparations this range may extend up to 50 mg./l. The following methods are used in estimating inhibitory action: in instances where the length of exposure of the enzyme to the inhibitor is of little or no consequence, the enzyme is introduced into a mixture containing both inhibitor and substrate, and the subsequent viscosity changes followed; where the extent of inhibition is dependent on the duration of exposure of the enzyme to the inhibitor, as in the case of inhibition by serum, the enzyme and the inhibitor are incubated together under the proper salt and pH conditions for a given length of time, then the substrate is introduced into the mixture, and the course of the reaction followed. The extent of inhibition is determined from the ratio of the half-time of the reaction with inhibitor to that without. Thus a threefold inhibition would be obtained if a half-time of 600 sec. is obtained with inhibitor as compared with 200 sec. without. The reciprocal of this ratio is the fraction of the initial enzyme concentration that is active. Partially purified bull-testis enzyme (Schering) was used in all these experiments.

Inhibition by hyaluronic acid esters. Nitrates and acetates that have been made from viscous hyaluronic acid retain much of their initial viscosity. The effect of hyaluronidase on them can therefore be

tested in the usual way. The viscosity of nitrates is not reduced by hyaluronidase and the viscosity of acetates often falls at first and then stays constant. We look on acetates behaving in this way as mixtures of material that can be attacked and material that is not attacked and does not inhibit the reaction. The stability of these hyaluronic acid esters towards the enzyme contrasts with the susceptibility to attack of the *p*-aminobenzyl ether (Humphrey, 1943). In Humphrey's preparations, however, less than one ether linkage seems to be present for each glucosamine in the hyaluronic acid, and no indication is given of the extent to which hydrolysis, which was recognized by the appearance of *N*-acetylglucosamine, took place.

with nitric esters are more systematic; all of them inhibit. Attempts to get less nitration by using a smaller ratio of nitric acid to hyaluronic acid or by allowing the nitration to proceed for a shorter time have led to less active products. The acid-soluble fractions from the nitration of viscous material and the acid-soluble nitrates of non-viscous material are stronger than the acid insoluble nitrates. By suitably controlled removal of nitrate groups with alkali and ethanol the inhibitory power is substantially increased; but it is clear from the table that the intensity of the alkali treatment is of importance and that too drastic a treatment leads to a weaker inhibitor. For comparison the results of experiments with non-viscous hyaluronic acid and

Table 1. *Effects of various inhibitors on the hyaluronidase-hyaluronic acid reaction*

Description of inhibitor	Concentration (g./l.)	Inhibition
Viscous hyaluronic acid acetylated by sulphuric method for 60 min.; 33% acetyl	0·1	1·5
Above product deacetylated for 15 min. with 2 g. KOH/l.; 14% acetyl	0·1	6
Viscous hyaluronic acid acetylated by sulphuric method for 10 min.; 18% acetyl	0·1	4
Viscous hyaluronic acid acetylated by formamide method for various times; 15, 25 or 30% acetyl	0·1	Inactive
Acid-soluble product from nitration of viscous hyaluronic acid	0·01	3·5
Acid-insoluble part of the same nitration	0·1	7·3
Acid-soluble product from nitration of viscous hyaluronic acid	0·03	3·0
Acid-insoluble part of the same nitration	0·03	2·2
Above insoluble product denitrated for 2 hr. with: 0·7 g. KOH/l.	0·005	5
2·5 g. KOH/l.	0·004	7·5
7·5 g. KOH/l.	0·03	2·2
Nitrated non-viscous hyaluronic acid	0·01	5
The same non-viscous hyaluronic acid before nitration	0·1	2
Heparin	0·1	2

In these experiments 2 ml. of a 0·6 g./l. solution of hyaluronic acid in 0·1 M-NaCl and 0·1 M-phosphate buffer pH 7 were mixed with 1 ml. of a solution of the inhibitor at four times the concentration specified in the second column. 1 ml. of a 50 mg./l. solution of hyaluronidase was then added and the flow time in an Ostwald viscosimeter was measured at various intervals. The half-time was found in the usual way, and the amount of inhibition set out in the third column is derived by dividing the half-time found in the presence of the inhibitor by that found in a control experiment with enzyme and substrate alone.

The inhibition is measured by mixing suitable amounts of inhibitor with hyaluronic acid and then adding the enzyme. There is a slight increase in the inhibition if the enzyme and inhibitor are mixed first, but in this instance the extent of inhibition is not increased by allowing the enzyme and esfer to be in contact for more than the time necessary for mixing. Some comparable results are collected in Table 1. Values are given for only one concentration of each inhibitor because the inhibition is proportional to the concentration. The table shows that acetates inhibit weakly, or not at all, and the most active are partially acetylated products the preparation of which is a matter of some uncertainty. On partial de-acetylation the inhibition always increases, but conditions that would lead to regularly repeatable results have not been found. The results

heparin are also included in the table. Many non-viscous preparations of varied origin have been tested and none has been found with a significantly greater inhibitory power than the one cited. Only one sample of heparin was tried; the inhibition found here is comparable to that found by McClean (1942) and Rogers (1946*a*) using different experimental procedures. Our tests were made at 25° with hyaluronic acid having a relative viscosity of 7–8 for a 1 g./l. solution in 0·05 M-NaCl and 0·05 M-phosphate buffer pH 7, although experiments described in the next paragraph show that there is more complete inhibition with smaller concentrations of phosphate. The same sample of dried, partially purified hyaluronidase from bull testes (Schering) was used throughout at 12·5 mg./l. Similar experiments have been made with larger and smaller concentrations of

enzyme, at 37° and with samples of hyaluronic acid having a relative viscosity as low as 2 for the 1 g./l. solution. These variations in the conditions have not significantly affected the amount of inhibition observed.

Variations in phosphate and NaCl concentration, however, have a marked effect on inhibition by the esters. Several of these, including an acid-soluble nitrate, an acetate, a denitrated nitrate and a de-acetylated acetate, were tested in 0·05M-NaCl with phosphate concentrations varying between 0·013 and 0·24M; and in 0·05M-phosphate with NaCl concentration varying within the same range. Within this range there is an increasing inhibitory activity with decreasing salt concentration. None shows a significant inhibitory activity in 0·05M-NaCl and 0·24M-phosphate and all show maximum activity in 0·05M-NaCl and 0·013M-phosphate; but they all follow different courses between these extremes. Products with identical inhibitory activity under our standard testing conditions may differ by a factor of 2, at lower phosphate concentrations. Thus the figures given in Table 1 would not necessarily indicate the relative effectiveness of these substances under physiological conditions.

For convenience of writing, the operations that have been performed on hyaluronic acid in making these inhibitors have been called acetylation, nitration and de-esterification. It is certain that the bulk of the material is undergoing these changes, for the observed acetyl content can be increased and diminished as can the Dumas nitrogen content and the inflammability of the nitrates. It is less certain that these are the changes primarily responsible for the inhibition. All of them are associated with changes in viscosity and so presumably in particle size, and it is possible that this is the significant change rather than the extent of esterification. The difficulty found in getting reproducible results from experiments on acetylation and de-acetylation slightly favours this interpretation. On the other hand, the stability of these inhibitors in the presence of hyaluronidase suggests that a graded breakdown of the molecule is not alone sufficient to make a good inhibitor. Furthermore, our attempts to degrade the molecule by other means, e.g. acid, alkali and partial enzyme digestion, have resulted in only feebly active products. It is probable that inhibitors have both an optimum particle size and an optimum degree of substitution. The high activity found with nitrated non-viscous products suggests that this size may be fairly small; but active material does not diffuse through cellophan.

Serum as an inhibitor. McClean (1942), in the course of studying the *in vivo* decapsulation of streptococci, discovered a factor in the sera of several species which inhibits enzyme from different sources, and noted that they varied greatly in their inhibitory activity. He considered the possibility that this inhibition is due to the presence in the serum of a polysaccharide which acts by competing with the substrate for the enzyme. Haas (1946), on the basis of some by no means conclusive evidence, has claimed that this inhibitory action of serum is due to the presence in it of an enzyme which inactivates hyaluronidase.

The inhibition of hyaluronidase by serum at 25° proceeds at a measurable rate, as shown by Haas (1946). The method used for its study by Haas, and with some modification by us, has been to expose the enzyme to the action of serum for a given length of time, then mix it with hyaluronic acid solution containing enough phosphate buffer to obtain a final concentration of 0·05–0·1M-phosphate. The validity of this method rests upon the fact, discovered by Haas and confirmed by us, that in the presence of such concentrations of phosphate (the exact concentration varies with different serum samples) the activity of hyaluronidase in reducing the viscosity of hyaluronic acid is unaltered by incubation with serum. One cannot, however, assume on such evidence that interaction between hyaluronidase and the serum factor ceases in the presence of phosphate.

If one introduces phosphate into a mixture of hyaluronidase and serum that has been incubated for a time sufficient to inactivate a part of the hyaluronidase, and then removes samples at various intervals for testing their hyaluronidase content, one finds that in low phosphate concentrations (0·01–0·05M) the inhibition of hyaluronidase will continue, and in high phosphate concentrations (0·05–0·1M) it will be reversed. However, at about 0·05M the rate of change in either direction is very slow. Since the process of estimating the hyaluronidase after the inactivation reaction seldom lasts longer than 30 min., this method of studying the inhibitory action of serum seems valid so long as significance is not attached to small changes in activity.

Like the reaction between hyaluronidase and hyaluronic acid, the reaction of dialyzed serum with hyaluronidase does not proceed in the absence of salt. It is activated by small amounts, with an optimum between 0·03 and 0·1M-NaCl. The exact optimum varies somewhat with the amount of serum used, but variations within the range specified are slight. In 0·2M-NaCl the inactivation of hyaluronidase by serum at the end of 10 min. of incubation is about one fifth of the maximum. Phosphate will activate this reaction to a lesser extent. However, it is much more effective relatively in this reaction than in the hyaluronidase-hyaluronic acid reaction. In the presence of 0·025M-phosphate the inhibition attains 75% of the maximum obtainable in NaCl. With higher concentrations of phosphate, as of NaCl,

the serum-hyaluronidase reaction is apparently inhibited, 0·075M-phosphate giving full inhibition. The serum used in these experiments was dialyzed for 4–5 days against several changes of distilled water. For the most part we have used pig serum in this study, but most of the experiments described have been repeated with human, rabbit, chicken or bovine serum. In view of the statement of Haas (1946) that serum obtained by clotting is less active than that obtained from blood defibrinated by stirring, all sera used were obtained in the latter manner. However, in subsequent experiments with sera obtained by clotting and by stirring from the same specimen of blood, we have been repeatedly unable to demonstrate under optimal salt conditions any significant difference between the two.

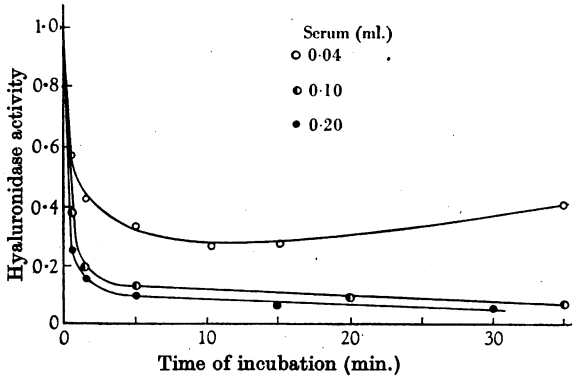

Fig. 2. Rate of inactivation of hyaluronidase by varying amounts of serum. 0·04, 0·1 and 0·2 ml. dialyzed pig serum, pH 7; incubation at 25° with 50 μg. of testis enzyme in 2 ml. of 0·05N-NaCl containing 0·3 ml. borate buffer pH 6·9. 2 ml. of 0·6 g./l. solution of a high viscosity hyaluronic acid in 0·1M-phosphate buffer and 0·05M-NaCl added to the serum-hyaluronidase mixture at the end of incubation and the residual hyaluronidase activity determined viscosimetrically. Hyaluronidase activity $= \frac{\text{half-time without inhibition}}{\text{half-time with inhibition}}$. Owing to the non-linearity of the relationship between enzyme concentration and the rate of hydrolysis of this particular hyaluronic acid preparation the actual amount of hyaluronidase, expressed as fraction of the original amount remaining active after incubation is about a third more than the activity indicated.

As a result of these findings, the following procedure was adopted for testing the inhibitory activity of serum, with such modifications as were required by varying conditions. The enzyme (50 μg.) is incubated with 0·05–0·3 ml. of dialyzed, neutralized serum for 10 min. in 2 ml. of 0·1M-NaCl buffered with 0·2 ml. of 0·2M-borate buffer at pH 6·9. To this is then added 2 ml. of hyaluronic acid solution, 0·6 g./l. in 0·1M-phosphate, and the course of the depolymerization of hyaluronic acid followed viscosimetrically.

Having established beforehand the range of enzyme concentration over which the relationship between hyaluronidase concentration and the rate of loss of viscosity by the hyaluronic acid preparation to be used does not deviate too markedly from linearity, one can follow the course of apparent inactivation of the enzyme by the serum. At 25° with 50 μg. of enzyme and 0·10 ml. of dialyzed pig serum in 2 ml. of buffered 0·05M-NaCl, only about 50% of the enzyme remains active 30 sec. after mixing, and 25% after 1 min. (Fig. 2). Maximum inhibition is reached within 10–500 min. depending upon the serum concentration. With high concentrations of serum for the next 5–10 hr. no apparent change occurs, but eventually, in every instance the hyaluronidase activity begins to return (Fig. 3).

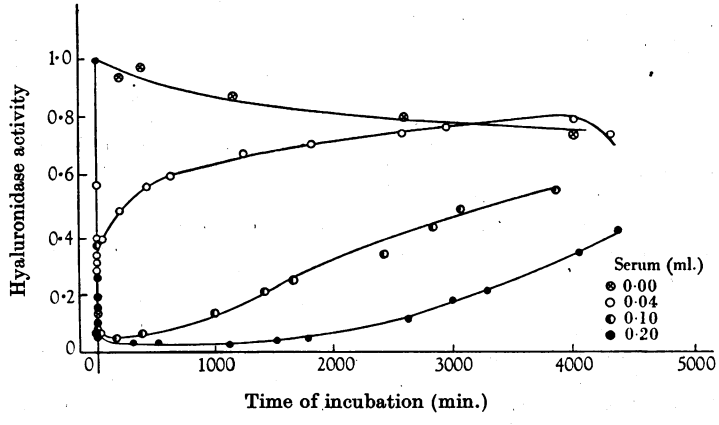

Fig. 3. Recovery of hyaluronidase activity after its inhibition by varying amounts of serum. Conditions of experiment are the same as specified under Fig. 2, where the course of the reaction for the first 35 min. is shown in detail. Serum incubated under the same conditions, but without hyaluronidase, had lost none of its inhibitory activity at the end of the experiment when tested on a fresh enzyme solution.

When there is no excess of serum, with 0·04 ml., recovery of hyaluronidase activity immediately follows its maximum inhibition. When there is an excess, apparently a condition of equilibrium is reached and is maintained as long as there is an excess of the serum factor available. The recovery process in every instance is slow, and is complete within 72 hr. only with low concentrations of serum. A hyaluronidase solution kept under the same temperature, salt and pH conditions may lose a quarter of its activity during this period. Serum kept under identical conditions loses very little, if any, of its original activity. In repeated experiments, the final hyaluronidase activity was never more than that at the beginning of the experiment, thus making it unlikely that this phenomenon is due to the production of hyaluronidase by contaminating bacteria. Experiments were also carried out in the presence of 0·05M-NaF and 0·05M-benzoate to prevent any possibility of bacterial contamination. Both interfere to some extent with hyaluronidase activity but the reappearance of hyaluronidase after inhibition can be demonstrated in their presence.

In these experiments inactivation and re-activation proceed in a solution that is not otherwise altered. By changing the environment the re-activation process can be hastened. Dilution of the mixture without change in salt concentration causes a 15% increase in the rate for fourfold dilution. The same dilution doubles the rate of re-activation if it is made with NaCl containing 0·03M-phosphate and quadruples it if made with NaCl containing 0·1M-phosphate.

We find the reaction between serum and hyaluronidase much faster than is manifest from the data of Haas (1946). This in part is due to the fact that Haas uses no salt in his reaction mixture except borate buffer and whatever salt there is present in the serum, in part to the fact that he has no determinations at less than 5 min., and in part to his presentation of the course of the reaction as an increase in the 'activity' of serum. Thus, according to his data, 0·1 ml. chicken serum has an 'activity' of 2 at the end of 5 min., and 4 at 20 min. The former corresponds to an inhibition of 65% of the hyaluronidase, and the latter to 80%, i.e. only an additional 15% of the original hyaluronidase is inhibited between 5 and 20 min. How much of the 65% inhibition at the end of 5 min. takes place in the first few seconds there is no way of determining from Haas's data, but from our own data it is evident that the reaction is slowed abruptly after about 1 min. when an excess of serum is present.

The inhibition of hyaluronidase by derivatives of hyaluronic acid resembles the serum inhibition, in a general way, in its dependence on the ionic environment, although the serum inhibition is suppressed by concentrations of phosphate at which all esters tested have retained some activity. The actions differ in two significant respects; ester inhibition reaches its maximum more quickly than serum inhibition and there is no recovery on prolonged incubation. It is obvious that the hyaluronic acid esters cannot be called enzymes, but the terminology most suitable for the serum inhibitor is uncertain. Haas (1946) has called it an enzyme because the reaction proceeds at a measurable rate and because the inhibitor is destroyed at 55°, but these properties, which we confirm, are not exclusively the properties of enzymes and the fact that the inhibited hyaluronidase can recover its activity is a strong argument against the applicability of the word enzyme to the inhibitor. Essentially, an enzyme is a substance that combines with its substrate in such a way that, when dissociation occurs, the substrate is altered; the serum inhibitor combines with hyaluronidase and dissociates from it without affecting the only property of hyaluronidase of which anything is known. Under these circumstances the analogy would seem to be with the antigen : antibody and toxin : antitoxin reactions rather than with the enzyme : substrate reactions. The indiffusibility of the inhibitor and its inactivation by heating suggest that it is, or that it is associated with, a protein and this is borne out by McClean's observation, which we confirm, that it precipitates in the globulin fraction of serum. The destruction of the inhibitor on incubation with a hyaluronidase preparation could be due to a protease present in the enzyme, but it is also possible that it is another manifestation of hyaluronidase activity. On this view the inhibitor is a substrate for hyaluronidase on which the enzyme acts slowly; it is this that distinguishes it from the esters for they are apparently not attacked at all by the enzyme. In an attempt to get evidence for this point of view we have estimated the glucosamine in hydrolysates of the ultrafiltrate from dialyzed serum that had been incubated with hyaluronidase for sufficiently long to destroy a part of its inhibitory power. No glucosamine could be detected. This suggests that the inhibitor does not contain glucosamine, that this is not liberated during its destruction, or that the quantity is too small for detection by our methods. There is therefore still no evidence on the nature of the inhibitor or the mechanism of its action.

SUMMARY

1. The effect of various salts on the action of hyaluronidase on hyaluronic acid has been studied viscosimetrically. There is an optimum molarity for each salt, but its value and the rate of the reaction at the optimum vary with different salts.

2. Methods of preparing nitrates and acetates of hyaluronic acid are given. These esters inhibit the

enzyme action and this inhibition is compared with that brought about by a constituent of normal serum.

3. The extent of the inhibition by these agents is dependent on the salt concentration.

4. The serum inhibitor, unlike the inhibitors made by esterifying hyaluronic acid, is destroyed by the testicular hyaluronidase used.

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The Fate of Certain Organic Acids and Amides in the Rabbit

3. HYDROLYSIS OF AMIDES BY ENZYMES *IN VITRO*

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In the first two papers of this series it was shown that the rabbit is able to hydrolyze the carbamyl group in benzamide and phenylacetamide (Bray, Neale & Thorpe, 1946), but that the introduction of a hydroxyl group in the position *para* to the carbamyl renders the latter stable, less than 7% hydrolysis occurring *in vivo* (Bray, Ryman & Thorpe, 1947). Further investigations are in progress of the effect of hydroxyl groups in other positions and also of amino and nitro groups. This paper reports a parallel study in which we have examined the activity of various enzyme preparations, especially those from rabbit liver, in hydrolyzing the carbamyl group.

Similar investigations are those of Gonnermann (1902, 1903), in which the action of several enzyme preparations, including pepsin, trypsin and sheep's liver and kidney, on a number of amides and anilides was studied, of Geddes & Hunter (1928) and of Grassmann & Mayr (1933) who examined the action of yeast asparaginase on several amides. The criterion used by Gonnermann as an indication of hydrolysis was the isolation of the unchanged amide or of products of its hydrolysis, whilst Geddes & Hunter estimated the ammonia liberated. Relevant results from these investigations are summarized in Table 1. Geddes & Hunter claim that asparaginase is present in calf liver and also that yeast asparaginase will hydrolyze glutamine. Glutaminases hydrolyzing this amide but not asparagine have been shown to be present in various tissues, including liver and kidney (Krebs, 1935).

In the present work we have determined quantitatively the degree of hydrolysis by a formol titration method based on that used by Balls & Lineweaver (1939) for assessing the activity of papain with hippurylamide as substrate. We have also compared the stability of the amides used to acid and alkaline hydrolysis.

METHODS

Materials

Amides. The amides which could not be purchased were prepared by the action of aqueous ammonia upon the corresponding acid chloride or upon the methyl ester of the parent acid. Aminobenzamides were prepared by the reduction of the nitrobenzamides with ferrous sulphate (Jacobs & Heidelberger, 1917).