their behaviour as substrates may be fortuitous because the number of samples was not large and their small amounts did not permit their thorough analysis. The kinetic treatment is empirical. No simple theoretical interpretation is possible, first, because the polymeric substrate is likely to be susceptible to enzymic attack at a large number of sites and, secondly because the effect of the products of this reaction upon the formation of turbidity is not known. However, under suitable conditions, useful comparisons of enzymic activities can be made.

SUMMARY

1. The turbidity-reducing activity of hyaluronidase follows first-order kinetics under standard conditions of assay, provided that the period of incubation is limited to 10 min. and the enzyme concentration to six turbidity-reducing units (Meyer, 1947).

2. Under the above conditions, the following relationship holds: $S_d = a(E - e) S_o^b$, wherein S_d = the amount of substrate destroyed, S_o = the initial concentration of the substrate, and E = the concentration of the enzyme, a , b and e are constants, the first two dependent principally upon the protein content and specific viscosity of the substrate, respectively.

3. While maximal hyaluronidase activity can be attained, the conditions are not feasible for the purpose of assay.

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The Turbidimetric Estimation of Hyaluronate

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The most convenient of the several techniques that have been devised for the assay of hyaluronidase is the turbidity-reduction technique of Kass & Seastone (1944), which has been modified in various respects by several investigators (Leonard, Perlman & Kurzrok, 1946; Meyer, 1947; Warren, Durso & Levin, 1948; Dorfman & Ott, 1948; Tolksdorf, McCready, McCullagh & Schwenk, 1949; Caselli &

Cutinelli, 1949; Harris & Harris, 1949; Escaron & Delfino, 1950; Alburn & Whitely, 1951).

Despite the widespread application of this procedure, the reactions upon which the assay is based have been given little critical attention. Difficulties were encountered when an attempt was made to measure hyaluronate and hyaluronidase in cultures of vaccinia virus by turbidimetric means. The formation of turbidity by the reaction of hyaluronate with protein was therefore investigated, as well as the effect of the enzyme, testicular

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hyaluronidase, upon this reaction. The former aspect of this investigation is described here.

MATERIALS

In order to acquire hyaluronate with as wide a range of properties as possible, it was prepared from human umbilical cords by several procedures:

(a) *Digestion technique.* The following procedure was devised following a suggestion of Hadidian (1950). Acetone-dried umbilical cords were taken up in water and digested with pepsin and trypsin. The crude hyaluronate was precipitated with ethanol, dissolved, freed of protein by treatment with CHCl_3 : amyl alcohol and zinc hydroxide, then re-precipitated with ethanol, washed and dried.

(b) *Alburn & Williams* (1950). Acetone-dried cords were taken up in water and digested with pepsin and trypsin. The digest was fractionated with $(\text{NH}_4)_2\text{SO}_4$ and pyridine and the hyaluronate precipitated by means of ethanol saturated with potassium acetate.

(c) *Hadidian & Pirie* (1948). Acetone-dried cords were extracted with saline solution following the removal of the soluble proteins by aqueous extraction. The saline extract was fractionated with $(\text{NH}_4)_2\text{SO}_4$ and pyridine and the hyaluronate fractions were purified with CHCl_3 : amyl alcohol and freeze-dried following dialysis.

(d) *Meyer* (1948). Deep-frozen umbilical cords were thawed, extracted with water and the hyaluronate of the extract precipitated as a mucin clot. The clot was dissolved and freed of protein by treatment with CHCl_3 : amyl alcohol and zinc hydroxide. The purified hyaluronate was precipitated with ethanol.

(e) *McClean, Rogers & Williams* (1943). Acetone-dried cords were extracted with water and the extract, after adjustment to pH 9.5, was treated with ethanol saturated with potassium acetate to precipitate the hyaluronate.

(f) *Harris & Harris* (1949). The crude product, obtained by the technique of McClean *et al.* (1943), was extracted with hot water. The extract was clarified and re-precipitated at pH 9.5 with ethanol saturated with potassium acetate.

Analyses

The hexosamine content of each of the preparations of hyaluronate was determined as follows. The polysaccharide was hydrolysed according to the conditions of Palmer, Smyth & Meyer (1937). The hydrolysate was dried, as suggested by Hadidian & Pirie (1948) and the amino sugar was estimated by a procedure modified from that of Blix (1948). A correction was made for the hexosamine destroyed during the hydrolysis and, from the corrected values, the purity of the preparation of hyaluronate was calculated, assuming pure hyaluronate to contain equimolar amounts of glucuronic acid and *N*-acetylglucosamine combined through glycosidic linkages. The nitrogen was estimated by the micro-Kjeldahl nesslerization procedure of Koch & McMeekin (1924) and the protein content calculated from the non-glucosamine nitrogen, assuming that the protein contains 16% N. Specific viscosities were determined according to the conditions of Hadidian & Pirie (1948), with a preliminary adjustment of the concentrations of hyaluronate according to the results of the hexosamine analyses.

EXPERIMENTAL AND RESULTS

The effect of various preparations of hyaluronate upon turbidity

The following modification of the procedure of Dorfman & Ott (1948) was employed: to 1 ml. of 0.1 M-acetate buffer, pH 6.0, containing 0.15 M-NaCl and 20–200 μg . of hyaluronate, was added 5 ml. of a reagent consisting of 0.1% (w/v) of albumin (bovine plasma fraction V, Armour Laboratories) in 0.1 M-acetate buffer, pH 4.2, adjusted to pH 3.75 by the addition of 4 N-HCl. Five min. (± 15 sec.) after the addition of the reagent, the optical density was read in a Klett-Summerson photoelectric colorimeter using the green filter (maximal transmission at 540 $m\mu$).

The turbidity increased with the concentration of the hyaluronate, but neither in direct proportion, as found by Dorfman & Ott (1948), nor according to the sigmoidal relationship reported by Meyer (1947) and others. Rather, the relationship was described by a power function over the range 20–200 μg . of hyaluronate/ml. As an example, the experimental findings obtained with the hyaluronate prepared by the digestion technique (*a*, above) fitted closely the equation $T = 2.25S^{0.725}$, as shown in Fig. 1. (T = colorimeter scale reading $\approx 500 \times$ optical density; S = concentration of hyaluronate, in μg ./ml.)

Fig. 1. The turbidity (T) with various concentrations of hyaluronate (S). The experimental points are compared with a plot of $T = 2.25S^{0.725}$.

The other preparations also yielded data that fitted well a power function, as evidenced by the standard error of estimate of turbidity, which ranged from 0.88 to 3.69 for turbidity values from 10 to 110 (see Table 1). This accuracy was as good as was expected since the colorimeter was read to the nearest unit of the scale.

The values observed for the exponent and coefficient with the various preparations of hyaluronate are recorded in Table 1. The values of the exponent did not differ for the various preparations

Table 1. *The constants of the equation, $T = aS^b$, relating turbidity (T) to the concentration of hyaluronate (S)*

Preparation and expt. no.		b	a^*	S.E. of estimate of turbidity*
Digestion technique	I	0.762	1.921	1.39
	II	0.711	1.898	1.00
	III	0.730	1.899	2.46
	IV	0.764	1.806	1.49
	V	0.723	1.815	2.18
Meyer (1948)	I	0.746	1.825	1.58
	II	0.721	1.833	1.43
McClean <i>et al.</i> (1943)	I	0.748	1.864	0.88
	II	0.828	1.834	1.44
Harris & Harris (1949)	I	0.731	2.006	1.76
	II	0.747	2.066	1.75
Hadidian & Pirie (1948)		0.760	1.898	3.69
Alburn & Williams (1950)		0.836	1.255	1.17

* The coefficients and S.E.'s of estimate were calculated using a mean value, 0.754, for the exponent b .

Table 2. *The relation of the coefficient to the properties of the hyaluronate*

Preparation	Coefficient		Specific viscosity	Purity (% w/w)	Protein (% w/w)
	Observed	Corrected*			
Digestion technique	1.868	2.747	0.16	60	3.3
Harris & Harris (1949)	2.036	3.132	0.26	57	6.1
Hadidian & Pirie (1948)	1.898	2.833	0.96	59	8.1
McClean <i>et al.</i> (1943)	1.849	3.773	0.90	39	17.7
Meyer (1948)	1.829	2.771	0.14	58	2.4
Alburn & Williams (1950)	1.255	2.561	1.17	39	3.4
Coefficient of correlation with					
Observed coefficient			-0.612	0.656	0.255
Corrected* coefficient			0.074	—	0.908†

* $a' = a/P^b$ where a and a' are observed and corrected coefficients. P is the purity of the hyaluronate.

† $P < 0.05$.

but the values of the coefficient did ($P < 0.01$). The mean coefficient for each preparation was calculated, using the mean exponent for all the preparations and provided a measure of the turbidity development with each preparation.

The mean coefficient for each preparation is compared with the measured properties of the hyaluronate in Table 2. No significant correlation was found to exist between any of the properties and the observed coefficient. However, the preparations differed appreciably in purity as indicated by their differing contents of hexosamine. Therefore, the coefficient for each preparation was corrected for the lack of purity by the expression $a' = a/P^b$, which was derived from the series of equalities

$$T = aS^b = (a/P^b)(PS)^b = a'S'^b,$$

in which S' = the corrected concentration of hyaluronate, a and a' = the observed and corrected coefficients respectively, b = the exponent and P = the purity of the preparation, calculated from its hexosamine content and expressed as a decimal fraction. The values of the corrected coefficients were slightly correlated with the protein contents of the preparations ($P < 0.05$).

Kinetics

One ml. portions of 0.15M-NaCl, buffered with 0.1M-acetate at pH 6.0 and containing 100, 250, 750 or 1000 μ g. of hyaluronate, were treated with 5 ml. of the albumin reagent of Dorfman & Ott (1948). The turbidity at 540 $m\mu$. was measured as soon as possible thereafter and at intervals up to 100 min. The hyaluronate used in this and subsequent experiments was prepared by the digestion technique (*a*, above).

The turbidity rose rapidly to a maximum and then declined gradually, as shown in Fig. 2. The law of mass action evidently applied to the initial reaction, since the rate of formation of turbidity was greater with 250 than with 100 μ g. and was too rapid to follow with higher concentrations.

Effect of pH

Solutions of 0.5M-sodium acetate and 0.5M-acetic acid, both containing 0.5% (w/v) of albumin, were mixed in various proportions to give pH values from 3.0 to 5.0. To 1 ml. portions of each of these buffers, 4 ml. of 0.1% (w/v) of hyaluronate were added and the turbidity was measured as described in the first paragraph of the Experimental section. The resultant pH in each tube was checked electrometrically using a glass electrode.

An appreciable turbidity developed only between pH 3.8 and 4.4 with the high values close to 4.2, as shown by Fig. 3. The turbidity was negligible above pH 4.6 and below 3.5.

Fig. 2. The kinetics of turbidity formation for 100 ($\square$), 250 ($\blacksquare$), 750 ($\circ$) and 1000 ($\bullet$) μ g. of hyaluronate. The scale is compressed tenfold after 10 min.

Fig. 3. The effect of pH upon turbidity development.

Effect of ionic strength

Acetate. M-Acetate buffer, pH 4.2, and water, both containing 0.5% (w/v) of albumin, were mixed to give concentrations of acetate from 0.1 to 1.0M. A solution of hyaluronate was added to 1 ml. portions of each solution and the turbidity was measured as already described.

The results, presented in Fig. 4, revealed that the elevation of the concentration of acetate interfered with the development of turbidity.

Sodium chloride. To 1 ml. portions of 0.1M-acetate buffer, pH 4.2, containing 0.5% (w/v) of albumin and from 0.1 to 1.0M-NaCl, hyaluronate was added and the turbidity measured.

Sodium chloride was much more effective than acetate in inhibiting the development of turbidity and, with concentrations of sodium chloride above 0.5N, the turbidity fell to low values (see Fig. 4).

Fig. 4. The effect of the concentration of acetate buffer, pH 4.2, ($\bullet$) and NaCl ($\circ$) upon the development of turbidity.

Fig. 5. The effect of the concentration of albumin upon the development of turbidity. The mean values are joined by the solid line, but the individual observations are plotted to illustrate the increasing irregularities at the high concentrations.

Effect of concentration of albumin

A 0.5M-acetate buffer, pH 4.2, and 2% (w/v) solution of albumin in the buffer were mixed to give concentrations of albumin from 0.2 to 2.0% (w/v). One ml. portions of these solutions were treated with hyaluronate and the turbidity measured.

The turbidity increased as the concentration of albumin was elevated but the values became irregular with high concentrations due to the formation of visible clot-like aggregates (Fig. 5).

Recommended procedure

These experiments suggested two methods for increasing the sensitivity of the estimation of hyaluronate. First, the total concentration of electrolytes could be reduced by using acetic acid in the protein reagent to bring the pH to 4.2, rather than acetate buffer. Secondly, the relative proportions of the protein reagent and of the solution of hyaluronate in the final mixture might be reversed.

The application of these principles is illustrated by the following procedure: to 4 ml. of 0.05M-acetate buffer, pH 6.0, containing 0.05M-NaCl and 20–200 μ g. of hyaluronate/ml., was added 1 ml. of 0.568N-acetic acid, containing 0.5% (w/v) of albumin. The turbidity was measured at 540 m μ . 5 min. after mixing. The pH of the mixture was 4.20. The electrolyte content of the solution of hyaluronate was reduced from that used previously in order to permit the development of turbidity.

Fig. 6. Standard curves for the new procedures applied to 0.05M-acetate buffer, pH 6.0, containing 0.05M-NaCl (●) and to virus culture medium, buffered to pH 6.0 with 0.0791M-NaH₂PO₄ (○).

A plot of the turbidity against the concentration of hyaluronate yielded a smooth uninflected curve which, however, did not fit a power function (Fig. 6). The four lower points corresponded to the equation $T = 9.47S^{0.607}$ with a standard error of estimation of 3.1 units of the colorimeter scale. The fourfold increase in sensitivity was relative rather than absolute, since four times the weight of hyaluronate was required. A procedure of this sort has value only if relatively large volumes of a dilute solution of hyaluronate, low in electrolytes, are to be analysed.

A solution of hyaluronate in 0.1M-acetate buffer, pH 6.0, containing 0.15M-NaCl could be analysed by treating a 1 ml. portion with 5 ml. of 0.0491N-acetic acid, containing 0.1% (w/v) of albumin. This solution yielded a final pH of 4.22. The data fitted a simple power function of the concentration of hyaluronate, $T = 2.92S^{0.703}$, with a standard error

of estimate of 1.37. This procedure was only 8% more sensitive than the usual technique, but the reagent was simpler in composition.

A slight modification of the procedure recommended above could be applied to the analysis of hyaluronate dissolved in the virus culture medium of Simms & Sanders (1942). The medium was buffered to pH 6.0 by the addition of an equal volume of 0.0791M-NaH₂PO₄ and 4 ml. of the mixture were treated with 1 ml. of 0.179N-acetic acid containing 0.5% (w/v) of albumin. The final pH was 4.10.

A smooth curve, similar to that obtained with the dilute acetate buffer, was also found under these conditions (Fig. 6) and, again, only concentrations of 60 μ g. of hyaluronate, or less, fitted to a power function. In this instance, the equation of best fit was $T = 1.87S^{0.855}$ with a standard error of estimate of 0.21 unit.

DISCUSSION

The mechanism of the turbidity-forming reaction has not been clearly demonstrated. Most investigators have assumed that a carbohydrate anion combines with a protein cation by an electrostatic bond to form a mucoprotein. The optimal pH and the effect of electrolytes provide some additional evidence for the hypothesis. Acidification of the system has the double effect of enhancing the cationic ionization of the protein and depressing the ionization of the hyaluronate anion. Thus, the maximal ionization of both hyaluronate and protein cannot be attained simultaneously, and the observed optimal pH of the reaction represents the best compromise. The interference of electrolytes with the formation of turbidity can be understood in terms of their effect on interionic forces.

In a practical procedure, the minimal concentration of electrolyte attainable is determined by the requirement for stable buffering on the one hand and the electrolyte content of the solution to be analysed on the other. An increased sensitivity of the reaction can be attained by the use of a weak acid, rather than a buffer, in the protein reagent, so that the cations of the solution containing hyaluronate provide the other component of the buffering system.

The increased sensitivity of the reaction, achieved by increasing the concentration of albumin, is offset by the irregular values that result from the formation of grossly visible particles in the mixture. The concentration recommended appeared to be a reasonable compromise between sensitivity and reproducibility.

The kinetics of the development of turbidity confirm the reported necessity for timing the period between the addition of the albumin reagent and the measurement of the turbidity. In choosing a time interval for routine assays, the increased sensitivity

attainable by making an early reading is offset by the additional effort involved in accurate timing. However, the time cannot be shortened beyond the period required for the maximal development of turbidity. The formation of turbidity apparently follows the law of mass action as judged by the time required to reach a maximum with various concentrations of hyaluronate. The decay phase of the reaction seems to be characteristic of the system, since the values for the different preparations of hyaluronate run parallel on a logarithmic plot.

Since the development of turbidity is dependent upon the properties of the hyaluronate, this reaction should be applied with caution to the estimation of hyaluronate of unknown composition. The possible difficulties encountered in the application of this reaction to systems not under strict control is illustrated by our experience with the analysis of virus cultures. Although smooth curves and excellent precision were found upon addition of solutions of hyaluronate to the medium for virus culture, the recovery of hyaluronate from the same medium containing, in addition, growing chick-embryo mince and vaccine virus was variable and averaged only 66%. This low recovery was not attributable to any of the factors studied.

The turbidity-forming reaction between hyaluronate and protein therefore provides a satisfactory method for the estimation of the polysaccharide in controlled systems, such as are commonly employed in enzyme assays but cannot, at the present, be applied with confidence even to simple biological systems.

SUMMARY

1. The effects of pH, and of the concentrations of hyaluronate, protein and electrolyte upon the turbidity-producing reaction between hyaluronate and protein have been studied, as well as the kinetics of the reaction.
2. The turbidity produced is a simple power function of the concentration of hyaluronate and depends upon the properties of the preparation, principally its purity and content of protein.
3. The optimal pH of the reaction is 4.1-4.2.
4. Electrolytes interfere with the formation of turbidity, sodium chloride being more active in this respect than acetate.
5. The increased sensitivity achieved by raising the concentration of albumin is offset by a loss of precision.
6. The turbidity develops rapidly at a rate dependent upon the law of mass action and decays at a diminishing rate over a period of several hours.
7. The application of these observations to some practical analytical problems is illustrated.

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