THE MECHANISM OF HYALURONIDASE ACTION*

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Bovine testicular hyaluronidase has been shown to depolymerize both hyaluronate and chondroitin sulphate A[1] by hydrolyzing the β-hexosamidic bond of either substrate[2]. Numerous workers have claimed that since the ratio of the activities of hyaluronidase upon both these substrates was unaffected by enzyme purification[3,4] only one enzyme site was involved in the hydrolysis of both substrates. Final proof for this contention has been provided by employing a kinetic analysis of the rates of reaction of hyaluronidase preparations ranging over 400-fold in purity acting upon mixtures of both substrates.

By studying the effect of temperature upon the kinetic constants of reaction, the thermodynamics of hyaluronidase action has been determined. These results were interpreted with regard to a possible mechanism of action.

MATERIAL AND METHODS

The preparation and analysis of bovine tracheal chondroitin sulphate A and human umbilical cord hyaluronate has been published elsewhere[5]. Bovine testicular hyaluronidase was prepared by ammonium sulphate fractionation of a 0.1 *M* acetic acid extract of decapsulated testes. Each fraction was dialyzed free of salt and the precipitated globulins removed. No mucolytic activity was found in the globulin fraction. The assay of the albuminoid fractions is shown is Table I. The 512 g/l fraction was further treated with ethanol according to the procedure of FREEMAN, ANDERSON, OBERG AND DORFMAN[6]. The assay of the three enzyme preparations resulting from this step, as well as a highly purified preparation kindly provided by Dr. MARTIN B. MATHEWS of the University of Chicago, is shown in Table II.

TABLE I

THE MUCOLYTIC ENZYME ACTIVITIES OF AMMONIUM SULPHATE FRACTIONS OF BOVINE TESTES

Assay	Activity of fractions per mg N precipitated by salt concentration (g/l) of				
	0	212	312	412	512
Hyaluronidase[15]	2841	260	158	(no ppt)	326
β-Glucuronidase[16]	630	2100	0		0
β-Hexosaminidase[17]	1280	1100	522		65
β-Galactosaminidase[18]	1180	580	2850		0

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TABLE II

THE MUCOLYTIC ENZYME ACTIVITIES OF ETHANOL FRACTIONS OF BOVINE TESTES EXTRACT

Assay	Activity of fractions per mg N precipitated by ethanol			
	I	II	III	IV
Hyaluronidase[15]	326	1,333	6,515	116,000
β-Glucuronidase[16]	0	0	0	0
β-Hexosaminidase[17]	65	0	0	0
β-Galactosaminidase[18]	0	0	0	0

Solutions of enzyme and substrate were separately dissolved in 0.1 M acetate buffer pH 5.0 containing 0.15 M sodium chloride. Duplicate mixtures containing 0.50 ml of enzyme and 0.50 ml of substrate were incubated together for 10 minutes. During this period the reaction was zero-order. Enzymic action was halted by the addition of the reagents used in the determination of reducing groups according to a modification of the PARK AND JOHNSON procedure[7]. This modification permitted the determination of reducing sugars at pH 9.0, thus eliminating the alkaline hydrolysis of chondroitin sulphate occurring at the higher pH usually required for reaction. The release of reducing substances due to enzymic action was translated via standard curves into μg or μmoles of N-acetyl hexosamine. These values were used statistically according to HOFSTEE[8] to calculate the Michaelis-Menten constant (K_m) and the maximum velocity (V_m).

EXPERIMENTAL AND RESULTS

Identity of hyaluronidase action

The velocity of the action of a single enzyme upon a mixture of two substrates may be calculated from the equation of THORN[9] by using the V_m and K_m determined upon the two substrates separately as follows:

$$v = \frac{K^{c}_{m}V^{H}_{m}\,(H) + K_{m}{}^{H}V_{m}{}^{c}\,(C)}{K^{H}_{m}K^{c}_{m} + K_{m}{}^{H}\,(C) + K^{c}_{m}\,(H)}$$

where H and C refer to hyaluronate and chondroitin sulphate A respectively. The K_m and V_m (using 0.5 TRU/ml of enzyme) for hyaluronate were found to be $0.86 \pm 0.03 \cdot 10^{-3}$ M and $4.1 \pm 0.21 \cdot 10^{-8}$ M per sec^{-1} respectively. The K_m for chondroitin sulphate was three times that for hyaluronate, while the V_m for the two substrates was the same. Using these values, v was calculated for equimolar mixtures of the two substrates containing a total of one to eight micromoles of hexosamine per ml of reaction mixture. These values are compared to the experimentally observed velocities resulting from the action of 0.50 TRU of a preparation of hyaluronidase assaying 6515 TRU/mg nitrogen upon an equimolar mixture of both substrates in Fig. 1. This figure also shows the velocity of 0.5 TRU of the enzyme acting upon similar concentrations of each substrate separately.

Table III shows the close correlation between the predicted and observed velocities resulting from the action of one TRU of enzyme preparation I, II, III, and IV which ranged in purity over 400-fold upon an equimolar mixture of both substrates containing 2.0 micromoles of hexosamine per ml. This close correlation strongly indicates the identity of the hyaluronate and chondroitin sulphate depolymerases; a conclusion which is further strengthened by the fact that this correlation is not affected by 400-fold purification of the enzyme.

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TABLE III

A COMPARISON OF THE CALCULATED AND OBSERVED VELOCITIES OF HYALURONIDASE ACTION UPON AN EQUIMOLAR MIXTURE OF SUBSTRATES

Enzyme number	*Activity (TRU/mgN)*	*Calculated*	*Observed*
I	326	6.4	6.6
II	1,333	6.2	6.2
III	6,515	6.4	6.3
IV	116,000	6.3	6.4

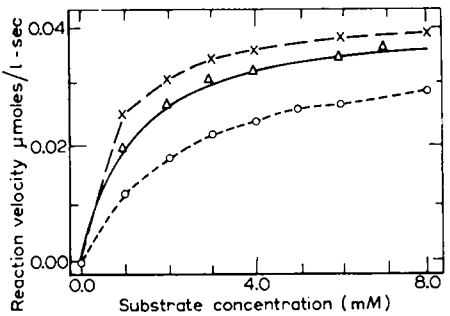

Fig. 1. The action of 0.50 TRU of hyaluronidase upon chondroitin sulphate A (o), hyaluronate (×), and equimolar mixtures of the two (△). The solid line represents the theoretical equation for the action of one enzyme upon two substrates.

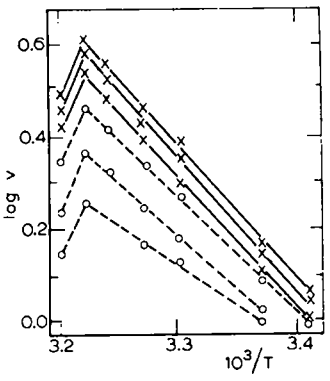

Fig. 2. The effect of absolute temperature (T) upon the velocity of hydrolysis (V) (µg of N-acetyl-hexosamine/ml/10 minutes) by 0.50 TRU of enzyme acting upon various concentrations of hyaluronate (×) and chondroitin sulphate (○). Reading from top to bottom the concentrations of substrate are as follows: 2.40, 1.44, and 0.96 mg/ml.

Effect of temperature

The action of 0.50 TRU of hyaluronidase upon 0.46, 1.44 and 2.40 mg of both chondroitin sulphate A and hyaluronate at temperatures ranging from 20°C to 39°C is shown in Fig. 2. The logarithm of the reaction velocity was linearly related to the reciprocal of the absolute temperature. The change in slope above 37° C was probably due to thermal inactivation of the enzyme, since the slope of each curve above this temperature was the same for all concentrations of either substrate. The slope of the remainder of the curves decreased with substrate concentration, particularly in the case of chondroitin sulphate A. The K_m and V_m calculated from the data of Fig. 2 for each temperature is shown in Table IV. Although there were only three substrate concentrations, these calculated values had less than a ten percent standard error.

Thermodynamics

The change in enthalpy (ΔH^*) of the enzyme-substrate mixture with the activation of the enzyme-substrate complex can be calculated from the effect of temperature upon the velocity constant of reaction according to the following equation:

$$\frac{d\ln K}{d(1/T^0)} = \Delta H^* - RT^0$$

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TABLE IV

THE EFFECT OF TEMPERATURE UPON HYALURONIDASE ACTION

Temperature (°C)	Hyaluronate		Chondroitin Sulphate A	
	V_m*	K_m**	V_m*	K_m**
37	4.59 ± 0.15	0.81 ± .03	4.60 ± 0.18	2.16 ± .01
32	4.22 ± 0.15	0.78 ± .03	4.18 ± 0.15	1.88 ± .01
27	3.64 ± 0.10	0.74 ± .03	3.55 ± 0.12	1.64 ± .01
22	3.06 ± 0.10	0.70 ± .02	2.92 ± 0.12	1.37 ± .01
20	1.80 ± 0.05	0.69 ± .03	1.46 ± 0.10	1.29 ± .01

* $\times 10^{-8}$ M/sec. ** $\times 10^{-3}$ M.

where K is the velocity constant, $T°$ the absolute temperature, and R the gas constant. Since the purity of the enzyme preparation employed in this investigation was unknown, the velocity constant K could not be calculated from the maximal velocity ($V_m = K(E)$). However a plot of ln V_m versus the reciprocal of the absolute temperature gave lines of slope ΔH^*–$RT°$, since the enzyme concentration was constant in these experiments, and the additive factor for the ordinant, ln $1/(E)$, would only shift the curve without changing its slope. This slope was calculated statistically from the data in Table IV. The change in free energy (ΔF^*) with activation may be calculated from the equation of EYRING[10]:

$$K = \frac{K_b T^0}{h} e^{-\frac{\Delta F^*}{RT^0}}$$

where K_b is Boltzmann's constant and h Plank's constant. If V_m again be substituted for K in this equation, the value for ΔF^* will be in error due to the contribution of the enzyme concentration. Since the enzyme concentration was the same for the reaction with both chondroitin sulphate and hyaluronate, a comparison between the values for the two substrates may be made. The entropy of activation calculated from these values would, of course, be subject to the criticism described above. These values are presented in Table V.

TABLE V

THE THERMODYNAMICS* OF HYALURONIDASE ACTION

Substrate	ΔF (Cal/mole)	ΔH (Cal/mole)	ΔS (Cal/mole)
		Complex formation	
Hyaluronate	−4,400 ± 90	−1,760 ± 200	+8.5 ± 0.7
Chondroitin Sulphate A	−3,860 ± 30	−5,500 ± 400	−5.3 ± 1.3
		Activation	
Hyaluronate	24,300 ± 1,500	12,800 ± 1,400	−37.3 ± 9.4
Chondroitin Sulphate A	24,300 ± 1,200	12,600 ± 1,600	−38.0 ± 9.0

* (Calculated for 36°C).

If K_m be considered an equilibrium constant, then the enthalpy change of the system due to enzyme-substrate complex formation may be calculated from the effect of temperature upon the reciprocal of K_m.

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$$\frac{\mathrm{d}\ln(1/K_m)}{\mathrm{d}(1/T^0)} = \frac{-\Delta H^0}{R}$$

The slope of the plot of $\ln 1/K_m$ versus $1/T^0$ was determined statistically from the data presented in Table IV. The change in free energy with complex formation may be calculated from the equilibrium constant,

$$\Delta F^0 = -RT^0 \ln 1/K_m$$

and the entropy change during complex formation may be calculated from these two values in the usual way. These values are presented in Table V, and indicate that the thermodynamics of complex formation differed markedly, while the thermodynamic parameters of activation were identical for both substrates.

TABLE VI

THE ANALYSIS IN μMOLES/mg OF PARTIALLY DESULPHATED PREPARATIONS

Ethanol fraction	*Hexosamine*[19]	*Nitrogen*[20]	*Sulphate*[21]
0	2.00	2.10	1.60
I	1.85	2.31	0.81
II	1.90	2.31	0.52
III	1.93	2.43	0.30

Effect of substrate sulphation

Partially desulphated chondroitin sulphate was prepared by incubating 400 mg of the substrate with 200 ml of 5*N* HCl at 36° for about three hours. After this period, the pH was brought to neutrality with concentrated barium hydroxide, and the barium sulphate precipitate was removed by centrifugation. The solution was then dialysed for 48 hours against distilled water at 4°C and the polysaccharide was precipitated with four volumes of cold ethanol containing one per cent (w/v) glacial acetic acid. The precipitate, after further washing and drying *in vacuo* was dissolved in isotonic saline — 0.1 molar acetate buffer, pH 5.0, at a concentration of 5 mg/ml. This solution was fractionated with ethanol and three fractions differing in alcohol solubility were prepared. All three preparations were subsequently refractioned with ethanol. The sulphate analysis of each fraction, indicated in Table VI, showed that the alcohol solu-

TABLE VII

THE EFFECT OF THE SUBSTRATE SULPHATION UPON THE THERMODYNAMICS OF HYALURONIDASE ACTION

Preparation	*ΔF*	*ΔH*	*ΔS*
		Complex formation	
I	−3700 ± 60	−3800 ± 400	−0.3 ± 1.6
II	−3840 ± 60	−2900 ± 400	+3.0 ± 1.5
III	−3900 ± 60	−2200 ± 300	+5.5 ± 1.4
		Activation	
I	24,000 ± 2300	13,000 ± 2900	35.5 ± 16.8
II	25,000 ± 2200	12,500 ± 2500	−40.5 ± 15.2
III	23,000 ± 2000	12,150 ± 1800	−35.0 ± 12.3

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bility was inversely related to substrate sulphation. The polymer size was determined by the ratio of reducing activity to total hexosamine content and was found to decrease with sulphate content. During desulphation, a small amount of de-acetylated material was found, but this material precipitated out at lower ethanol concentrations.

The thermodynamics of hyaluronidase activity upon these three partially desulphated preparations was calculated as described above, and the results are shown in Table VII. Again there was no change in the energies of activation with substrate sulphate content. The thermodynamics of complex formation tend to change from the pattern presented by chondroitin sulphate to that shown by hyaluronate with decreasing substrate sulphation.

DISCUSSION

The discovery of the transglycosidative activity of hyaluronidase complicated the interpretation of hydrolytic reaction rates[11]. The zero-order reaction rates occurring under the conditions described above however, mean that any interactions between enzyme and reaction products were minimized to the point where the kinetic constants of hydrolysis may be calculated from an equation in which only the interaction between the enzyme and substrate was postulated *i.e.*[12].

$$\text{velocity} = \frac{K(E)\ (S)}{(K_m + (S))}$$

The energetics of the activation of the enzyme-substrate complex was the same for the two substrates. This might be expected since the energy of the β-hexosamidic bond would not be electromerically affected by a sulphate group some three carbons away[13].

A combination of enzyme and substrate should demonstrate a loss in the entropy of the system, as occurred when chondroitin sulphate was the substrate. When hyaluronate was used as a substrate however, the system demonstrated a gain in entropy with complex formation. There are at least two explanations for this difference: (1) The entropy changes were derived from the K_m of enzymic action by assuming that k_3 was much smaller than k_2 from the equation described below:

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_3} E + P$$

The increase in the value of K_m for chondroitin sulphate over that of hyaluronate could not be due to differences in k_3, since both substrates presented identical maximum velocities of enzymic hydrolysis. The sulphate group however, might sterically hinder the rate of complex formation and thus decrease k_1 as well as rendering the complex less stable and so increasing k_2. This larger K_m would then more closely approximate an equilibrium constant. The loss of entropy with complex formation would then be an expression of the more accurate equilibrium constant used for calculation. (2) Positive entropies of complex formation are also explicable by reference to the displacement of bound water from both enzyme and substrate. This difference between the entropies of the two substrates reacting with hyaluronidase would then result from the fact that the more highly ionic chondroitin sulphate does not bind as much water as hyaluronate[13].

Negative enthalpies of complex formation involving highly charged molecules

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are well known. The steric accommodation of the substrate sulphate group might require a greater distortion of the internal enzyme bonds involved in complex formation than would be necessary for hyaluronate. The three-fold difference in enthalpy change described above would indicate that the complex formed with hyaluronate at a rate faster than that with chondroitin sulphate. This greater ease of complex formation with hyaluronate was reflected also in the free energy values.

The enthalpy change with complex formation decreased as the ester sulphate was removed from chondroitin sulphate, since in the absence of the sulphate group the distortion of enzyme bonds required for complex formation would be reduced. The entropy of formation was related inversely to the sulphate content of the substrate, and thus reflected the greater ability of substrates low in sulphate to bind water molecules. The presence of the sulphate group would also tend to decrease the free energy of complex formation by sterically hindering the union of enzyme and substrate as mentioned above. An examination of Fischer-Hirschfelder-Taylor models of the repeating units of hyaluronate and condroitin sulphate A indicated that the free rotation of the sulphate group allowed it to extend over the pyran ring of the hexosamine and effectively cover the labile glycosamidic bond. Under this condition, no "tight fit" between the enzyme and the substrate around the area of this bond would be possible. The rate of reaction between hyaluronidase and chondroitin sulphate A would thus be dependent upon an extra factor, the rate of the covering and uncovering of the labile bond. Therefore the rate of formation would be lower and K_m larger for chondroitin sulphate than hyaluronate. The final equilibrium between enzyme, substrate and complex would be the same for both substrates, however, and so the V_m of enzymic action would be unaffected by the sulphate content of the substrate. It would seem therefore that the presence of the sulphate group on the chondroitin sulphate polymer was responsible for the differences in the rate of reaction between hyaluronidase and the two substrates. This idea is consistent with the observation of DAVIDSON AND MEYER[11] that chondroitin was as reactive to hyaluronidase as hyaluronate.

Although it would be tempting to conclude that only k_1 would be affected by this sulphate group, and hence k_2 would be the same for both substrates, the thermodynamics of complex formation do not establish this point.

SUMMARY

1. The identity of the enzyme site involved in the hydrolysis of hyaluronate and chondroitin sulphate A was demonstrated.

2. The thermodynamics of hyaluronidase action were determined. These parameters indicated that the energies of complex activation were identical for both hyaluronate and chondroitin sulphate A, while the energies of complex formation markedly differed with the substrates used.

3. The difference was shown to be caused by the steric hindrance of the sulphate group.

4. Partial removal of the ester sulphate of the substrate reduced these differences in the thermodynamics of complex formation.

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DIE BINDUNG VON ADENOSINDIPHOSPHAT, VON ANORGANISCHEM PHOSPHAT UND VON ERDALKALIEN AN DIE STRUKTURPROTEINE DES MUSKELS

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I

Die Untersuchungen BOZLERS[1] haben gezeigt, dass Muskelfasern vom Psoas des Kaninchens nach Glycerin-Wasser-Extraktion noch etwa 0.6 μMol Ca^{++}/g Nassgewicht und etwa die gleiche Menge Magnesium enthalten. Diese Erdalkaliionen sind sehr fest an die Proteinstrukturen der extrahierten Fasern gebunden. Selbst länger dauernde EDTA-Behandlung entfernt aus diesen Fasern praktisch kein Magnesium und nur etwa 50% des Calciums. Auch Aktomyosinlösungen[2] und Aktinlösungen[3] enthalten Erdalkaliionen, die bei üblicher Reinigung nicht zu entfernen sind.

Auf Grund der grossen Bedeutung der Erdalkaliionen für die ATP-Kontraktion und die ATP-Spaltung durch die Proteine der kontraktilen Strukturen werden die Bindungsverhältnisse im folgenden im einzelnen untersucht. Insbesondere wird festgestellt, wie sich die Erdalkaliionen und das gebundene ADP PERRY's[4] auf die verschiedenen Proteine der Fibrille verteilen.

II

Wird Muskelbrei mit Latapie und Starmix zerkleinert und gründlich mit 0.1 *M* KCl gewaschen, so lassen sich nach etwa fünf Waschungen – bei jeder Waschung wird der Muskelbrei achtfach verdünnt – in der Waschflüssigkeit weder Eiweiss, Phosphat noch Erdalkaliionen nachweisen. Bei dieser Waschprozedur verliert der Muskel etwa

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