3. Thoroughly dialysed DF^{32}P treated human sera as well as sera obtained from humans a few days after the injection of DF^{32}P were submitted to zone electrophoresis. No radioactivity could be detected on the paper electrophoresis strips, but after column electrophoresis it was possible to localise the radioactivity between the α-2 and the β-peaks.

4. The conclusion is reached that in human sera, which have been in contact with DF^{32}P, only one component, the pseudo-cholinesterase, is irreversibly labelled by ^{32}P. The values obtained for the turnover of serum proteins by means of DF^{32}P therefore clearly reflect the turnover of the pseudo-cholinesterase component.

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THE EFFECT OF SUBSTRATE SIZE UPON HYALURONIDASE ACTION*

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WEISSMANN[1] has shown that the activity of hyaluronidase increased with substrate size. This paper describes in quantitative terms the effect of substrate size upon the thermodynamics of hyaluronidase action, as well as providing a measure of the size of the active center of the enzyme.

MATERIAL AND METHOD

Partially degraded substrate

250 mg of human umbilical cord hyaluronate and bovine tracheal chondroitin sulphate A were dissolved separately in 50 ml of 0.10 *M* acetate buffer (pH 5.0) containing 0.15 *M* sodium chloride. To five 10 ml portions of each solution 0.10 ml of buffer containing 40 TRU of a preparation of hyaluronidase, assaying 6500 TRU/mg N was added. The enzymic reaction was stopped after 0, 4, 8, 12 and 15 minutes incubation at 36° C by thermal inactivation, and the solutions were pooled. This pooled material contained a mixture of degraded substrate of greatly varying chain length. Ethanol was then added until the first traces of a precipitate occurred. The solution was then stored at 4° C for 30 minutes. After storage the precipitate was collected by centrifugation and more ethanol was added to the clear supernatant. This procedure was repeated until no more centrifugable material formed. The precipitates were washed in ethanol, dried, and redis-

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solved in saline-buffer. The ethanol fractionation procedure was repeated. After a third fractionation each precipitate separated sharply over a narrow range of ethanol concentration. The comparative homogeneity of the five fractions was demonstrable by recording the optical density at 400 mμ during the titration of 6 ml solutions containing 3 mg of each preparation with ethanol. These data, after correction for volume changes, indicated that each preparation was relatively homogeneous (Fig. 1). The analysis of these materials and their mean chain length, as determined by the ratio of reducing endgroups to total hexosamine is shown in Table I. The reducing endgroups were determined by a modification of the PARK AND JOHNSON procedure[2], which eliminated the alkaline hydrolysis of chondroitin sulphate usually found with this substrate[3].

Degraded substrate

The tetra-, hexa-, and octa-saccharides resulting from the prolonged action of large amounts of hyaluronidase upon hyaluronate were isolated by the chromatographic method of WEISSMANN, MEYER, SAMPSON AND LINKER[4]. Extra octasaccharide was kindly provided by Dr. BERNHARD WEISSMANN, Mt. Sinai Hospital, New York City. The hexa- and octa-saccharide of hyaluronate were reduced by 0.10 *M* sodium borohydride according to WEISSMANN[1].

Both enzyme and substrate were dissolved in saline buffer pH 5.0 and incubated together for 10 minutes at 37°C. During this period, the reaction rate was zero-order for the range of substrate concentration used. The reaction rate was determined by translating the reducing activity of a given solution into moles of N-acetyl-hexosamine per unit time via a standard curve. The K_m and V_m of reaction was calculated statistically according to HOFSTEE[5].

TABLE I

THE ANALYSIS OF THE PARTIALLY DEGRADED SUBSTRATES

	Concentration (μmole/mg) of		*Reducing Activity (μmole N-acetyl-hexosamine/mg)*	*Approximate mean chain length**
	Hexosamine[a]	*Nitrogen*[b]		
Hyaluronate				
1	2.00	2.4	0.0156	256
2	2.00	2.4	0.0320	125
3	1.98	2.5	0.0620	64
4	1.95	2.5	0.1180	33
5	1.90	2.6	0.1520	25
Chondroitin sulphate A				
1	1.65	2.1	0.0260	127
2	1.65	2.1	0.0386	86
3	1.62	2.3	0.0550	59
4	1.60	2.2	0.0890	36
5	1.60	2.3	0.1334	24

* Expressed as monosaccharide units per molecule and calculated:

$$\frac{2 \times \text{total hexosamine (μmole/mg)}}{\text{Reducing activity (μmole N-acetylhexosamine/mg)}}$$

EXPERIMENTAL AND RESULTS

One-half TRU of enzyme was made to react at 36°C with 0.25, 0.50, 0.75, and 1.00 mg/ml of the various substrates and the Michaelis-Menten constant K_m and maximum velocity V_m were determined. A similar substrate curve was prepared at 32, 27, and 22°C. Table II shows the K_m and V_m at 36°C for the various sized substrates. The amount of reducing materials released by 100 TRU of enzyme from 1 mg/ml of the tetra-, hexa- and octa-saccharides as well as their reduced derivatives after two hours incubation at 36°C was also determined.

These results, expressed qualitatively, are shown in Table II. Reduction of the

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hexasaccharide completely inhibited enzymic action upon this substrate, while the reduced derivative of the octasaccharide was still enzymically labile.

The free energy, enthalpy and entropy of activation were calculated from the effects of temperature upon the maximal velocity. The thermodynamic parameters were also calculated for the formation of the enzyme-substrate complex from the effect of temperature upon the K_m of enzymic action. The technique and implicit assumptions for these calculations have been described previously[3]. Table III shows that substrate size has no effect upon the thermodynamics of activation. Table IV however, illustrates the marked effect of substrate size upon the free energy and entropy of complex formation.

Five mg of hyaluronate and chondroitin sulphate A were incubated for 48 hours at 36°C under toluene with 200 TRU of enzyme. At this time 200 TRU of enzyme was again added and the incubation was continued for another 48 hours. The addition of 100 TRU of enzyme after this time resulted in no further release of reducing groups. Aliquots were removed from each solution, diluted, partially deproteinized by trichloracetic acid, and the reducing activity quantitatively determined. Similar concentrations of substrate and inactivated enzyme were used as controls. The comparatively high concentration of protein rendered the precise determination of chain length from the ratio of reducing activity to total hexosamine difficult, but the apparent equilibrium products of reaction are reported in Table V along with their standard error.

TABLE II

THE ACTION OF HYALURONIDASE UPON DEGRADED ACID MUCOPOLYSACCHARIDES

Preparations	*Enzymic activity**	K_m ($\times 10^{-3}$ M)	V_m ($\times 10^{-6}$ M sec)
Hyaluronate			
1	+	0.81 ± 0.02	4.1 ± 0.26
2	+	0.98 ± 0.02	4.0 ± 0.15
3	+	1.30 ± 0.04	4.4 ± 0.40
4	+	2.00 ± 0.02	4.1 ± 0.20
5	−	4.00 ± 0.04	4.2 ± 0.25
Octasaccharide	+		
Reduced octasaccharide	·	–	
Hexasaccharide	+		
Reduced hexasaccharide	o		
Tetrasaccharide	o		
Chondroitin sulphate A			
1	+	2.1 ± 0.20	4.1 ± 0.15
2	+	2.4 ± 0.20	4.3 ± 0.20
3	+	3.3 ± 0.15	4.1 ± 0.30
4	+	6.6 ± 0.18	4.4 ± 0.40
5	+	9.9 ± 0.25	3.9 ± 0.25

* Determined from the relative release of reducing groups from one mg substate by 100 TRU of enzyme after two hours at 36°C.

A plot of the standard free energy of complex formation as presented in Table IV versus the reciprocal of chain length is shown in Fig. 2. The extrapolation to zero free energy provides a measure of the chain length at which, for every mole of complex

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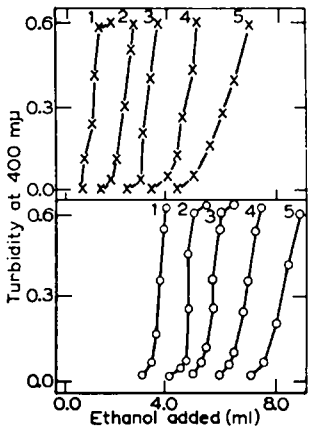

Fig. 1. The absorbance resulting from the addition of ethanol to solutions of preparations 1 through 5 of hyaluronate (×) and chondroitin sulphate A (○).

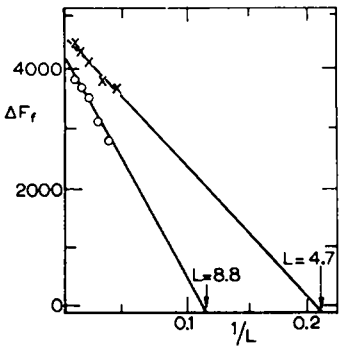

Fig. 2. The standard free energy of complex formation (ΔF_f) for hyaluronate (×) and chondroitin sulphate A (○) at various chain lengths (L).

TABLE III

THE EFFECT OF SUBSTRATE SIZE UPON THE THERMODYNAMICS OF ACTIVATION*

Substrate	*Chain length*	*ΔF (Cal/mole)*	*ΔH (Cal/mole)*	*ΔS (Cal/degree mole)*
Hyaluronate				
1	256	24,300 ± 350	12,700 ± 1500	−38 ± 3.5
2	125	24,200 ± 350	12,000 ± 1400	−38 ± 3.5
3	64	24,000 ± 400	11,800 ± 1800	−38 ± 3.5
4	33	24,600 ± 400	11,900 ± 1400	−38 ± 3.5
5	25	24,500 ± 300	12,600 ± 1500	−38 ± 3.5
Chondroitin sulphate A				
1	127	24,400 ± 400	12,500 ± 1600	−38 ± 4.0
2	86	24,000 ± 400	12,900 ± 1400	−38 ± 4.0
3	59	24,000 ± 400	11,400 ± 1400	−38 ± 3.5
4	36	24,000 ± 400	12,000 ± 1400	−38 ± 4.0
5	24	25,000 ± 500	11,700 ± 1400	−38 ± 4.0

* Calculated for 36°C.

formed, there is one mole each of enzyme and substrate released. A comparison of the extrapolated chain length with the polymer size of the actual equilibrium products demonstrated a striking correspondence.

DISCUSSION

Throughout the course of this research, two major assumptions have been utilized, viz: (1) the measured rates of hydrolysis were unaffected by transglycosidation; (2) the calculated value of K_m represented the dissociation constant of the enzyme-substrate complex. The first assumption has been discussed previously[3], the second will be dealt with here.

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TABLE IV

THE EFFECT OF SUBSTRATE SIZE UPON THE THERMODYNAMICS OF COMPLEX FORMATION*

Substrate	Chain length	ΔF^0 (Cal/mole)	ΔH (Cal/mole)	ΔS (Cal/mole degree)
Hyaluronate				
1	256	4400 ± 70	−1800 ± 200	+8.4 ± 0.9
1	125	4280 ± 70	−1800 ± 200	+8.3 ± 0.9
3	64	4120 ± 50	−1800 ± 200	+7.9 ± 0.9
4	33	3800 ± 60	1800 ± 200	+6.6 ± 0.8
5	25	3610 ± 60	1800 ± 200	+5.9 ± 0.8
Chondroitin sulphate A				
1	127	3800 ± 60	5500 ± 500	−5.5 ± 1.7
2	86	3700 ± 60	5500 ± 500	−5.5 ± 1.7
3	59	3500 ± 80	−5500 ± 500	−7.7 ± 1.5
4	36	−3100 ± 60	5500 ± 500	−9.4 ± 1.8
5	24	−2850 ± 50	5500 ± 500	−8.9 ± 1.7

* Calculated for 36° C.

TABLE V

THE PRODUCTS OF PROLONGED HYALURONIDASE ACTION

Substrate	Product chain length
Hyaluronate	4.8 ± 0.85
Chondoritin sulphate A	9.0 ± 1.88

For many enzyme systems, k_3 is sufficiently large that K_m is a steady state constant. Our treatment of K_m assumes that $k_2 \gg k_3$. Extrapolation of the plot of ΔF^0 versus the reciprocal of chain length to zero standard free energy gave values of chain length corresponding closely to the composition of the reaction mixture after prolonged incubation. Under these conditions

$$K_m = \frac{k_2 + k_3}{k_1} = \frac{(E)(S)}{(ES)} = 1$$

Because both (E) and (S) have appreciable values, (ES) must also be appreciable. Since the velocity of enzymic activity at this chain length was zero, k_3 must be extremely small ($v = k_3(ES)$), and therefore k_2 must be greater than k_3. At this substrate size, K_m is the dissociation constant of the enzyme-substrate complex.

For greater chain lengths the value of K_m decreased approximately a 1000-fold. Since V_m was constant for substrates of various chain length, k_3 must be relatively unaffected by substrate size. Therefore the changes in K_m observed must be due to changes in k_1 and/or k_2 with substrate chain length. If the change occurred only in k_2, the value of K_m would differ markedly from that of the dissociation constant, whereas if only k_1 changed, K_m would still closely approximate an equilibrium constant. The result of both rate constants changing would be intermediate between these two extremes. The observation that the standard free energy of complex formation was simply related to chain length strongly indicated that the modifications of K_m with sub-

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strate size were predominantly due to changes in k_1. In addition, the entropy change with complex formation calculated from the assumption that K_m is an equilibrium constant corresponded to the known water binding characteristics of both substrates. For these reasons, K_m appears to closely approximate the dissociation constants of both the hyaluronidase-hyaluronate and the hyaluronidase-chondroitin sulphate complexes.

Since alterations in the terminal group of the octasaccharide of hyaluronate have comparatively little effect upon enzyme action, while a similar occurrence with the hexasaccharide completely inhibited enzymic hydrolysis, it would appear that the smallest reactive substrate unit of hyaluronate was three disaccharides in length. In view of the poly-ionic nature of the active center of hyaluronidase[6], it would appear that the hexasaccharide is the whole reactive unit of the substrate.

If the hexasaccharide is the reactive substrate unit, then reduction of the end-group of the hexasaccharide would effectively degrade the substrate to the non-reactive tetrasaccharide. Similar reduction of the octasaccharide would leave a substrate equivalent in chain length to a hexasaccharide, which would react. Table II illustrates these effects. The enzyme could therefore be pictured as requiring a substrate unit six sugar units in length or, since the glucuronic bond is not reactive, three disaccharide units long. Fischer-Taylor-Hirschfelder models indicate that this unit would be about 50 A long and 10 A wide.

A decrease in the molecular weight of either substrate reduced its affinity for the enzyme, although the maximal velocity was unchanged. According to FLORY[7], the rate of a polymer reaction depends upon the rate of collision between reactive units. Thus a given weight of substrate composed of a large number of small molecules might be expected to react faster than a similar weight composed of small numbers of large molecules. FLORY, however, also pointed out that the reactive units of a polymer were the labile bonds rather than the whole molecule. The shape of a polyelectrolyte in a solution of constant ionic strength would not influence the reaction rates directly since the configuration of a flexible molecule would change at sufficient speed that all the reactive bonds would be equally exposed to attack. If the reactive unit in the case of hyaluronidase was three disaccharides in length, the number of these reactive units would decrease with substrate chain length. Therefore, since a collision between the active center of hyaluronidase and its substrate would be less likely to involve these reactive mid-chain groups with decreasing polymer size, the reaction rate and equilibrium constants of complex formation would decrease and increase respectively with decreasing substrate size.

Similarly the maximal velocity of the enzymic reaction with substrates of various size was constant, since an increase in substrate concentration would eventually provide the maximal number of reactive sites for enzymic action despite the greater number of non-reactive collisions between the enzyme and the smaller substrate molecules.

The thermodynamics of complex activation were independant of substrate size, since the energies of the complex would not be effected appreciably by the 'loose' ends of the polymer. Similarly the enthalpy of complex formation was not effected by chain length since the same amount of internal bond rupturing would be required for the reactive unit regardless of the overall length of the polymer. Since the shorter polymer would bind less water, the entropy of complex formation decreased with substrate size. The free energies of complex formation also decreased with substrate size however,

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since an increasing number of collisions would occur too close to the terminal group of the polymer to be reactive. Therefore a decrease in the rate of complex formation was observed with decreasing substrate chain length. The constant enthalpy of complex formation with changes in substrate size could mean that since the nature of the complex is uneffected by the unbound portions of the polymer, therefore the reaction rate constant k_2 also remains unchanged.

The linear relation between ΔF^0 and the reciprocal of substrate chain length followed the general form $\Delta F^0 = \Delta Q^0 - a/L$, where L is chain length, a a constant and ΔQ^0 equals the free energy of complex formation at infinite chain length. The difference between the ΔQ^0 of the two substrates is due to the steric hindrance of the sulphate group in complex formation[3] and is an energy barrier equal to about 800 calories.

SUMMARY

1. Evidence has been presented indicating that the reactive unit of hyaluronate is a hexasaccharide.

2. This fact has been used to explain the changes in the free energy and entropy of complex formation with changing substrate chain length. A similar argument has been presented to explain the constant value for the thermodynamics of activation and the enthalpy of complex formation with changing substrate size.

3. The decrease in the activity of hyaluronidase with substrate molecular weight is related to the free energy of complex formation. The chain length of the substrate when the change in the free energy of formation is zero agreed closely with the observed substrate size in equilibrium mixtures.

4. The relationship between the free energy of complex formation and chain length has been used to show that the *Km* of hyaluronidase closely approximates an equilibrium constant, and hence the validity of the previous work is indicated.

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