A PROTON MAGNETIC RESONANCE KINETIC APPROACH TO THE STEREOCHEMISTRY OF CARBOHYDRATE ENZYMIC HYDROLYSIS. HYDROLYSIS OF HYALURONIC ACID BY TESTICULAR HYALURONIDASE

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ABSTRACT

The determination of the anomeric configuration of the products resulting from carbohydrase hydrolysis is based on the observation that the sign of the accumulation rate of the P_1 anomer, in the process $S \xrightarrow{(E)} P_1 \rightleftharpoons P_2$, could change under certain conditions, whereas the accumulation rate of the P_2 anomer remains positive. This was confirmed by a p.m.r. kinetic study of two stereochemically known enzymic reactions: the hydrolysis of amylopectin by barley β-amylase and the hydrolysis of benzyl 2-acetamido-2-deoxy-β-D-glucopyranoside by boar epididymis *N*-acetyl-β-D-glucosaminidase. This method was applied to hyaluronic acid hydrolysis by testicular hyaluronidase, which was found to cleave the 2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→4) bond of the substrate with retention of the anomeric configuration.

INTRODUCTION

Carbohydrases can be classified on the basis of the anomeric configuration of the sugars liberated in the reaction[1]. Identification of the configuration can conveniently be performed by p.m.r. spectroscopy[2]. However, in spite of definite advantages over other methods[3,4], a direct p.m.r. procedure has some limitations, for example, in the hydrolysis of hyaluronic acid by testicular hyaluronidase. Therefore, we have based our determination on the kinetics of the anomer accumulation by use of p.m.r. spectroscopy.

MATERIALS AND METHODS

Substrates. — The potassium salt of hyaluronic acid was prepared from human umbilical cords as described by Aronson[5]. It contained no sulfated polysaccharides. *Anal.* Calc. for $(C_{14}H_{21}O_{11}N)_n$: N, 3.69; found: N, 3.70; ratio hexosamine[6] to uronic

acid (carbazole)[7]: 1.06:1.0. The relative viscosity of a 0.1% (wt./v) solution in 0.15M acetate buffer, at pH 3.8, was 4.3–4.5 at 25°. Hyaluronic acid was thrice dissolved in D_2O and lyophilized.

Benzyl 2-acetamido-2-deoxy-β-D-glucopyranoside was synthesized as previously described[8], m.p. 207–208°, $[\alpha]_D^{25}$ −50° (*c* 0.3, water). Amylopectin (Gee Lowson Chemical Ltd.) was dissolved in D_2O and lyophilized.

Enzymes. — Bovine testicular hyaluronidase (hyaluronate glycanohydrolase, E.C. 3.2.1.35) was obtained by purification of crude hyaluronidase (Reanal, Hungary), as previously described[9]. The purified enzyme (specific activity 13,500 TRU per mg) was free of β-D-glucuronidase and *N*-acetyl-β-D-glucosaminidase activity. β-*N*-Acetylglucosaminidase (2-acetamido-2-deoxy-β-D-glucoside 2-acetamido-2-deoxy-β-D-glucohydrolase, E.C. 3.2.1.30) was isolated from bear epididymis[10] (specific activity 400 U per mg with *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside). Barley β-amylase [α-D-(1→4)-glucanmaltohydrolase, E.C. 3.2.1.2] was obtained from Schuchardt (Germany) and was free from α-amylase activity, 2000° Linter units/g.

Buffer. — The sodium potassium phosphate buffer (0.1M, pH 5.1) was prepared by dissolving, in deuterium oxide, potassium dihydrogen phosphate and sodium dihydrogen phosphate recrystallized from deuterium oxide.

Hydrolysis of hyaluronic acid by testicular hyaluronidase. — Deuterated hyaluronic acid (50 mg) was dissolved in 400 μl of phosphate buffer (D_2O) and the solution was preincubated for at least 10 min at 45°. Lyophilized enzyme (2 mg) was added and the mixture was incubated at 45°. Quantitative determination of *N*-acetylglucosamine was performed on aliquots with the method of Reissig *at al.*[11]. After 24 h, the incubation mixture was desalted, concentrated and analyzed by paper chromatography on Filtrak, FN 17, in butyl alcohol–acetic acid–water (10:7:3, v/v), descending, for 72 h. Detection with the chlorine–*o*-toluidine reagent[12] showed the hyaluronic acid tetrasaccharide to be the main product of hydrolysis.

Hydrolysis of benzyl 2-acetamido-2-deoxy-β-D-glucopyranoside by N-*acetyl-β-D-glucosaminidase.* — The substrate (17.9 mg) was dissolved in phosphate buffer (400 μl), enzyme (2 mg) was added, and the mixture was incubated at 31°. *N*-Acetyl-D-glucosamine was determined according to Reissig *et al.*[9].

Hydrolysis of amylopectin by β-amylase. — Amylopectin (50 mg) and enzyme (6 mg) in phosphate buffer (400 μl) were incubated at 45°. The reducing sugar was determined according to Bernfeld[13].

P.m.r. spectra. — They were recorded on a Varian HA-100D spectrometer with 2-methyl-2-propanol as the internal standard. Chemical shifts δ are given in p.p.m. relative to tetramethylsilane. Samples were prepared by dissolution of the substrate in phosphate buffer (400 μl), or deuterium oxide for amylopectin, and addition of the enzyme; as soon as the latter dissolved, the mixture was placed into the p.m.r. sample tube. The spectra were recorded at 45° for hyaluronidase and β-amylase, and at 31° for *N*-acetyl-β-D-glucosaminidase. The change of intensity of the H-1 signals of the reducing sugars was determined from the change of amplitude. The accumulation of α- and β-anomer was plotted in percent of the total intensity of the H-1 signals, taken

as one proton intensity unit in the spectrum obtained after exhaustive hydrolysis. The accuracy of the determinations of relative intensity was 7% for hyaluronidase, and 5% for β-amylase and *N*-acetyl-β-D-glucosaminidase hydrolysis.

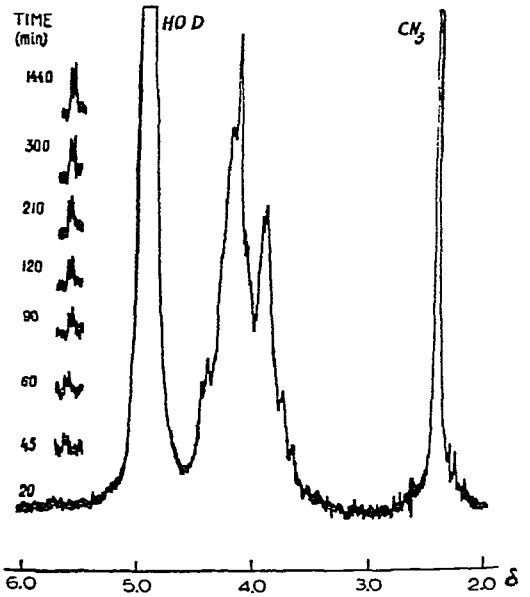

Fig. 1. P.m.r. spectra recorded during the enzymatic hydrolysis of hyaluronic acid by testicular hyaluronidase. The α-signals at 5.57 p.p.m. are due to H-1 of the α-D anomers of the hyaluronic acid oligosaccharides released.

In the p.m.r. spectra of the products of the enzymic hydrolysis of hyaluronic acid (Fig. 1), the signal due to the H-1 of a β-D anomer could not be distinguished, and consequently the total intensity of the H-1 signals of the α- and β-D anomers could not be determined. It was also not possible to use the signals due to the methyl group of the *N*-acetyl residue of hyaluronic acid as a reference of intensity since the hydrolysis had resulted in a mixture of oligosaccharides. Thus, the relative intensity of the H-1 signal due to the α-D anomer was calculated on the basis of the signal recorded after a 24-h hydrolysis and of the anomer ratio after mutarotation. This ratio was taken as equal to that observed in a solution of *N*-acetyl-D-glucosamine in equilibrium after mutarotation under the same conditions, *i.e.* [α] to [β] 29:21, as derived from the p.m.r. spectrum.

RESULTS AND DISCUSSION

Testicular hyaluronidase degrades hyaluronic acid by cleavage of the (1→4) 2-acetamido-2-deoxy-β-D-glucopyranoside bonds of the polymer chain, the main end-product (approximately 80–90%) being the tetrasaccharide *O*-β-D-GA*p*-(1→3)-

O-β-D-GNAc*p*-(1→4)-*O*-β-D-GA*p*-(1→3)-D-GNAc. As far as we know, no data on the stereochemistry of enzymic hydrolysis products of hyaluronic acid have been published.

The anomeric configuration of the products of hydrolysis can be studied by polarimetry[14], g.l.c.[3,4], or p.m.r. spectroscopy[2,8]. Since the various products of hyaluronic acid digestion have very similar specific rotations and a low volatility, the first two methods are inappropriate. For the direct application of p.m.r. spectroscopy, it is necessary to record the signals of both the α- and β-D anomers simultaneously. However, only the signal attributable to H-1 of the α-D anomers of hyaluronic acid oligosaccharides (δ 5.57 p.p.m., $J_{1,2}$ 2.9 Hz) is observed at room temperature (Fig. 1), and it is necessary to raise the temperature to 80° to detect the H-1 signal of the β-D anomer (δ 4.93 p.p.m.). Therefore, another approach was attempted to study the stereochemistry of the products of enzymic hydrolysis of hyaluronic acid.

The enzymic hydrolysis by a carbohydrase and the mutarotation process can be represented by the following scheme:

$$S + E \underset{k_{-1}}{\overset{k_{+1}}{\rightleftarrows}} ES \xrightarrow{k_{+2}} E + P_1 \underset{k_{-3}}{\overset{k_{+3}}{\rightleftarrows}} P_2 \qquad (1)$$

where P_1 and P_2 are the products of hydrolysis and of mutarotation, respectively, while k_{+3} and k_{-3} are the rate constants of anomerisation of P_1 and P_2, respectively.

When $[S]_0 \gg [E]_0$ (a condition satisfied in our p.m.r. experiment), the process (*1*) can be represented by the following simplified equation:

$$S \xrightarrow[(E)]{k_0} P_1 \underset{k_{-3}}{\overset{k_{+3}}{\rightleftarrows}} P_2 \qquad (1a)$$

where k_0 is the apparent rate constant of hydrolysis. Application of the Briggs–Haldane equation gives:

$$k_0 = \frac{k_{+2}[E]_0}{K_s + [S]_0} \qquad (2)$$

The rates of the enzymic hydrolysis and accumulation of anomers (*1a*) can be described by a system of linear differential equations:

$$-\frac{d[S]}{dt} = k_0[S]$$

$$\frac{d[P_1]}{dt} = k_0[S] - k_{+3}[P_1] + k_{-3}[P_2]$$

$$\frac{d[P_2]}{dt} = k_{+3}[P_1] - k_{-3}[P_2]$$

Solving for $[P_1]+[P_2]$, $[P_1]$ and $[P_2]$ we obtain

$$[P_1]+[P_2] = [S]_0 \{1-\exp(-k_0 t)\} \quad (3)$$

$$[P_1] = [S]_0 \left\{\frac{k_{-3}}{r} + \frac{k_{-3}-k_0}{k_0-r}\exp(-k_0 t) + \frac{k_0 k_{+3}}{r(k_0-r)}\exp(-rt)\right\} \quad (4)$$

$$[P_2] = [S]_0 \left\{\frac{k_{+3}}{r} + \frac{k_{+3}}{k_0-r}\exp(-k_0 t) - \frac{k_0 k_{+3}}{r(k_0-r)}\exp(-rt)\right\} \quad (5)$$

where $r = k_{+3}+k_{-3}$.

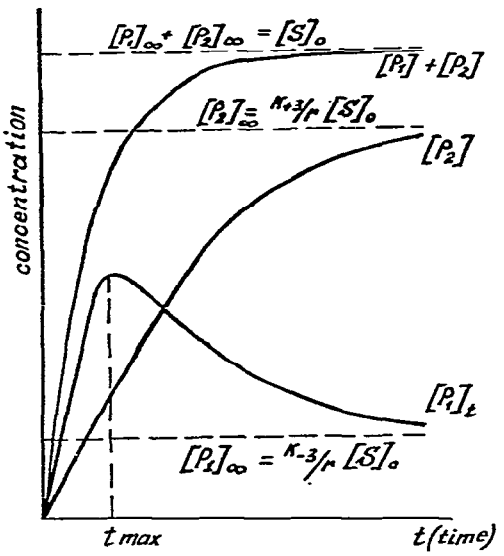

Fig. 2. Time–concentration curves corresponding to equations (3)–(5); $k_0:k_{+3}:k_{-3} = 10:5:1$.

Analysis of the equations (3)–(5) (Fig. 2) indicates that $[P_2]$ and $[P_1]+[P_2]$ aie increases monotonically with time, whereas the concentration–time relationship of the hydrolysis product P_1 can reach a maximum at

$$t_{max} = \frac{1}{k_0-r}\ln\frac{k_0-k_{-3}}{k_{+3}}$$

This maximum exists when

$$k_0 > k_{+3}+k_{-3} \quad (6)$$

On the basis of the equation just described, we studied the kinetics of the accumulation of anomers in both types of enzymic reactions: hydrolysis of amylopectin by barley β-amylase as an example of a reaction involving inversion[2] and

hydrolysis of benzyl 2-acetamido-2-deoxy-β-D-glucopyranoside by boar epididymis *N*-acetyl-β-D-glucosaminidase as a reaction with retention of the configuration[10]. The change of concentration of $[P_1]$ and $[P_2]$ was followed by examination of the intensities of the H-1 signals arising from the primary product P_1 (resulting from the hydrolysis) and from the secondary product P_2 (formed on mutarotation of P_1).

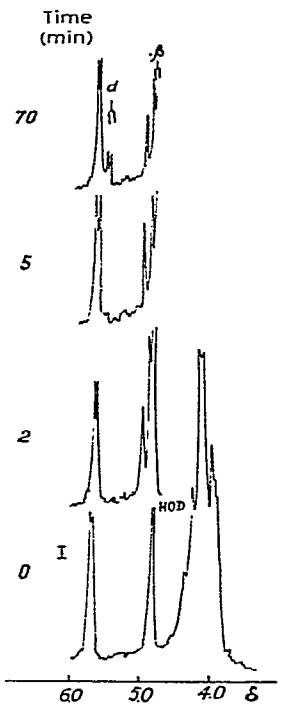

Fig. 3. P.m.r. spectra recorded during the enzymic hydrolysis of amylopectin by barley β-amylase: G, signal due to H-1 of the α-D-glucopyranosyl units of the substrate; α and β, the signals due to H-1 of α- and β-maltose, respectively.

The hydrolysis of amylopectin by β-amylase gives β-maltose, which has an anomeric configuration opposite to that of the original glycoside bond. The p.m.r. spectrum observed during the early stages of the reaction (Fig. 3), shows a doublet (δ 5.05 p.p.m., $J_{1,2}$ 7.8 Hz) attributable to H-1 of the β-anomer (P_1) which rapidly increases, reaches a maximum, and then gradually decreases (Fig. 4, Curve 1), indicating a reversal of the rate of β-D anomer accumulation. In contrast, the intensity of the H-1 doublet (δ 5.63 p.p.m., $J_{1,2}$ 3.8 Hz) due to α-maltose (P_2) increases monotonically (Fig. 4, Curve 2) until the equilibrium is established. The time dependence of the H-1 signal intensity of the α-anomer shows the mutarotation kinetics. Curve 3 of Fig. 4, which is a sum of Curves 1 (β-anomer) and 2 (α-anomer), illustrates the total intensity increase of the H-1 protons, thus describing the kinetics of the hydrolysis (P_1+P_2).

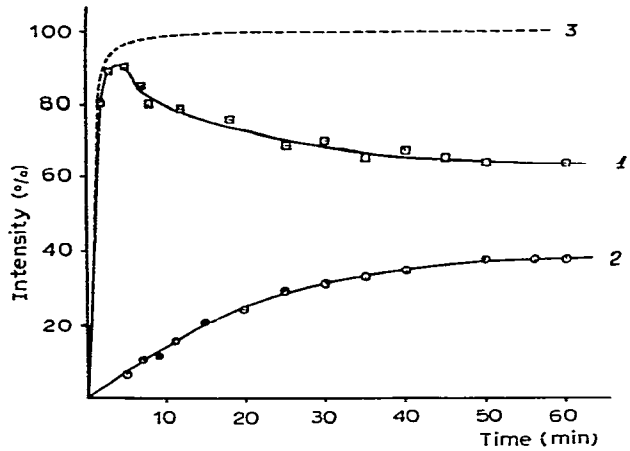

Fig. 4. Time dependence of the p.m.r.-signal intensity (in percent relative to one proton unit) in the enzymic hydrolysis of amylopectin by barley β-amylase: Curve 1, signal due to H-1 of β-maltose; Curve 2, signal due to H-1 of α-maltose; Curve 3, signal due to H-1 of both anomers.

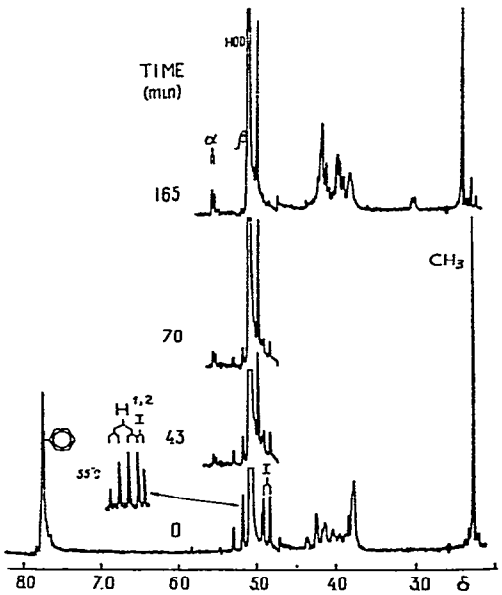

Fig. 5. P.m.r. spectra recorded during the enzymic hydrolysis of benzyl 2-acetamido-2-deoxy-β-D-glucopyranoside by boar epididymis *N*-acetyl-β-D-glucosaminidase: G, signal due to H-1 of the β-D-glycopyranosyl residue of the substrate; α and β, H-1 signals due to the α- and β-D anomers of *N*-acetyl-D-glucosamine, respectively.

Enzymolysis of benzyl 2-acetamido-2-deoxy-β-D-glucopyranoside by *N*-acetyl-β-D-glucosaminidase from boar epididymis gives *N*-acetyl-D-glucosamine. P.m.r. spectra recorded during the reaction (Fig. 5), show a steadily growing H-1 doublet

(δ 5.58 p.p.m., $J_{1,2}$ 2.7 Hz) due to the α-anomer (P_2) produced by mutarotation. The signals of the magnetically nonequivalent CH_2 protons of the $C_6H_5CH_2$ group (AB quartet, center δ 5.18 p.p.m., J_{gem} 12 Hz) and the signal of the CH_3 group (a singlet, δ 2.33 p.p.m.) of the substrate gradually decrease and then disappear. At the same time, the proton signals of the benzyl alcohol CH_2 group (δ 5.01 p.p.m.) and CH_3 group of *N*-acetyl-D-glucosamine (δ 2.42 p.p.m.) monotonically increase.

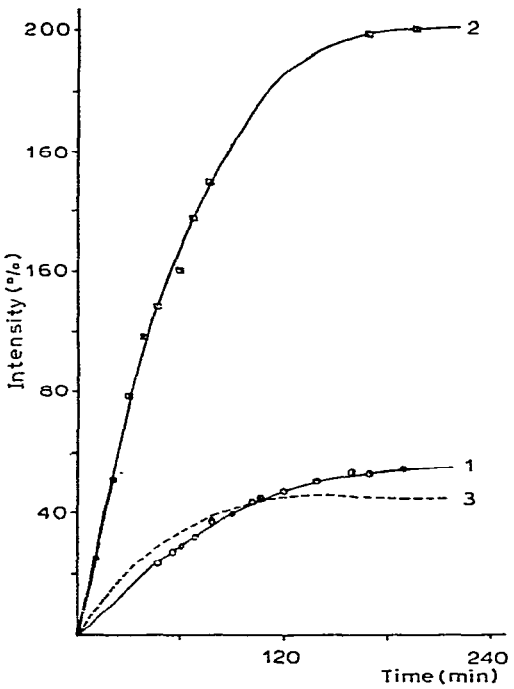

Fig. 6. Time dependence of the p.m.r.-signal intensity in the enzymic hydrolysis of benzyl 2-acetamido-2-deoxy-β-D-glucopyranoside by boar epididymis *N*-acetyl-β-D-glucosaminidase: Curve 1, signal due to H-1 of 2-acetamido-2-deoxy-α-D-glucopyranose; Curve 2, signal due to the protons of the benzyl alcohol methylene group; Curve 3, calculated curve from Curves 1 and 2 indicating the H-1 signal of 2-acetamido-2-deoxy-β-D-glucopyranose.

The time-intensity dependencies of the H-1 signals due to the α-D anomer (mutarotation) and those of the benzyl alcohol methylene group (hydrolysis) (Fig. 6, Curves 1 and 2, respectively) show that the rates of the two processes remain positive. Curve 3 of Fig. 6, calculated from Curves 1 and 2, gives the intensity change of the H-1 signal of the β-D anomer hydrolysis product (P_1) and indicates that the concentration of the β-D anomer passes through a maximum.

Consistency of the just described results with the theoretical equations (*3*)–(*5*) forms the basis for a p.m.r. kinetic approach to the determination of the anomeric configuration of enzymic hydrolysis products. In this approach, the substrate concentration to be used should be such as to be easily recorded by p.m.r. spectroscopy,

whereas the enzyme concentration is chosen so that the k_0-value determined by equation (*2*) would satisfy the expression (*6*). The maximum on the $[P_1]$–time curve under the condition $[S]_0 \gg [E]_0$ is evidence that the expression (*6*) is satisfied.

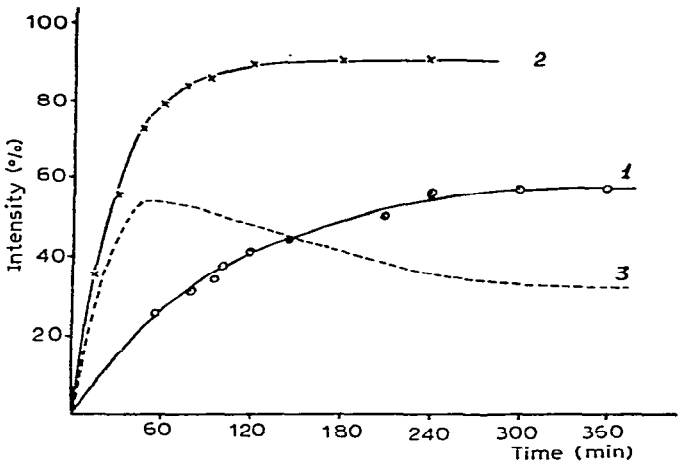

Fig. 7. Products obtained in the hydrolysis of hyaluronic acid by testicular hyaluronidase: Curve 1, intensity of the H-1 signal due to the α anomers of the oligosaccharides; Curve 2, total concentration of the amino sugars in the incubation mixture relative to that obtained after 24 h of incubation (in percent); Curve 3, concentration of the β anomers of the oligosaccharides calculated as a difference between Curves 2 and 1.

The p.m.r. kinetic approach has been applied to the hydrolysis of hyaluronic acid by testicular hyaluronidase. The p.m.r. spectra recorded during this reaction (Fig. 1) show a doublet (δ 5.57 p.p.m., $J_{1,2}$ 2.9 Hz) which is attributable to the α-D anomers of the oligosaccharides. The intensity of this signal monotonically increases with time (Fig. 7, Curve 1). Since the H-1 signal of the β-anomer was obscured by the signal of the solvent, it was not possible to detect the β-D anomer accumulation by the p.m.r. technique. Therefore, the problem was approached as follows. The kinetics of the enzymic reaction *in toto* were obtained by the determination of the totality of the aminosugars in the incubation mixture. The time–concentration dependence of the reaction products is represented by Curve 2 of Fig. 7. It should be noted that, already after a 15-min incubation, the reaction mixture contained mainly octa-, hexa-, and tetra-saccharides (as shown by paper chromatography), and the amount of oligosaccharides of higher molecular weight did not exceed 40%. Curve 3 of Fig. 7 is the difference between Curves 2 and 1 and, therefore, describes the kinetics of the accumulation of the β-D anomer. The time-concentration curve of this anomer has a strongly pronounced maximum, which shows that this anomer is the direct product of enzymolysis. Thus, it can be concluded that the enzymolysis of hyaluronic acid by testicular hyaluronidase proceeds with retention of the configuration; these findings, coupled with the literature data[15] on the composition of the reaction mixture, show hyaluronidase to be an endoglycosidase.

The approach, described in this paper which is based on the kinetic studies of the anomer accumulation using p.m.r. techniques, can serve as a convenient method for the determination of the stereochemistry of enzymolysis by carbohydrases.

REFERENCES

1 E. T. REESE, A. H. MAGUIRE, AND F. W. PARRISH, *Can. J. Biochem.*, 46 (1968) 25.
2 D. E. EVELEIGH AND A. S. PERLIN, *Carbohyd. Res.*, 10 (1969) 87.
3 R. BENTLEY AND N. BOTLOCK, *Anal. Biochem.*, 20 (1967) 312.
4 G. SEMENZA, H.-CH. CURTIUS, O. RAUNHARDT, P. HORE, AND M. MÜLLER, *Carbohyd. Res.*, 10 (1967) 417.
5 N. ARONSON JR. AND E. A. DAVIDSON, *J. Biol. Chem.*, 242 (1967) 437.
6 A. G. OGSTON, *Anal. Biochem.*, 8 (1964) 337.
7 J. D. GREGORY, *Arch. Biochem. Biophys.*, 89 (1960) 157.
8 A. YA. KHORLIN, S. E. ZURABYAN, N. I. DUBROVINA, V. F. BYSTROV, G. V. VIKHA, AND E. D. KAVERZNEVA, *Carbohyd. Res.*, 21 (1972) 316.
9 I. V. VIKHA, N. V. FEDURKINA, AND A. YA. KHORLIN, *Biokhimiya*, 36 (1971) 216.
10 G. V. VIKHA, E. D. KAVERZNEVA, AND A. YA. KHORLIN, *Biokhimiya*, 36 (1971) 33.
11 J. L. REISSIG, J. L. STROMINGER, AND L. F. LELOIR, *J. Biol. Chem.*, 217 (1955) 959.
12 F. REINDEL AND W. HOPPE, *Chim. Ber.*, 87 (1954) 1103.
13 P. BERNFELD, *Methods Enzymol.*, 1 (1955) 159.
14 R. KUHN, *Ber.*, 57 (1924) 1965.
15 B. WEISSMANN, K. MEYER, P. SAMPSON, AND A. LINKER, *J. Biol. Chem.*, 208 (1954) 417.