Effect of Hyaluronidase Treatment of Intact Cells on Hyaluronate Synthetase Activity†

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ABSTRACT: Hyaluronidase treatment of mouse oligodendroglioma cells in monolayer culture resulted in a 4–5-fold stimulation of hyaluronate synthetase, assayed in washed membrane preparations [Philipson, L., & Schwartz, N. B. (1984) *J. Biol. Chem. 259*, 5017–5023]. We now report studies on the mechanism of the hyaluronidase-induced increase in the specific activity of the membrane-bound synthetase complex. The stimulation was dependent on the concentration of hyaluronidase but not on the particular bond cleaved or the nature of the product generated. Analysis of chain growth during cell-free synthesis by the disaccharide ratio method suggested that substantial internal labeling of hyaluronate chains had occurred. With both treated and untreated membranes, greater than 90% of incorporated (and recovered) radioactivity appeared in unsaturated disaccharides. Further analysis showed that hyaluronidase treatment increased both the rate of elongation and the rate of release of elongated chains from the enzyme complex. Hyaluronidase treatment also caused a change in the apparent steady-state kinetic patterns of double-reciprocal plots from intersecting lines for membranes from control cells to a family of parallel lines. Both the overall stimulation of synthesis and the change in apparent kinetic pattern were reversed by brief incubation of washed cells in the absence of hyaluronidase. These results have led to the development of an explicit kinetic model for hyaluronate synthesis which suggests an explanation for the switch in apparent kinetic patterns based on changing concentrations of a postulated key intermediate.

The elucidation of the molecular events in the biosynthesis of hyaluronate has presented unique problems in the study of polysaccharide production (Roden, 1980). More than 20 years ago, Markovitz et al. (1959) proposed a model of synthetase activity for the steps occurring during elongation of hyaluronate at the nonreducing end in a prokaryotic system. No such model has been postulated in similar detail for eukaryotic systems, and the events in de novo synthesis, elongation, and termination of hyaluronate chains in such systems have remained obscure. Recently Prehm (1983a,b) has presented evidence for elongation occurring at the reducing end in the hybrid cell B6 line, and this interesting finding awaits confirmation in other eukaryotic systems.

In a previous report (Philipson & Schwartz, 1984), we presented evidence for the localization of hyaluronate synthetase at the inner surface of the plasma membrane from mouse oligodendroglioma cells in culture. In an attempt to "clear" membranes of endogenous contaminating hyaluronate, cells were treated with testicular hyaluronidase. Homogenates and membrane fractions prepared from washed cells so treated showed a specific stimulation (4–5-fold) of hyaluronate synthetase activity which cofractionated with plasma membrane markers. The hyaluronidase effect provided additional evidence for the localization of the synthetase at the cell surface. This localization has been independently confirmed by Prehm (1984).

To better understand the mechanism responsible for this stimulation, the effect of hyaluronidase on the synthetase activity was examined in greater detail. Part of this investigation involved a steady-state analysis of apparent kinetic parameters, which has led for the first time to a kinetic model of hyaluronate synthetase in a eukaryotic system. The formal mechanism predicts the existence of an intermediate and the participation of at least two and possibly three activities in the complex. The model suggests that the effect of hyaluronidase at the cell surface is to modulate the concentration of the intermediate, which determines the level of synthetase activity and the apparent pattern obtained in double-reciprocal plots.

Materials and Methods

Materials. The mouse oligodendroglioma cell line (strain G26-24) was generously provided by Dr. G. Dawson (The University of Chicago). The strain was originally isolated by Sundarraj et al. (1975) from an immature glial cell tumor induced by methylcholanthrene treatment of C57BL/6 inbred mice (Zimmerman, 1955). Modified Eagle's medium (Matalon & Dorfman 1966), fetal calf serum, Hank's balanced salt solution (HBSS),[1] and calcium–magnesium-free Hank's solution were supplied by GIBCO. Gentamicin sulfate (sterile, 10 mg/mL) was purchased from Schering Corp. Human umbilical cord hyaluronate was a gift from Dr. Martin B. Mathews (The University of Chicago). [^{14}C]Hyaluronate, internally labeled with [^{14}C]glucuronic acid, was prepared with isolated streptococcal membranes (Sugahara et al., 1979). Riker Laboratories supplied heparin (157 USP units/mg). Bovine γ-globulin and protein assay dye reagent concentrate were obtained from Bio-Rad Laboratories. Streptococcal

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[1] Abbreviations: HBSS, Hank's balanced salt solution; $NaDodSO_4$, sodium dodecyl sulfate; TRU, turbidity reducing units; IU, international units; Cl_3CCOOH, trichloroacetic acid; USP, United States Pharmacopeia; Tris, tris(hydroxymethyl)aminomethane.

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hyaluronidase was a gift from Dr. A. Horwitz (The University of Chicago). Bovine testicular hyaluronidase (15 000–22 000 IU/mg) was purchased from Leo, Helsinborg, Sweden. Leech hyaluronidase (crude) was a gift from Dr. B. Jacobson (Institute for Biological Sciences, Boston, MA). Hyaluronidase from *Streptomyces hyalurolyticus* (2000 TRU/mg) was obtained from Miles Laboratories. The G-26-24 cells were grown as described previously (Dawson & Kernes, 1979). Hyaluronate synthetase was assayed in washed crude membrane preparations, as described (Philipson & Schwartz, 1984).

Kinetics of Hyaluronate Synthetase. Steady-state kinetic data were processed on a DEC-20 computer with the Basic program ENZKIN. Briefly, this program fits initial velocity data to the best rectangular hyperbola by an iterative least-squares method. Curve parameters from a family of such lines are then replotted against the concentration of the other substrate, again by hyperbolic fitting. The apparent kinetic constants were obtained from the appropriate curve parameters of these replots.

Hyaluronidase Assay. Membrane fractions prepared from intact cells treated with hyaluronidase were assayed for residual hyaluronidase. In a volume of 0.25 mL, 0.1 M acetate (final concentration), pH 5.0, membrane protein (0.5 mg), and [^{3}H]hyaluronate were combined and incubated for 18 h at 37 °C. Control tubes also contained 0.6 mg of heparin or boiled membranes. Reactions were terminated by the addition of $NaDodSO_4$ to a final concentration of 1%. The mixtures were chromatographed in Sepharose CL-2B (0.5 × 27 cm) or Sephacryl S-100 (0.5 × 90 cm) and eluted with 50 mM Tris/0.1% $NaDodSO_4$, pH 8. Fractions (0.5 mL) were collected for determination of radioactivity. The excluded volume of the Sephacryl S-1000 column was determined with phage DNA.

Disaccharides. Hyaluronate disaccharides obtained by streptococcal hyaluronidase digestion were characterized by a modification of the method of Ro[illegible]en et al. (1972). Briefly, the mixture to be analyzed was sp[illegible]ted on Whatman 3MM paper with authentic material and first subjected to electrophoresis in 0.1 M formate, pH 1.9, for 90 min at 2000 V. The paper was allowed to dry, cut off approximately 5 cm from the origin, and sewn on to a fresh sheet. Descending paper chromatography was then performed in solvent A: 1-butanol/pyridine/acetic acid/water (15:10:3:12). Compounds were visualized with a silver nitrate dip reagent (Partridge & Westall, 1948), or the paper was cut into 0.5-cm segments for liquid scintillation counting.

Precipitation of Hyaluronate Synthetase Assay Mixtures. Hyaluronate synthetase assays were separated into soluble and particulate fractions by the method of Stoolmiller & Dorfman (1969). Cold trichloroacetic acid was added to a final concentration of 10%, and the tubes were kept on ice for at least 1 h. Carrier umbilical cord hyaluronate (100 μg) was added immediately prior to the acid. The precipitate, washed 3 times with cold 5% trichloroacetic acid and brought to neutral pH with NaOH, was solubilized with 1% $NaDodSO_4$. The washes were combined with the supernatant. [^{14}C]Hyaluronate in each fraction was then determined by Sephadex G-50 chromatography.

Protein Determination. Protein was determined by the dye binding method of Bradford (1976). Sonication of homogenates and membrane fragments was necessary to eliminate large debris which did not react uniformly.

RESULTS

Stimulation of Hyaluronate Synthetase Activity. Results presented previously (Philipson & Schwartz, 1984) suggested that hyaluronidase treatment of cells in monolayer culture caused a 4–5-fold stimulation of hyaluronate synthetase activity. As an initial step in characterizing this effect, cells in culture were incubated with increasing concentrations of testicular hyaluronidase, and the hyaluronate synthetase was assayed in the washed, crude membrane pellet. Synthetase activity was stimulated with hyaluronidase concentrations up to 100 units/mL, as shown in Figure 1. Above this concentration, the synthetase assay was no longer reliable, as the hyaluronate product began to appear in the included volume of the Sephadex G-50 column.

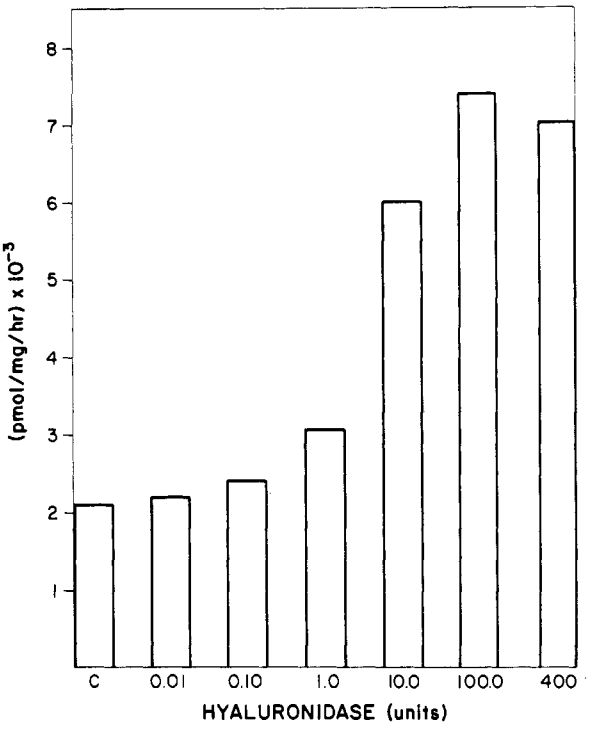


FIGURE 1: Hyaluronate synthetase activity after treatment with various concentrations of hyaluronidase. Cells were treated with the indicated concentrations of testicular hyaluronidase (international units per milliliter in HBSS, 15 min at 37 °C) before harvest from tissue culture plates. After harvest, washed cells were homogenized by sonication, and hyaluronate synthetase was assayed in the washed 100000*g* (1 h) pellet.

Table I: Effect of Hyaluronidases and Heparin on Hyaluronate Synthetase

hyaluronidase treatment[a]	hyaluronate synthetase[b] (pmol mg^{-1} h^{-1} × 10^{-2}) control	heparin[c]	% inhibition[d]
none	1.9 (1.0)[e]	1.2 (1.0)	37
testicular			
10 IU/mL	4.6 (2.4)	3.6 (3.1)	21
100 IU/mL	5.5 (2.9)	4.3 (3.7)	22
Streptomyces			
0.33 TRU/mL	5.3 (2.8)	4.0 (3.4)	25
1.67 TRU/mL	7.6 (4.1)	6.0 (5.2)	21
leech			
3.3 μg/mL	3.1 (1.7)	2.0 (1.7)	36
16.7 μg/mL	2.9 (1.5)	1.8 (1.6)	36

[a] Cells were treated in monolayer culture (see Materials and Methods). [b] Assayed in washed crude membrane pellet. [c] Added to in vitro assay mixture (2.7 μg/mL). [d] In the presence of heparin. [e] Relative activity.

The stimulation of the synthetase was determined not to be dependent on the type of hyaluronidase used (Table I). *Streptomyces* hyaluronidase cleaves hyaluronate at the same glucosamine–glucuronic acid bond as testicular hyaluronidase

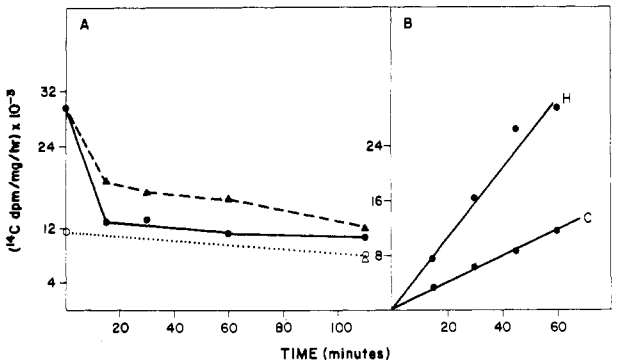


FIGURE 2: (A) Time course of recovery of hyaluronate synthetase activity in intact cells. (B) Time course of synthetase activity in isolated membranes. (A) Cells were treated with testicular hyaluronidase (10 IU/mL) as indicated in the legend to Figure 1. After digestion, duplicate plates were either harvested immediately or reincubated in HBSS with (●) or without (▲) glucose for the times indicated. Cells, cooled to 4 °C, were homogenized, and the hyaluronate synthetase was assayed in the 100000*g* pellet. Open symbols indicate cells processed identically but without hyaluronidase treatment. (B) Hyaluronate synthetase activity plotted against time of assay: H, hyaluronidase-treated cells (see above); C, control cells.

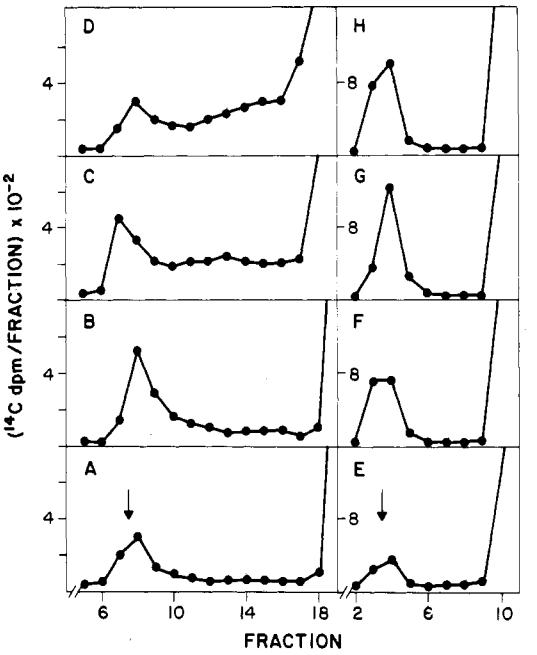


FIGURE 3: Gel filtration profiles of cell-free hyaluronate. Hyaluronate synthetase was assayed in the 100000*g* pellets from homogenized cells. The solubilized incubation mixtures were chromatographed on columns of Sepharose CL-2B (panels A–D) or Sephadex G-50 (E–H) and fractions collected for determination of radioactivity. Arrows indicate the void volume. (A and E) Control cells. Other panels show the results obtained with cells incubated with the following concentrations of testicular hyaluronidase as indicated in the legend to Figure 2: panels B and F, 20 IU/mL; panels C and G, 50 IU/mL; panels D and H, 100 IU/mL.

but results in the production of tetra- and hexasaccharides with a double bond between carbons 4 and 5 in the nonreducing terminal glucuronic acid. Leech hyaluronidase cleaves the glucuronic acid–glucosamine bond, resulting in the production of oligosaccharides with glucosamine residues at the nonreducing terminii. Thus, the particular bond cleaved and the state of the nonreducing ends of the resulting cleavage products are not important to the stimulation of hyaluronate synthetase by hyaluronidase.

Reversibility of Hyaluronidase Stimulation. The treatment of cells in monolayer culture with hyaluronidase under the conditions described did not result in the detachment of cells from the tissue culture dish. It was observed that cell layers, washed after hyaluronidase treatment, showed normal viability when reincubated with media or could then be subcultured. When cells were washed and reincubated with HBSS, or HBSS without glucose, the specific activity of hyaluronate synthetase rapidly diminished to control levels as shown in Figure 2A. The $t_{1/2}$ of the recovery was on the order of 10–15 min at 37 °C. Figure 2A also shows that recovery was slowed when cells were incubated in media without glucose. The cell-free assay for hyaluronate synthesis was linear with time regardless of pretreatment of cells with 10 IU/mL hyaluronidase, shown in Figure 2B. Again, reversible stimulation of the synthetase was observed only with viable cells and not with isolated membranes.

Hyaluronate Synthesized in Vitro. Treatment of cells in monolayer culture with high concentrations (greater than 100 IU/mL) of testicular hyaluronidase resulted in an increased percentage of the cell-free product that was included in a Sephadex G-50 column. As indicated previously (Philipson & Schwartz, 1984), hyaluronate that was normally synthesized by the G26-24 cell line was sufficiently large to be excluded from large porosity gels such as Sephacryl S-1000. To determine whether hyaluronidase treatment might reduce the size of primer chains to be extended during cell-free synthesis, the cell-free products were examined by gel filtration in Sepharose CL-2B or Sephacryl S-1000 columns. A comparison of the elution profiles of the cell-free product from membranes of control and hyaluronidase-treated cells is shown in Figure 3. While treatment of cells with concentrations of testicular hyaluronidase up to 100 IU/mL did not change the Sephadex G-50 profiles (right-hand panels), the cell-free product was increasingly included in Sepharose CL-2B. Only 44% of the [^{14}C]hyaluronate synthesized by membranes from cells treated with hyaluronidase at 100 IU/mL, that was excluded from the Sephadex G-50 column, was also excluded from the Sepharose CL-2B column (panels H and D).

To determine whether residual hyaluronidase had contaminated the membrane preparations during cell-free synthesis, a sensitive assay for hyaluronidase was devised. Partially purified [^{3}H]hyaluronate (metabolically labeled with [^{3}H]-glucosamine) was incubated with approximately 0.5 mg of crude membrane protein for 16 h followed by Sepharose CL-2B or Sephacryl S-1000 chromatography of the solubilized assay mixture. These experiments revealed that membranes from cells treated with only 10 IU/mL testicular hyaluronidase contained enough hyaluronidase to decrease the proportion of radioactivity which eluted in the void volume by approximately 20%. Membranes from control cells, heat-inactivated membranes, or membranes incubated with heparin, a potent inhibitor of hyaluronidase activity, showed no hyaluronidase activity.

The competitive inhibition of testicular hyaluronidase by heparin was therefore employed to investigate the possibility that stimulation of hyaluronate synthetase was due to the presence of hyaluronidase in the cell-free assay. Heparin at a concentration (2.6 mg/mL) sufficient to completely inhibit the residual hyaluronidase retained in membranes was included in the hyaluronate synthetase assay. As shown in Table I, heparin had essentially no inhibitory effect on the relative stimulation of hyaluronate synthetase by testicular hyaluronidase, even though the synthetase itself was inhibited 20–30% by this concentration of heparin.

Effect of Hyaluronidase Treatment of Cells on Soluble and Precipitable Hyaluronate Synthesized in Vitro. Cold trichloroacetic acid has been utilized previously to partition the

Table II: Cl_3CCOOH Precipitation of Hyaluronate-Synthesizing System

treatment	time (min) 10	30	60
control			
supernatant	1590[a]	3040	4250
pellet	1375	2170	2520
soluble (%)	54	58	65
hyaluronidase[b]			
supernatant	4190	8680	11010
pellet	1520	1880	2360
soluble (%)	74	82	82

[a] ^{14}C dpm/assay tube, average of duplicate experiments (±10%). [b] Cells in monolayer culture were treated with 10 IU/mL testicular hyaluronidase, and hyaluronate synthetase was assayed, as described in the legend to Figure 1.

Table III: Disaccharide Ratios

treatment	I[a]	II[b]	ratio
control	119[c]	1363	1:12
hyaluronidase[d]	508	9369	1:18

[a] Saturated disaccharide peak. [b] Unsaturated disaccharide. [c] ^{14}C dpm/min. [d] Cells in culture treated with testicular hyaluronidase; see Materials and Methods.

cell-free synthetase system into soluble and precipitable (membrane-bound) hyaluronate (Sugahara et al., 1979). In the streptococcal system, the precipitable hyaluronate was demonstrated to be the precursor of the soluble hyaluronate (Sugahara et al., 1979). When the control membranes from G26-24 glioma cells were partitioned with Cl_3CCOOH, the percentage of soluble radioactivity increased over time from 54% at 10 min to 65% at 60 min (Table II). In contrast, hyaluronidase treatment of cells increased the percentage of hyaluronate that was soluble to 74% at 10 min and 82% at 60 min, while the amount of precipitable radioactivity remained comparable at the various time points. Therefore, the increased rate of synthesis also involved an increased rate of release of chains from cell membranes.

Addition of Disaccharide Units during Cell-Free Synthesis. The ratio of saturated to unsaturated labeled disaccharides following complete digestion of [^{14}C]hyaluronate with streptococcal hyaluronidase has been used both to determine the direction of synthesis and to quantitate the average number of disaccharides added during cell-free synthesis (Stoolmiller & Dorfman, 1969). We first determined the disaccharide ratios of labeled hyaluronate that remained membrane-bound after brief (10 min) incubation in the presence of one or both UDP-sugar precursors. Only the unsaturated disaccharide was found when membranes from either treated or control cells were incubated with UDP-[^{14}C]glucuronic acid in the absence of added UDP-GlcNAc (Figure 4, data shown for control cells only). This result is not consistent with simple sequential addition of monosaccharides from either end and is further considered under Discussion.

This disaccharide analysis was also applied to the total [^{14}C]hyaluronate synthesized by membranes prepared from control and treated cells in the presence of both nucleotide sugars after 60 min of incubation (Philipson & Schwartz, 1984). As shown in Table III, the recovered radioactivity was increased in both disaccharide peaks obtained from membranes prepared from treated cells. Neither increased time of synthesis nor higher concentrations of nucleotide sugar substrates resulted in substantially increased unsaturated to saturated disaccharide ratios (data not shown), as would have been predicted from the prokaryotic model (Stoolmiller & Dorfman, 1969).

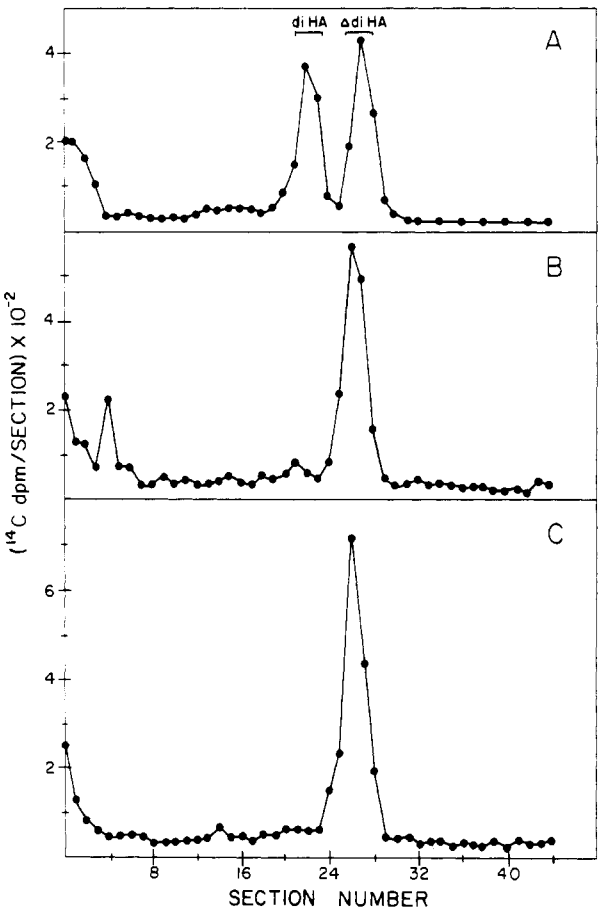


FIGURE 4: Chromatographic profiles of streptococcal hyaluronidase digestions. Cell-free hyaluronate assays were performed and hyaluronate disaccharides were generated and separated as described under Materials and Methods. Control digest of HA tetrasaccharide purified by Bio-Gel P-2 chromatography (panel A); digest of membrane-bound HA purified by Sephadex G-50 chromatography labeled in the presence of UDP-[^{14}C]glucuronic acid and UDP-GlcNAc (panel B); digest of membrane-bound HA purified by Sephadex G-50 chromatography labeled in the presence of UDP-[^{14}C]glucuronic acid (panel C); ΔdiHA, unsaturated disaccharide; diHA, saturated disaccharide.

Kinetics of Hyaluronate Synthetase. In an attempt to further define the basis for the observed increase in synthetase activity upon hyaluronidase treatment, a detailed kinetic analysis was undertaken to determine whether the affinity of substrates for enzyme or the reaction rate (V_{max}) was increased. In all the previous investigations of hyaluronate synthetase, the apparent Michaelis constants were determined by holding one substrate concentration constant while the other concentration was varied (Markovitz et al., 1959; Sugahara et al., 1979; Ishimoto et al., 1966; Appel et al., 1979). This approach can be misleading because it sheds no light on kinetic relationships of the substrates. We therefore determined the effects of hyaluronidase treatment on the apparent steady-state kinetics of the synthetase. Experiments for kinetic consideration were typically conducted with four concentrations of each substrate. The concentration ranges were chosen from preliminary experiments to ensure that several concentrations larger than the K_m^{app} were included for each substrate. The appropriate ranges were found to be 50 μM–0.5 mM for UDP-GlcUA and 100 μM–1.4 mM for UDP-GlcNAc.

Double-reciprocal plots of E_0/v_0 vs. 1/substrate concentration typically exhibited the forms shown in Figure 5. The consistent result found with control membranes was a family of intersecting lines. Unexpectedly, treatment with hyal-

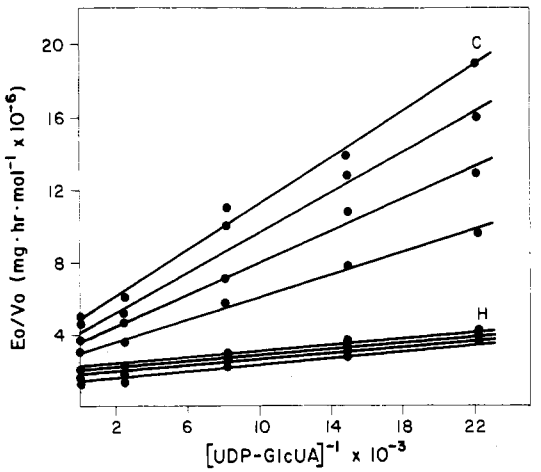


FIGURE 5: Double-reciprocal plots: hyaluronate synthetase activity in membranes from control and hyaluronidase-treated cells. Each family of lines was generated with four concentrations of each UDP-sugar substrate. The points on the y intercept are extrapolated values. The plots of the reciprocal velocities against the other substrate, [UDP-GlcNAc]$^{-1}$, have identical patterns. C, data obtained with membranes from control cells; H, data obtained with cells treated with testicular hyaluronidase as described in the legend to Figure 1.

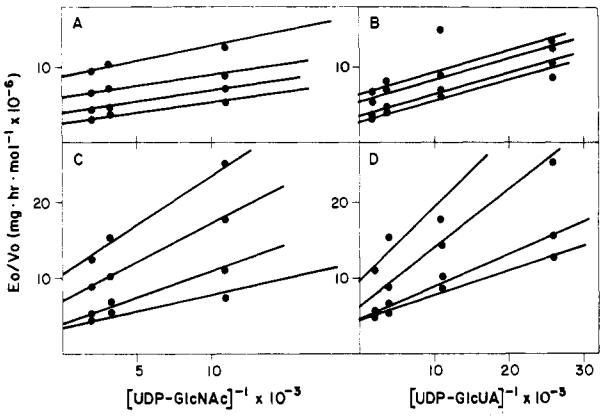


FIGURE 6: Kinetic reversibility: hyaluronate synthetase in membranes from hyaluronidase-treated and recovered cells. The same data are shown plotted with respect to the reciprocal concentrations of both substrates. (Panels A and B) Values obtained with membranes treated with testicular hyaluronidase as described in the legend to Figure 1. (Panels C and D) Additional tissue culture plates were treated with hyaluronidase, as above, and then rinsed and reincubated with media for 30 min before harvest and preparation of membranes ("recovered" cells; see legend to Figure 2).

uronidase not only increased the reaction velocity in a substrate-dependent manner but also changed the apparent kinetic pattern to a family of parallel lines. A pattern of parallel lines is indicative of a double-displacement mechanism, while a pattern of intersecting lines, such as that obtained with control membranes, is generally consistent with a single-displacement mechanism.

As discussed previously, reincubation of hyaluronidase-treated cells resulted in the "recovery" of the hyaluronate synthetase to control levels. As shown in Figure 6, the qualitative kinetic pattern obtained in double-reciprocal plots was also restored to intersecting lines in membranes from recovered cells. This result suggests that the recovery period allowed the synthetase to return to the initial state and that the fall in enzyme activity was not simply due to inactivation.

The kinetic values obtained in three representative experiments are shown in Table IV. Experiments 1 and 2 of Table IV were comparisons of control and hyaluronidase-treated cells, while experiment 3 compared hyaluronidase-treated with recovered cells. The significant difference is the mathematical equivalent of the change in pattern: hyaluronidase treatment resulted in the disappearance of the cross term, and the cross term reappeared when cells were allowed to recover. A kinetic interpretation that is consistent with these observations is presented under Discussion.

Table IV: Representative Kinetic Values

expt		k_{+1}^{app} ($h^{-1} \times 10^6$)	k_{+2}^{app} ($h^{-1} \times 10^6$)	cross-term constant ($h^{-1}\ \mu M^{-1} \times 10^{10}$)	V_{max} (nmol mg^{-1} $h^{-1} \times 10^{-1}$)
1	control[a]	3.6 ± 0.4[d]	7.4 ± 1.5	1.4 ± 0.2	3.7 ± 0.1
	hyaluronidase[b]	9.7 ± 0.5	11.4 ± 0.4	0.0	9.9 ± 0.5
2	control	5.9 ± 0.9	2.4 ± 0.5	4.4 ± 0.2	10.1 ± 0.5
	hyaluronidase	16.1 ± 0.8	14.5 ± 1.4	0.0	17.5 ± 0.7
3	hyaluronidase	3.0 ± 0.2	3.6 ± 0.3	0.0	8.4 ± 0.9
	recovered[c]	2.9 ± 0.1	1.9 ± 0.2	3.7 ± 0.5	3.7 ± 0.4

[a] Cells were incubated and washed exactly as hyaluronidase-treated cells. [b] Cells in monolayer culture were treated with 10 IU/mL testicular hyaluronidase. [c] Cells in culture were treated with hyaluronidase, washed, and reincubated as described. [d] Values are given with their standard errors.

Discussion

We have observed that treatment of oligodendroglioma cells in culture with the nonpenetrating agent hyaluronidase resulted in a substantial increase in hyaluronate synthetase activity. The effect was a property only of intact viable cells and not demonstrable after similar treatment of cell homogenates or membrane preparations exhibiting hyaluronate synthetase activity. Furthermore, the effect was rapidly reversible by incubating washed cells in media without hyaluronidase. These observations, as well as the direct demonstration of both control and stimulated hyaluronate synthetase activity in plasma membrane enriched fractions, suggested that the synthetase is located at or near the cell surface in eukaryotic cells (Philipson & Schwartz, 1984). The pronounced stimulation of the synthetase activity was considered sufficiently unusual and novel to warrent further investigation, especially with the expectation that the stimulated system may be valuable in extending our understanding of the mechanism of hyaluronate synthesis.

Several explanations of this effect were considered including (1) that hyaluronidase treatment caused a clearing of membrane-associated hyaluronate which allowed increased substrate availability at the active site, (2) that hyaluronidase was not completely removed by several washes of cells and membranes, and subsequently allowed oligosaccharides to be generated in situ which were then elongated by the synthetase, and (3) that hyaluronidase specifically cleaved high molecular weight chains at the cell surface that remained attached at one end to the synthetase complex. The modified complex, with shorter hyaluronate chains, elongated the bound chains at a higher rate.

The least likely possibility was that oligosaccharides generated in situ were elongated by the synthetase. In all previous studies [reviewed by Roden (1980)], exogenously added mono- or oligosaccharides have not resulted in stimulation of the synthetase. It may be mentioned that in contrast to the suggestion of Prehm (1983a), this observation does not relate in any way to the direction of chain growth. At the low

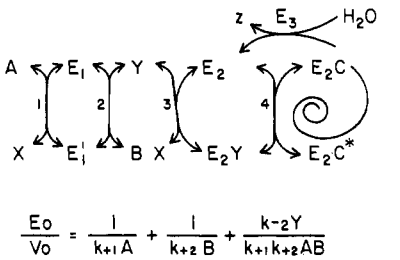
concentrations of hyaluronidase used for these studies, the [^{14}C]hyaluronate synthesized in vitro was still of very large molecular size. In the presence of heparin, an inhibitor of testicular hyaluronidase, there was no decrease in the molecular size of the product, and the relative stimulation of the synthetase was unaffected. This finding indicates that low levels of residual contaminating hyaluronidase in the isolated membranes were not responsible for the increase in specific activity. Furthermore, treatment of cells with other types of hyaluronidase which produce oligosaccharides with glucosamine or unsaturated glucuronic acid at the nonreducing end also resulted in stimulation of the synthetase. In this context, it should be emphasized that no glycosyltransferase has been reported with the ability to transfer monosaccharides to unsaturated acceptor residues. These results therefore do not support the suggestion of in situ generation of endogenous acceptors for the synthetase.

Simple "clearing" of surface-associated hyaluronate to increase the accessibility of substrate is likewise not a plausible explanation since trypsinization, which also removes hyaluronate from the surface of eukaryotic cells (Philipson & Schwartz, 1984; Mikuni-Takagaki & Toole, 1980), did not cause a stimulation of synthetase activity. Moreover, we have shown that nucleotide binding sites and protease-sensitive synthetase sites are not exposed at the outer membrane surface (Philipson & Schwartz, 1984) and therefore are not significantly affected by these treatments.

Attempts were then made to estimate the degree of hyaluronate chain elongation in vitro in order to determine whether a substrate was produced by hyaluronidase treatment that was more efficiently elongated. The ratios of saturated to unsaturated disaccharides following complete digestion with streptococcal hyaluronidase to measure the elongation of hyaluronate chains during cell-free synthesis (Stoolmiller & Dorfman, 1969) suggested that simple addition of monosaccharide units to the nonreducing terminus could not be demonstrated directly. In particular, digestion of newly elongated hyaluronate (membrane-bound) in the absence of one of the nucleotide sugars yielded only unsaturated disaccharides. Neither nonreducing-end nor reducing-end labeling by monsaccharides should give this result, and suggests either extensive rearrangement or addition of a preformed unit.

Rearrangement of chains would represent an immense complication of the biosynthetic process, which is not supported by the kinetic analysis (see below). Furthermore, such an enzyme activity has never been described except by the transferase activity of bovine testicular hyaluronidase (Weissman, 1955). Neither the expected array of disaccharides produced by this enzyme nor endogenous hyaluronidase activity has ever been found during the course of our experiments. We also carefully considered the possibility of contamination with UDP-GlcNAc bound in our washed membrane preparations. However, in recent experiments (unpublished) in which cells were labeled with radioactive uridine, phosphate, or glucosamine, no labeled UDP-GlcNAc (nor UDP-GlcUA) was recovered from washed membranes.

The increased amount of radioactivity incorporated following hyaluronidase treatment of cells was also associated with stimulation of chain release. Trichloroacetic acid partition of labeled products showed that within minutes of incubation, the percentage of membrane-bound radioactivity was substantially decreased. The severalfold-increased amounts of hyaluronate produced by treated membranes were recovered exclusively in the acid-soluble fractions. These results suggest that chains are "completed" and released throughout an incubation and are similar to the findings we previously reported (Sugahara et al., 1979). This has also been recently confirmed by Van der Rijn (1983) in the streptococcal system. Previous observations (confirmed in the present work) also indicate that there are no small or intermediate size hyaluronate chains produced by the hyaluronate synthetase in vitro, again pointing to the elongation of existing, bound polymers and to the absence of de novo synthesis in vitro (Sugahara et al., 1979; Appel et al., 1979). These observations imply that a pool of high molecular weight primers is available to the synthetase for elongation and that a larger pool is available after hyaluronidase treatment.



$$\frac{Eo}{Vo} = \frac{1}{k_{+1}A} + \frac{1}{k_{+2}B} + \frac{k_{-2}Y}{k_{+1}k_{+2}AB}$$

FIGURE 7: Kinetic scheme: a model hyaluronate-synthesizing system. The exact form of this transfer does not affect the equation shown. See text for discussion. A, UDP-GlcUA; B, UDP-GlcNAc; X, UDP; E, enzyme; Y, UDP-GlcUA-GlcNAc; Y′, C, C*, bound hyaluronate (growing chain); Z, completed chain.

In an attempt to understand the increase in synthetase activity in terms of the relationship between products and substrates, a detailed kinetic analysis was carried out comparing the two forms of the enzyme complex. A consideration of the kinetics of such reactions often provides a powerful tool for defining the properties and possible modes of action of the enzyme itself. Furthermore, when dealing with impure preparations, a kinetic approach is often the only means for studying enzyme function. While steady-state kinetics have been applied to a great variety of enzymes in solutions, we are aware that the application of the theory to membrane-bound enzymes (two-state systems) must be approached with caution. However, the general conclusions obtained here are clear and unambiguous. Hyaluronate synthetase assayed in membranes from untreated cells gave families of intersecting lines, while the membranes from hyaluronidase-treated cells yielded patterns of parallel lines. Further, the effect was reversible simply by reincubation of cells after hyaluronidase treatment. This is important since the explanation of the effect of hyaluronidase treatment must also be consistent with a biosynthetic system located in the cytoplasmic face of the plasma membrane (Philipson & Schwartz, 1984).

A formal kinetic mechanism for hyaluronate biosynthesis, shown in Figure 7, was formulated to account for the behavior described earlier.[2] At steady state, the initial velocity (v_0) is equal to the rate of appearance of elongated primer hyaluronate (C*), the disappearance of the substrates (A and B), and the appearance of UDP (X) (Figure 7). A hydrolytic step, denoted by E_3, which releases "mature" chains (z) is necessary to cleave the enzyme–hyaluronate complex. The intermediate Y (UDP-disaccharide) has some small, steady-state concentration, which provides a reversible pathway connecting the entry of the substrates (A and B). With the kinetic isolation broken, due to the reversibility of reaction 2, the overall result

[2] An alternate scheme with a single-displacement ("sequential") formal mechanism for E would fit the observations only if the entry of substrate A into a transient noncovalent complex with the free enzyme were irreversible. This is conceivable but seems much less likely than the double-displacement ("ping-pong") form.

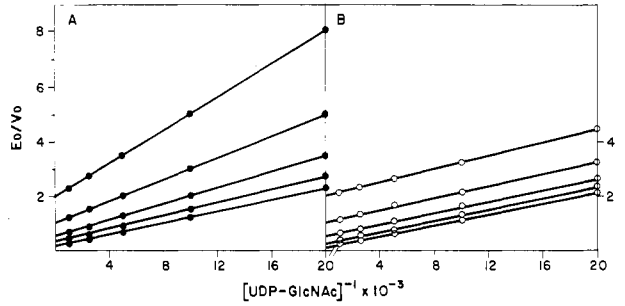


FIGURE 8: Kinetic patterns generated with the equation in Figure 7. The values were obtained with an enzyme modeling program (MODEL). Values of constants were chosen to reflect the apparent constants. Identical concentrations were used for both substrates; the choice of UDP-GlcNAc as the varied substrate was arbitrary. Panel A: $Y = 10^{-3}$ M; panel B, $Y = 10^{-4}$ M. Other constants were the same for both: $k_{+1} = k_{+2} = 10^7$; $k_{-2} = 10^6$.

is that initial velocity patterns with respect to substrate A or B are intersecting. If hyaluronidase treatment converts the primer hyaluronate to a better acceptor, k_{+4} increases, and the steady-state concentration of A and B will decrease. As Y concentration decreases, the pathway for the entrance of B becomes less reversible. The next effect is that the overall velocity will be increased and the initial velocity patterns will tend to be more nearly parallel.

These properties do not depend only on the formal mechanism of transfer of the intermediate Y to the acceptor C and the final release of Z as a "completed" chain. Additional participatory activities may be involved. The disaccharide moiety of Y may also be secondarily transferred to another acceptor [for example, a poly(isoprenol)lipid] before final transfer to the growing chain. The change of the initial velocity pattern does not result from shifting the rate-controlling step between the half-reactions but only from the decrease in the steady-state concentration of Y and the increase in k_{+4}. This can be seen by examination of the steady-state rate equation written only in terms of A, B, and Y, shown in Figure 7. In these equations, Y is the steady-state concentration of the intermediate. As the concentration of Y approaches 0, the cross term constant in the equation disappears (Figure 6), v_0 increases, and the lines appear more nearly parallel.

The characteristics of this system are also relevant to the experimental observation of reversibility in kinetic form. If the addition of intermediate (Y) to the modified acceptor eventually results in its conversion back to an acceptor with the original characteristics, the steady-state concentration of Y will rise. The net effect will be reversal of the hyaluronidase-induced modification. Patterns of lines generated with the equation in Figure 7 to illustrate the properties of the model are shown in Figure 8. The constants were chosen with the same orders of magnitudes as found experimentally, and only the concentration of Y was changed, as indicated.

Parenthetically, the model contains no predictions for the direction of synthesis. Elongation of primers at either the reducing end or the nonreducing end of the growing chain is compatible with the postulated kinetic mchanism. However, the existence of an intermediate such as a UDP-disaccharide could readily explain the enigma of internal labeling. Further work is needed to conclusively resolve this problem, perhaps along the lines suggested by Robbins et al. (1967). It is interesting to note that the model system also suggests a mechanism for de novo synthesis. In the absence of sufficient amounts of primer (C), intermediates (Y) would accumulate. These then could be serially concatenated to once again result in a partitioned system with small Y and large C.

The model presented in Figure 7 is a minimal scheme that strives to avoid speculative overspecification, which is especially hazardous in multiphase systems. We omitted transient complexes of enzymes with substrates and products, which undoubtedly occur, because there is not yet sufficient information to indicate which of these are kinetically significant. The rate constants considered in the model are therefore composite constants representing only the limiting values of the true expressions. The only formal distortion that this simplification causes is the absence of maximal velocity terms in the rate equation, occasioned by the absence of unimolecular steps in the formal mechanism. In this representation, then, the term that includes Y exerts a pure slope effect. It is likely, however, that in the actual reaction, at least one unimolecular step at or after step 4 in Figure 7 is accelerated by the same process that converts C to a better acceptor and increases k_{+4}. The effect of this change would be to increase the maximal velocity, in addition to the slope effect, reproducing the pattern of change seen in the real data of Figure 5 more exactly than can Figure 8, which is based on the equation for the simplified scheme.

Finally, in regard to the existence of the hypothetical intermediate, we have previously described such a molecule in an investigation of the hyaluronate-synthesizing system of group A streptococci (Sugahara et al., 1979). Actually, two molecules with the charcteristics of a UDP-disaccharide were purified, but one was considered to be a participant in cell wall biosynthesis, as it could not be radiolabeled with UDP-[^{14}C]glucuronic acid. The other molecule was purified from membranes incubated with UDP-GlcNAc and UDP-[^{14}C]-glucuronic acid and added back to membranes, but no transfer of radioactivity to hyaluronate was detected. Our earlier conclusion that the UDP-disaccharide was probably not an intermediate in hyaluronate biosynthesis may have been premature. Just as hyaluronate oligosaccharides are not utilized as acceptors by the synthetase, so too the UDP-disaccharide intermediate may have to occupy a privileged site in the synthetase complex and be channeled through the system. The existence and participation of the intermediate in hyaluronate synthesis remain to be demonstrated in a eukaryotic system.

In conclusion, we have presented biochemical and kinetic data which suggest that the treatment of intact cells with hyaluronidase does indeed exert its effect through the modification of enzyme activity. This modification has occurred to hyaluronate chains still bound to the membrane-bound enzyme complex, rather than by the creation of exogenous acceptors and results in an acceleration of substrate flow through the synthetase complex, as shown by the reversible change in the kinetic pattern.

Registry No. Hyaluronidase, 9001-54-1; hyaluronate synthetase, 39346-43-5; hyaluronic acid, 9004-61-9.

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Deuterium and Phosphorus Nuclear Magnetic Resonance Studies on the Binding of Polymyxin B to Lipid Bilayer–Water Interfaces†

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ABSTRACT: Deuterium and phosphorus NMR methods have been used to study the binding of polymyxin B to the surface of bilayers containing lipids that were deuterated at specific positions in the polar head-group region. The binding of polymyxin B to acidic dimyristoylphosphatidylglycerol (DMPG) membranes induces only small *structural* distortions of the glycerol head group. The deuterium spin–lattice relaxation times for the different carbon–deuterium bonds in the head group of the same phospholipid are greatly reduced on binding of polymyxin B, indicating a restriction of the motional *rate* of the glycerol head group. Only very weak interactions were detected between polymyxin B and bilayers of zwitterionic dimyristoylphosphatidylcholine (DMPC). In mixed bilayers of the two phospholipid types, in which either of the two phospholipids was deuterated, the presence of polymyxin B caused a lateral phase separation into DMPG-enriched phospholipid–peptide clusters and a DMPG-depleted phase. Complete phase separation did not occur: peptide-containing complexes with charged phosphatidylglycerol contained substantial amounts of zwitterionic phosphatidylcholine. Exchange of both phospholipid types between complexes and the bulk lipid matrix was shown to be fast on the NMR time scale, with a lifetime for phospholipid–peptide association of less than 1 ms.

It has been well established that the antibiotic action of the cationic peptide polymyxin B is due to its binding and subsequent disruption of bacterial membranes. The outer membrane and the cytoplasmic membrane of Gram-negative bateria are attacked by the peptide (Teuber & Bader, 1976; Teuber, 1974), resulting in a highly increased membrane permeability and the release of cytoplasmic material (HsuChen & Feingold, 1973; Newton, 1953; Imai et al., 1975; Feingold et al., 1974). Studies on model systems have revealed a high affinity of polymyxin B for acidic phospholipids (HsuChen & Feingold, 1973; Sixl & Galla, 1981; El Mashak & Tocanne, 1980; Hartmann et al., 1978). Evidence has been presented to show that the cationic antibiotic induces lateral phase separation of charged lipids in mixtures of acidic and zwitterionic lipids (Sixl & Galla, 1981), and the presence of mono- and divalent cations was shown to have a strong effect on the peptide–lipid interaction (Sixl & Galla, 1979, 1981; Burt & Langer, 1983).

Although a detailed model for the structure of phosphatidic acid–polymyxin B complexes was proposed on the basis of calorimetric and fluorescence polarization experiments (Sixl & Galla, 1982), little is known about the mutual associations of membrane-active antibiotics and phospholipids at a molecular level. Proton NMR[1] studies of polymyxin B on binding to *Escherichia coli* membranes have revealed a relative immobilization of the peptide acyl chain that was attributed to its penetration into the hydrophobic membrane interior (Barrett-Bee et al., 1972).

No information is available about the interactions at the membrane surface between the peptide and the polar lipid head groups. This is mainly due to the inability of most experimental methods to detect local structural details and motions of lipid molecules, particularly in multicomponent systems. Using the unique potential of ^2H and phosphorus-31 NMR

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[1] Abbreviations: NMR, nuclear magnetic resonance; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DMPG, 1,2-dimyristoyl-*sn*-glycero-3-phospho-*rac*-glycerol; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; CSA, chemical shift anisotropy; EDTA, ethylenediaminetetraacetic acid; TEMPO, 2,2,5,5-tetramethylpiperidine-1-oxyl; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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