Kay, E., Fareed, G. C., Ruoslahti, E., & Fessler, J. H. (1979) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 38*, 817.
Kleinman, H. K., Murray, J. C., McGoodwin, E. B., & Martin, G. R. (1978) *J. Invest. Dermatol. 41*, 9–11.
Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.
Laskey, R. A., & Mills, A. D. (1975) *Eur. J. Biochem. 56*, 335–341.
Lewis, L. J., Hoak, J. C., Maca, R. D., & Fry, G. L. (1963) *Science 181*, 453–454.
Limeback, H. F., & Sodek, J. (1979) *Eur. J. Biochem. 100*, 541–550.
Loskutoff, D. J., & Edgington, T. S. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 3903–3907.
Macarak, E. J., Kirby, E., Kirk, T., & Kefalides, N. A. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 2621–2625.
McAuslan, B. R., & Reilly, W. (1979) *J. Cell. Physiol. 101*, 419–430.
Monson, J. M., Click, E. M., & Bornstein, P. (1975) *Biochemistry 14*, 4088–4092.
Ross, R., Nist, C., Kariya, B., Rivest, M. J., Raines, E., & Callis, J. (1978) *J. Cell. Physiol. 97*, 497–508.
Sage, H., Crouch, E., & Bornstein, P. (1979a) *Biochemistry 18*, 5433–5442.
Sage, H., Woodbury, R. G., & Bornstein, P. (1979b) *J. Biol. Chem. 254*, 9893–9900.
Sage, H., Pritzl, P., & Bornstein, P. (1981a) Biochemistry (in press).
Sage, H., Pritzl, P., & Bornstein, P. (1981b) *Collagen Relat. Res. 1* (in press).
Schwartz, S. M. (1978) *In Vitro 14*, 966–980.
Sinclair, R. A., Antonovych, T. T., & Mostofi, F. K. (1976) *Hum. Pathol. 7*, 565–588.
Stein, Y., & Stein, O. (1976) *Expo. Annu. Biochim. Med. 33*, 131–135.
Stenman, S., & Vaheri, A. (1978) *J. Exp. Med. 147*, 1054–1064.
Studier, F. W. (1973) *J. Mol. Biol. 79*, 237–248.
Timpl, R., Martin, G. R., Bruckner, P., Wick, G., & Wiedemann, H. (1978) *Eur. J. Biochem. 84*, 43–52.
Wechezak, A. R., Holbrook, K. A., Way, S. A., & Mansfield, P. B. (1979) *Blood Vessels 16*, 35–42.
Weksler, B. B., Marcus, A. J., & Jaffe, E. A. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 3922–3926.
Zweifach, B. W. (1973) in *The Inflammatory Process* (Zweifach, B. W., Grant, L., & McCluskey, R. T., Eds.) pp 3–46, Academic Press, New York.

Characterization of a Platelet Endoglycosidase Degrading Heparin-like Polysaccharides†

Åke Oldberg, Carl-Henrik Heldin, Åke Wasteson, Christer Busch, and Magnus Höök*

ABSTRACT: An endoglycosidase (heparitinase) acting on heparin and heparan sulfate was partially purified (~300 times) from human platelets by affinity chromatography on heparan sulfate substituted Sepharose. Only heparin-like polysaccharides were degraded by the enzyme. The susceptibility of various biosynthetic heparin intermediates indicated that the platelet heparitinase had a requirement for sulfamino but not ester sulfate groups. No activity toward other uronic acid containing glycosaminoglycans could be demonstrated. Glucuronidic but not glucosaminidic linkages in heparin or heparan sulfate were attacked by the enzyme as shown by analysis of the reducing sugar moiety in oligosaccharide products. The anticoagulant activity of heparin, determined in an antithrombin III activation assay, was markedly reduced after treatment with the heparitinase. The enzyme was released from its storage site in platelets after induction of the platelet release reaction. The physiological function of platelet heparitinase is not known but may be to modify extracellular heparin-like polysaccharides in the vascular system.

A number of exoenzymes have been demonstrated to participate in the degradation of glycosaminoglycans in mammals [for review, see, e.g., Dorfman & Matalon (1976)]. By the concerted action of these enzymes, inorganic sulfate and monosaccharide units are released from the nonreducing terminal of the polysaccharide. An endoglycosidase would presumably facilitate the degradation process by cleaving internal linkages in the polysaccharide and thus increase the number of nonreducing terminals which can serve as potential substrates for the exoenzymes. Recent work in our laboratory (Höök et al., 1975a, 1977; Wasteson et al., 1976) has demonstrated the presence of a family of endoglycosidases degrading heparin and heparan sulfate. By analyzing the reducing termini of heparan sulfate oligosaccharides stored intracellularly in cultured human skin fibroblasts, Klein and co-workers suggested that these oligosaccharides had been formed by endoenzymatic cleavage of both glucosaminidic and glucuronidic linkages in heparan sulfate chains (Klein & von Figura 1976a; Klein et al., 1976), and, later, the presence of an endoglucuronidase degrading heparan sulfate was demonstrated in human placenta (Klein & von Figura, 1976b). This work deals with a heparan sulfate degrading endoglycosidase

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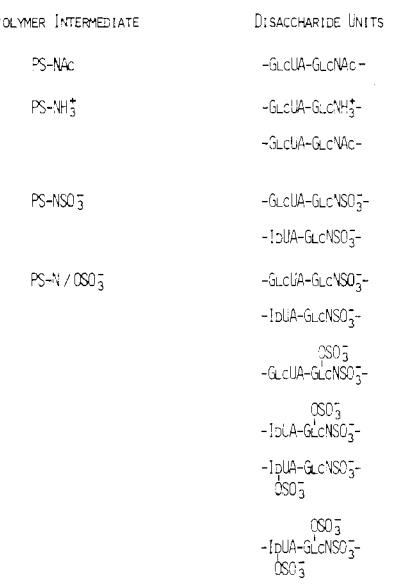

FIGURE 1: Representative disaccharide units of polymer intermediates in heparin biosynthesis. In addition to the N-sulfated glucosamine residues indicated as hexosamine constituents of PS-NSO_3^- and PS-N/OSO_3^-, these polysaccharides also contain small amounts of N-acetylated glucosamine units. The intermediates PS-NAc, PS-NH_3^+, PS-NSO_3^-, and PS-N/OSO_3^- are formed in the indicated order during biosynthesis of heparin (Höök et al., 1975b). (Further structural details of the polysaccharides are discussed in Jacobsson et al., 1979.)

demonstrated in human platelets. We report on the partial purification and characterization of the enzyme, including an investigation of its substrate specificity. The possible physiological function of the enzyme is discussed.

Materials and Methods

[^{35}S]Sulfate (carrier-free), [^{125}I]iodine (carrier-free), [^{3}H]acetic anhydride (500 mCi/mmol), and [^{3}H]borohydride (8 Ci/mmol) were obtained from the Radiochemical Center, Amersham, Bucks, U.K. Heparin, isolated from pig intestinal mucosa (stage 14), was obtained from Inolex Pharmaceutical Division, Park Forest South, IL, and purified as described (Lindahl et al., 1965). Heparin exhibiting a high affinity for antithrombin III (HA-heparin) was isolated by affinity chromatography on antithrombin–Sepharose (Höök et al., 1976). Heparan sulfate was isolated from human aorta essentially as described (Iverius, 1971), chondroitin sulfate was isolated from bovine nasal septa (Wasteson, 1971), and hyaluronic acid was isolated from rooster combs (Laurent et al., 1960). Dermatan sulfate isolated from pig intestinal mucosa was kindly given by Dr. L. Rodén, Birmingham, AL, and purified as described (Teien et al., 1976).

^{3}H-Labeling of the polysaccharides mentioned above was achieved by chemical ^{3}H-acetylation of free amino groups of the polysaccharide either directly (heparin and heparan sulfate) or after partial deacetylation of the polysaccharides (hyaluronic acid, chondroitin sulfate, and dermatan sulfate) (M. Höök, J. Riesenfeld, and U. Lindahl, unpublished experiments).

^{35}S-Labeled heparan sulfate was isolated from cultures of rat liver cells grown in the presence of [^{35}S]sulfate (Oldberg et al., 1977). Radioactively labeled microsomal heparin precursor polysaccharides were synthesized by incubating a mouse mastocytoma fraction with UDP-[^{14}C]glucuronic acid, UDP-*N*-acetylglucosamine, and phosphoadenylyl sulfate. After isolation of the labeled polysaccharides these were fractionated by anion-exchange chromatography [the details of the procedure have been published elsewhere, Jacobsson et al. (1979)]. By this procedure four classes of polysaccharides with different structural composition can be obtained (see Figure 1). Heparan sulfate was coupled to CNBr-activated Sepharose as described (Iverius, 1971). The gel contained 1.2 mg of polysaccharide/mL.

Human serum albumin (AB Kabi, Stockholm), denatured by incubating a solution at 100 °C for 2 min, was labeled with ^{125}I as described (Hunter & Greenwood, 1962). The labeled protein isolated by chromatography on Sephadex G-25 had a specific activity of 2×10^6 cpm/μg.

Outdated (3–8-days-old) platelets obtained from the Blood Center, University Hospital, Uppsala, were washed and lysed as described previously (Heldin et al., 1977). Particulate material was removed by centrifugation at 100000*g* for 60 min.

Methods for the determination of uronic acid, protein, and radioactivity have been described (Oldberg et al., 1977). Gel electrophoresis of proteins in sodium dodecyl sulfate was carried out on 10% polyacrylamide gels by using the buffer system described by Laemmli (1970). Paper electrophoresis at pH 5.3 and paper chromatography in ethyl acetate–acetic acid–water (3:1:1) were performed as described (Höök et al., 1974a).

Deaminative degradation of polysaccharides with nitrous acid was performed at pH 1.5 (Shively & Conrad, 1976). Acid hydrolysis of polysaccharides was performed in 4 M HCl at 100 °C for 17 h. Degradation of polysaccharides, essentially to the monosaccharide level, was accomplished by a combination of acid hydrolysis and nitrous acid deamination (Höök et al., 1974b).

Reduction of Oligosaccharides with Borohydride. The monosaccharide unit located at the reducing end of an oligosaccharide was labeled by reduction of the oligosaccharide with borotritide essentially as described (Ögren & Lindahl, 1975). The sample was dissolved in 0.5 mL of H_2O to which 50 μL of 1 M glycine, pH 7.0, and 50 μL of 0.5 M KOH containing 5 mCi of [^{3}H]borohydride (sp act. 1 ci/mmol) were added. After 3 h at room temperature excess borohydride was destroyed by adding 4 M acetic acid to pH 5.0 until bubbling ceased. The samples were passed through columns (1 × 3 cm) of Dowex 50-X8 (H^+ form) and evaporated to dryness 4 times in the presence of methanol. The labeled oligosaccharides were further purified by two successive gel chromatography runs on columns (1 × 90 cm) of Sephadex G-15 eluted with 1 M NaCl and 10% ethanol, respectively.

Assay of Heparitinase. An assay was developed for measuring the activity of the heparan sulfate degrading endoglycosidase. The procedure is based on the decreased precipitability with cetylpyridinium chloride of glycosaminoglycan oligosaccharides compared to undegraded polysaccharide. A standard incubation containing 25 μL of enzyme solution, 125 μL of 75 mM 2-(*N*-morpholino)ethanesulfonic acid (Mes), pH 6.0, and 5000 cpm of [^{3}H]heparan sulfate was incubated at 37 °C for 2 h. After addition of 50 μg of heparin in 50 μL of H_2O as a carrier, the samples were digested with papain; 200 μL of 0.8 M NaCl and 0.1 M acetate buffer, pH 5.5, containing 0.75 mg of papain was added, and the mixtures were incubated at 60 °C for 6 h. Polysaccharides in the papain digest were precipitated by adding 100 μL of 5% cetylpyridinium chloride and incubating the samples at 37 °C for 30 min. A final concentration of NaCl of 0.32 M was chosen since initial experiments showed that at this ionic strength a major portion (85%) of the [^{3}H]heparan sulfate used was precipitated by cetylpyridinium chloride. After centrifugation at 2000*g* for 10 min the ^{3}H radioactivity in the supernatant was determined. The amount of degraded and hence nonprecipitable [^{3}H]heparan sulfate showed a linear relationship

to the amount of enzyme in the range of 35–85% of ^3H radioactivity recovered in the supernatant.

Determination of Anticoagulant Activity. The anticoagulant activity of heparin was determined by an antithrombin activation assay (Björk & Nordenman, 1976). Assays were carried out in the presence of 2.7 μg of bovine antithrombin, 2 NIH units (0.8 μg) of thrombin, and 50 ng of high-affinity heparin. Thrombin activity was determined with D-phenylalanyl-L-pipocolyl-L-arginine-*p*-nitroanilide dihydrochloride (S-2238, AB Kabi, Diagnostica, Stockholm, Sweden) as substrate. High-affinity heparin (500 μg) was incubated for 6 h at 37 °C with 1 mL of enzyme solution (partly purified by affinity chromatography on heparan sulfate–Sepharose and dialyzed against 0.15 M NaCl and 0.02 M phosphate buffer, pH 6.0) or enzyme solution heat inactivated at 100 °C for 5 min.

Platelet Release Experiments. Human platelet-rich plasma (PRP) was obtained as described (Busch et al., 1976). To 2.5 mL of PRP was added 0.2 mL of 0.154 M Tris-HCl buffer, pH 7.4, and 0.3 mL of release-inducing substance dissolved in phosphate buffered saline, pH 7.3 ($NaCl/P_i$; 3.4 mM KCl, 137 mM NaCl, and 10 mM phosphate): thrombin (human, purified, AB Kabi, Stockholm), 1 NIH unit/mL; collagen, 3 μg/mL (Collagen-Reagent-Horm, Hormon-Chemie, Munich), ADP, 10 μM (adenosine 5′-diphosphate, Sigma No. A-0127, Grade 1, Sigma Chemical Co., St. Louis, MO); adrenaline, 5 μM (Adrenalin, ACO, Stockholm).

The experiments were performed in a Payton Single Channel Aggregation Module (Payton Associates, Ltd., Scarborough, Ont., Canada). The aggregation mixture was stirred at 37 °C for 5 min at 800 rpm. The reaction was interrupted by addition of 100 μL of 0.15 M ethylenediaminetetraacetic acid (EDTA) to the mixture, which was chilled on ice and centrifuged at 17500*g* for 10 min at 4 °C.

Samples of the supernatant were assayed for heparitinase activity as described above. In control mixtures $NaCl/P_i$ was substituted for the respective inducers. Control PRP was frozen and thawed 5 times and centrifuged as above. The enzyme activity of this supernatant was set at 100%.

Results

Partial Purification of Heparan Sulfate Degrading Endoglycosidase. Chromatography of platelet lysate on a column of heparan sulfate coupled to Sepharose was found to be an efficient step in the purification of the heparitinase. The platelet lysate, kept at pH 7.4, was applied to a column of heparan sulfate substituted Sepharose. The column was washed with a glycine buffer, pH 9.0, and eluted with a linear gradient of sodium chloride from 0.04 to 0.4 M at pH 9.0 (Figure 2; for further details see the legend to Figure 2). Most of the platelet protein did not bind to the affinity matrix or was eluted early in the gradient, whereas the enzyme activity required a sodium chloride concentration of ~0.25 M for elution from the column. An enzyme preparation obtained by this method (pooled as indicated in Figure 2) and dialyzed against 0.15 M NaCl and 0.02 M phosphate buffer, pH 6.0, contained ~0.07% of the protein and 20% of enzyme activity (when preserved by addition of albumin, see below) of that found in the platelet lysate corresponding to an increase in specific activity of ~300 times. Analysis of the purified preparation by gel electrophoresis in polyacrylamide revealed the presence of at least five components stainable with Coomassie Brilliant blue R-250, indicating that the preparation contained several proteins. The attempts to pursue the purification of the heparitinase beyond the initial affinity chromatography step were hindered by the rapid decline in enzyme activity in partially purified material; in contrast, the enzyme activity was preserved in unfractionated platelet lysate for more than a year at −20 °C or for more than 4 weeks at 4 °C. Various substances were investigated for their ability to stabilize the activity of the partially purified material (Table I). Serum albumin and to a lesser extent also ethylenediamine acetate (0.1 M) acted as stabilizing agents. Other substances tested were essentially inactive in this respect. In view of this finding the eluate emerging from the column of heparan sulfate–Sepharose was collected in test tubes to which human serum albumin and ethylenediamine acetate had been added (Figure 2).

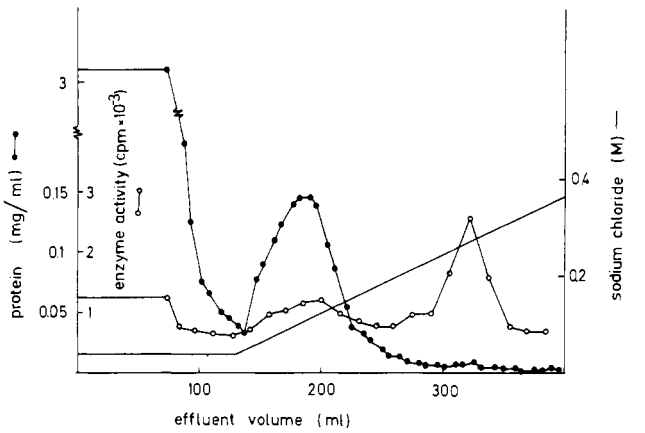

FIGURE 2: Chromatography of platelet lysate on heparan sulfate Sepharose. Forty milliliters of platelet lysate (280 mg of protein) was dialyzed against 0.075 M NaCl and 0.01 M phosphate buffer, pH 7.0, and applied to a 2 × 5 cm column (15 mL) of heparan sulfate–Sepharose equilibrated with the same buffer. The column was operated at 4 °C and at a flow rate of 15 mL/h. After being washed with ~80 mL of 0.04 M NaCl and 0.02 M glycin buffer, pH 9.0, the column was eluted with a gradient of NaCl (300 mL) from 0.04 to 0.4 M in 0.02 M glycin buffer, pH 9.0. Every third 5-mL fraction was collected in a test tube which had previously received 0.5 mL of human serum albumin, 10 mg/mL in 0.2 M phosphate buffer, pH 6.0, and 0.25 mL of 2 M ethylenediamine acetate. These fractions were assayed for enzyme activity (○) and the others for protein (●).

Table I: Stability of Enzyme Activity[a]

stabilizing agent, final concn	remaining enzyme act. (%)[b] day 1	day 4
serum albumin, 1 mg/mL	100	90
ethylenediamine acetate, 0.1 M	97	13
ammonium sulfate, 20%	30	NT[c]
protamin sulfate, 1 mg/mL	27	NT
lysine, 0.04 M	10	NT
glycerol, 20%	0	NT
no addition	10	0

[a] Platelet lysate was chromatographed on heparan sulfate–Sepharose as described. Fractions (5 mL) were collected in test tubes containing 0.5 mL of solutions of the stabilizing agents listed, resulting in the indicated final concentrations. Samples were left at 4 °C for 1 or 4 days before analysis of enzyme activity. [b] The activity of enzyme preparations stabilized with serum albumin was set to 100%, although the recovery of activity in this case was ~20% of the total amount applied to the column. [c] NT = not tested.

It is essential that the preparation does not contain protease activity, to be able to use the partially purified enzyme as an analytical tool in studies of the functions of heparin-like polysaccharides. To detect the presence of possible contaminating protease activity, we incubated the partially purified heparitinase (100 μL) at pH 6.0 and 37 °C for 12 h with 50 000 cpm of ^{125}I-labeled human denatured serum albumin. The incu-

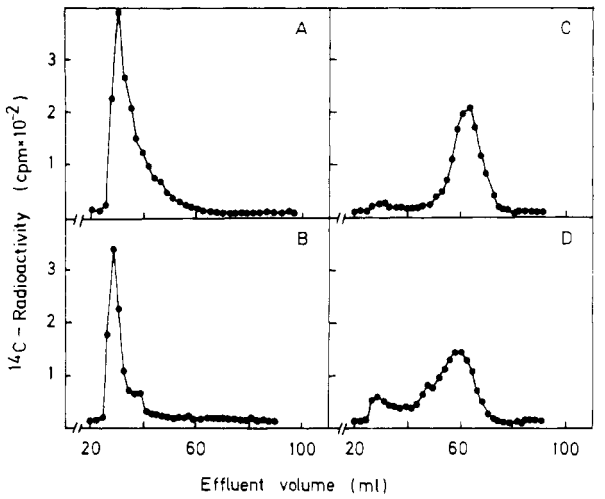

FIGURE 3: Gel chromatography on Sephadex G-100 of heparin precursor polysaccharides after incubation with the platelet heparitinase. Heparin precursor ^{14}C-labeled polysaccharides (A) PS-NH_3^-, (B) PS-NAc, (C) PS-NSO_3^-, and (D) PS-N/OSO_3^- were incubated with the platelet heparitinase as described under Materials and Methods. A small amount of [^{3}H]heparan sulfate was included as a susceptible control substrate to guarantee the activity of the enzyme. The incubation mixtures were applied to a column (1 × 90 cm) of Sephadex G-100 and eluted with 1 M NaCl at a flow rate of ~5 mL/h. Fractions of ~2 mL were collected and analyzed for ^{3}H and ^{14}C radioactivity. In all the experiments the [^{3}H]heparan sulfate control was susceptible to the enzyme. However, only partial degradation of the control substrate was obtained in the experiment shown in (D). Polysaccharides incubated with heat-inactivated enzyme were not degraded, all labeled material emerging with the void volume of the column (data not shown).

bation mixture was analyzed by electrophoresis on polyacrylamide gels in the presence of sodium dodecyl sulfate. The gels were sliced and analyzed for ^{125}I radioactivity. By this technique it was not possible to demonstrate any degradation of [^{125}I]albumin, indicating the absence of active proteases in the heparitinase preparation.

Substrate Specificity of Platelet Heparitinase. The substrate specificity of the platelet heparitinase was investigated by incubating the partially purified enzyme with various ^{3}H-labeled polysaccharides. The incubations were performed at 37 °C for 16 h, and degradation was monitored by gel chromatography of the incubation mixtures on a column of Sephadex G-100. A small amount of ^{35}S-labeled heparan sulfate was included in each incubation as a susceptible control substrate to indicate the activity of the enzyme. Of the various glycosaminoglycans tested as potential substrates, only heparin and heparan sulfate were degraded by the enzyme, whereas chondroitin sulfate, dermatan sulfate, and hyaluronic acid were left intact. These results demonstrate that the enzyme acts only on heparin-like polysaccharides.

When ^{14}C-labeled polysaccharides, acting as intermediates in the biosynthesis of heparin (see Figure 1), were incubated with the enzyme, only those polysaccharides containing sulfamino groups, PS-NSO_3^- and PS-N/OSO_3^-, were degraded whereas PS-NAc and PS-NH_3^+ resisted the treatment (Figure 3). These results implicate sulfamino groups as important features of the polysaccharide structure recognized by the enzyme. The susceptible polymer intermediate PS-NSO_3^- contains sulfate substituents only in the form of sulfamino groups, suggesting that the presence of ester sulfate groups in the substrate polysaccharide is not required for degradation by the enzyme.

Identification of Susceptible Linkages. Heparin and heparan sulfate prereduced with unlabeled sodium borohydride

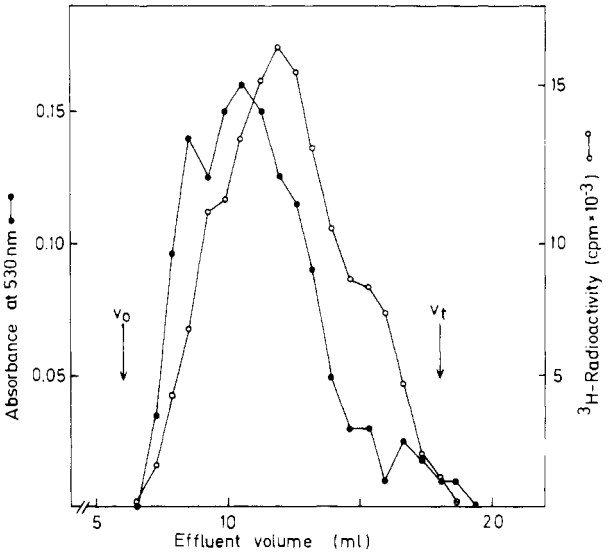

FIGURE 4: Gel chromatography on Sephadex G-100 of heparan sulfate incubated with the platelet heparitinase and reduced with [^{3}H]-borohydride. Three milliliters of a heparitinase solution (partly purified by affinity chromatography on heparan sulfate–Sepharose and dialyzed against 0.15 M NaCl and 0.02 M phosphate buffer, pH 6.0) was incubated with 3 mg of heparan sulfate for 48 h at 37 °C. The sample was diluted 10-fold with 0.05 M acetate buffer, pH 4.0, and applied to a column (1 × 5 cm) of DEAE-cellulose. The column was washed with 10 volumes of 0.2 M NaCl in 0.05 M acetate buffer, pH 4.0, and subsequently the heparan sulfate oligosaccharides (monitored by carbazole analyses) were eluted with 1.5 M NaCl in 0.05 M acetate buffer, pH 4.0. The oligosaccharides were desalted by chromatography on a column of Sephadex G-15 in 10% ethanol. The sample of oligosaccharides was evaporated to dryness and dissolved in 50 μL of 1 M glycin buffer, pH 7.0, and reduced with B^3H_4. The labeled oligosaccharides were isolated as described (see Materials and Methods). The recovery of heparan sulfate throughout the degradation, labeling, and isolation procedure was 50% as estimated by uronic acid determinations. ^{3}H-Labeled heparan sulfate oligosaccharides were applied to a column (0.5 × 90 cm) of Sephadex G-100 eluted with 1 M NaCl at a flow rate of ~2 mL/h. Fractions of ~0.7 mL were collected and analyzed for uronic acid (●) and ^{3}H radioactivity (○). Undigested heparan sulfate eluted as a single sharp peak at the void volume of the column. Similar results were obtained with heparin as the substrate polysaccharide.

were incubated at 37 °C for 48 h with the partly purified enzyme. The degradation products were isolated and labeled at their reducing ends by tritiated borohydride as described under Materials and Methods. In control experiments heparin and heparan sulfate were incubated with heat-inactivated (100 °C, 5 min) enzyme prior to radiolabeling. The material incubated with the inactive enzyme was not degraded, as shown by gel chromatography, and the specific activity of this material was considerably lower than that of the material radiolabeled after incubation with active enzyme. Gel chromatography of the latter material indicated that this polysaccharide had been degraded to oligosaccharides (Figure 4). Furthermore, the specific activity (cpm/uronic acid) increased toward fractions containing material of lower molecular weight (Figure 4) as expected for a polydisperse population of oligosaccharides containing one labeled end group per molecule.

To identify the labeled monosaccharide at the reducing end, we degraded the labeled oligosaccharides by a combination of acid hydrolysis and nitrous acid deamination. In this process labeled aldonic acid (corresponding to uronic acid units in the unreduced material) residues should be liberated as unmodified monosaccharides, which spontaneously may undergo lactonization to form an uncharged compound. If the oligosaccharides contained glucosaminitol as the reduced end sugar, this residue should probably be converted mainly to uncharged

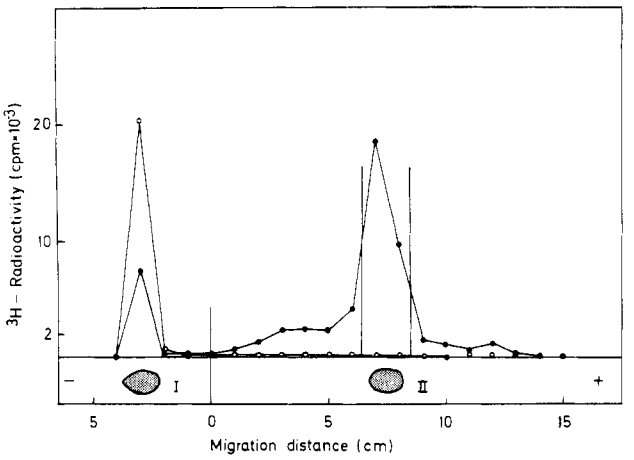

FIGURE 5: Paper electrophoresis of labeled monosaccharides derived from heparan sulfate, reduced with [^{3}H]borohydride after heparitinase digestion. The labeled oligosaccharides were further degraded by a combination of acid hydrolysis and nitrous acid deamination (see legend to Figure 4 and Materials and Methods) and subjected to paper electrophoresis. A guide strip of the paper was cut in 1-cm pieces and analyzed for ^{3}H radioactivity (●). Standards were (I) the lactone of D-gulonic acid, (II) D-gulonic acid, and (○) [^{3}H]anhydromannitol.

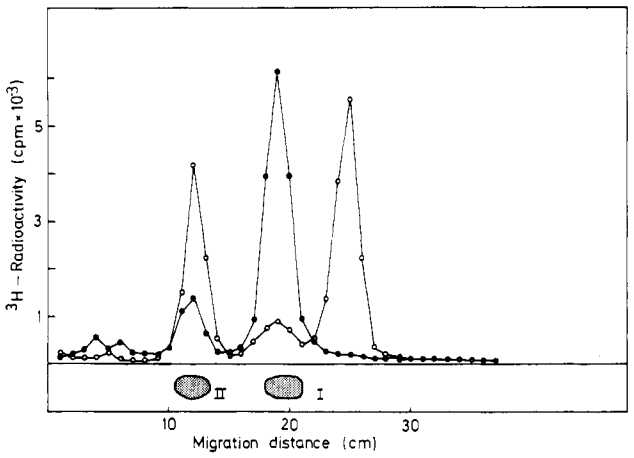

FIGURE 6: Paper chromatography of labeled components derived from heparan sulfate, reduced with [^{3}H]borohydride after digestion with heparitinase. The labeled monosaccharides migrating as uronic acid on paper electrophoresis (obtained as indicated in Figure 5) were eluted from the paper with water, concentrated, and subjected to paper chromatography. After 24 h the paper chromatogram was dried and cut into 1-cm pieces and analyzed for ^{3}H radioactivity (●). Standards were (I) L-gulonolactone, (II) free L-gulonic acid, and (○) a mixture of free L-[^{3}H]idonic acid and L-[^{3}H]idonolactone.

2-deoxy-D-*arabino*-hexose unit.[1]

Paper electrophoresis of the labeled monosaccharide derived from heparan sulfate (Figure 5) showed two major components with migration properties similar to those of an uncharged component and an aldonic acid standard, respectively. These results suggest that at least part of the oligosaccharides obtained after digestion of the heparin-like polysaccharides with the platelet endoglycosidase contained a uronic acid residue at the reducing end. The nature of the uncharged component is not clear; it could represent an aldonolactone or originate from glucosaminitol. Hence, the presence of an endohexosaminidase cannot be excluded from these data.

[1] As discussed by Ögren & Lindahl (1975) and by Aspinall et al. (1980) several products may be formed during nitrous acid deamination of *N*-acetylglucosaminitol. In some of these products the ^{3}H at Cl has been lost. Consequently cleavage of glucosaminidic linkages in heparin by the endoglycosidase may have escaped detection by procedures involving nitrous acid deamination.

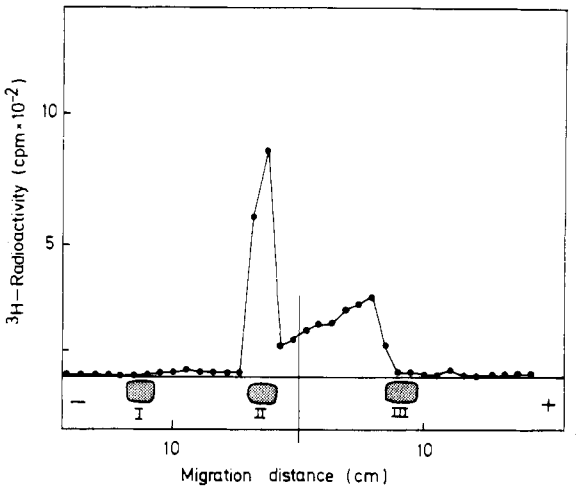

FIGURE 7: Paper electrophoresis of labeled components obtained after acid hydrolysis of heparitinase-digested and [^{3}H]borohydride-reduced heparan sulfate. After electrophoresis the paper strip was cut into 1-cm pieces and analyzed for ^{3}H radioactivity (●). Standards were (I) glucosamine hydrochloride, (II) D-glucuronolactone, and (III) D-glucuronic acid.

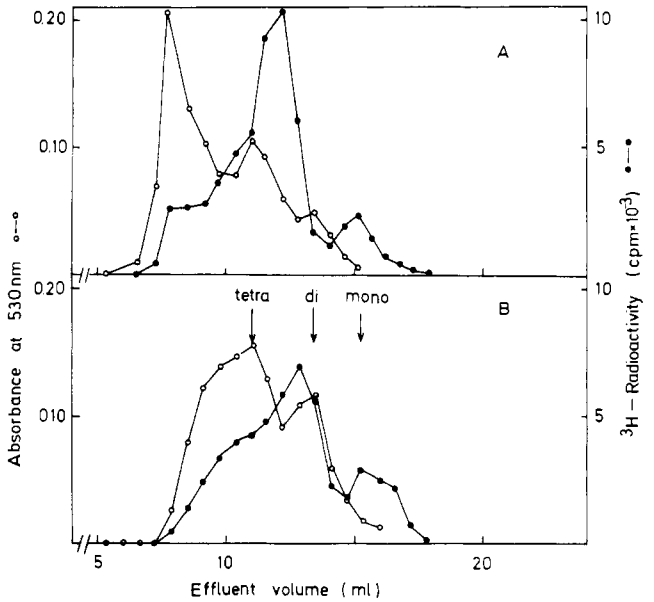

FIGURE 8: Gel chromatography on Sephadex G-25 of nitrous acid degraded heparan sulfate (A) and heparin (B) reduced with [^{3}H]-borohydride after partial depolymerization with platelet heparitinase. The samples were applied to a column (0.5 × 90 cm) of Sephadex G-25 eluted with 1 M NaCl at a rate of 2 mL/h. Fractions of 0.7 mL were collected and analyzed for ^{3}H radioactivity (●) and uronic acid (○). Tetra-, di-, and monosaccharides, respectively, migrated as indicated by arrows.

To establish the nature of the aldonic acid, we eluted the charged material in the paper electropherogram as indicated in Figure 5 and subjected it to paper chromatography. Prior to application on the paper, the labeled samples were treated with hydrochloric acid (Perry & Hulyalkar, 1965) to facilitate lactonization of the aldonic acids. On paper chromatography the labeled material comigrated with a gulonolactone standard, clearly separated from idonolactone (Figure 6), demonstrating that glucuronidic linkages were cleaved by an endoglycosidase in the enzyme preparation.

The degradation products, reduced with [^{3}H]borohydride, were hydrolyzed at 100 °C in 4 M HCl for 17 h and subjected to paper electrophoresis at pH 5.3 to investigate the possibility that also glucosaminidic linkages in the polysaccharide had been split. During the hydrolysis, glucosaminitol residues

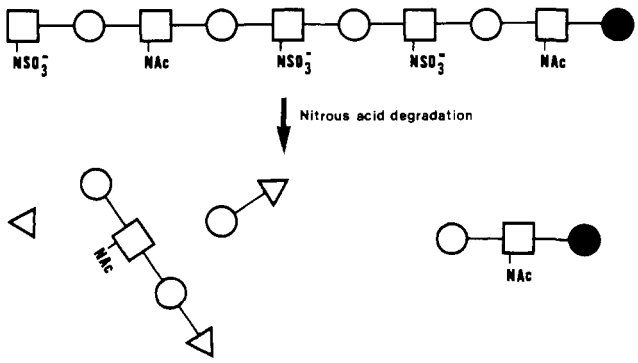

FIGURE 9: Nitrous acid degradation of ^{3}H-labeled oligosaccharide (schematic representation). A hypothetical oligosaccharide detained after heparitinase digestion of heparin and ^{3}H-labeled at the reducing end group was degraded by nitrous acid treatment. The deamination procedure employed converts N-sulfated (but not N-acetylated) glucosamine residues to anhydromannose units with concomitant cleavage of the glucosaminidic linkages. Note that only fragments originating from the reducing end of the oligosaccharide are labeled and will be detected in the fractions from the gel chromatography in Figure 8.

should be released as positively charged monosaccharides. However, no labeled material migrated toward the cathode on paper electrophoresis, indicating that no endoglucosaminidase is present in the enzyme preparation (Figure 7). The N-substitution pattern of glucosamine residues in the vicinity of the susceptible glucuronic linkage was investigated by subjecting the ^{3}H-reduced oligosaccharides to nitrous acid deamination and analyzing the size of the deamination products by gel chromatography on Sephadex G-25 (Figure 8). The deamination procedure used attacks N-sulfated but not N-acetylated glucosamine residues. Most of the labeled material obtained from the enzymatically digested heparin and heparan sulfate eluted as a trisaccharide, but radioactive material also appeared as larger oligosaccharides and as monosaccharides. The observation that odd-numbered labeled oligosaccharides were obtained by this degradation technique supports the idea that the enzymatic depolymerization of the polysaccharides involved cleavage of glucuronidic linkages (Figure 9). Furthermore, the preponderence of the trisaccharides among the deamination products indicates that the glucosamine residues located next to the [^{3}H]gulonic acid unit preferentially are N-acetylated. However, the enzyme may also cleave the glucuronidic linkage if this glucosamine unit carries sulfamino groups; this is a prerequisite for cleavage and generation of monosaccharides in the employed deamination procedure.

The possibility that the anticoagulant activity of heparin is affected by endoglycosidase degradation was investigated by analyzing the activity of HA-heparin in an antithrombin activation assay after incubation of the polysaccharide with active and heat-inactivated enzyme (Table II). The results showed that after a 6-h incubation with heparitinase the antithrombin activating potency of heparin was markedly reduced and hence the thrombin in the assay system remained active. On the other hand, when heparin was incubated with heat-inactivated enzyme, the polysaccharide retained its ability to enhance the activity of antithrombin resulting in an almost complete inhibition of the thrombin in the assay system.

Release of Heparitinase from Platelets. Treatment of intact platelets with certain inducers of the platelet-release reaction led to the liberation in soluble form of heparitinase activity (Table III) indicating that the enzyme is among the platelet-release products. Thrombin and collagen released the heparitinase more efficiently than did adrenalin or ADP. This finding is compatible with the view that the storage site of platelet heparitinase is in the α granules or in the vesicle fraction containing acid hydrolases (Bentfeld & Bainton, 1975) rather than in the dense bodies (Holmsen et al., 1969; Gordon & Milner, 1976). However, the exact localization of the enzyme activity has to await a more detailed analysis of the release reaction and/or subcellular fractionation experiments.

Table II: Inactivation of Thrombin by Antithrombin in the Presence of High-Affinity Heparin, Treated with Active or Heat-Inactivated Enzyme[a]

	inactivated thrombin (nkat)	thrombin act. (%)
buffer without HA-heparin	0	100
HA-heparin incubated with enzyme	0.295	82
HA-heparin incubated with heat-inactivated enzyme	1.425	0.1

[a] See Materials and Methods.

Table III: Release of Heparitinase from Platelets Treated with Release Inducers[a]

releasing agent	enzyme act. cpm	%
thrombin, 1 unit/mL	3.247	109
collagen, 3 μg/mL	2.541	66
adrenaline, 5 μM	1.863	25
ADP, 10 μM	1.648	12
freezing and thawing[b]	3.098	100
buffer	1.441	0

[a] Human platelets were purified by repeated centrifugations and incubated with different release inducers at 37 °C for 5 min or subjected to repeated freezing and thawing. After platelets had been spun down, the enzyme activity in the supernatants was determined. [b] The enzyme activity obtained by freezing and thawing was set at 100%.

Discussion

In the present paper we report a novel assay for quantitation of the activity of the heparan sulfate degrading endoglycosidase. The assay was based on the difference in precipitability with cetylpyridinium chloride of intact polysaccharides as compared to depolymerized ones. It made possible the simultaneous handling of a large number of samples and was highly reproducible. A similar approach has been used in the study of a different glycosaminoglycan-degrading endoglycosidase (Wasteson et al., 1975).

A 300-fold purification of the heparan sulfate degrading endoglycosidase was obtained by affinity chromatography of the platelet lysate on a Sepharose column, substituted with heparan sulfate of low sulfate content. Also, heparan sulfate of higher sulfate content or heparin could be used as affinity matrices although Sepharose columns substituted with the latter polysaccharides yielded less efficient purification, presumably because more protein bound to the columns in a nonspecific fashion due to the strong polyanionic properties of these polysaccharides. The enzyme obtained after affinity chromatography rapidly lost activity on storage, hampering further purification. The enzyme preparation did not contain protease activity detectable by our method and showed no activity on other glycosaminoglycans. After stabilization of enzyme activity by addition of albumin, it may, therefore, be used as a specific degradative tool in studies of heparan sulfate structure and function (Wasteson et al., 1977; Oldberg et al.,

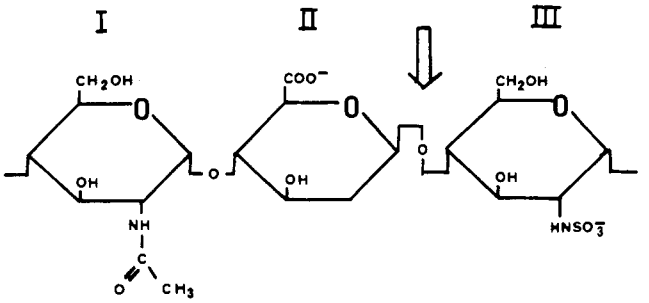

FIGURE 10: Hypothetical carbohydrate structure of heparin and heparan sulfate susceptible to the platelet heparitinase. The trisaccharide shown in the figure represents the structure of lowest sulfate content recognized by the enzyme. A sulfamino group essential for cleavage by the enzyme has hypothetically been located at glucosamine III since glucosamine I is preferentially N-acetylated. The presence of ester sulfate at glucosamine I or III is apparently not required for enzymatic attack. However, it is not known whether ester sulfate groups at glucosamine I or II or at both locations influence the reaction.

1979; L. Kjellén, Å. Oldberg, and M. Höök, unpublished experiments).

Analysis of the reducing end group of the product oligosaccharides obtained after enzymatic degradation of heparin and heparan sulfate identified the enzyme as an endoglucuronidase. No endoglucosaminidase activity could be detected in the partially purified enzyme preparation.

Investigation of the substrate specificity of the platelet enzyme showed that only heparin-like polysaccharides (e.g., heparin and heparan sulfate) were degraded. Furthermore, only those heparin precursor polysaccharides containing sulfamino groups were susceptible to the enzyme, suggesting that a sulfamino group is an essential part of the polysaccharide structure recognized by the enzyme (hypothetically located at position III, Figure 10). In view of the importance of the sulfamino groups, it is somewhat surprising to find that the penultimate glucosamine residue of the newly formed oligosaccharide (located at position I in Figure 10) preferentially appears to be N-acetylated. However, this *N*-acetyl group is presumably not part of the substrate structure recognized by the enzyme since, apparently, this glucosamine unit may also be N-sulfated. Rather, its preponderance is probably a consequence of the substrate specificity of the uronic acid epimerase participating in the biosynthesis of heparin-like polysaccharides. Only if the glucosamine residue at position I in Figure 10 is N-sulfated will the glucuronic acid at position II be recognized by the epimerase and converted to iduronic acid (Jacobsson et al., 1979). The amino substituent of the glucosamine unit at position I in Figure 10 thus governs to some extent the C-5 configuration of the uronic acid residue at position II; the requirement of a glucuronic acid in position II is thus in agreement with an abundance of N-acetylated glucosamine units at position I. The finding that PS-NSO_3^- was degraded by the enzyme suggests that ester sulfate groups are not necessary for recognition of the substrate by the enzyme. However, it remains unclear if the presence of O-sulfated glucosamine residues at position I or III (Figure 10) is compatible with enzymatic cleavage. The substrate specificity of the heparan sulfate degrading endoglycosidase from rat liver was found to be identical with that of the platelet enzyme by using the methodology described in the present paper (Å. Oldberg and M. Höök, unpublished experiments), suggesting that the heparitinases from the two sources are related or identical.

An intracellular degradation of heparin or heparan sulfate has not been demonstrated in platelets, and it is thus unclear if the enzyme has a degradative function within the platelets. The finding that the enzyme is among the platelet-release products raises the interesting possibility that its main physiological role is to depolymerize extracellular polysaccharides. The present experiments showed that the antithrombin-enhancing activity of HA-heparin was destroyed by the heparitinase. A glucuronic acid linked to an N-sulfated glucosamine residue, proposed to be the essential component in the polysaccharide structure recognized by the enzyme, is frequently found in the antithrombin binding sequence in heparin (Lindahl et al., 1979; Rosenberg & Lam, 1979). However, the polysaccharide structures recognized by the two proteins are not identical since heparan sulfate which is cleaved by the enzyme does not bind to antithrombin with high affinity (M. Höök and U. Lindahl, unpublished observation). Thus additional components are required in the heparin molecule for binding to antithrombin. Another tentative substrate is the heparan sulfate associated with the endothelial cells lining the vessel wall, and it was previously demonstrated that heparan sulfate of cultured endothelial cells was susceptible to the enzyme (Wasteson et al., 1977). Platelet heparitinase together with PF-4 may thus constitute a system for neutralization of heparin and heparin-like polysaccharides.

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References

Aspinall, G. O., Gharia, M. M., & Wong, C. O. (1980) *Carbohydr. Res. 78*, 275–285.

Bentfeld, M. E., & Bainton, D. F. (1975) *J. Clin. Invest. 56*, 1635–1649.

Björk, I., & Nordenman, B. (1976) *Eur. J. Biochem. 68*, 507–511.

Busch, C., Wasteson, Å., & Westermark, B. (1976) *Thromb. Res. 8*, 493–500.

Dorfman, A., & Matalon, R. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 630–637.

Gordon, J. L., & Milner, A. J. (1976) *Res. Monogr. Cell Tissue Physiol. 1*, 3–22.

Heldin, C.-H., Wasteson, Å., & Westermark, B. (1977) *Exp. Cell Res. 109*, 429–437.

Holmsen, H., Day, H. J., & Stormorken, H. (1969) *Scand. J. Haematol. Suppl. No. 8*, 1–26.

Höök, M., Lindahl, U., Bäckström, G., Malmström, A., & Fransson, L.-Å. (1974a) *J. Biol. Chem. 249*, 3908–3915.

Höök, M., Lindahl, U., & Iverius, P.-H. (1974b) *Biochem. J. 137*, 33–43.

Höök, M., Wasteson, Å., & Oldberg, Å. (1975a) *Biochem. Biophys. Res. Commun. 67*, 1422–1428.

Höök, M., Lindahl, U., Hallén, A., & Bäckström, G. (1975b) *J. Biol. Chem. 250*, 6065–6071.

Höök, M., Björk, I., Hopwood, J., & Lindahl, U. (1976) *FEBS Lett. 66*, 90–93.

Höök, M., Pettersson, I., & Ögren, S. (1977) *Thromb. Res. 10*, 857–861.

Hunter, W. M., & Greenwood, F. C. (1962) *Nature (London) 194*, 495–496.

Iverius, P.-H. (1971) *Biochem. J. 124*, 677–683.

Jacobsson, I., Bäckström, G., Höök, M., Lindahl, U., Feingold, D. S., Malmström, A., & Rodén, L. (1979) *J. Biol. Chem. 254*, 2975–2982.

Klein, U., & von Figura, K. (1976a) *FEBS Lett. 71*, 266–268.

Klein, U., & von Figura, K. (1976b) *Biochem. Biophys. Res. Commun. 73*, 569–576.

Klein, U., Kresse, H., & von Figura, K. (1976) *Biochem. Biophys. Res. Commun. 69*, 158–166.
Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.
Laurent, T. C., Ryan, M., & Pietruszkiewicz, A. (1960) *Biochim. Biophys. Acta 42*, 476–485.
Lindahl, U., Cifonelli, J. A., Lindahl, B., & Rodén, L. (1965) *J. Biol. Chem. 240*, 2817–2820.
Lindahl, U., Bäckström, G., Höök, M., Thunberg, L., Fransson, L.-Å., & Linker, A. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 3198–3202.
Ögren, S., & Lindahl, U. (1975) *J. Biol. Chem. 250*, 2690–2697.
Oldberg, Å., Höök, M., Öbrink, B., Pertoft, H., & Rubin, K. (1977) *Biochem. J. 164*, 75–81.
Oldberg, Å., Kjellén, L., & Höök, M. (1979) *J. Biol. Chem. 254*, 8505–8510.
Perry, M. B., & Hulyalkar, R. K. (1965) *Can. J. Biochem. 43*, 573–584.
Rosenberg, R. D., & Lam, L. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 1218–1222.
Shively, J. E., & Conrad, H. E. (1976) *Biochemistry 15*, 3932–3942.
Teien, A., Abildgaard, U., & Höök, M. (1976) *Thromb. Res. 8*, 859–867.
Wasteson, Å. (1971) *Biochem. J. 122*, 477–485.
Wasteson, Å., Amadô, R., Ingmar, B., & Heldin, C.-H. (1975) *Protides Biol. Fluids 22*, 431–435.
Wasteson, Å., Höök, M., & Westermark, B. (1976) *FEBS Lett. 64*, 218–221.
Wasteson, Å., Glimelius, B., Busch, C., Westermark, B., Heldin, C.-H., & Norling, B. (1977) *Thromb. Res. 11*, 309–321.

Spinach Calmodulin: Isolation, Characterization, and Comparison with Vertebrate Calmodulins†

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ABSTRACT: Calmodulin is the name proposed for a multifunctional, calcium binding protein whose presence has been detected in a number of eukaryotic cells. In the studies summarized here, calmodulin has been isolated from spinach leaves (*Spinacea oleracea*), characterized, and compared to vertebrate calmodulins. Quantitative recovery data for a rapid-isolation protocol demonstrate that calmodulin is a major constituent of spinach leaves. Spinach calmodulin is indistinguishable from vertebrate calmodulins in phosphodiesterase activator activity using vertebrate brain phosphodiesterase and in quantitative immunoreactivity using antiserum made against vertebrate calmodulin. However, spinach calmodulin is readily distinguished from vertebrate and invertebrate calmodulins in electrophoretic mobility and in amino acid composition. Spinach calmodulin, like vertebrate calmodulins, lacks tryptophan and contains 1 mol each of N^{ϵ}-trimethyllysine and histidine per 17 000 g of protein. In contrast to vertebrate calmodulins, spinach calmodulin has only one tyrosinyl residue and has a threonine/serine ratio of 1.3. While amino acid compositions indicate differences between spinach and vertebrate calmodulins, isolation and characterization of tryptic peptides containing the single histidinyl and N^{ϵ}-trimethyllysyl residues and both prolinyl residues indicate that these regions in spinach calmodulin are similar to the corresponding regions in vertebrate calmodulin. These studies more fully define the general and specific characteristics of calmodulins and indicate that calmodulin structure is not as highly conserved among all eukaryotes as it is among vertebrates and invertebrates.

Calcium-modulated proteins are a subgroup, or family, of calcium-binding proteins. It has been proposed [for a review see Kretsinger (1980)] that the biochemical activity, as well as the tertiary structure, of these proteins is modulated by calcium. Members of this group include parvalbumins, troponin C's, and calmodulins. Calmodulin is a multifunctional calcium modulated protein that has been isolated from a variety of eukaryotes [for a review, see Kretsinger (1980); Klee et al., 1980].

Phylogenetic studies of calmodulins and other calcium-modulated proteins have provided information on the common as well as unique structural features of these biological signal transducers. Alignment of the amino acid sequence of bovine brain calmodulin and striated muscle troponin C's clearly demonstrates the structural relationships within this class of calcium-modulated proteins (Watterson et al., 1980a). Comparison of the amino acid sequence repeats found in the primary structures of calmodulin and troponin C suggests that these proteins have arisen by duplication of a two-domain precursor (Watterson et al., 1980a). Using algorithms for the comparison of calcium-modulated proteins, Barker et al. (1978) and Erickson et al. (1980) have suggested that calmodulin is closely related to the original four-domain precursor of calmodulin, troponin C, and the myosin light chains. Therefore, structural and functional comparisons of calmodulins from closely related as well as distant species might provide insight into the evolution of this class of biologically important macromolecules.

Reports of amino acid sequences for calmodulins from a limited number of sources (Watterson et al., 1980a; Dedman et al., 1978; Grand & Perry, 1978) as well as detailed comparative characterization studies (Watterson et al., 1980b) indicate that the calmodulin molecule has been highly conserved during vertebrate evolution. Calmodulin or calmodulin-like proteins have been isolated from only a few invertebrate

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