Sulfated Proteoglycans and Sulfated Proteins in Guinea Pig Megakaryocytes and Platelets in Vivo

**RELEVANCE TO MEGAKARYOCYTE MATURATION AND PLATELET ACTIVATION**

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This study has examined changes in proteoglycan synthesis during megakaryocyte maturation in vivo. Guinea pigs were injected with Na$_2$SO$_4$. Megakaryocytes and platelets were isolated from 3 h to 5 days later. The proteoglycans and other sulfated molecules in both cells were characterized at each time point by gel filtration, ion-exchange chromatography, gel electrophoresis, and chemical and enzymatic digestions. Two populations of chondroitin 6-sulfate proteoglycans were found by DEAE-Sephard chromatography. The major fraction was eluted with 4 M guanidine hydrochloride and the minor fraction with 4 M guanidine HCl, 2% Triton X-100. The $K_v$ of the major proteoglycan peak in the platelets at 1 day after injection was 0.19–0.20 on Sepharose CL-6B and decreased gradually to 0.12 by 3 days, when proteoglycan radioactivity per cell was maximal. The peak for megakaryocyte proteoglycans at 3 h was broad, with $K_v = 0.1–0.2$. The appearance of different portions of the proteoglycan peak in platelets coincided with their disappearance from megakaryocytes. Proteoglycan size was a function of glycosaminoglycan chain length. The proteoglycans eluted with Triton X-100 from DEAE-Sephard ($K_v = 0.04–0.07$ on Sepharose CL-6B) were not labeled in platelets until 2 days after injection. Our data suggest that megakaryocytes synthesize different-sized chondroitin sulfate proteoglycans at different stages of development. The proteoglycans of the major fraction were released from platelets in response to thrombin, and a small amount was released by ADP. The proteoglycans of the Triton X-100 eluate were not released by thrombin or ADP. About 20% of the sulfate radioactivity was incorporated into molecules that appear to be sulfated proteins and were not released by thrombin or ADP.

Megakaryocytes are the bone marrow cells that produce the circulating blood platelets. The development of megakaryocytes is unique. They are polyploid cells, predominantly 8N, 16N, and 32N, which produce 1000–5000 platelets/cell through an extraordinary process of cytoplasmic and membrane formation and differentiation. Platelets are devoid of nuclei, Golgi, and endoplasmic reticulum and therefore have virtually no capacity for protein or proteoglycan synthesis. The platelet proteins and proteoglycans appear to be derived by their synthesis by the megakaryocyte during platelet production (1). The proteoglycans of megakaryocytes and platelets are of interest to study for several reasons. First, they may be important for megakaryocyte development. Changes in proteoglycans with cell maturation have been reported in several types of cells, both in terms of glycosaminoglycan composition and proteoglycan size (2–6). Proteoglycans have been shown to be important for tissue differentiation (7, 8). Megakaryocytes appear to synthesize proteoglycans or other large sulfated molecules at all stages of development, according to autoradiographic studies (9, 10); and it is possible that the nature of the molecules synthesized could change as the cells mature. This might be important for migration of the cells within the marrow stroma, as has been shown for marrow granulocytes (11); for the sorting out of the developing platelet plasma membranes into appropriate territories for the individual platelets, analogous to tissue differentiation (7, 8); and for granule formation since the $\alpha$ granules of the platelets contain proteoglycans (9, 12–15). In addition, proteoglycans appear to be involved in platelet activation. The platelet proteoglycans appear to be found predominantly in the $\alpha$ granules and to a lesser extent in the plasma membrane. They are found in the analogous structures in megakaryocytes, i.e. the $\alpha$ granules and the demarcation membrane system, which is presumably the source of the surface membrane of the platelet (9, 12, 13). The proteoglycans are released from the $\alpha$ granules by thrombin (14, 15) and presumably from the platelet surface by ADP treatment (15, 16). Release of surface proteoglycans could affect platelet-platelet or platelet-vessel wall interactions. Understanding the timing of synthesis of proteoglycans in the megakaryocyte could help us to understand the processes of $\alpha$ granule and membrane synthesis in these cells that are critical for platelet function.

Two major difficulties in studying the biochemistry of developing megakaryocytes are the inability to isolate pure populations of very young cells from the marrow mononuclear cells of other lineages and the inability to separate the complex array of the isolatable, relatively mature "recognizable" megakaryocytes into their several ploidy and maturation classes (17). The isolatable megakaryocytes represent predominantly the more mature spectrum of megakaryocytes (18). We have tried to circumvent these problems by in vivo studies. Since the product cell, the blood platelet, is completely separated from the precursor cell compartment, a rationale could be established for interpretation of the radiolabeling of
molecules found in both cells at various times after administration of a single injection of sodium \[^{35}S\]sulfate to guinea pigs. The assumptions were that the single injection would serve as a pulse, with the \[^{35}S\]sulfate taken up at a high specific activity during a brief period by megakaryocytes at all stages of development, and that some or all of the molecules synthesized during this time would be found eventually in the platelets. Thus, molecules synthesized by relatively mature megakaryocytes would appear in the platelets at early time points after the pulse injection, and molecules synthesized by the younger megakaryocytes would appear in the platelets at later times.

This approach has permitted us to study proteoglycan metabolism over the time required for megakaryocyte maturation and platelet production and has, in addition, provided evidence for the presence of other types of sulfated molecules in megakaryocytes and platelets. The in vivo labeling has also enabled us to explore the role of these different molecules in platelet activation.

A preliminary account of this work has been presented (19).

EXPERIMENTAL PROCEDURES

Materials—Sodium \[^{35}S\]sulfate was obtained from Amersham Corp. or ICN Radiochemicals (Irvine, CA). Sepharose CL-6B, DEAE-Sepharose Fast Flow columns (Sepharose CL-6B) were from Pharmacia LKB Biotechnology Inc. Bio-Gel P-2 was from Bio-Rad. Chondroitinases ABC and AC-II, chondro-4-sulfatase, chondro-6-sulfatase, and glycosaminoglycan and unsaturated disaccharide standards were obtained from Miles Laboratories. Chondroitinase and chondrosulfatase enzymes were obtained also from Sigma and gave the same results as the enzymes from Miles Laboratories. Trypsin, papain, CHAPS, \(^1\) and guanidine hydrochloride (Grade 1) were obtained from Sigma; urea (ACS-certified) from Fisher; and Zweigenichte 3-12 from Behring Diagnostics. Thin-layer chromatography plates (Avicel, 250 μm thick) were obtained from Analtech Inc. (Newark, DE). Centricon-10 filters were from Amicon. Thrombin (bovine) was from Armour. All reagents for electrophoresis were purchased from Bio-Rad.

Animals—Male guinea pigs of the Hartley strain were obtained at 350 g from Hazleton Research Products (Denver, PA) and killed at 350-500 g. Animals were housed individually in wire-bottomed cages and fed and watered daily.

Injection of Animals with \[^{35}S\]Sulfate—The radiosulfate was obtained as a lyophilized salt, and dissolved in saline, and sterilized by filtration through a 0.2-μm Millipore filter. The material was administered intraperitoneally. The amount of radiolabel incorporated into megakaryocytes and platelets in different animals had a variability of ±20% at any given time point, but duplicate determinations on platelets obtained from separate syringes of blood from the same animal and duplicate determinations on megakaryocytes from the same preparation were always identical. The dose response was linear over a range of 1-5 μCi/g, most experiments were performed with 3 μCi/g, which provided sufficient radioactivity for analysis of molecules in the megakaryocytes and platelets of individual animals.

Cell Preparations—Guinea pig platelets were prepared as described previously (20). Blood was drawn by cardiac puncture into a 10-ml syringe containing 1 ml of NaHCO/sodium citrate/dextran anticoagulant. Anesthesia was either light ether or Vetalar/acepromazine. Platelet-rich plasma was prepared by centrifuging the blood at room temperature at 250 × g for 15 min. Platelets were pelleted from the platelet-rich plasma at 750 × g at 4 °C for 15 min and washed twice under these conditions with calcium- and magnesium-free Hanks' balanced salt solution, pH 6.5. This washing was sufficient to remove the plasma-associated \[^{35}S\]sulfate from the cells since the second wash contained only 2-4% of the total radioactivity of the platelet pellet. Aliquots of the platelet suspension were removed before the final centrifugation for platelet counts and for radioactivity determinations. Platelets were counted on a Becton-Dickinson model CC-3 platelet counter. Radioactivity determinations were made by mixing 100 μl of the platelet suspension with 5 ml of Scintiverse II. The data were in excellent agreement with radioactivity determinations of aliquots of the platelet extracts. The platelets were free of red and white cell contamination as determined by phase-contrast microscopy. Approximately 2 × 10⁷ platelets were obtained per animal.

Human platelets were prepared as described previously (21). Blood was collected in one-time use plastic syringes of 3.5% citrate and washed with Tris/EDTA. Contamination with white cells was less than 1/10,000.

Guinea pig megakaryocytes were prepared by the method of Levine and Fedorko (18) with subsequent modifications (22). Marrow was obtained from the femora, humeri, and tibiae of each animal. The cells were dispersed in a medium containing guanidine hydrochloride, 2 mM theophylline, and 0.32% sodium citrate in calcium- and magnesium-free Hanks' balanced salt solution, washed in this same solution but without citrate and with 5% bovine serum albumin, and passed through a density gradient centrifugation and two successive velocity sedimentations on bovine albumin gradients. Adenosine and theophylline were included in all gradient solutions to optimize cell morphology. The yield of megakaryocytes was 4-8 × 10⁴/animal, at a purity of 80-90% by cell number. At this degree of purity, the megakaryocyte mass accounts for at least 97% of the cell suspension because of the large size of the megakaryocytes relative to the contaminating cells (25). The viability as judged by trypan blue exclusion was at least 90%.

Other Cells—In some experiments, the radiolabeling of cells from the upper layers of the velocity sedimentation gradients, which are normally discarded during the megakaryocyte isolation, was analyzed for comparison to the megakaryocytes.

Extraction of Cellular Proteoglycans—The megakaryocytes, platelets, and other narrow cells were completely solubilized by the procedure of Kimura et al. (23). The cells were treated first with 8% Zweigenichte 3-12 in 50 mM sodium acetate, pH 5.8, containing the protease inhibitors 2 mM phenylmethylsulfonyl fluoride, 20 mM EDTA, 5 mM benzamide, and 0.1 M 6-aminohexonic acid for 3 hr at 4 °C. An equal volume of 8 M guanidine HCl in the same buffer was then added. The radiolabeled material was stable in these extracts for at least 1 month at 4 °C. Alternatively, the cells were dissolved completely in 8 M urea, 50 mM Tris-HCl, 0.1 M NaCl, 0.2% Triton X-100, and this was the method of choice for analyses beginning with ion-exchange chromatography.

Gel Filtration of Megakaryocyte and Platelet Extracts—The extracts were applied directly to Sepharose CL-6B columns (60 × 0.9 cm) equilibrated and eluted with 50 mM sodium acetate, 4 M guanidine HCl, 0.2% Triton X-100, pH 7.0 (24). Elution was performed at about 3.5 ml/hr. Fractions of about 0.5 ml were collected directly into 7-ml vials for radioactivity determinations. The void and active volumes of each column were determined with blue dextran and phenol red or sodium \[^{35}S\]sulfate. Inclusion of the dyes did not alter the migration of the \[^{35}S\]-labeled molecules on the columns. Platelets which were solubilized directly in the 8 M urea solvent behaved identically to the Zweigenichte/guanidine HCl extracts on the Sepharose columns.

Ion-exchange Chromatography—The initial experiments were performed using DEAE-Sephaloc columns (8 × 0.9 cm), equilibrated with 8 M urea, 50 mM Tris-HCl, 0.1 M NaCl, 0.2% Triton X-100, pH 8.0, and eluted with this solvent system, followed by a gradient of 0.1-0.8 M NaCl (24). The Zweigenichte/guanidine extracts were either diluted to a final concentration of 0.1 M chloride ion and applied directly to the column or first chromatographed on a PD-10 column with 4 M guanidine HCl, 50 mM sodium acetate, 0.1 M sodium sulfate, 0.2% Triton X-100, after which the excluded fractions were concentrated and resuspended in the 8 M urea saline solution and then applied to a DEAE-Sephaloc column. A major problem was precipitation of \[^{35}S\]-labeled material when any of these preparations was exposed to 8 M urea. This problem was circumvented by solubilizing the platelets directly in the DEAE-Sephaloc column buffer, avoiding use of Zweigenichte 3-12. Then, since most of the proteoglycan eluted in a sharp peak at 0.5 M NaCl during the gradient elution, the protocol was modified to a batch procedure. The DEAE-Sephaloc was equilibrated in the 8 M urea buffer, but without Triton X-100. The sample was applied to the column and eluted with 3 bed volumes each of 8 M urea, 50 mM Tris-HCl, 0.1 M NaCl, pH 8.0; 0.23 M NaCl in the same buffer, 4 M guanidine HCl, 50 mM sodium acetate, pH 8.0 and the same buffer without NaCl, 50 mM sodium acetate, 2% Triton X-100 or 2% CHAPS. After the first fraction of the 2% Triton (or CHAPS) solvents was collected, the column flow was stopped for several hours before elution of the final fractions. Nearly quantitative recovery (at least 97%) of radioactivity was achieved by this procedure.
Chemical and Enzymatic Digestion of Proteoglycans—Glycosaminoglycans were released from the proteoglycans by digestion with 1 M NaOH, 1 M NaBH₄, for 18 h at 42 °C (25). Papain and trypsin digests were done as described previously (24) except that a 10-fold greater enzyme concentration was used. Pronase digestion was performed as described (26). Chondroitinase ABC and II and chondroitin 4-sulfate or chondroitin 6-sulfate as carrier. Digestion products were separated by thin-layer chromatography using 20 × 20-cm plates (28), bands were visualized under UV light, and the lanes were scraped in 0.5-cm portions for radioactivity determinations. Alternatively, digestion products were separated by chromatography on 0.9 × 20-cm columns of Sephadex G-25, eluted with 50 mM NaAc, 0.2 M NaCl, or Bio-Gel P-2, eluted with 0.4 M ammonium acetate (29). Uronic acid was quantitated by spectral methods.

Core Protein Analysis—The 35S-labeled proteoglycans obtained by DEAE-Sepharose chromatography were dialyzed and lyophilized. They were digested with chondroitinase ABC as described by Oike et al. (31), except that the digestion was stopped by adding 3 volumes of 1.3% KOH in 95% ethanol. The digest was allowed to precipitate at −20 °C overnight. The precipitate was collected by centrifugation at 15,000 × g for 5 min and then was analyzed by polyacrylamide gel electrophoresis in the Laemmli system (32) as modified by Rosenberg et al. (33) using 10% acrylamide gels. The gels were stained with Coomassie Blue to identify proteins, and the radioactive bands were identified by fluorography. The gels were treated with ENanhance (Du Pont-New England Nuclear) and kept at −70 °C in the presence of Kodak XAR film. The film was developed by an automated X-Omat processor.

Electrophoresis of Intact Proteoglycans and Glycosaminoglycans—Intact proteoglycans were electrophoresed as described by Rosenberg et al. (33) except that a polyacrylamide gradient of 4-8 or 4-10% was used. Glycosaminoglycan electrophoresis was performed as described by Min and Cowman (34) using either 32- or 20-cm gels of 10% polyacrylamide run in a continuous buffer system. The chondroitin sulfates obtained from Miles Laboratories were run as standards. The gels were stained with Alcian blue, and the radioactive bands were visualized by fluorography.

Analysis of 35S-Labeled Molecules Released from Platelets by Thrombin and ADP Treatment—Platelets were washed as described above. Platelets were treated with 2 units/ml thrombin in 1 M CaCl₂ in magnesium-free Hanks' balanced salt solution, pH 7.4, for 5 min with stirring. The suspension was cooled to 4 °C and centrifuged for 15 min at 750 × g. The platelet pellet was resuspended in 0.25 M sucrose, 75 mM NaCl, 5 mM MgCl₂, 10 mM EDTA, pH 7.4, for 10 min at 37 °C. ADP was then added to a final concentration of 8-60 μM, and the suspension was stirred in an aggregometer (Payton Scientific Inc.) to determine aggregation. The supernatant was analyzed by gel filtration on Sepharose CL-6B in 1 M guanidine HCl without further treatment, and the pellet was solubilized in the guanidine HCl eluate and gel filtration.

RESULTS

Time Course of Incorporation of [35S]Sulfate into Megakaryocytes and Platelets following Injection of Sodium [35S]Sulfate

The time courses of total incorporation of radioactivity per cell for megakaryocytes and platelets and the incorporation of radioactivity into macromolecules and proteoglycans per cell for platelets are shown in Fig. 1. These and other data in this paper have been normalized to compare one megakaryocyte to 1000 platelets, reflecting the average relative protein content of the populations of the two cells (20). The earliest time point obtained was 3 h following the injection. Maximal radioactivity was observed at this time in the megakaryocytes, with a rapid fall through the first 3 days and a much slower decline thereafter. More than 90% of the whole cell radioactivity at all times was in macromolecules (see Fig. 3). Platelet radioactivity was very high at 3 h, but was predominantly due to material at the V₀, which was probably adherent free [35S]sulfate; this has been reported previously in mouse platelets (35). In contrast, in platelets at all other time points, activity of the radioactivity was contained in macromolecules (see also Figs. 2 and 3). The radioactivity in platelets increased from 1 to 3 days after injection and then declined slowly. The decrease in radioactivity is most likely due to degradation of the platelets during their time in circulation and to the removal of platelets from the circulation (10, 36). The time course of appearance and disappearance of total cell radioactivity in guinea pig platelets is the same as that reported by others for rat (36) and rabbit (37) platelets. The labeling of megakaryocytes has not been quantitated previously.

Determination of Incorporation of [35S]Sulfate into Various Molecules in Platelets and Megakaryocytes by Sepharose CL-6B Chromatography

The elution patterns of 35S-labeled material in the whole cell extracts of platelets and megakaryocytes from 3 h to 5 days after injection of [35S]sulfate are shown in Figs. 2 and 3. At 3 h, only trace amounts of label were observed in platelets in high molecular weight molecules (about 1000 cpm/10⁵ cells); the peaks were at Kᵥ = 0.25, 0.35, and 0.55. Most of the radioactivity was at the Vₒ of the column. The major peak in the platelet extract 1 day after labeling had a Kᵥ of 0.18-0.20. At 2 days, the peak was around Kᵥ = 0.14; at 3 days, was broader, centering around Kᵥ = 0.12; and at 4 days, was about 0.10. The appearance of the various portions of this
Fig. 2. Pattern of \textit{in vivo} labeling of molecules in whole cell extracts of platelets over 5 days after $^{35}$S sulfate injection. Washed cells were solubilized in Zwittergent and guanidine as described in the text, and extracts were chromatographed on Sepharose CL-6B columns equilibrated and eluted with 4 M GdnHCl, 50 mM NaAc, 0.2% Triton X-100. A peak in platelets until 3 days after injection could be accounted for by the disappearance of similar material from megakaryocytes over a given time period. At 3 h, the megakaryocyte extract had a broad asymmetrical peak ranging from $K_v = 0.1$ to 0.2. At 1 day, the lower molecular weight portion of this peak was greatly diminished; and the $K_v$ at this point was less than 0.1. Over the following 48 h, the material under the $K_v = 0.1$ peak was lost, corresponding to the increased labeling of the platelets and the shift of the $K_v$ to higher molecular weight in the platelet extract.

A second region of the eluates that is of interest is the portion from $K_v = 0.3$ to 0.6, which was also seen in megakaryocytes and platelets, but followed a different time course from that of the major peak. At 3 h after injection, very little was found in the isolated megakaryocytes. However, this portion of the eluate increased in megakaryocytes for at least 2 days and gradually increased in platelets over 3 and, in some experiments, 4 days. There was a net loss of radioactivity in these molecules from the megakaryocytes only after 3 or 4 days after labeling. This peak increased gradually in platelets and was usually maximal at 4 days. Considerable labeling of molecules in this size range was found 3 h after injection in the cells remaining in the upper layers of the velocity sedimentation gradients (Fig. 4), which are discarded during

Fig. 3. Pattern of \textit{in vivo} labeling of molecules in whole cell extracts of megakaryocytes over 5 days after $^{35}$S sulfate injection. Cells were solubilized and columns were run as described for Fig. 2.

Fig. 4. \textit{In vivo} labeling of megakaryocytes and other marrow cells at 3 h after $^{35}$S sulfate injection. Cells were obtained from the upper layers of the velocity sedimentation gradients to compare with purified megakaryocytes. $\circ$ and $\boldsymbol{\Delta}$, cells from the first and second velocity sedimentations, respectively. Cells were solubilized and chromatographed as described for Fig. 2. The three curves represent cells obtained from the same animal.
megakaryocyte purification and which contain many of the small, young megakaryocytes, along with the erythroid and myeloid cells. One possible interpretation of the time course of labeling of these molecules in megakaryocytes and platelets is that they are synthesized primarily by megakaryocytes that are too small to be isolated by our methodology at the early time points following sulfate injection, but that after 1 or 2 days, these cells have matured to the point where they can be isolated, and then after 2–3 days, to the point where they have released platelets. Thus, the amount of labeling of the molecules between \( K_w = 0.3 \) and 0.6 in megakaryocytes at any point from 2 to 5 days after injection would be the net result of \(^{35}\)S-labeled molecules in cells entering and leaving the isolatable cell population.

A third peak running just before the \( V_c \) of the Sepharose column was also seen in all megakaryocyte and platelet extracts. The changes in height of this peak in the platelet extracts generally paralleled the increase and decrease of the height of the major peak, and this peak declined with time in the megakaryocytes. As discussed below, about one-third to one-half of this material is released when platelets are stimulated with ADP or thrombin. We thus suggest that this material may not be free sulfate, but may include sulfated amines such as serotonin sulfate or dopamine sulfate, which have been found by others in platelets (38, 39). The pool of these molecules in the platelets also appears to be derived from the megakaryocytes.

Thus, all the labeling of molecules observed in platelets by means of Sepharose CL-6B chromatography of whole cell extracts can be accounted for by their appearance first in megakaryocytes.

The order in which the radiolabeled molecules are lost from the platelets appears to follow the order in which they appear in the cells: the portion of the major peak at \( K_w = 0.18 \) disappears first, followed by the higher molecular weight material. We cannot determine from our data whether this represents a partial slow release of a granule contents by a last-in-first-out mechanism, complete release of granule contents, or removal of the whole platelet from the circulation.

Characterization of Sulfated Molecules Synthesized by Guinea Pig Megakaryocytes in Vivo

The sulfated molecules found in the megakaryocytes and platelets in vivo were characterized by ion-exchange chromatography, alkaline borohydride digestion, and enzymatic treatments as described below.

Characterization of the \(^{35}\)S-Labeled Molecules Separated by Ion-exchange Chromatography

The Zwittergent/guanidine HCl extract of platelets labeled in vivo for 4 days was passed through a PD-10 column, concentrated by Centricon-10 filtration, and diluted to 0.1 M NaCl with 8 M urea, 50 mM Tris HCl, 0.2% Triton X-100 before application to the DEAE-Sephacel column. The very low molecular weight \(^{35}\)S-labeled molecules were lost during Centricon filtration and so were not applied to the column. About 25% of the remaining radioactivity was eluted in the wash-through with 0.1 M NaCl in the 8 M urea buffer, a few percent in the early fractions of the gradient, and about 5% at 0.5 M NaCl. The remainder could not be eluted from the column even with 2 M NaCl.

A similar distribution of radioactivity was observed when platelets were solubilized directly in the 8 M urea, 50 mM Tris HCl, 0.1 M NaCl, 0.2% Triton X-100 buffer and eluted by the batch procedure described above. A typical elution pattern is shown in Fig. 5 for platelets obtained 3 days after \(^{35}\)S-sulfate injection. About 30% of the radiolabel in the extract eluted in the wash-through. The Sepharose CL-6B profile of each fraction of the DEAE-Sephacel column is shown in Fig. 6. The material in the four fractions differed significantly from each
other, and all fractions are described below.

0.1 M NaCl Eluate—The material in this fraction had a broad distribution of radioactivity from the V, to about \( K_v = 0.5 \). This material was completely resistant to chondroitinase digestion. Hydrolysis of this fraction with mild alkaline borohydride, Pronase, or papain resulted in nearly complete release of \(^{35}S\) radioactivity as fragments which eluted at \( K_v \approx 0.9 \) on Sepharose CL-6B and were found mostly in the included volume of Bio-Gel P-2 columns. On this basis, the 0.1 M NaCl eluate from the DEAE-Sephacel column appeared to contain some sulfated proteins, and the small fragments released by alkaline borohydride or Pronase digestion may be small sulfated oligosaccharides. The very low molecular weight material near the \( V_c \) on the Sepharose columns of the whole cell extracts also eluted in this fraction and could be seen when the Sepharose column was run without prior dialysis (data not shown).

0.23 M NaCl Eluate—This fraction contained a high molecular weight material as well as some molecules in the size range found in the 0.1 M NaCl eluate. These molecules appear to be sulfoproteins by the same criteria described for the 0.1 M NaCl eluate.

4 M Guanidine HCl Eluate—The material which eluted with 4 M guanidine HCl in the absence of Triton X-100 produced a single broad peak on Sepharose CL-6B. The \( K_v \) and the shape of the peak varied in the same direction as did the major peak in the whole cell extracts (cf. Fig. 2) depending upon the time after \(^{35}S\)sulfate injection that the cells were isolated. This difference could also be shown by SDS-PAGE of the isolated proteoglycans (Fig. 7). About 96% of the material in this fraction could be digested with chondroitinase ABC, and further characterization using chondro-4-sulfatase, chondro-6-sulfatase, and chondroitinase AC-II demonstrated that the glycosaminoglycans consisted entirely of chondroitin 6-sulfate. This finding was obtained by analysis of the digestion products by Sephadex G-25 and Bio-Gel P-2 column chromatography and by thin-layer chromatography (data not shown).

Mild alkaline borohydride treatment of the proteoglycans produced large fragments migrating with \( K_v \approx 0.85 \) consistent with glycosaminoglycans (Fig. 8), which were digested to 99% with chondroitinase AC-II. No degradation was detected with nitrous acid. Subdivision of the Sepharose CL-6B eluate of the proteoglycans as shown in Fig. 8 and digestion of each subfraction with mild alkaline borohydride demonstrated that the size of the proteoglycans was a function of the glycosaminoglycan chain length. This same relationship was observed when proteoglycans from megakaryocytes obtained 3 h after sulfate injection were analyzed by the same protocol. Electrophoresis of the glycosaminoglycans demonstrated that the chain length was in the same range as the chondroitin 4-sulfate standard obtained from Miles Laboratories, approximate \( M_r \), 4,000–80,000 (Fig. 9). Comparison of the size of the radiolabeled glycosaminoglycans found in platelets at 1 and 3 days after sulfate injection demonstrates that only the lower molecular weight glycosaminoglycans are labeled in the platelets at 1 day after injection, but the whole spectrum of glycosaminoglycans seen with the Alcian blue stain is labeled at 2 and 3 days (see also Fig. 12). These data, together with Fig. 7, demonstrate that the most mature megakaryocytes (i.e., those cells which will produce platelets within 24 h after the sulfate injection) synthesize smaller proteoglycans with smaller glycosaminoglycan chains than do the younger megakaryocytes.

In addition to the glycosaminoglycans, borohydride digestion produced small fragments migrating at \( K_v \approx 0.85 \) on Sepharose CL-6B which may be small sulfated oligosaccharides.

The proteoglycans were totally resistant to trypsin digestion. Papain digestion produced a broad spectrum of fragments with a peak at \( K_v = 0.30–35 \) (data not shown), and Pronase digestion also produced fragments similar in size to those of the alkaline borohydride digest. The peak at \( K_v = 0.85 \) was generated by both papain and Pronase.

![Fluorography of SDS-PAGE of platelet proteoglycans](image)

**Fig. 7.** Fluorography of SDS-PAGE of platelet proteoglycans. Samples were run on gradient gels as described in the text. *First to third lanes,* proteoglycans from the 4 M GdnHCl fraction of the DEAE-Sephacel sodium comparing 1- and 3-day labeled platelets (4–8% polyacrylamide gel); *fourth and fifth lanes,* proteoglycans from the 4 M GdnHCl and the 4 M GdnHCl, 2% CHAPS eluates from 3-day labeled platelets (4–10% gel).

![Relationship of glycosaminoglycan chain length to proteoglycan size](image)

**Fig. 8.** Relationship of glycosaminoglycan chain length to proteoglycan size. Proteoglycans from the 4 M GdnHCl eluate as shown in Fig. 6 were subfractionated as shown (a), and each fraction was digested with NaOH/NaBH₄ to release the glycosaminoglycans (b).
3 days after [35S]sulfate injection. The higher molecular weight band is more prominent in the 3-day than in the 1-day sample, suggesting that this band is associated preferentially with higher molecular weight proteoglycans and the smaller core protein with the smaller proteoglycans. A similar experiment in which the proteoglycans were obtained from the three portions of the Sepharose CL-6B eluate as shown in Fig. 8 demonstrated the same core protein and proteoglycan size relationship. We cannot conclude unambiguously from these experiments that the different proteoglycan size components have different core proteins, but the data suggest that there are some small differences in molecular weight. We speculate that these differences may be due to different degrees of glycosylation, possibly due to the small sulfated oligosaccharide. The multiple bands do not appear to be an artifact of the length of digestion since a 24-h incubation produced the same banding pattern.

4 M Guanidine HCl + 2% Triton X-100 (or 2% CHAPS) Eluate—The molecules eluting in this fraction were larger than those eluting with 4 M guanidine HCl without detergent. This finding did not appear to be an artifact of the presence of detergent since the same profile on Sepharose CL-6B was obtained when the material was chromatographed with 0.2% Triton X-100, when the proteoglycans were precipitated from the 2% Triton solution to remove most of the detergent, when the sample was dialyzed extensively against 4 M guanidine HCl to remove the Triton (confirmed by monitoring $A_{280}$), or when Triton was replaced by 2% CHAPS for the DEAE-Sephacel column. This material migrated at a higher molecular weight on SDS-PAGE than did the proteoglycans from the 4 M guanidine HCl fraction of the DEAE-Sephacel column at different time points, stained with Alcian blue; right, fluorography of samples in center panel.

**Fig. 9. Electrophoresis of platelet glycosaminoglycans.** Samples were electrophoresed as described in the text. Left, chondroitin 4-sulfate (C-4-S) and chondroitin 6-sulfate (C-6-S) (Miles Laboratories) stained with Alcian blue; center, glycosaminoglycans from the proteoglycans of the 4 M GdnHCl fraction of the DEAE-Sephacel column at different time points, stained with Alcian blue; right, fluorography of samples in center panel.

**Fig. 10. Analysis of platelet proteoglycan core proteins by SDS-PAGE.** Lanes 1–3, chondroitinase ABC digest of 4 M GdnHCl fraction from DEAE-Sephacel columns from 3-day labeled platelets, high $M$, standards, intact proteoglycan, Coomassie Blue stain; lanes 4–6, same samples, fluorography; lane 7, chondroitinase ABC digest from 3-day labeled platelets; lane 8, chondroitinase ABC digest of 1-day labeled platelets; lane 9, protein standards, Coomassie Blue stain; lanes 10–12, fluorography of lanes 7–9; lane 13, chondroitinase ABC control; lane 14, protein standards, Coomassie Blue stain. Protein standards are $M_x \times 10^{-3}$.

Fig. 10 demonstrates the core proteins obtained from the proteoglycans of the 3-day labeled platelets by chondroitinase ABC digestion. Two distinct bands are present at $M_x$, 39,000 and 36,000, with several minor bands. None of these bands are present in the digested material. Fig. 10 also compares the core proteins from platelet proteoglycans obtained 1 and 3 days after [35S]sulfate injection. The higher molecular weight band is more prominent in the 3-day than in the 1-day sample, suggesting that this band is associated preferentially with higher molecular weight proteoglycans and the smaller core protein with the smaller proteoglycans. A similar experiment in which the proteoglycans were obtained from the three portions of the Sepharose CL-6B eluate as shown in Fig. 8 demonstrated the same core protein and proteoglycan size relationship. We cannot conclude unambiguously from these experiments that the different proteoglycan size components have different core proteins, but the data suggest that there are some small differences in molecular weight. We speculate that these differences may be due to different degrees of glycosylation, possibly due to the small sulfated oligosaccharide. The multiple bands do not appear to be an artifact of the length of digestion since a 24-h incubation produced the same banding pattern.

4 M Guanidine HCl + 2% Triton X-100 (or 2% CHAPS) Eluate—The molecules eluting in this fraction were larger than those eluting with 4 M guanidine HCl without detergent. This finding did not appear to be an artifact of the presence of detergent since the same profile on Sepharose CL-6B was obtained when the material was chromatographed with 0.2% Triton X-100, when the proteoglycans were precipitated from the 2% Triton solution to remove most of the detergent, when the sample was dialyzed extensively against 4 M guanidine HCl to remove the Triton (confirmed by monitoring $A_{280}$), or when Triton was replaced by 2% CHAPS for the DEAE-Sephacel column. This material migrated at a higher molecular weight on SDS-PAGE than did the proteoglycans from the 4 M guanidine HCl fraction of the DEAE-Sephacel column at different time points, stained with Alcian blue; right, fluorography of samples in center panel.

**Fig. 10. Analysis of platelet proteoglycan core proteins by SDS-PAGE.** Lanes 1–3, chondroitinase ABC digest of 4 M GdnHCl fraction from DEAE-Sephacel columns from 3-day labeled platelets, high $M_x$ standards, intact proteoglycan, Coomassie Blue stain; lanes 4–6, same samples, fluorography; lane 7, chondroitinase ABC digest from 3-day labeled platelets; lane 8, chondroitinase ABC digest of 1-day labeled platelets; lane 9, protein standards, Coomassie Blue stain; lanes 10–12, fluorography of lanes 7–9; lane 13, chondroitinase ABC control; lane 14, protein standards, Coomassie Blue stain. Protein standards are $M_x \times 10^{-3}$.

In order to determine whether the effects of radiation on the marrow might be responsible for the changes in the sulfated proteoglycan size which we observed over time, several guinea pigs were injected with [35S]sulfate twice before isolation of platelets and megakaryocytes. The injections were made 5 days and again 1 day before death. The first injection was 3 mCi/kg, and the second was 2 mCi/kg. The pattern of labeling was what would have been anticipated by adding together the eluates of Sepharose CL-6B columns from animals injected once at each time point. Therefore, the radioactivity did not appear to alter proteoglycan synthesis in the megakaryocytes.

Control Experiment for the Effect of [35S]Sulfate Radioactivity on Megakaryocytes

In order to determine whether the effects of radiation on the marrow might be responsible for the changes in the sulfated proteoglycan size which we observed over time, several guinea pigs were injected with [35S]sulfate twice before isolation of platelets and megakaryocytes. The injections were made 5 days and again 1 day before death. The first injection was 3 mCi/kg, and the second was 2 mCi/kg. The pattern of labeling was what would have been anticipated by adding together the eluates of Sepharose CL-6B columns from animals injected once at each time point. Therefore, the radioactivity did not appear to alter proteoglycan synthesis in the megakaryocytes.
Labeling of Megakaryocyte-poor Fractions Obtained during Megakaryocyte Purification

Fig. 4 shows the labeling profile on Sepharose CL-6B of megakaryocytes obtained at 3 h after sulfate injection in comparison with the cells in the upper layers of the two velocity sedimentation gradients. All three samples were obtained from the same animal. The cells from the first velocity sedimentation contain some erythroid, but mostly myelocytic cells, and the cells from the second gradient appear to be mostly myelocytic. Each of these fractions contains a small percentage, but significant number of recognizable megakaryocytes (Table I), most of which are small, i.e. 15-20 μm in diameter, or quite large and apparently at terminal stages of development with pycnotic nuclei. The megakaryocyte number in these cell populations is probably underestimated because of the difficulty in distinguishing immature 4N and 8N cells from the large precursor cells of other hematopoietic cell lineages by phase-contrast microscopy (17). The amount of radiolabel per 5 × 10⁶ purified megakaryocytes is compared to 25 × 10⁶ other marrow cells. The purified megakaryocytes contain much more total radioactivity per cell than do the other cell populations. There are striking differences in the profiles of the molecules synthesized by the megakaryocytes compared to the other cell fractions. The other cells show a much more disperse pattern of labeling than the megakaryocytes, with a much greater amount between Kₖₑᵥ = 0.3 and 0.6. The peak near the V is a smaller percentage of the total labeling of the purified megakaryocytes than of the extracts from the other cell populations. The radioactivity per cell in the upper layers of the velocity gradient decreased steadily with time over 5 days (data not shown). Whereas our data do not permit us to conclude with certainty that the young megakaryocytes contribute to the synthesis of the molecules migrating at Kₖₑᵥ = 0.3-0.6 in the cells from the upper layers of the velocity gradients, the disappearance of these molecules from this cell population follows a time course consistent with their appearance in the isolatable mature megakaryocytes.

Labeling of Plasma after [³²S]Sulfate Injection

The Sepharose CL-6B elution profile of plasma after sulfate injection showed that a small amount of the labeling coincides with the material found in platelets, with peaks near Kₑᵥ = 0.2 and a shoulder at Kₑᵥ = 0.3-0.4 leading to a major peak at Kₑᵥ = 0.55 on columns run without guanidine HCl and detergent (data not shown). Whereas one must consider the possibility that some of the radioactivity in the platelet extracts was due to contamination with plasma, we consider this unlikely for several reasons. First, the second wash of the platelets contained no more than 2-4% of the total radioactivity found in the final platelet pellet, consistent with a previous report (35). Second, molecules in the same size range were labeled in megakaryocytes, and the time course of their disappearance from megakaryocytes could be correlated with their appearance in platelets. It is unlikely that the megakaryocytes were contaminated with plasma since the cells are washed or passed through gradients at least seven times before they are extracted. The labeling profile of plasma was the same from 1 to 5 days after injection for molecules migrating with Kₑᵥ up to 0.55 on Sepharose CL-6B. The lower molecular weight peaks at Kₑᵥ = 0.85 and the V, predominated only at the very early time points, accounting for most of the radioactivity in the plasma. At 24 h, the peak at Kₑᵥ = 0.85 had only 28% of the total radioactivity, and this declined rapidly thereafter. The total sulfate radioactivity in plasma at 3 h was about 6 μCi/ml, at 48 h 0.12 μCi/ml, and at 5 days 0.06 μCi/ml. This is consistent with other reports of the decay of plasma sulfate-specific activity in man (41) and mice (10).

Release of [³³S]-Labeled Molecules by Thrombin and ADP

From 55 to 70% of the radioactivity in the cells was released to the medium by thrombin treatment, i.e. accord with the findings of Ward and Packham (15) with rabbit platelets and Riddell and Bier (42) with pig platelets. Similar data were obtained with guinea pig platelets obtained 1, 2, or 3 days after [³³S]sulfate injection.

Fig. 11 shows the difference between the labeling of molecules that are released from 4-day labeled thrombin-treated platelets and the labeling pattern of molecules that are retained in the cell. Both the supernatant and pellet were fractionated by DEAE-Sepharose chromatography, and the proteoglycans were chromatographed on Sepharose CL-6B. Almost all the radiolabeled material which was found in the

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**Table I**

Comparison of [³²S]sulfate incorporation in vivo into megakaryocyte-poor fractions of marrow from velocity sedimentation compared to purified megakaryocytes

| Top layers of: | Velocity Gradient I | Velocity Gradient II | Purified megakaryocytes |
|---------------|---------------------|----------------------|-------------------------|
| Total cells   | 2.4 × 10⁶           | 2.55 × 10⁶           | 7.88 × 10⁶               |
| No. of megakaryocytes | 0.53 × 10⁶ | 0.50 × 10⁶ | 5.4 × 10⁶                  |
| % megakaryocytes | 0.23                | 1.2                  | 69                       |
| Total cpm in fraction | 31,175     | 15,625               | 30,950                   |
| cm³/10⁶ cells | 130                 | 613                  | 3,915                    |
| % cpm in Kₑᵥ < 0.4 on | 27.3             | 32.8                 | 86.8                     |
| Sepharose CL-6B | 21.7              | 22.9                 | 7.9                      |
| % cpm in V, of Sepharose CL-6B | 36            | 201                  | 3,350                    |

* Data are from the experiment shown in Fig. 4.
supernatant was retained on the DEAE-Sephacel column and could be eluted with 4 M guanidine HCl. The small peak in the wash-through fraction contains mostly the low molecular weight material found at the \( V_c \) of the Sepharose column shown in Fig. 2. When the 4 M guanidine HCl eluate was chromatographed on the Sepharose CL-6B column, a single symmetrical peak with \( K_o \sim 0.12 \) was obtained. In contrast, most of the radiolabel from the resolubilized platelet pellet eluted in the wash-through fraction of the DEAE-Sephacel column. The pellet itself could be completely resolubilized after thrombin treatment; however, if the 0.1 M NaCl eluate from the DEAE-Sephacel column was dialyzed, 40–60% of the radiolabeled material could not be resolubilized subsequently. This problem was not encountered using material from unstimulated cells and may be the result of formation of a cytoskeleton-like material. The resolubilized material eluted from Sepharose CL-6B primarily between \( K_o = 0.3 \) and 0.5. The proteoglycan in the 4 M guanidine HCl eluate from the pellet was larger than that which was released to the supernatant. The \( K_o \) of this molecule ranged from 0.04 to 0.07 in four experiments. It should be noted that all solvents used in this experiment contained Triton X-100, in contrast to the detergent-free solvent systems used for the unstimulated whole cells. This molecule appears to be the same as the one described above which was eluted from the DEAE-Sephacel column only with high detergent in the whole cell experiments. These data suggest that the sulfated macromolecules released from stimulated platelets are primarily proteoglycans, and the material retained consists of mostly sulfoproteins and a small amount of a proteoglycan that is larger than the released proteoglycans.

It should be noted that in all experiments about half the radiolabeled material near the \( V_c \) of the Sepharose CL-6B column was released from the thrombin-treated platelets. Studies are in progress to determine the identity of these molecules; they are likely to include sulfated amines such as serotonin sulfate and dopamine sulfate, which have been reported to be present in platelets.

Fig. 12 compares the proteoglycans released by thrombin from platelets labeled for 1 and 4 days and the glucosaminoglycans generated from both. The relationship between proteoglycan size and glucosaminoglycan chain length observed in the proteoglycans purified from whole cell extracts of 3-day labeled platelets (Fig. 8) is also seen in the released proteoglycans. Also seen is the small peak at \( K_o = 0.85 \) which was found in the borohydride digests of the DEAE-purified proteoglycans from whole cells and the peak at the \( V_c \) which represents released small molecules.

ADP treatment of platelets caused release of 10–14% of the sulfated material in platelets (data not shown), again in agreement with Ward et al. (15, 16) with rabbit platelets. The proteoglycans and the material near the \( V_c \) were the only molecules released from the platelets after treatment with 8, 20, or 60 \( \mu \)M ADP. The \( K_o \) of the released material was representative of the average size of the proteoglycans in the whole cell extract and thus did not appear to differ from that of the proteoglycans released by thrombin, although the amount released was much less. This was true of platelets obtained 1–4 days after \( [^{35}S] \) sulfate injection. About 20–30% of the low molecular weight material was released in all experiments.

**Comparison of the Molecular Weight of Human and Guinea Pig Platelets**

Proteoglycans were isolated from human and guinea pig platelets by DEAE-Sephacel chromatography and were then cochromatographed on Sepharose CL-6B. Platelets were taken from animals 3 days after \( [^{35}S] \) sulfate injection because this was the time of maximal labeling and was expected to be most representative of the overall proteoglycan population. The number of human platelets was 10 times that of guinea pig platelets. Aliquots of each column fraction were taken for uronic acid determinations (30) and radioactivity determinations. The molecular weight distribution of the guinea pig platelet proteoglycans was virtually identical to that of the human platelets.

**DISCUSSION**

Our experimental approach has differed from those of previous studies of platelet proteoglycans by analyzing metabolic aspects of proteoglycans in both megakaryocytes and platelets. By monitoring the pattern of incorporation of \( [^{35}S] \) sulfate into specific molecules in both megakaryocytes and platelets in vivo over the 5-day maturation period of megakaryocytes, a metabolic diversity has been demonstrated in what otherwise appears to be a homogeneous or heterodisperse population of proteoglycan molecules. The time course of appearance and disappearance of radiolabel in specific molecules in megakaryocytes and platelets has provided the first biochemical evidence that the platelet proteoglycans are derived from the megakaryocytes. Unfortunately, our protocol was unable to monitor proteoglycans which might be specific for very young megakaryocytes and which would be lost during the early stages of development. The chondroitin sulfate proteoglycans produced by the most mature megakaryocytes are smaller than those produced by the less mature cells, suggesting a relationship between the degree of megakaryocyte maturation and the size of the proteoglycans and glucosaminoglycan chains synthesized by the cells. This pattern is consistent with reports of synthesis of proteoglycans with shorter chondroitin sulfate chains by mature cells in studies with chondrocytes (3) and bone cells (43). The importance of this finding with regard to cell development is not understood. The significance to platelet physiology of a diverse popu-
lation of sulfated proteoglycans also is not understood. Most of the \[^{35}S\]sulfate-labeled proteoglycans are released from the platelets by thrombin, including both the molecules at \(K_r = 0.18-0.20\) in the 1-day labeled platelets and the larger molecules near \(K_r = 0.1-0.13\) which are found at 3 and 4 days after labeling. These proteoglycans are most likely stored in the \(\alpha\) granules. One role for the platelet proteoglycan is thought to be as a carrier for platelet factor 4 (14, 44).

However, it is likely that other basic proteins in the \(\alpha\) granules could also bind to the proteoglycans, and specific proteoglycans could bind preferentially to specific proteins within a given mixture. The data of Huang et al. (44) would argue against this possibility since their proteoglycan did not bind to platelet-derived growth factor or to other molecules in platelet lysates and the glycosaminoglycans did not bind to platelet factor 4. However, it is possible that the binding properties of their proteoglycan were altered by long exposure to plasma proteases. Barber et al. (14) reported that several glycosaminoglycans could bind to platelet factor 4 as well as or better than chondroitin 4-sulfate. It is intriguing to consider the findings of Cramer et al. (45) that several \(\alpha\) granule proteins are arranged in an orderly fashion within the granule and the apparent location of mucopolysaccharide in a “nu-
cleoid” within the \(\alpha\) granule as described by Behnke (12). The diversity of proteoglycan size may contribute toward developing and maintaining an orderly arrangement of the \(\alpha\) granule constituents.

It is of interest that the thrombin-releasable platelet proteo-
glycans, which are probably derived from the \(\alpha\) granule, are degraded by Pronase and papain, in contrast to the mast cell lysosomal proteoglycans which are resistant to these proteases (46). This suggests that the core proteins of the platelet proteoglycans are quite different from those of the mast cells and may reflect the different nature and function of the proteins within the granules.

The proteoglycan that is found associated with the platelet pellet after thrombin treatment is larger than the proteogly-
cans secreted from the cells. It is probably the same molecule which requires high detergent concentration for elution from DEAE-Sephadex in our studies and which did not elute from DEAE-Sephadex with high salt in a study with human platelets (40). We suggest that this may be the membrane proteoglycan. Ward et al. (15, 16) proposed that the proteoglycan released by ADP is a membrane proteoglycan, but only the nature of the glycosaminoglycan chain rather than the intact molecule was identified in their studies. However, we found that the proteoglycans released by ADP and thrombin appear to be the same molecules. This large proteoglycan appears to be synthesized 2–3 days before platelet release.

The analysis of whole cell extracts has demonstrated the presence of molecules which appear to be sulfated proteins or sulfated glycoproteins in both megakaryocytes and platelets in vivo. To our knowledge, sulfation of platelet proteins has not been reported previously. The function of these proteins remains to be established. It is possible that they form a portion of the glyocalyx. The platelet surface and the demarcation membrane system of the megakaryocytes can be labeled with reagents which are known to complex with proteoglycans but can also react with other negatively charged substances (13, 47), and these membranes are also labeled metabolically with \[^{35}S\]sulfate (15). However, Barber et al. (14) found only 2% of platelet uronic acid associated with the plasma membrane. The rather low amount of uronic acid detected in membranes by the biochemical study could result from loss of the proteoglycans during membrane purification; however, the apparent discrepancy between the biochemical and morphological studies could also represent an alternative source of sulfate labeling in the plasma membrane, possibly the sulfoproteins. Since each protein would contain only one or several sulfate residues, in contrast to 100–200/proteo-
glycan molecule, these proteins may be more abundant on a molar basis. The characterization of these molecules is in progress in this laboratory and will be reported in a future publication.2 Sulfoproteins have been identified in several types of cells (e.g. Refs. 47–50).

Activated platelets are known to release substantial amounts of protease activity. Thus, it was of interest to note that the sulfate-labeled molecules, both the proteoglycans and the putative sulfoproteins, appeared not to be degraded as a result of platelet activation or by the thrombin in the medium. This conclusion is based on our ability to account for all portions of the whole cell eluate in the sum of the elution curves for the pellet and supernatant and the absence of detectable new peaks which would represent extensive deg-
gradation products. Therefore, any degradation of these mole-
cules in vivo would result from the action of enzymes in plasma or tissues in which the platelets become activated.

We have found that the molecular weight range of the guinea pig platelet proteoglycans is the same as that of human platelet proteoglycans, although the former contain chon-
droitin 5-sulfate and the latter chondroitin 4-sulfate (14, 44). The glycosaminoglycan chains have molecular weights of 40,000–80,000 based on electrophoresis and on elution posi-
tion on Sepharose CL-6B (51), and the core proteins are \(M_r\) 36,000–39,000. These data suggest a higher molecular weight than those of previous studies. The calibration of Heinegard and Hascall (52) for cartilage proteoglycan digests would suggest that the proteoglycans in our study have an average \(M_r\) of about 200,000. However, two previous studies have characterized a human platelet proteoglycan as having \(M_r\) 56,000 with four chondroitin 4-sulfate chains of \(M_r\) 12,000. These molecules were derived from the releasate of thrombin-
treated platelets (14) or from outdated frozen-thawed platelet-
rich plasma (44). In contrast, a recent study (49) which used methods similar to ours described the human platelet proteo-
glycan as a molecule of \(M_r\) 136,000 with chondroitin sulfate chains of \(M_r\) > 22,000 and presented evidence for the existence of other proteoglycans of different size or charge density; however, no reason for the diversity was established. The similarity of the guinea pig and human platelet proteoglycans suggests that our work is relevant to human platelets as well.

In conclusion, our studies suggest that megakaryocyte and platelet sulfated metabolism is more complex than previous studies have suggested. It will now be of interest to determine the specific roles of the sulfated molecules in megakaryocyte development and platelet function.

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