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# Quantification of $^{35}$S-Labeled Proteoglycans Complexed to Alcian Blue by Rapid Filtration in Multiwell Plates

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**This paper describes a rapid filtration assay for the quantification of $^{35}$S-labeled proteoglycans and/or $^{35}$S-labeled glycosaminoglycans in a large number of samples. Separation of $^{35}$S-labeled proteoglycans and $^{35}$S-labeled glycosaminoglycans from unincorporated [$^{35}$S]sulfate is effected by forming insoluble complexes between alcian blue and the glycosaminoglycan moieties of the proteoglycans and then filtering the solutions through "Durapore membrane" discs (0.45 μm pore size) fitted in a 96-well plate. Following brief rinsing steps, the discs are punched out and $^{35}$S-labeled macromolecules retained on the membrane are then quantified by scintillation counting. In this rapid filtration assay, the relationship between the amount of [$^{35}$S]-aggrecan applied and radioactivity measured was linear over a broad range of concentrations (2–800 μg aggrecan/ml). The amount of $^{35}$S-labeled proteoglycans measured in media and 4 M guanidine HCl extracts of articular cartilage and three different chondrocyte culture systems (monolayer, agarose gel, and alginate bead) ranged between 90 and 101% of the value obtained by sieve chromatography on Sephadex G-25. The presence in samples of unlabeled proteoglycans (up to 1 mg/ml), bovine serum albumin (up to 4 mg/ml), DNA (up to 20 μg/ml), serum (up to 30%), or guanidine hydrochloride at 4 M did not affect recovery of $^{35}$S-labeled proteoglycans measurably. CPM values obtained for $^{35}$S-labeled proteoglycans or $^{35}$S-labeled glycosaminoglycans quantified by chromatography on Sephadex G-25 and the filtration assay showed a strong linear relationship ($r > 0.99$) irrespective of the type of culture medium, extract, or digest used.** © 1994 Academic Press, Inc.

Much of what is known today about the structure and metabolism of proteoglycans (PGs)[2] was learned from *in vitro* studies of chondrocytes which synthesize PGs in larger amounts than other cell types (1–5). These studies have taken advantage of the fact that [$^{35}$S]sulfate added in the medium is incorporated almost exclusively (>98%) into the chondroitin sulfate and keratan sulfate moieties of the newly synthesized PGs (1). The use of [$^{35}$S]sulfate as a radiolabeled precursor offers a unique advantage over other precursors (radiolabeled amino acids and monosaccharides) in that quantification of the $^{35}$S-labeled PGs does not require that they first be purified. Various methods currently are available to quantify radioactivity present in $^{35}$S-labeled PGs. As only a small proportion of the [$^{35}$S]sulfate added to culture medium is incorporated, all methods must be able to separate the $^{35}$S-labeled PGs from the unincorporated isotope present in large excess. Sieve chromatography on Sephadex G-25 is very effective in achieving that goal and has proven to be the most popular method of quantification (1–5) but a variety of approaches which take advantage of one or more physicochemical properties of the PGs also have been proposed. Separation of $^{35}$S-labeled glycosaminoglycans ($^{35}$S-labeled GAGs) or $^{35}$S-labeled PGs from unincorporated [$^{35}$S]sulfate by precipitation with ethanol (2) or cetylpyridinium chloride (6) has not retained favor. In part, this reflects the time-consuming aspects of the quantification process: precipitation reaches equilibrium only after several hours (2,6,7). Further, exogenous PGs or GAGs must be added to yield a stable pellet and the latter must be resuspended and recentrifuged to ensure the removal of all unincorporated [$^{35}$S]sulfate (2).

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[2] Abbreviations used: PGs, proteoglycans; GAGs, glycosaminoglycans; BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; DMB, dimethylmethylene blue; EDTA, ethylenediaminatetraacetate.

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Methods which take advantage of the ability of cationic dyes such as dimethylmethylene blue (DMB) or alcian blue to bind with good specificity to sulfated groups on GAGs have gained in popularity as tools to quantify nonradiolabeled GAGs and PGs (7–13). The cationic dyes carry hydrophobic side chains which cause the GAG–dye complexes to aggregate and precipitate from aqueous solutions (7,13). After centrifugation to recover the precipitate or entrapment of the complex on a membrane, quantification of the dye is used as a measure of GAG or PG content. Binding of a cationic dye to DNA or carboxyl groups on proteins present in samples can occur but this is minimized by lowering the pH to protonate these groups. In practice, however, this does not always help as proteins tend to block the binding of the dye to the GAGs at low pH (14). A major problem with most of these dye-binding methods is that predigestion of molecules in biological fluids and cell or tissue extracts with proteases, nuclease digestion, and/or hyaluronidase digestion is a necessity. With the exception of alcian blue, the cationic dyes used in these assays are monovalent. Alcian blue carries up to four cationic groups (15,16) and thus binds more firmly to polysulfates at high ionic strength as it coordinates four sulfate groups at the same time (13). Björnsson (13) recently modified a previously published quantitative assay which measures alcian blue in precipitated alcian blue–GAG or alcian blue–PG complexes as a measure of GAG and/or PG content (7). In this modified assay, there is no need for pretreatment of samples with enzymes. The author used low pH, detergent, and high salt concentrations to minimize interference by proteins, hyaluronate, and guanidine HCl, a denaturing solvent often used to extract PGs from tissues.

Modified dye-binding approaches also have been used to quantify $^{35}$S-labeled PGs or $^{35}$S-labeled GAGs. In 1988, Hronowski and Anastassiades (17) quantified $^{35}$S-labeled PGs by applying an aliquot of the PG-containing solution on cellulose acetate, staining the membrane with alcian blue, and then measuring the radioactivity present on the membrane. A major limitation of this approach is that it required extensive purification of the PGs by sieve and diethylaminoethyl-Sephacel chromatography. Using an alternative approach, Rapraeger and Yeaman (18) captured $^{35}$S-labeled PGs on a single cationic nylon blot. However, there was no attempt to determine if quantification is affected by the presence of nonphysiological salt concentrations and recovery of the PGs was significantly affected by the presence of guanidine HCl. Further, saturation of cationic sites on the membrane with DNA and PGs restricted the analyses to samples containing limiting amounts of either component.

In this paper, we have taken advantage of a commercially available disposable multiwell filtration system (19) to develop a simple and rapid assay for the quantification of $^{35}$S-labeled PGs and/or $^{35}$S-labeled GAGs in a large number of samples. Most importantly, there is no need to pretreat the samples to remove or degrade non-PG-related molecules or reagents to obtain a reliable measure of the radiolabeled molecules present. We provide evidence that recovery of $^{35}$S-labeled PGs or $^{35}$S-labeled GAGs in undiluted labeling media and 4 M guanidine HCl extracts from three different types of chondrocyte culture systems and cartilage explants is excellent.

## MATERIALS AND METHODS

### *Materials*

Alcian blue was purchased from Bio-Rad (electrophoresis purity reagent, Cat. 161-0401, Batch 12H-4375). Alcian blue 8GX (Cat. A 3157, Batch 14705) was purchased from Sigma Chemical Co. Components of the MultiScreen system are as follows: disposable 96-well plate assemblies, hydrophilic Millipore Durapore membranes of different pore sizes (0.22, 0.45, and 0.65 μm), and a vacuum manifold and disposable punch tip assemblies. All were purchased from Millipore. Bovine serum albumin (BSA), DNA from calf thymus, and papain and guanidine HCl also were obtained from Sigma Chemical Co. Pronase E was purchased from Calbiochem, collagenase P (*Clostridium histolyticum*) from Boehringer Mannheim, Dulbecco's modified Eagle's medium (DMEM) and Ham's F12 medium from Gibco, and fetal bovine serum (FBS) from Hyclone. Hydrofluor was from National Diagnostics and [$^{35}$S]sulfate (0.9–1.5 TBq/mg) from Amersham Corp. Sephadex G-25 (prepacked in PD-10 columns) was from Pharmacia. Low-temperature gelling sea plaque agarose was obtained from FMC Corp. and low-viscosity alginate (Keltone LV) was a gift from Kelco (Chicago, IL). The dye 1,9-dimethylmethylene blue was purchased from Polysciences. All other chemicals and reagents used were analytical grade.

### *Articular Cartilage Explant Cultures*

Slices were removed from the full thickness of articular cartilage from the metacarpophalangeal joints of 18-month-old bovine steers (20). The slices were washed several times at 4°C with Ham's F12 medium, cut into small pieces (3 × 3 × 1 mm), and placed in DMEM/Ham's F12 (50/50) supplemented with 10% FBS, 50 μg/ml gentamycin, and 25 μg/ml ascorbic acid, pH 7.2. The explants were maintained in this medium at 37°C in the presence of 5% $CO_2$ in air. The medium was changed daily.

### *Chondrocyte Cultures*

Chondrocytes were isolated from the slices of articular cartilage after sequential digestion of the matrix

with 0.4% pronase E and 0.025% bacterial collagenase P as previously described (5). The cells were cultured in three different ways.

*A. Monolayers.* The cells were plated at high density ($2 \times 10^5$ cells/cm$^2$ in 60-mm plastic dishes) and maintained with daily changes of Ham's F12 medium containing 10% FBS, 50 μg/ml gentamycin, and 25 μg/ml ascorbic acid, pH 7.2 (20).

*B. Suspension in agarose.* The cells were seeded in 1% agarose at a density of $4 \times 10^6$ cells/ml in 35-mm plates as previously described (4) and maintained using daily changes of Ham's F12 medium containing 10% FBS, 50 μg/ml gentamycin, and 25 μg/ml ascorbic acid, pH 7.2.

*C. Suspension in alginate gel.* The cells were encapsulated in alginate beads at a density of $4 \times 10^6$ cells/ml of gel as previously described (5). These beads were maintained using daily changes of DMEM/Ham's F12 (50/50) medium containing 10% FBS, 50 μg/ml gentamycin, and 25 μg/ml ascorbic acid, pH 7.2.

### *Labeling of $^{35}$S-labeled PGs*

The same labeling protocol was used for all cultures. On Days 4–6 of culture, the medium was removed and replaced with fresh medium. One hour later, this medium was replaced by fresh medium containing [$^{35}$S]-sulfate at 20 μCi/ml. After incubation for 15 min–18 h, the labeling medium was removed and explants, monolayers, or gels were rinsed briefly with cold fresh medium. For each type of culture, $^{35}$S-labeled PGs in the extracellular matrix (explant), cell-associated matrix (monolayer), or gel (agarose or alginate) then were extracted for 4 h at 4°C with 4 M guanidine HCl, 0.05 M sodium acetate, pH 6.0, containing protease inhibitors (0.1 M 6-aminohexanoic acid, 0.01 M disodium ethylenediaminetetraacetate (EDTA), 0.005 M benzamidine hydrochloride, 0.01 M *N*-ethylmaleimide, and 0.001 M phenylmethylsulfonyl fluoride), as previously described (4,5,20,21). In some experiments, after removal of the labeling medium, the cell-associated matrix of monolayers was digested with papain (25 μg/ml in 0.01 M sodium acetate, 0.05 M disodium EDTA, 0.005 M L-cysteine HCl, pH 5.8) at 60°C for 16 h or treated with 1 N NaOH at 25°C for 16 h to solubilize $^{35}$S-labeled GAGs followed by rapid neutralization with an equal volume of 1 N HCl.

### *Purification of Radiolabeled Aggrecan*

In order to obtain purified $^{35}$S-labeled aggrecan, the 4 M guanidine HCl extract of cartilage explants cultured for 16 h in the presence of [$^{35}$S]sulfate was subjected to equilibrium density gradient centrifugation (48 h, 37,000 rpm, 10°C) in the presence of cesium chloride (starting density = 1.45 g/ml) (20). At the end of the run, the bottom $\frac{1}{4}$ of the tube containing purified aggrecan (D1 fraction) was recovered, dialyzed against 0.05 M sodium acetate and then extensively against distilled water, lyophilized, and stored at −20°C. Analysis of this sample on Sephadex G-25 showed that the sample was free of unincorporated $^{35}$S-labeled sulfate: all the radioactivity (>99.9%) was recovered in the void volume fractions. Unlabeled aggrecan also was purified from a 4 M guanidine HCl extract of bovine nasal cartilage using the same protocol.

### *DMB Assay*

The concentration of sulfated GAGs in samples was measured using a previously described assay which measures DMB complexed to sulfated GAGs by spectrophotometry (22). Because the majority (>90%) of the mass of sulfated GAGs in all samples was present in aggrecan molecules (data not shown and Refs. 5,20,21), PG content was expressed in terms of equivalents of the purified standard of bovine nasal aggrecan.

### *Quantification of $^{35}$S-labeled PGs by Sieve Chromatography on Sephadex G-25*

A 200-μl aliquot of each sample in 4 M guanidine HCl was subjected to sieve chromatography on Sephadex G-25 (prepacked in PD-10 columns) equilibrated and eluted with 4 M guanidine HCl, 0.05 M sodium acetate, 0.1 M sodium sulfate, 0.5% Triton X-100, pH 7.5. Each fraction (0.5 ml each) then was mixed with ethanol (0.5 ml) and Hydrofluor scintillation fluid (2.5 ml). Radioactivity in the first peak, corresponding to the void volume of the column, was taken as a measure of $^{35}$S-PG present, as previously described (20).

### *Preparation and Storage of Alcian Blue Dye Solution*

Alcian blue was prepared fresh every 2 weeks as a 0.2% (w/v) solution in 0.05 M sodium acetate, pH 5.8, containing 0.085 M $MgCl_2$. Unless indicated, the alcian blue preparation from Bio-Rad was used in all experiments. The solution was filtered at room temperature through Whatman Paper No. 1. The solution was stored at room temperature; the dye remained soluble and showed no loss of ability to bind to PGs over a period of 20 days.

### *Standard Protocol for the Rapid Filtration of Alcian Blue–PG Complexes in Multiwells*

For each sample to be analyzed, 75 μl of the dilution buffer (0.05 M sodium acetate, pH 5.8, containing 0.5% Triton X-100) was pipetted into a well of the 96-well MultiScreen filtration plate assembly. Twenty-five microliters of the sample to be analyzed and 150 μl of the alcian blue dye solution then were sequentially added into the well. The plate was gently agitated for 1 h at

room temperature. The liquid then was filtered through the Millipore Durapore membrane (0.45 μm pore size, unless indicated otherwise) using a gentle vacuum source attached to the vacuum manifold of the plate assembly. Unincorporated [$^{35}$S]sulfate was removed by washing each well three times with 200 μl of buffer (0.05 M sodium acetate, pH 5.8, containing 0.1 M sodium sulfate, 0.05 M $MgCl_2$) followed by vacuum filtration through the membrane. The bottom of the underdrain then was blotted using absorbent paper. After removal of the underdrain, the bottom of the plate was blotted again. Although the manufacturer recommends complete drying of the test material, this was not done in our assay in order to facilitate dissociation of the $^{35}$S-labeled PGs from the dye and their solubilization. The membrane disc in each well then was punched out manually into a 4-ml scintillation vial, using the disposable punch tip assembly. (The multiple-punch assembly provided by the manufacturer could not be used as it requires the membrane to be fully dried.) Five hundred microliters of dissolving buffer (4 M guanidine HCl, 33% isopropanol) then was added to each scintillation vial followed by gentle shaking for 1 h to dissociate and solubilize the $^{35}$S-labeled macromolecules bound to alcian blue. Finally, 2.5 ml of Hydrofluor scintillation fluid was added to each vial followed by counting of radioactivity in a scintillation counter. All samples were analyzed in duplicate.

### *Statistical Analyses*

Regression analysis was used to evaluate the relationship between counts per minute values obtained by the filtration assay and sieve chromatography on Sephadex G-25. Regression analysis also was used to evaluate the CPM values obtained for different dilutions of the same sample.

## RESULTS

### *Selection of the Membrane*

One of the initial studies was performed to compare three types of membranes differing in porosity (0.22, 0.45, and 0.65 μm) but with the same low direct protein-binding capacity (19). This initial study, performed on $^{35}$S-labeled PGs in labeling medium from explant cultures, identified the 0.45-μm membrane as the membrane of choice. Once the optimum conditions for the quantification of $^{35}$S-labeled PGs were established for the other parameters in the assay, the three membranes once again were compared to one another. Labeling medium containing $^{35}$S-labeled PGs and a large excess of unincorporated [$^{35}$S]sulfate was diluted with cold medium to yield solutions containing 20–80% of the molecules present in the original solution. A 25-μl aliquot from undiluted labeling medium and each dilution was then analyzed using the standard protocol described under Materials and Methods. The 0.45- and 0.22-μm membranes yielded superposable curves: the CPM values for individual samples varied by 5%. The relationship between amount of labeling medium used and radioactivity measured was linear throughout the range of dilutions (0.45 μm, $r > 0.99$; 0.22 μm, $r > 0.99$). Importantly, the relationship was also linear when the labeling medium was diluted with the dilution buffer rather than with medium (0.45 μm, $r > 0.99$; 0.22 μm, $r > 0.99$). The 0.65-μm membrane consistently yielded lower values for $^{35}$S-labeled PGs, irrespective of the dilution used. The decision to select the 0.45-μm membrane as the membrane of choice was made for two reasons. First, it was considerably easier to punch out by hand to yield circular discs for scintillation counting. Second, the 0.22-μm membrane showed a tendency to clog, although this did not occur consistently in all experiments.

### *Development of Optimum Conditions for the Quantification of $^{35}$S-labeled PGs*

Several experiments were performed to optimize the separation of $^{35}$S-labeled PGs from unincorporated [$^{35}$S]sulfate and maximize the recovery of $^{35}$S-labeled PGs present. In all of the optimization experiments described below, the conditions described in the standard protocol were followed rigorously with the exception of the parameter under study. The volume of sample (25 μl) subjected to analysis in each well was kept small to minimize the possibility of clogging of the membrane by proteins and other molecules and because our preliminary findings had indicated that recovery of the alcian blue/aggrecan complex on the membrane remained high (>90%) even when the total amount of aggrecan present was as low as 50 ng, i.e., 2 μg/ml (see below). A larger volume of sample can however be used, provided the final concentrations of buffer, dye, and Triton X-100 are kept at the same level within the well. Whiteman (7) showed that the initial rate of complex formation between alcian blue and PGs is extremely rapid in the presence of $MgCl_2$ and reaches a maximum after 40–60 min. Our own findings showed that a 60-min period of incubation of labeling medium with the alcian blue dye was sufficient to consistently give maximum recovery of the $^{35}$S-labeled PGs precipitated on the Durapore membrane. Triton X-100, a detergent sometimes used in the extraction of intracellular and membrane-bound PGs, recently was shown to be essential for the quantification of unlabeled PGs by precipitation with alcian blue (13). In that study, this detergent was particularly effective in minimizing interactions between the dye and proteins. Measurement of $^{35}$S-labeled PGs in labeling medium by the rapid filtration assay showed that this detergent improves recovery. Thus, Triton

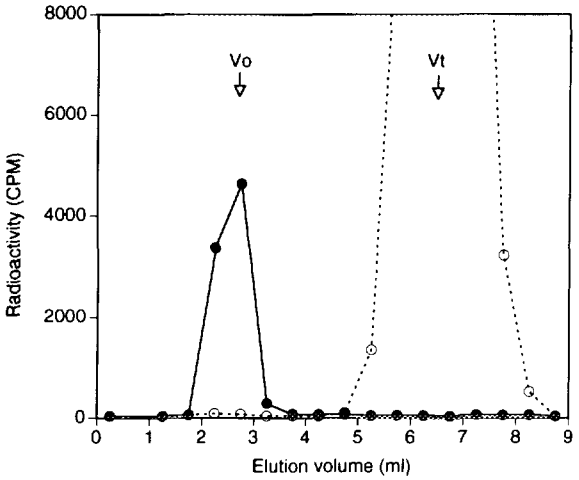


**FIG. 1.** Sephadex G-25 chromatography of $^{35}$S-labeled molecules retained on the membrane and present in the filtrate. After analysis of a sample of labeling medium, the $^{35}$S-labeled molecules present on the disc (●) were recovered by shaking in 4 M guanidine HCl and then subjected to chromatography on Sephadex G-25. The elution profile of $^{35}$S-labeled molecules present in the filtrate is shown for comparison (○). The positions of the void ($V_0$) and total column ($V_t$) volumes are shown.

X-100 at 0.375% (final concentration in the sample prior to the addition of 150 μl of the alcian blue solution) improved recovery of $^{35}$S-labeled PGs in labeling medium by 7%. Higher concentrations of Triton X-100 were not more effective (no Triton X-100, 8883 CPM; 0.375%, 9488 CPM; 0.75%, 9450 CPM). Triton X-100 thus was incorporated routinely by adding 75 μl of dilution buffer containing Triton X-100 at 0.5% to the 25-μl sample to make the concentration of Triton X-100 = 0.375% prior to the addition of the alcian blue solution and =0.15% in the sample–alcian blue mixture. At this concentration, Triton X-100 particularly was useful in preventing clogging of the membrane, which can cause a marked decrease in the rate of filtration in assays of undiluted labeling medium samples rich in protein (data not shown).

$^{35}$S-labeled molecules present on the membrane and in the filtrate after analysis of a labeling medium sample by the standard protocol were examined to determine how effective the method is in separating the macromolecular $^{35}$S-labeled PGs from the unincorporated [$^{35}$S]-sulfate. After the washing steps, the $^{35}$S-labeled molecules on the disc were immediately solubilized by incubation at 25°C for 1 h with shaking in 4 M guanidine HCl while the filtrate was mixed with an equal volume of 8 M guanidine HCl. The results of chromatography of these samples on Sephadex G-25 showed that 99% of the $^{35}$S-labeled molecules on the membrane were of high molecular size, eluting in or near the void volume of the column in fractions normally selected for quantification of $^{35}$S-labeled PGs or $^{35}$S-labeled GAGs by this method (Fig. 1). In contrast, less than 1% of the radioactivity in the filtrate was detected in these fractions: all of the radiolabel eluted as a single peak in the total volume of the column. Separate experiments showed that the filtrates from the three washing steps contained 0.11, 0.03, and <0.01% of the unincorporated [$^{35}$S]sulfate. Additional rinsing steps were not found to be needed to remove unincorporated [$^{35}$S]sulfate, even when using labeling medium which contained unincorporated [$^{35}$S]sulfate/$^{35}$S-labeled PGs in a 1000/1 ratio. These additional rinsing steps did not cause a detectable loss in the $^{35}$S-labeled PGs present on the membrane. Importantly, radiolabeled PGs are not retained on the membrane when alcian blue is omitted from the assay.

Experiments also were devised to select the optimal concentration of alcian blue for effective precipitation and recovery of the $^{35}$S-labeled PGs. Magnesium chloride has been shown to be very important in maximizing interaction between alcian blue and GAGs (7). It was kept constant at 50 mM while the alcian blue dye solution was varied. Recovery was optimum when the dye was present in the final PG–alcian blue mixture at a concentration of 0.12% (95.0% of $^{35}$S-labeled PGs recovered from the void volume of a PD-10 column). This concentration of alcian blue thus was selected for the assay: it is in the midrange of concentrations used by others to form precipitable alcian blue–GAG (7) or alcian blue–PG complexes (13) from solution. Recovery also was good at half this concentration of the dye (94.4%) but, as shown previously (7), there was a significant decrease in complex formation when the concentration was raised to 0.3% (91.8%) or 0.6% (83.0%). It should be noted that not all alcian blue preparations give such high recoveries in the assay. Two other batches of the "electrophoresis purity" alcian blue dye (Cat. 161-0401) which had been purchased from Bio-Rad in previous years also yielded excellent recoveries of $^{35}$S-labeled PGs on a consistent basis, i.e., >90% of $^{35}$S-labeled PGs were recovered on the membrane. On the other hand, the alcian blue 8GX preparation from Sigma Chemical Co. gave recoveries that were consistently below 50%, irrespective of the concentration of alcian blue used.

### *Effect of Varying the Concentration of PGs*

In one experiment, the standard protocol was used to determine the range of PG concentrations over which linearity, between the amounts of $^{35}$S-labeled PGs measured and actually present, is maintained. In order to obtain high concentrations of purified bovine articular $^{35}$S-labeled aggrecan, a 4 M guanidine HCl extract of explants cultured for 16 h in the presence of [$^{35}$S]sulfate was subjected to equilibrium density centrifugation. The $^{35}$S-labeled aggrecan molecules sedimenting in fractions of highest buoyant density and isolated as a

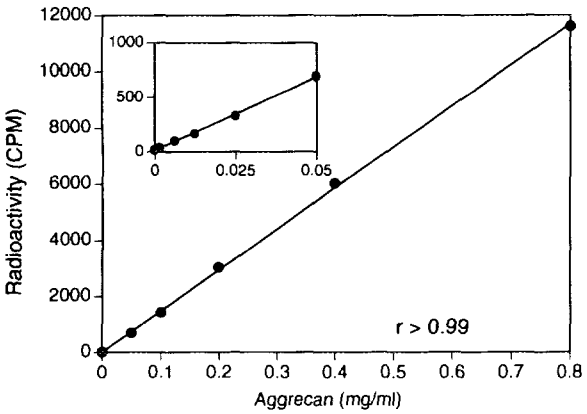


**FIG. 2.** Analysis of purified $^{35}$S-labeled aggrecan by the rapid filtration assay. After labeling of articular cartilage explants with [$^{35}$S]-sulfate, the tissue was extracted with 4 M guanidine HCl. Radiolabeled aggrecan in the extract was purified by equilibrium density gradient centrifugation and resuspended in the dilution buffer at concentrations ranging from 2–800 μg PG/ml. For each concentration of the $^{35}$S-labeled aggrecan, the results represent the mean of the analyses of two 25-μl aliquots. The inset shows the results obtained at the lowest concentrations.

lyophilized powder, as described under Materials and Methods, were dissolved in the dilution buffer and then diluted to yield a broad range of aggrecan concentrations. A 25-μl aliquot from each sample then was analyzed. The results shown in Fig. 2 demonstrate that the relationship between radioactivity and aggrecan concentration was linear in the range 2–800 μg aggrecan/ml. In a separate experiment, purified unlabeled bovine nasal aggrecan at different concentrations was mixed with an equal volume of labeling medium. A 25-μl aliquot from each mixture was analyzed in duplicate using the standard protocol. The results showed that the presence of unlabeled bovine nasal aggrecan at concentrations up to 1 mg/ml does not affect to any significant extent the precipitation and detection of $^{35}$S-labeled PGs present in labeling medium (no aggrecan added = 8846 ± 213 CPM; 1 mg aggrecan/ml = 8823 ± 111 CPM).

*Effects of DNA and Protein on Quantification*

As DNA (18) and proteins (14) have been shown to interfere with the quantification of the GAG moieties of PGs in dye-binding assays, experiments were performed to determine if modifications to the standard protocol were required when samples contained relatively large amounts of these molecules. The results showed that the addition of DNA (up to 20 μg/ml labeling medium) does not significantly affect recovery (no DNA added, 9563 ± 175 CPM; 20 μg DNA/ml, 9822 ± 171 CPM). Likewise, BSA at up to 4 mg/ml in labeling medium has little effect on the quantification of $^{35}$S-labeled PGs (no BSA added, 2290 ± 20 CPM; 4 mg BSA/ml, 2192 ± 9 CPM).

*Effects of Guanidine HCl on Quantification*

Previous studies have shown that guanidine HCl can, when used at high concentrations, inhibit the binding of alcian blue to sulfated GAGs. As 4 M guanidine HCl commonly is used as a dissociative solvent to extract PGs from cartilage and other connective tissues, experiments were performed to determine what effect this denaturing agent has upon recovery. Analysis of samples of labeling medium made 2–8 M with guanidine HCl revealed that guanidine HCl causes a minor concentration-dependent decrease in the precipitation and retention of $^{35}$S-labeled PGs on the membrane (Fig. 3). A decrease of 4–6% was observed consistently when the concentration of guanidine HCl was raised to 4 M, the molarity used to dissociate and extract cartilage PGs (1,2). Investigators using the rapid filtration assay to quantify $^{35}$S-labeled PGs in samples containing different concentrations of guanidine HCl thus may wish to bring the concentration of guanidine HCl in all samples to the same level prior to the assay proper. In separate experiments, the presence of guanidine HCl at 4 M in samples containing $^{35}$S-labeled GAGs solubilized by papain (Fig. 3) or 1 N NaOH treatment of chondrocyte monolayers was found to cause a similar minor decrease in the amount of $^{35}$S-labeled GAGs recovered on the disc. However, this decrease became much more significant at higher concentrations of guanidine HCl.

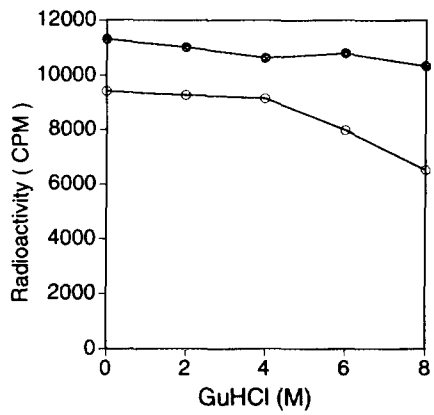


**FIG. 3.** Effects of different concentrations of guanidine HCl on the quantification of $^{35}$S-labeled PGs and $^{35}$S-labeled GAGs by the rapid filtration assay. Labeling medium (●) or a papain digest (○) of chondrocyte monolayers was first mixed with an equal volume of a solution containing guanidine HCl at different molarities. A 25-μl aliquot from each mixture then was analyzed in duplicate by the rapid filtration assay. In each case, the result represents the mean of the CPM values obtained for the two analyses. The concentrations on the *X* axis represent the molarity of guanidine HCl in the 25-μl aliquots analyzed.

*Analyses of Media and Extracts from Different Cultures*

Analyses of labeling media from the three chondrocyte culture systems (monolayers, suspension in agarose gel, and suspension in alginate beads) and the articular cartilage explant culture system showed that quantification by the standard protocol outlined under Materials and Methods can be performed on undiluted medium. The proteins present in serum do not appear to interfere to any measurable extent with quantification: the relationship between CPM measured and amount of labeling medium used remained linear throughout the whole range of dilution of the labeling medium. The correlation coefficient was greater than 0.99 in all cases. Analysis of a sample of undiluted labeled medium in 12 wells of the same plate yielded a coefficient of variation of 1.9%. Interassay variation was similarly low (2.2%).

Analyses of associative (Fig. 4A) or dissociative (Fig. 4B) extracts of alginate beads also yielded a linear relationship throughout the dilution range of each extract ($r > 0.985$ in both cases). The very negatively charged alginate present at relatively high concentration in the 4 M guanidine HCl extract of alginate beads did not in any way interfere with the quantification of the radiolabeled PGs. This is worth noting since alginate at this concentration shows a reactivity with the DMB dye in the DMB assay (data not shown). The relationship between the CPM value and the dilution factor also showed a high degree of linearity ($r > 0.98$) when 4 M guanidine HCl extracts from the other types of cultures were analyzed. Digestion of the cell-associated matrix of chondrocyte monolayers with papain degrades $^{35}$S-labeled PGs into peptidoglycans bearing one chondroitin sulfate chain or one to three keratan sulfate chains. Analysis of this papain digest showed that the enzyme and other reagents present in the digestion mixture do not interfere with quantification (Fig. 4C). Analyses of papain digests from the other culture systems showed that the relationship between amount of digest used and CPM detected also remains constant throughout the dilution range ($r > 0.985$ in all cases). A similar linear relationship also was obtained for radiolabeled molecules released by treatment of the cell-associated matrix of chondrocyte monolayers with 1 N NaOH (at 25°C for 16 h), a procedure which releases all GAGs as single chains (Fig. 4D).

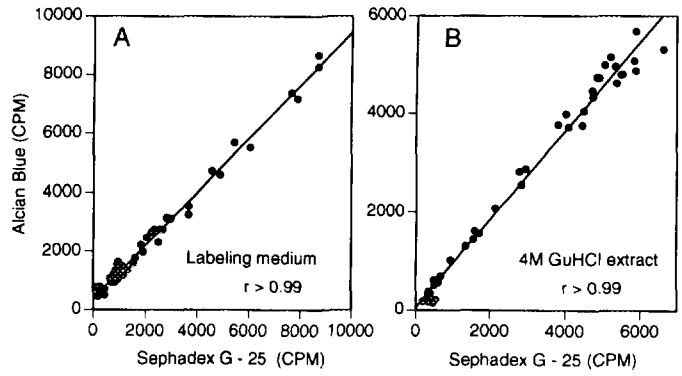


**FIG. 5.** Comparison of the rapid filtration assay with sieve chromatography on Sephadex G-25. Labeling media and 4 M guanidine HCl extracts from several different cultures of chondrocytes in alginate beads were quantified by chromatography on Sephadex G-25 ($X$ axis) and by the rapid filtration assay ($Y$ axis). Each sample containing $^{35}$S-labeled PGs was analyzed at full strength and after a 2- to 10-fold dilution. The relationship between CPM detected in the two assays was evaluated by linear regression: the correlation coefficient ($r$) is given in each case.

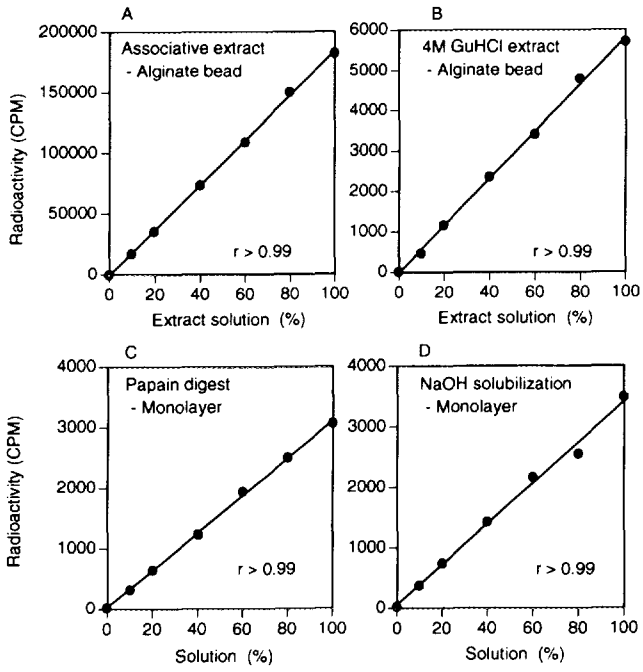


**FIG. 4.** Measurements of matrix-derived $^{35}$S-labeled macromolecules solubilized by various treatments of chondrocyte cultures and analyzed by the rapid filtration assay. With the exception of the 4 M guanidine HCl extract (B), the samples were first diluted using the dilution buffer prior to analysis by the standard protocol: % refers to the relative amount of sample analyzed with 100% representing the undiluted sample. The various dilutions of the 4 M guanidine HCl extract (B) were prepared using 4 M guanidine HCl as the diluent; these then were processed in the same manner as the other samples. The relationship between CPM measured and concentration of sample assayed was evaluated in each case by linear regression: the correlation coefficient ($r$) is shown.

*Comparison of Multiwell Rapid Filtration Assay with Sieve Chromatography on Sephadex G-25*

Radiolabeled PGs in samples of undiluted labeling medium and associative or dissociative extracts from the different cultures were quantified by the rapid filtration assay as well as by chromatography on Sephadex G-25. The rapid filtration assay yielded in all cases

mean CPM values that ranged between 90 and 101% of the mean value obtained by chromatography on Sephadex G-25 [labeling medium of monolayers, 96%; associative extract of alginate beads, 101%; 4 M guanidine HCl extracts, 90% (agarose), 94% (alginate), and 93% (cartilage explant)]. Linear regression showed in all cases a high degree of correlation ($r > 0.99$) between the values obtained for different samples by the two methods: Figure 5 shows the results of the statistical evaluation of the CPM values obtained for samples of labeling medium (Fig. 5A) and 4 M guanidine HCl extracts (Fig. 5B). Analyses of $^{35}$S-labeled GAGs generated by papain digestion or 1 N NaOH treatment of chondrocyte monolayers also showed a high degree of correlation between the two methods ($r > 0.99$ in both cases): in both cases, the mean value obtained using the rapid filtration assay was 90% of the value obtained by chromatography.

## DISCUSSION

The rapid filtration assay we have developed represents an attractive alternative to other methods of $^{35}$S-labeled PG or $^{35}$S-labeled GAG quantification. It consists of three basic steps. First, the sample containing the $^{35}$S-labeled PGs is mixed with alcian blue dye in the presence of guanidine HCl, Triton X-100, and $MgCl_2$ within a well of a commercially available 96-well plate. Second, the solution containing the alcian blue–PG complexes is filtered through a membrane disc made of polyvinylidene difluoride which covers the bottom of the same well. Finally, the preformed disc is punched out and the $^{35}$S-labeled PGs complexed to alcian blue on the disc are first solubilized and then quantified by scintillation counting. The assay was shown to yield excellent recovery of $^{35}$S-labeled PGs in very small samples (25 $\mu$l) representing media and extracts of articular cartilage explants and three different types of chondrocyte cultures. Measurement of the $^{35}$S-labeled PGs was not affected by the presence of relatively large amounts of protein (4 mg/ml) or DNA (20 $\mu$g/ml).

Although all alcian blue preparations available on the market have the same colored compound, copper phthalocyanin, they differ with respect to their cationic groups (16) and ability to precipitate PGs quantitatively (13). Our finding that alcian blue 8GX from Sigma was not as effective in precipitating the PGs on the membrane as the alcian blue preparation sold by Bio-Rad as an "electrophoresis purity" reagent is consistent with this view. It is worth noting, however, that the three different batches of Bio-Rad alcian blue dye we tested yielded excellent recoveries on a consistent basis. This strongly suggests that the effectiveness of this dye preparation in precipitating the PGs on the membrane does not reside in an unusual property that cannot be obtained reproducibly during the manufacturing process. Most importantly, the rapid filtration assay overcomes some of the limitations inherent in other methods of quantification. While methods which use sieve chromatography to eliminate the unincorporated [$^{35}$S]sulfate prior to quantification are very effective in measuring $^{35}$S-labeled PGs in samples containing these molecules at very high concentrations (>1 mg/ml), unlabeled PG must be added as a carrier when the concentration of PGs reached below 100 $\mu$g/ml. In our rapid filtration assay, recovery was not affected significantly when the PG concentration was reduced to 2 $\mu$g/ml. The very broad range of PG concentrations over which the relationship between CPM and PG concentration is maintained represents a clear advantage over most methods. For example, quantification of $^{35}$S-labeled PGs after capture on a single cationic blot is limited to the analysis of samples containing lower amounts of PGs, DNA, and other anionic molecules which can saturate the cationic binding sites. It is worth noting that, unlike this previously published solid-phase assay based upon the binding of $^{35}$S-labeled PGs to a cationic nylon blot in a 96-well plate, the discs are set individually within the membrane, thereby reducing the risk of cross-contamination.

With the exception of the associative extracts of alginate bead cultures, the amount of $^{35}$S-labeled PGs or $^{35}$S-labeled GAGs measured by the rapid filtration assay ranged consistently between 89 and 96% of the values obtained by chromatography on Sephadex G-25. This was true for all labeling media and associative or dissociative extracts as well as for samples containing $^{35}$S-labeled GAGs solubilized by papain digestion or 1 N NaOH treatment. This observation strongly suggests that the rapid filtration assay can be used to quantify most, if not all, types of $^{35}$S-labeled PGs and $^{35}$S-labeled GAGs. This is not surprising since at pH 5.8 the charged carboxyl groups interfere with binding of the dye to the sulfate groups to such an extent that the position of the sulfate groups has little effect upon the critical electrolyte concentration, which becomes similar for all GAGs. Papain makes more than 100 cleavages at distinct sites along the core protein of bovine steer articular aggrecan; this releases most chondroitin sulfate as single chains ($M_r$ = 8600 Da) and keratan sulfate as single chains ($M_r$ = 3000 Da) or clusters bearing two or three chains (23). As papain digestion did not cause any measurable loss in the amount of $^{35}$S-labeled PGs detected in the rapid filtration assay, one may conclude that sulfated GAG chains of small to moderate size ($M_r > 5000$ Da) were effectively retained on the disc after interaction with the alcian blue dye. This is consistent with the recent findings of Björnsson (13), who showed that keratan sulfate and short chondroitin sulfate chains bind effectively to the alcian blue dye in the presence of Triton X-100 and guanidine HCl at the concentrations used in our assay. There should, however, be no difficulty in modifying the assay, i.e., using a lower pH and/

or higher salt concentration, to selectively precipitate individual GAGs or PGs as has been shown by Björnsson recently (13).

The ability to quantify $^{35}$S-labeled GAGs and $^{35}$S-labeled PGs of small size is worth noting as the assay is likely to be of interest to those investigators who have found that complete separation of these molecules from unincorporated [$^{35}$S]sulfate is sometimes difficult to achieve when using the commercially available Sephadex G-25 columns. The rapid filtration assay thus should prove useful to the many investigators who are interested in quantifying $^{35}$S-labeled heparin, heparan sulfate, or dermatan sulfate PGs synthesized by a wide variety of different cell types and/or $^{35}$S-labeled GAGs derived from these. The assay can probably also be used with some minor modifications to quantify PGs or GAGs synthesized in the presence of other radiolabeled precursors, i.e., $^{3}$H- or $^{14}$C-labeled glucosamine or glucose, which are incorporated into GAG chains. Although the formation of complexes between alcian blue dye and radiolabeled glycoproteins can be a problem, this readily is circumvented by pretreating the sample with *Streptomyces* hyaluronidase and then papain prior to analysis (data not shown).

In summary, the assay that we have developed should become an attractive tool for the quantification of $^{35}$S-labeled PGs for two reasons. First, it is very rapid: it takes approximately 3 h to process 96 samples (in duplicate) for scintillation counting. The rapidity of the assay could prove most useful when dealing with the analysis of a large number of samples generated in a single experiment since this could alleviate the need to take into consideration decay of radioactivity. Second, the total costs of performing the analyses are no higher than those of other assay systems. The cost of the permanent equipment needed to perform the assay routinely is low: while a vacuum manifold must be purchased, it only additionally requires access to a vacuum line. The overall costs of performing the assays are kept low by cutting down on scintillation fluids and volumes of radioactive waste for disposal.

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