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# THE QUANTITATIVE SPECTROPHOTOMETRIC ESTIMATION OF TOTAL SULFATED GLYCOSAMINOGLYCAN LEVELS

## FORMATION OF SOLUBLE ALCIAN BLUE COMPLEXES

EDWARD W. GOLD

*Orthopedic Research Laboratory, Ohio State University, College of Medicine, Columbus OH 43229 (U.S.A.)*

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## Summary

The formation of soluble complexes between alcian blue dye and sulfated glycosaminoglycans provides the basis for the quantitative spectrophotometric estimation of the total concentration of these polysaccharides. Samples containing μg quantities of sulfated glycosaminoglycan are mixed with a stable dye solution prepared in 15% phosphoric/2% sulfuric acids and absorbance readings at 480 nm are compared to an appropriate standard curve. The method is rapid, convenient, and reproducible. Analyses are performed under conditions in which there is no interference from the non-sulfated glycosaminoglycan hyaluronic acid, or most other anionic macromolecules. In addition, estimations are not affected by small anions or individual monosaccharides. The method has been used for the determination of the purity of commercially available preparations of hyaluronic acid and for the estimation of the sulfated glycosaminoglycan content of various biological fluids including normal human urine and the synovial fluid of individuals with rheumatoid arthritis and osteoarthritis.

## Introduction

Several methods based on the formation and isolation of insoluble alcian blue dye-glycosaminoglycan complexes have been developed for the estimation of total glycosaminoglycan concentrations [1,2]. A technique for making these estimations by direct spectrophotometric measurement, following the formation of soluble alcian blue-glycosaminoglycan complexes, which have altered absorption spectra compared to that of the dye alone, has also been described

[3]. In that procedure samples are mixed with freshly prepared dye solution and absorbance readings at 480 nm are compared to an appropriate standard curve. The method possesses the distinct advantages of simplicity and rapidity, and employs equipment readily available in most laboratories.

A modification of the method is described in this report in which alcian blue complexes are formed with the various sulfated glycosaminoglycans, but not with non-sulfated glycosaminoglycans, such as hyaluronic acid. This permits the rapid and reproducible estimation of the concentration of sulfated glycosaminoglycans in a sample in the presence or absence of other polyanionic macromolecules. The modified method, like the original, has a sensitivity at the μg level and appears to be well suited for estimations of glycosaminoglycan levels in biological fluids. It has been used in the present study to estimate sulfated glycosaminoglycan levels in commercially available preparations of hyaluronic acid, and in the urine of normal individuals and the synovial fluid of individuals with rheumatoid arthritis and osteoarthritis.

## Materials and Methods

*Reagents.* Alcian blue 8GN (or 8GX) was obtained from Matheson, Coleman and Bell, hyaluronic acid grades I and III (human umbilical cord), grade IV (bovine vitreous humor), chondroitin sulfate (mixed isomers), DNA (type VII), RNA (type IV), poly(L-glutamic acid) (type IIB) and poly(galacturonic acid) (grade III) were purchased from Sigma. A purified hyaluronic acid from human umbilical cord was purchased from Miles. Samples of purified dermatan sulfate, keratan sulfate, heparin, heparan sulfate, hyaluronic acid and chondroitin 4- and 6-sulfates were obtained from M.B. Mathews and J.A. Cifonelli, University of Chicago. Concentrated phosphoric (85—87%) and sulfuric acids were reagent grade. Protein content of glycosaminoglycan preparations was determined by the method of Lowry et. al. [4] and uronic acid by the method of Bitter and Muir [5]. All glycosaminoglycan solutions were dialyzed against distilled water and then adjusted to the desired concentration.

*Preparation of dye solution.* Alcian blue dye was dissolved in a solution of 15% concentrated phosphoric acid/2% concentrated sulfuric acid to produce a final dye concentration of 1.0 mg/ml. The dye dissolves completely and should not be filtered. The dye solution, which will be referred to as the acid-dye, can be stored at room temperature. This contrasts with the limited stability of the dye solution prepared in sodium acetate which undergoes progressive aggregation of dye with a corresponding increase in background absorbance, thereby requiring fresh preparation prior to use. The dye solution in sodium acetate (pH 8.6) will be referred to as the basic dye, and is prepared prior to use by dissolving alcian blue in 0.5 M sodium acetate to produce a final dye concentration of 1.0 mg/ml. Analysis of total glycosaminoglycan in certain samples of synovial fluid required that the sodium acetate concentration in the basic dye solution be increased to 0.65 M to prevent precipitation of complex.

*Sulfated glycosaminoglycan assay.* The assay is performed in the same manner as previously described for the spectrophotometric estimation of total glycosaminoglycan [3]. Samples containing 5—75 μg sulfated glycosaminoglycan are adjusted to a volume of 0.10 ml. A standard curve is prepared at the same

time by adjusting aliquots of a sulfated glycosaminoglycan standard to 0.10 ml. Chondroitin sulfate (mixed isomers) functions as a convenient and inexpensive standard. A blank containing 0.10 ml of water or buffer is also prepared. To each tube 1.2 ml of dye solution is added and the contents are thoroughly mixed. After at least 15 min the absorbance at 480 nm of each tube is measured against the blank in cuvettes with a capacity of 2.5 ml or less. The concentration of sulfated glycosaminoglycan in samples is estimated by comparison to the standard curve. The blue color imparted to glassware by the analyses can be removed by rinsing with concentrated sulfuric acid.

*Collection of biological samples.* Random samples of urine were collected from normal individuals. A few drops of toluene were added to each specimen as a preservative. Synovial fluid was obtained from the knee joints of individuals with rheumatoid arthritis or osteoarthritis at the time of surgery for total joint replacement. Fluids were centrifuged to remove cellular and particulate matter and diluted 1:4 with distilled water before analysis. All biological samples were refrigerated if not analyzed immediately.

## Results

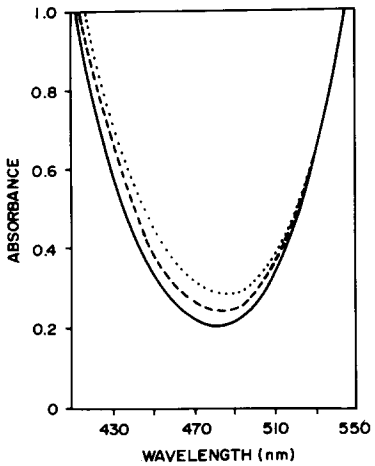
*Interaction of alcian blue dye with glycosaminoglycans*

The absorbance spectra from 410—550 nm of alcian blue dye and alcian blue-chondroitin sulfate complexes in the acid-dye solution are shown in Fig. 1. The illustrated spectra are similar to those obtained using the basic dye solution. The spectrum of dye alone exhibits an absorbance minimum at 480 nm while the spectra of the dye-chondroitin sulfate complexes are shifted so that absorbances below 530 nm are increased and minima appear just below 490 nm. Alcian blue complexes with other sulfated glycosaminoglycans exhibit similar spectra. The spectrum of a mixture of dye and hyaluronic acid however, is the same as that of dye alone, indicating the absence of complex formation.

The alcian blue dye assay of $\mu$g quantities of several purified glycosaminoglycans in the basic dye and acid dye are shown in Fig. 2A and 2B, respectively. In both assays the absorbances of the dye-glycosaminoglycan complexes increase linearly as a function of glycosaminoglycan concentration. Both assays exhibit moderate variability in the intensities of the various dye-glycosaminoglycan complexes.

The formation of the alcian blue-sulfated glycosaminoglycan complex in the acid-dye is complete within several minutes of mixing and the intensity of the increased absorbance produced by the complexes is stable for several hours. The complexes formed in the basic dye, it should be noted, had been found to undergo a slow progressive increase in absorbance attributable to aggregation of uncomplexed dye, which could be accounted for by appropriate use of the blank, but which introduced a time limitation on absorbance measurements.

The interference produced by the presence of various potential inhibitors of sulfated glycosaminoglycan estimations was examined. No interaction with the dye nor interference with the formation of dye-chondroitin sulfate complexes was found with 30 $\mu$mol of the monosaccharide sugar moieties glucose, glucosamine, glucuronic acid or *N*-acetyl glucosamine. This was true as well for albumin (300 $\mu$g), EDTA (40 $\mu$mol), acetate (50 $\mu$mol), sulfate (50 $\mu$mol), chloride



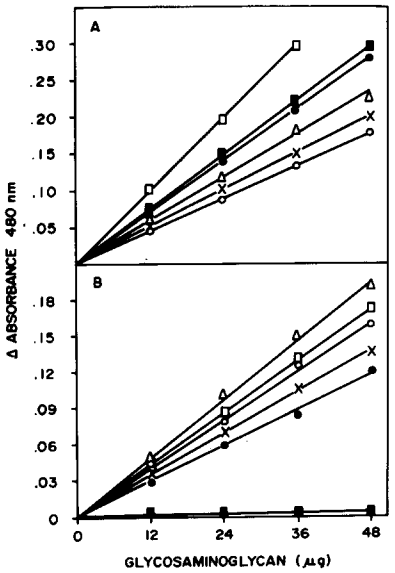




Fig. 1. Absorption spectra of alcian blue dye and dye-condroitin 4-sulfate complexes. The dye was dissolved in phosphoric/sulfuric acid at a concentration of 1.0 mg/ml and was mixed with a sample of water (———), 24 μg purified chondroitin 4-sulfate (- - -) or 48 μg purified chondroitin 4-sulfate (· · ·).

Fig. 2. Alcian blue dye complex formation with various purified glycosaminoglycans. Samples of keratan sulfate (□), heparin (△), dermatan sulfate (○), chondroitin 4- or 6-sulfates (●), hyaluronic acid (■) and heparan sulfate (X) were analyzed as described in Methods using: A, the basic dye solution; and B, the acid-dye solution. Absorbance measurements were read against a blank containing the appropriate dye solution and an aliquot of water.

(50 μmol) or NaOH (20 μmol). The estimation of total glycosaminoglycan using the basic dye solution had been found to be affected by the presence of 50 μmol of chloride or sulfate.

Hyaluronic acid does not interact with alcian blue in the acid-dye solution nor does it interfere with the formation of complexes between alcian blue and sulfated glycosaminoglycans. It was found that up to 150 μg hyaluronic acid (grade IV) had no effect on the estimation of as little as 10 μg quantities of chondroitin sulfate.

Alcian blue interacts with other anionic macromolecules in addition to glycosaminoglycans. The interactions of polyanions such as DNA, RNA, poly(L-glutamic acid) and poly(galacturonic acid) with the basic and acid alcian blue dyes are shown in Table I. The polyanionic compounds interact substantially with the basic dye, but their interaction with the more selective acid dye is very much reduced.

*Spectrophotometric determination of purity of hyaluronic acid preparations*

Sulfated glycosaminoglycans can be estimated in the presence of relatively large quantities of hyaluronic acid. The content of sulfated glycosaminoglycans existing as contaminants of preparations of hyaluronic acid can therefore be determined. The results of analysis of several commercially available preparations of hyaluronic acid are shown in Table II. Sulfated glycosaminoglycan

TABLE I

INTERACTION OF NON-GLYCOSAMINOGLYCAN MACROMOLECULES WITH ALCIAN BLUE DYE SOLUTIONS

Data represent μg (±S.E.) material appearing as glycosaminoglycan in analyses in duplicate of 50-μg quantities of the indicated macromolecule. The standard employed in both assays was chondroitin sulfate (mixed isomers).

| | Basic dye solution (μg) | Acid-dye solution (μg) |
|---|---|---|
| RNA | 21 ± 3 | 0 |
| DNA | 22 ± 3 | 4 ± 0.9 |
| Poly(galacturonic acid) | 14 ± 2 | 2 ± 0.8 |
| Poly(L-glutamic acid) | 28 ± 4 | 0 |

(percent, w/w) in grades I and III hyaluronic acid was 6.8 ± 0.6% and 29.6 ± 2.1%, respectively. Sigma grade IV and Miles hyaluronic acid were found to contain less than 2% sulfated glycosaminoglycan.

Using purified hyaluronic acid as standard, Sigma grade IV and Miles hyaluronic acid were found to consist almost entirely of glycosaminoglycan when assayed by the original spectrophotometric method. Grade I and grade III hyaluronic acid were found to contain approximately 93.5% and 84.5% glycosaminoglycan, respectively. The determination of uronic acid and protein contents in the various hyaluronic acid preparations were in agreement with the results of the glycosaminoglycan determinations. The uronic acid contents (percent, w/w) of Sigma grades I, III, and IV and Miles hyaluronic acid were 43.3 ± 1.4, 39.5 ± 1.6, 44.8 ± 1.8 and 45.5 ± 1.5, respectively. The protein contents (percent, w/w) of these same samples were 3.5 ± 0.6, 9.0 ± 1.0, less than 0.5 and less than 0.5, respectively.

*Estimation of glycosaminoglycans in biological fluids*

The suitability of the modified assay for the estimation of sulfated glycosaminoglycan levels in biological fluids was determined by examining the recoveries of 10—30-μg quantities of chondroitin sulfate (mixed isomers) added to samples of normal human urine and pathological synovial fluid. The recoveries were 90—96% and 89—110%, respectively. The recovery of grade IV hyaluronic

TABLE II

PURITY OF COMMERCIAL HYALURONIC ACID PREPARATIONS

Sulfated glycosaminoglycan levels were calculated by means of the modified spectrophotometric method using the acid dye solution with chondroitin sulfate (mixed isomers) as standard. Total glycosaminoglycan levels were calculated by means of the original spectrophotometric method using the basic dye solution with purified human umbilical cord hyaluronic acid as standard. Values are ±S.E.

| Hyaluronic acid type | Sulfated glycosaminoglycan (%, w/w) | Total glycosaminoglycan (%, w/w) | Uronic acid (%, w/w) | Protein (%) |
|---|---|---|---|---|
| Grade I | 6.8 ± 0.6 | 93.5 ± 2.5 | 43.3 ± 1.4 | 3.5 ± 0.6 |
| Grade III | 29.6 ± 2.1 | 83.5 ± 3.5 | 39.5 ± 1.6 | 9.0 ± 1.0 |
| Grade IV | 1.5 | 99.0 ± 1.7 | 44.8 ± 1.8 | <0.5 |
| Miles | 1.1 | 99.2 ± 1.3 | 45.5 ± 1.5 | <0.5 |

TABLE III

GLYCOSAMINOGLYCAN LEVELS IN HUMAN BIOLOGICAL FLUIDS

Concentrations ±S.E. of mean were estimated using grade IV hyaluronic acid and chondroitin sulfate (mixed isomers) as standards for total and sulfated glycosaminoglycan, respectively. The number of samples analyzed are in parentheses.

| Sample | Sulfated glycosaminoglycan (mg/ml) | Total glycosaminoglycan (mg/ml) |
|---|---|---|
| Normal urine (7) | 0.14 ± 0.02 | 0.13 ± 0.02 |
| Rheumatoid synovial fluid (10) | 0.25 ± 0.04 | 4.1 ± 0.3 |
| Osteoarthritic synovial fluid (10) | 0.28 ± 0.05 | 3.6 ± 0.1 |

acid added to synovial fluid in the spectrophotometric estimation of total glycosaminoglycan was 90—105%. The suitability of spectrophotometric estimation of total glycosaminoglycans in urine has previously been established [3].

The results of the spectrophotometric estimation of glycosaminoglycan concentration in urine and synovial fluid are tabulated in Table III. The average sulfated glycosaminoglycan level of random samples of normal human urine was 0.14 ± 0.02 mg/ml. The average total glycosaminoglycan content was 0.13 ± 0.02 mg/ml. In both sets of experiments the standard used was chondroitin sulfate (mixed isomers) which is believed to be the principal glycosaminoglycan excreted in normal urine [6]. Comparison of the results of the two analyses on each specimen indicated that there is little non-sulfated glycosaminoglycan excreted in normal urine which is consistent with an earlier report to that effect [7].

The sulfated glycosaminoglycan assay seems particularly well suited for analyses of samples of synovial fluid since it is not affected by the presence of relatively large amounts of hyaluronic acid. The calculated levels of sulfated glycosaminoglycan in the synovial fluid of individuals with rheumatoid arthritis was 0.25 ± 0.04 mg/ml and in osteoarthritic fluid was 0.28 ± 0.05 mg/ml. The estimated total glycosaminoglycan levels were 4.1 ± 0.3 mg/ml in rheumatoid fluid and 3.6 ± 0.1 mg/ml in osteoarthritic fluid. Sulfated glycosaminoglycan, therefore, constituted about 6.3% and 8.1% of the total glycosaminoglycan pool in rheumatoid and osteoarthritic synovial fluid, respectively.

The presence of acidic glycoproteins which could interact with alcian blue dye were considered to be a possible source of error in the estimations of sulfated glycosaminoglycan levels in samples of urine and synovial fluid. However, determinations of glycosaminoglycan content of samples of these biological fluids, before and after digestion of the samples with papain, revealed that interference by such glycoproteins was negligible.

## Discussion

The various methods relying on the formation of complexes with alcian blue dye for the estimation of glycosaminoglycan concentration [1—3] may suffer from a common drawback caused by the interaction of the dye with various non-glycosaminoglycan polyanionic macromolecules, such as nucleic acids. This

lack of specificity can seriously limit the usefulness and accuracy of such methods. Using an alcian blue dye solution prepared in phosphoric/sulfuric acids, stable, soluble complexes were quantitatively formed between the dye and μg quantities of sulfated glycosaminoglycans. Under the conditions of this analysis no complexes were formed with hyaluronic acid and the interaction of dye with non-sulfated polyanionic macromolecules was in most cases negligible. This procedure for glycosaminoglycan estimation is easier to use than the previously described spectrophotometric method because: (a) the dye solution is stable and does not require fresh preparation; (b) estimations are not affected by the presence of small anions such as chloride or sulfate; and (c) the dye-glycosaminoglycan complexes themselves are stable and therefore measurements are much less subject to limitations of time.

The interaction of alcian blue with macromolecules possessing negatively charged functional groups has been known for years [8]. In strongly acidic solutions only sulfate moieties appear to retain their negative charges and bind to the cationic alcian blue dye. Histochemical studies [9,10] have taken advantage of this behavior to selectively detect sulfated polysaccharides using alcian blue dye at pH 1 or below. In a less acidic environment weakly acidic polysaccharides are also stained. This then would appear to be the likely rationale behind the two different spectrophotometric assays. In the basic dye solution many weakly acidic groups are able to interact with alcian blue whereas in phosphoric/sulfuric acids only sulfate groups interact.

The intensities of absorbance of the dye-sulfated glycosaminoglycan complexes in acid-dye should be proportional to the content of hexose sulfate groups. Calculations of the sulfate content of the purified glycosaminoglycan samples based on their analytical and physical data, which are similar to published data [11], give the following results (percent, w/w): heparin, 34.6; dermatan sulfate, 19.1; keratan sulfate, 18.1; chondroitin 6-sulfate, 15.1; chondroitin 4-sulfate, 14.7; heparan sulfate, 14.5; hyaluronic acid, 0. These calculated sulfate levels agree well with the relative intensities of the individual dye-glycosaminoglycan complexes shown in Fig. 2B.

The absence of interference, in the estimation of sulfated glycosaminoglycan, by the presence of high concentrations of hyaluronic acid permits the relatively simple and convenient determination of the purity of hyaluronic acid specimens with respect to contaminating glycosaminoglycans (Table I). In addition, the method provides a useful technique for measuring the sulfated glycosaminoglycan content of certain biological fluids which contain very high concentrations of hyaluronic acid, such as synovial fluid and vitreous humor. Such analyses may be particularly useful in comparing normal and pathological fluids.

Although the predominant glycosaminoglycan in synovial fluid is hyaluronic acid, reports of the presenee of sulfated glycosaminoglycans have appeared [12—15]. The levels of 0.25—0.28 mg/ml found in this report represent the first quantitative estimate of the levels of total sulfated glycosaminoglycan in synovial fluid.

The spectrophotometric estimation of sulfated glycosaminoglycan is simple and reproducible. The major source of error appears to be in the choice of standard employed. If the type and purity of the standard are carefully chosen,

the simplicity and convenience of the spectrophotometric method outweigh its drawbacks.

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