The Effect of Zwitterionic Detergents on the Extraction and Functional Properties of Cartilage Proteoglycans*

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A range of structurally related zwitterionic detergents, Zwittergents 3-06, 3-08, 3-10, and 3-12, and a derivative of cholic acid (Chaps) were examined for their ability to enhance the extraction of newly synthesized, intracellular proteoglycans and for their effect on the functional properties of cartilage proteoglycan. Although none of the detergents could extract >4% of the intracellular proteoglycans when used alone, Zwittergents 3-10, 3-12, and Chaps proved equally as effective when used in combination with 4 M guanidine HCl extracting >90% of newly synthesized proteoglycans. Rate zonal centrifugation of aggregates containing either 3H-link protein or 3H-monomer, which had been incubated with 2% (w/v) detergent indicated that none of the test detergents caused a disassembly of intact aggregates. However, both Zwittergents 3-10 and 3-12 prevented the reaggregation of components dissociated with 4 M guanidine HCl. Similar to the finding with aggregate, none of the detergents caused a disassembly of monomer-link protein complexes prepared from purified 3H-link protein and proteoglycan monomer, while Zwittergents 3-10 and 3-12 prevented their assembly from free link protein and monomer. However, monomer-link protein complexes once formed were able to associate with hyaluronic acid to form link-stable ternary complexes in the presence of all detergents tested including Zwittergents 3-10 and 3-12.

The mechanical properties of articular cartilage (i.e. the transmission and distribution of tensile and compressive stresses) are largely dependent upon the organization of the macromolecular components of the extracellular matrix. In particular the presence of high concentrations of highly anionic proteoglycans trapped in a collagenous network are responsible for the resistance to compressive load observed in normal cartilage (1-4). This functional ability of proteoglycan, in turn, relies in part upon the organization of proteoglycans into aggregates consisting of numerous proteoglycan monomers bound to hyaluronic acid. The binding of monomers to hyaluronate is stabilized by a link protein, which binds to both the proteoglycan and to hyaluronic acid (5-7). Aggregation is known to occur only after all components have been secreted from the cell (8-11). Assembly of link-stabilized aggregate has been observed to occur in the nutrient medium of cultures of rat chondrosarcoma (8) as well as of normal chondrocytes (9). The assembly process can be completely blocked by the addition of hyaluronic acid oligosaccharides greater than 20 monosaccharides in length (8) and inhibited by about 50% with the addition of exogenous proteoglycan to the culture medium of chondrosarcoma chondrocytes (10). In the latter experiments (10), it was found that both link protein and proteoglycan were prevented from aggregating in a fixed proportion. The nonaggregated link was not recovered as free link protein but rather in complex with proteoglycan monomer leading to the suggestion that aggregate formation proceeds sequentially through the formation of a monomer-link protein complex (8, 11). However, only limited information is available about this process due in part to a lack of knowledge about the properties of the putative monomer-link intermediate.

Previous studies have shown that the sequential addition of the sulfobetaine detergent Zwittergent 3-12 followed by 4 M guanidine HCl to rat chondrosarcoma cultures solubilizes the majority of intracellular proteoglycans (12), while 4 M guanidine HCl alone was relatively ineffective (8, 10). However, the effect of this detergent on the binding capabilities of components of the proteoglycan aggregate was not examined. This study was designed to examine a range of related zwitterionic detergents (Zwittergents 3-12, 3-10, 3-08, and 3-06) and a zwitterionic derivative of cholic acid (Chaps)† for their ability to preserve the functional characteristics of the proteoglycan molecule and for their ability to enhance the extraction of intracellular proteoglycans. All of these detergents stabilize aggregates better than 4 M guanidine HCl alone.

The Zwittergents are a homologous series of N-sulfobetaine detergents (13) where R = CnH2n+1 and n ranges from 8 to 18. Chaps combines features of both the Zwittergents (same polar head group) and cholic acid (R) (14).

† The abbreviations used are: Chaps, 3-(3-cholamidopropyl)dimethylammonio-1-propane sulfonate; Zw, Zwittergent; Zw 3-12, N-dodecyl-N,N-dimethyl-3-ammonio-1-propanesulfonate; Zw 3-10, N-decyl-N,N-dimethyl-3-ammonio-1-propanesulfonate; Zw 3-08, N-octyl-N,N-dimethyl-3-ammonio-1-propanesulfonate; Zw 3-06, N-hexyl-N,N-dimethyl-3-ammonio-1-propanesulfonate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Tes, N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; Bes, N,N-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid; SDS, sodium dodecyl sulfate; HAA5,0, hyaluronic acid oligosaccharides between 60 and 70 monosaccharides in length. The abbreviations used to designate proteoglycan fractions (Al, AID1, etc.) follow the nomenclature of Heinegård (19).
gents exhibit an increased capacity to solubilize biologically active cellular proteins in other systems (13, 15–18) and have been chosen because of their compatibility with low temperature, guanidine HCl, CsCl, and Cs2SO4 solutions, conditions conventionally encountered in the purification of proteoglycans (12).

Of the detergents tested, Zw 3-12, Zw 3-10, and Chaps all markedly enhanced the extraction of intracellular proteoglycans above that observed in 4 M guanidine HCl alone. Chaps was the only one of these three that had no effect on the functional properties of the proteoglycan aggregate or monomer-link complex. The other two detergents, Zw 3-10 and Zw 3-12, both interfered with specific aspects of the aggregation process, seemingly exerting a greater effect on link protein to prevent it from binding to monomer or hyaluronic acid.

EXPERIMENTAL PROCEDURES

Materials

The Zwitters were purchased from Behring Diagnostics; Chaps was purchased from Sigma. Density gradient grade Cs2SO4 was purchased from Gallard-Schlesinger Chemical Manufacturing Corp. Guanidine HCl was from Research Plus Laboratories; [4,5-3H]Leu- cine (120 Ci/mmol) and [35S]sodium sulfate (1140 Ci/mmol) were purchased from Amersham Corp. All other chemicals were of reagent grade.

Methods

Extraction of Intracellular Proteoglycans Using Zwitierionic Deter- gents—Primary chondrocyte cultures (2 × 106 cells/35-mm dish) were prepared by enzymatic digestion of the Swarm rat chondrosarcoma as previously described (8). The cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 15 mM Hepes, 10 mM BES, and 10 mM Tes, pH 7.2, 100 units/ml penicillin, 50 µg/ml gentamycin sulfate, and 20% fetal calf serum, with daily medium changes.

On day 2 of tissue culture, the cells were exposed to 100 µCi/ml [35S]sulfate in 1 ml of the above medium for 5 min to label predominantly intracellular proteoglycans. Parallel cultures were exposed to 50 µCi/ml [3H]leucine in 1 ml of the above medium for 30 min at 37 °C to label newly synthesized protein. The medium was then discarded and the cell layers were extracted with 1 ml of the following detergent solutions made up in 50 mM sodium acetate, pH 5.8, containing 0.1 M 6-amino caproic acid, 10 mM Na2 EDTA, 5 mM benzamidine, 10 mM n-ethylmaleimide, and 0.5 mM phenylmethyl- sulfonil fluoride. The cell layers were initially extracted with 2% (w/v) detergent for 30 min at 4 °C. The extract was removed and stored frozen prior to analysis. Cell layers were subsequently extracted with 2% (w/v) detergent + 4 M guanidine HCl for a further 30 min at 4 °C. The cell residue was then solubilized with 1 ml of 0.5 M NaOH overnight at room temperature. The amount of 35S-radioleblamed macromolecules solubilized by each procedure was determined by frac- tionating an aliquot of each extract on a prepacked Sephadex G-25 (PD-10) column. The incorporation of [3H]leucine into protein was assayed after dialysis against 0.1 M Tris, 0.1 M sodium acetate, pH 7.2, overnight at 4 °C.

Preparation of Aggregate Containing H-Link Protein or H-Monom- omer—Rat chondrosarcoma chondrocyte cultures (20 × 106 cells/75- mm2 flask) were exposed to 50 µCi/ml [3H]leucine in low leucine Dulbecco’s modified Eagle’s medium (containing 10% of the normal leucine concentration) plus 20% fetal calf serum for 24 h to label both link protein and proteoglycan monomer. Carrier aAl, from the Swarm rat chondrosarcoma, was added to the culture medium to give a final concentration of 3 mg/ml. At the same time, culture medium from a similar batch of cells which had not been exposed to radiolabeled precursors was also made 3 mg/ml with carrier aAl. The labeled and unlabeled culture media were then subjected to associative CsCl density gradient centrifugation as described elsewhere (20) and the bottom one-fourth of the tube (A1 fractions) was collected. Guanidine HCl was added to the A1 fractions to a final concentration of 4 M and the samples were subjected to CsCl density gradient centrifugation under dissociative conditions. Both the labeled and unlabeled dissociative gradients were fractioned in half. The top half of the labeled gradient was combined with the bottom half of the unlabeled gradient and dialyzed against 0.1 M Tris, 0.1 M sodium acetate, pH 7.2, overnight at 4 °C to give aggregates containing [3H]leucine- labeled link protein. Likewise aggregates containing [3H]leucine- labeled monomer were prepared by combining the bottom half of the labeled dissociative gradient with the top half of the unlabeled gradient. Aggregated [3H]link and [3H]monomer were separated from unassociated molecules by rate zonal centrifugation on preformed cesium sulfate gradients as described below. Labeled aggregates were isolated by combining fractions 25–29 of the gradient (Fig. 1, shaded area) and dialyzing against 0.1 M Tris, 0.1 M sodium acetate, pH 7.2, overnight at 4 °C.

The purity of the [3H]link-labeled and [3H]monomer-labeled aggre- gate preparations was examined by chromatography on Sepharose CL-4B columns. Aliquots of labeled aggregate were incubated with 2% SDS and 5% β-mercaptoethanol for 30 min at 70 °C, applied to Sepharose CL-4B columns (0.6 × 100 cm), and eluted with 0.1% SDS in 0.1 M lithium acetate, pH 6.5. Fractions of 500-µl volume were collected and assayed for radioactivity; the profiles are shown in Fig. 2. The majority (94%) of the radioactivity associated with [3H]monomer-labeled aggregate eluted in the void volume of the column, indicating the absence of contaminating [3H]-link protein. Likewise, most of the radioactivity associated with [3H]-link-labeled aggregate (80%) was found near a Kn of 0.7 with less than 4% of radioactivity associated with monomer.

Preparation of H-Link Protein—On day 2 of tissue culture, rat chondrosarcoma chondrocytes (20 × 106 cells/75-mm2 flask) were labeled with 50 µCi/ml [3H]leucine in 20 ml of low leucine Dulbecco’s...
modified Eagle's medium plus 20% fetal calf serum overnight. Carrier aAl was added to the culture medium at a final concentration of 2 mg/ml. The culture medium was then subjected to associative CsCl density gradient centrifugation followed by dissociative centrifugation (20). The A1D5 fraction containing $^3$H-link protein was dialyzed first against 0.1 M Tris, 0.1 M sodium acetate, pH 7.2, and then exhaustively against $H_2O$ before lyophilization. It was then redisolved in 4 M guanidine HCl containing 0.1 M Tris, 0.1 M sodium acetate, pH 7.2. Link protein was applied to a 0.6 M X 100-cm Sephrose 6B column (1 mg of link protein in a total volume of 500 ml) and eluted with 4 M guanidine HCl, 0.1 M NaSO$_4$, 0.05 M Tris, 0.25% Chaps, pH 7.5. The use of Chaps was introduced to increase the recovery of link protein after it had been observed that this detergent had no effect on the reassocation of aggregates. Fractions of 500 $\mu$l were collected and assayed for radioactivity (Fig. 3a). Fractions 60–68 containing $^3$H-link protein were pooled and dialyzed against $H_2O$ overnight at 4°C, and then lyophilized and dissolved in 4 M guanidine HCl, 0.1 M Tris, 0.1 M sodium acetate, pH 7.2.

The purity of this $^3$H-link protein preparation was determined by polyacrylamide gel electrophoresis on 6.825% (w/v) acrylamide, 0.175% (w/v) bisacrylamide gel (21). Briefly, a sample of the link protein containing 30,000 cpm in 10 $\mu$l was dialyzed against $H_2O$ to remove guanidine HCl. SDS and bromphenol blue made up in 0.1 M Tris, 0.1 M sodium acetate, pH 7.2, were added to give a final concentration of 2 and 0.0025%, respectively, and the sample was reduced in 10 mM dithiothreitol by heating to 100°C for 3 min. Electrophoresis was then carried out on a 3-mm slab gel in 0.05 M Tris, 0.05 M glycine pH 8.9 buffer for 1 h at 20 mA/gel followed by 40 mA/gel for 2 h. After electrophoresis, the gel was washed twice with $H_2O$ and then soaked in 1 M sodium salicylate for 2 h. The gel was then dried and exposed to Kodak X-AR5 film at -70°C for 18 days.

The standard used was a core protein precursor-link protein preparation from the rat chondrosarcoma labeled with $^3$H]ietamine and prepared as described by Kimura et al. (22) (Fig. 3b, Lane 1). The $^3$H-link protein preparation (Fig. 3b, Lane 2) showed a major band corresponding to link protein. Exposure for different times indicated that the contaminant of lower mobility constituted less than 10% of the link band.

**Rate Zonal Centrifugation of Proteoglycan Aggregates**—Aggregated proteoglycans were separated from monomeric proteoglycans by centrifugation on preformed Cs$_2$SO$_4$ gradients essentially as described by Kimata et al. (23). Briefly, 15-ml linear gradients of cesium sulfate (0.15–0.5 M in 0.1 M Tris, 0.1 M sodium acetate, pH 7.2) were prepared on a 1.5-ml cushion of 2 M Cs$_2$SO$_4$ using a Buchler Auto Density-Flow II gradient maker. The sample (0.5 ml) was layered on top and the gradient was centrifuged in a Beckman SW-28 rotor at 27,000 rpm for 6 h at 10°C. Gradients were then fractionated, using the same gradient maker, into 500-ml fractions. Aggregated proteoglycans were recovered from the bottom of the gradients.

**Experiments involving the effect of detergent** were analyzed on gradients made up in 0.5% (w/v) of the appropriate detergent.

**Formation of Proteoglycan Aggregate in the Presence and Absence of Detergent**—$^3$H-Link-labeled aggregates and $^3$H-monomer-labeled aggregates were combined with carrier aAl (6–8 mg/ml final concentration) and dissociated by the addition of 4 M guanidine HCl in the presence of 2% (w/v) detergent. The components were then allowed to reassoclate by dialysis against 0.1 M Tris, 0.1 M sodium acetate, pH 7.2, in the presence or absence of 2% (w/v) detergent overnight at 4°C.

The percentage aggregation under these conditions was measured by rate zonal centrifugation on preformed Cs$_2$SO$_4$ gradients as previously described.

**Formation of Monomer-Link Complex**—Dissociatively extracted proteoglycan monomer (A1D1) from the rat chondrosarcoma was centrifuged on 32-m1 linear Cs$_2$SO$_4$ gradients as described above to remove any residual hyaluronic acid-monomer complexes in the preparation. The fractions containing monomer (as determined by uronic acid analysis) were combined, lyophilized, and then redisolved in 0.1 M Tris, 0.1 M sodium acetate, pH 7.2, at a concentration of 21 mg/ml.

Monomer was then combined with $^3$H-link protein in the presence of 4 M guanidine HCl. Monomer-link complexes were then formed by dialysis against 0.1 M Tris, 0.1 M sodium acetate, pH 7.2, overnight at 4°C. Monomer was present at a final concentration of 6–8 mg/ml and link protein was added to give an approximate 2:1 molar ratio of proteoglycan core protein:link protein. In those experiments which examined the effect of detergent on the formation of this complex the appropriate detergent was initially added to the monomer and link protein along with 4 M guanidine HCl at a final concentration of 2% (w/v) and was also added to the dialyzing medium at the same concentration.

**Stability of the Proteoglycan Aggregate and the Monomer-Link Complex in the Presence of Detergent**—Carrier aAl from the Swarm rat chondrosarcoma was added to the $^3$H-link protein-labeled or $^3$H-monomer-labeled aggregates prepared as described above to give a final concentration of 6–8 mg/ml. The aggregate was then combined with 2% (w/v) detergent in 0.1 M Tris, 0.1 M sodium acetate, pH 7.2 (total volume = 500 ml), and incubated for 1 h at 4°C. Similarly, preformed monomer-link complexes were also mixed with 2% (w/v) detergent in 0.1 M Tris, 0.1 M sodium acetate, pH 7.2, for 1 h at 4°C. The percentage of proteoglycans still existing in an aggregate or monomer-link complex form was then determined by rate zonal centrifugation on 16.5-m1 Cs$_2$SO$_4$ gradients containing 0.5% of the appropriate detergent. 500-$\mu$l fractions were collected and assayed for radioactivity and uronic acid.

**Formation of Aggregate from Monomer-Link Complex and Hyaluronic Acid**—Monomer-link complex was prepared as described above, hyaluronic acid was then added in the presence or absence of 2% (w/v) detergent such that the ratio of uronic acid in hyaluronic acid to uronic acid in monomer was 2% (w/v). The mixture was incubated for 3 h at 4°C to allow the components to aggregate. The percentage aggregation was then determined by rate zonal centrifugation on preformed Cs$_2$SO$_4$ gradients.

**Competition with HA$_{50,000}$—HA oligosaccharides** were prepared by testicular hyaluronidase digestion of macromolecular hyaluronate followed by P-30 column chromatography on 9.25 M pyridinium acetate, pH 6.7, as described previously (24). Aggregates were formed as above by the combination of monomer-link and hyaluronic acid in the presence or absence of detergent. The percentage of these aggregates existing in link-stabilized form was determined by the addition of HA$_{50,000}$ (1 mg = 25-fold excess (w/v)) to each sample for a further 3 h at 4°C. The sample was then applied to Cs$_2$SO$_4$ gradients and the percentage of link-stabilized aggregate was determined.

HA$_{50,000}$ (1 mg) was also added to monomer-link complex at the same time as macromolecular hyaluronic acid, in the presence and absence of detergent, to prevent aggregation from occurring. The mixtures were incubated for 3 h at 4°C before analysis of the components by rate zonal centrifugation.
RESULTS

Extraction of Intracellular Proteoglycans Using Zwitterionic Detergents—Primary cultures of rat chondrosarcoma chondrocytes were labeled on day 5 of tissue culture with $^{35}$SO$_4$ for 5 min or with $^3$H-leucine for 30 min to label predominantly intracellular proteoglycans and cell-associated proteins. Several zwitterionic, sulfobetaine detergents, Zw 3-12, Zw 3-10, Zw 3-08, and Zw 3-06, and a zwitterionic derivative of cholic acid, Chaps, were examined for their ability to enhance the extraction of these molecules above that observed with 4 M guanidine HCl.

None of the detergents tested were capable of solubilizing more than 4% of the newly synthesized, intracellular proteoglycans when used alone (Table I). In a subsequent extraction by detergent in combination with 4 M guanidine HCl, the majority (>90%) of $^{35}$S-labeled proteoglycans were solubilized in the presence of Zw 3-12, Zw 3-10, and Chaps while Zw 3-08 and Zw 3-06 still proved ineffective at enhancing the extraction of proteoglycans above the level observed in 4 M guanidine HCl alone (~6%). Likewise Zw 3-12, Zw 3-10, and Chaps in combination with 4 M guanidine HCl were more effective than the other zwittergents at extracting the newly synthesized proteins (>88% for Zw 3-12, Zw 3-10, and Chaps compared to <50% for the other Zwittergents, i.e. extract 1 + extract 2).

As the detergents alone did not solubilize a significant amount of intracellular proteoglycan, it was likely that Zw 3-12, Zw 3-10, and Chaps exerted their effect by disrupting the chondrocyte membrane, thus making the intracellular space more accessible to the guanidine HCl extraction solution. The possibility was then raised that these detergents might exert mild effects on the properties of the aggregate, and the following experiments therefore were designed to examine the functional characteristics of the various components of the proteoglycan aggregate in the presence of the test detergents.

Aggregate Stability in the Presence of 2% Detergent—Proteoglycan aggregates were formed, as described under "Experimental Procedures," containing either $^3$H-monomer or $^3$H-link protein so that the fate of both components could be followed. Each of the detergents was then examined for its effect on aggregate stability. The percentage aggregation in each case was determined by rate zonal centrifugation on preformed cesium sulfate gradients as described by Kimata et al. (23).

Under these conditions, aggregated proteoglycans will sediment to the bottom of the centrifuge tube where they are trapped in the 2 M Cs$_2$SO$_4$ cushion. Nonaggregated monomers sediment as a broad peak around fraction 10, while nonaggregated link proteins remain largely in the sample layer and are found in fractions 1-4.

As seen in the upper panel in Fig. 4, in the absence of added detergent 85% of the radioactive monomer (Fig. 4b) was found in the bottom aggregate fractions of the cesium sulfate gradient. A similar proportion of the radioactive link protein (Fig. 4a, 82%) was also associated with the aggregate fractions. The addition of 2% Zwwittergent 3-12 to aggregate preparations for 1 h prior to centrifugation (Fig. 4, lower panel) did not appear to cause any significant destabilization of the aggregate structure, with the majority of the radioactivity associated with either link protein (66%) or monomer (74%) recovered from the bottom of the density gradient. The apparent decrease in the amount of link protein in aggregate was probably due to the observed increase in recovery of link protein in the presence of this detergent (59% versus 51%) rather than a disruption of aggregate. This enhanced recovery of link protein was routinely observed in the presence of Zw 3-12, Zw 3-10, and Chaps.

Similarly, proteoglycan aggregate was stable in the presence of all other test detergents, with 74% ± 5% of the $^3$H-link protein and 75% ± 3% of $^3$H-monomer found in the aggregate fractions (mean ± S.D., where n = 4; profiles not shown).

Reaggregation in the Presence and Absence of Detergents—It was clear from the previous experiments that, once a stable ternary complex had formed, the addition of detergent did

**TABLE I**

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<th>2nd extract</th>
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<tr>
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<td>Zw 3-06</td>
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*Total proteoglycan represents the total radioactivity in macromolecules after a 5-min pulse with $^{35}$S-sulfate.

†Total protein represents the total radioactivity in nondialyzable macromolecules from cultures labeled for 30 min with $^3$H-leucine.

![Fig. 4. Stability of the proteoglycan aggregate.](image-url)
not cause its disassembly. The interference of the detergents with the reverse process, i.e. formation of aggregates, was tested by assessing the ability of disassociated components to reaggregate in the presence of 2% (w/v) detergent. Samples were dissociated with 4 M guanidine HCl and detergent added. The samples were then dialyzed to 0.4 M guanidine HCl in the presence of detergent, and the percentage capable of reaggregation was determined by CsSO₄ rate zonal centrifugation.

In the absence of detergent (Fig. 5, upper panel), 65% of the link protein and 74% of the monomer was capable of reassociating into aggregate. Zw 3-08 (Fig. 5, g and h) and Chaps (Fig. 5, i and j) appeared to have very little effect on this process with 56 and 47% of the radioactivity associated with link protein and 59 and 65% of the radioactivity associated with monomer, respectively, recovered from the gradient appearing in the bottom aggregate fractions. (Zw 3-06 gave the same result as Zw 3-08 and Chaps; data not shown.) Conversely, Zw 3-12 and Zw 3-10 interfered with the reaggregation process. In both cases monomer appeared to be partially capable of rebind to hyaluronic acid as some radioactivity associated with this molecule was recovered from the bottom of the gradient (27% in the presence of Zw 3-12 and 38% in the presence of Zw 3-10). Link protein, however, was recovered from the top of the cesium sulfate gradient (86% in the presence of Zw 3-12, 80% in the presence of Zw 3-10), indicating that it did not reassociate with monomer or hyaluronic acid under these conditions. Therefore, it appeared that Zw 3-12 and Zw 3-10 had a differential effect on the ability of proteoglycan monomer and link protein to rebind to hyaluronic acid. Monomer binding was diminished but not abolished while the binding of link protein to either hyaluronic acid or monomer appeared to be inhibited to a much greater extent.

The reversibility of the effects of Zw 3-12 and Zw 3-10 was examined by dialysis of samples containing the detergents against 0.1 M Tris, 0.1 M sodium acetate, pH 7.2, to remove both detergent and guanidine HCl simultaneously. Dialysis of the sample containing Zw 3-10 resulted in the reassociation of a greater percentage of monomer and link protein (59 and 34%, respectively) into aggregate (Fig. 5, f and e) than previously observed in the presence of detergent (Fig. 5, f and e). However, the same treatment of sample containing Zw 3-12 still did not permit the majority of monomer or any of the link protein to rebind to hyaluronic acid (Fig. 6, d and c). As the concentration of Zw 3-12 used in this experiment was well above the reported critical micelle concentration (0.12%, w/v) for this detergent, it is possible that the formation of micelles prevented the passage of Zw 3-12 through the dialysis membrane (13).

This was further examined by dialyzing samples of disassociated aggregates containing Zw 3-12 and 4 M guanidine HCl against an excess volume of 2% (w/v) Chaps in 0.1 M Tris, 0.1 M sodium acetate, pH 7.2. Chaps, as shown (Fig. 5, i and j), does not interfere with the reaggregation of either link protein or monomer with hyaluronic acid when present. When dialyzed against 2% Chaps, the majority of link protein (40%) and of monomer (53%) reassociated with hyaluronic acid and was recovered from the bottom fractions of the density gra-

![Fig. 5. Reaggregation in the presence of detergent.](image1)

![Fig. 6. Reaggregation after dialysis to remove detergent.](image2)
Detergent Effects on Proteoglycan Aggregation

The Effect of Zwitterionic Detergents on the Functional Properties of a Monomer-Link Complex—From the previous experiments it was clear that at least two of the test detergents, while not causing the disassembly of an intact aggregate, did interfere with aggregate formation from its individual components. Since it was postulated that extracellular aggregation might proceed through the formation of a monomer-link protein complex (10), this study was extended to examine detergent effects on the formation and stability of such a complex.

Initial attempts to isolate a monomer-link protein complex as a unit were based on our previous findings that a complex could be dissociated from hyaluronic acid by lowering the solvent pH below 3.6 (25). However, the resulting complex could not be adequately separated from hyaluronic acid to provide high yields of sufficient purity. Subsequently, purified 3H-link protein was prepared from cultures of rat chondrosarcoma cells labeled with [3H]leucine, as described under "Experimental Procedures," and combined with purified monomer (AID1) in the presence of 4 M guanidine HCl. The sample was dialyzed to 0.4 M guanidine HCl overnight to permit association of the monomer with link protein. The sedimentation position of proteoglycan monomer was monitored by the measurement of uronic acid while 3H radioactivity indicated the presence of link protein. After rate zonal centrifugation on preformed Cs2SO4 gradients, free link protein was observed in the top fractions 1-4 of the Cs2SO4 gradient (Fig. 8a); however, with the addition of proteoglycan monomer, the link protein sedimented at the same position as monomer (Fig. 8b). After a 3-h incubation in the presence of 2% hyaluronic acid, both uronic acid and radioactivity associated with a monomer-link complex were observed in the bottom aggregate fractions of the density gradient (Fig. 8c).

The presence of Chaps, Zw 3-10, and Zw 3-12 increased the total recovery of radioactivity (56-59% compared to 40%, Fig. 9) presumably because of their greater ability to keep link protein in solution. As expected, the presence of 2% (w/v) Chaps, 2 Zw 3-08, or Zw 3-06 did not interfere with the formation of monomer-link complexes with 57, 72, and 73% of radioactivity, respectively, associated with the complex. Conversely, Zw 3-12 and Zw 3-10 were observed to prevent the formation of this complex with 79 and 75%, respectively, of the radioactivity recovered from the gradient found in the top four fractions and therefore associated with free link protein. These results substantiate the observation that Zw 3-12 and Zw 3-10 interfered with the reformation of aggregates (Fig. 4).

These experiments clearly indicated that the initial formation of a monomer-link complex was prevented by the presence of Zw 3-12 or Zw 3-10. However, it was not clear whether or not this inhibition was due to an increase in the dissociation constant of the monomer-link complex itself or to the prevention of normal binding. This was examined by incubating preformed monomer-link complex in the presence of 2% detergent as outlined under "Experimental Procedures."

Te show decreased sedimentation and increased radioactivity when samples containing Zw 3-12 and 4 M guanidine HCl are dialyzed against 0.1 M Tris, sodium acetate, pH 7.2, alone (Fig. 7, upper panel, also Fig. 6). In the presence of Chaps, mixed micelles of the two detergents are likely to form having properties intermediate to micelles composed of either detergent alone. This would result in a reduction of the interaction between Zw 3-12 and components of the aggregate and facilitate the dialysis of Zw 3-12, leading to a reaggregation of proteoglycan components.

The increased ratio of uronic acid/link protein observed after fraction 10 (as compared to fractions 6-10) in Fig. 8 and subsequent figures is thought to result from the polydispersity of newly synthesized monomers. Fellini et al. (26) reported that this polydispersity resulted from differences in chondroitin sulfate chain length rather than core protein size. Accordingly, it is expected that larger monomers would contain more uronic acid/mol of hyaluronic acid binding region than would smaller monomers resulting in a displacement of the radioactivity representing link molecules towards smaller monomer sizes.

Chaps was observed to interfere with the uronic acid analysis resulting in a high base line. The uronic acid profiles presented in Figs. 9-13 have not been corrected for the high base line observed in the presence of this detergent.

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Detergent Effects on Proteoglycan Aggregation

Fig. 9. Formation of monomer-link protein complex. Monomer-link complexes were formed as described under "Experimental Procedures" in the presence and absence of 2% (w/v) detergent. Rate zonal centrifugation was then carried out and the radioactivity (—) and uronic acid (——) content of each fraction were determined. The percentages refer to the recovery of 3H-link protein under the experimental conditions.

Once formed, the monomer-link complex was stable in the presence of each of the test detergents (Fig. 10), indicating that destabilization of the complex had not occurred. It was noted that 20–30% of the recovered radioactivity appeared in the top four fractions of the gradient in the presence of Zw 3-10, Zw 3-12, and Chaps. This was most likely a reflection of greater recovery of link protein from the gradient routinely observed in the presence of these detergents, rather than a disassociation of the monomer-link complex, as the total counts/min associated with the complex remained constant in the presence or absence of these detergents (5678 ± 861 cpm, mean ± S.D., n = 4).

Zw 3-12 and Zw 3-10 therefore were capable of preventing both aggregate and monomer-link protein complex formation but could not destabilize either intact aggregate or preformed monomer-link complex. Preformed monomer-link complexes were then incubated with macromolecular hyaluronic acid for 3 h in the presence of 2% (w/v) Chaps, Zw 3-10, or Zw 3-12 to examine their effect on aggregate formation. As previously noted (Fig. 8c), preformed monomer-link complexes are capable of binding to hyaluronic acid to form aggregate structures. The presence of Zw 3-10, Zw 3-12, or Chaps appeared to have no effect on this process (Fig. 11). All of the radioactivity and uronic acid associated with the monomer-link complex now sedimented in the bottom aggregate fractions, although some radioactivity still appeared in the top fractions in the presence of detergent and probably represents the recovery of additional unbound link protein. The result in the presence of Chaps was as expected from previous observations (Fig. 5) in which it had no effect on the ability of individual components (link, monomer, and hyaluronic acid) to rebind into aggregate. In contrast, Zw 3-12 and Zw 3-10 prevented the individual components from aggregating just as they prevented the formation of a monomer-link complex. However, once a monomer-link complex had formed it was apparently capable of aggregating with hyaluronic acid despite the presence of Zw 3-10 or Zw 3-12.

Fig. 10. Stability of the monomer-link complex. Preformed monomer-link protein complexes were incubated in the presence and absence of 2% (w/v) detergent for 1 h prior to rate zonal centrifugation on Cs2SO4 gradients. The percentage of 3H-link protein recovered under these conditions is indicated in the figure. Both radioactivity (—) and uronic acid (——) were monitored.

Competition with HA20-50—Segments of hyaluronic acid 40-

Fig. 11. Aggregate formation was monitored by rate zonal centrifugation on Cs2SO4 gradients after the addition of 2% hyaluronic acid to preformed monomer-link protein complexes in the presence and absence of 2% (w/v) detergent. *, these percentages represent the ratio of radioactivity (X 100) associated with aggregate in the detergent-treated samples relative to that in the detergent-free sample (a). The recovery of link protein under these conditions was as follows: in the absence of added detergent, 34% (a); in the presence of Chaps, 43% (b); in the presence of Zw 3-12, 62% (c); and in the presence of Zw 3-10, 57% (d).
50 monosaccharides long have been shown to be long enough to accommodate both the hyaluronic acid binding region of a proteoglycan monomer and link protein (8). To determine whether the aggregates formed by the addition of hyaluronic acid to monomer-link complexes in the presence or absence of detergent were in a link-stabilized form, HA$_{60:70}$ oligosaccharides were added to the incubation after 3 h and allowed to compete for monomer not bound in a ternary structure with link protein.

In the absence of detergent (Fig. 12a) the addition of HA$_{60:70}$ did not appear to significantly displace link protein with the majority (81% of the counts/min reported in Fig. 11) still associated with aggregate. In contrast, the amount of monomer in aggregate decreased with only 38% of the uronic acid bound to macromolecular hyaluronate in Fig. 11 still present after the addition of oligosaccharides. As the monomer-link protein complexes were initially formed using an excess of monomer, these results were consistent with the selective displacement of the link-free monomer from aggregate by HA$_{60:70}$ A similar pattern was observed in the presence of Chaps with 89% of link protein and 47% of monomer remaining in aggregate upon the addition of oligosaccharides. The addition of HA$_{60:70}$ to samples containing Zw 3-12 or Zw 3-10 produced a very different pattern. The percentage of link protein which remained in aggregate was 66% in the presence of Zw 3-12 and 75% in the presence of Zw 3-10. A portion of the link protein therefore was displaced from the aggregate, indicating that it was not part of a ternary complex. The amount of monomer still present in aggregate after the addition of oligosaccharide was 66% in the presence of Zw 3-12 and 64% in the presence of Zw 3-10, indicating that less monomer was displaced from aggregate than in samples containing Chaps or in the absence of detergent. Both Zw 3-12 and Zw 3-10 were previously shown to interfere with the binding of monomer to hyaluronate (Fig. 5) while not affecting the binding ability of monomer-link complexes (Fig. 11). These results were therefore consistent with the majority of monomer initially binding to hyaluronic acid in a link-stabilized form.

The ability of HA$_{60:70}$ to interrupt available monomer-link protein complexes not yet bound to hyaluronate was tested by the simultaneous addition of oligosaccharide and hyaluronate to preformed complexes. After incubation for 3 h, the samples were analyzed on Cs$_2$SO$_4$ gradients. As expected, HA$_{60:70}$ completely prevented the aggregation of monomer-link with macromolecular HA both in the presence or absence of detergent (Fig. 13) with less than 5% of $^3$H-link protein observed in the aggregate fractions.

**DISCUSSION**

Detergents have been used in the extraction of intracellular and cell-associated proteoglycans from a variety of cell types although they do not appear to be necessary for all cells (12, 20, 27-30). For example, deoxycholate and Triton X-100 have been used to solubilize cell membrane-associated heparan sulfate proteoglycans (20, 28), SDS appeared to enhance the extraction of link protein from bovine nasal cartilage (29, 30), and Zw 3-12 in combination with 4 M guanidine HCl effectively extracted the majority of intracellular proteoglycans from rat chondrosarcoma chondrocytes (12). However, these detergents SDS was shown to cause denaturation of the proteoglycan aggregate (30) while deoxycholate interfered with purification procedures (28). Therefore, a range of structurally related, Zwitterionic, sulfobetaine-type detergents were examined for their ability to extract newly synthesized intracellular proteoglycans while preserving the functional characteristics of the proteoglycan molecules.

The relative extraction ability of the Zwittergents (Zw 3-12, 3-10, 3-08, and 3-06), in other systems has been shown to increase with their increasing alkyl chain length, due to a concomitant logarithmic decrease in critical micelle concentration (13). This correlated with the extraction results observed in this study. Zw 3-06 and Zw 3-08 were ineffective at enhancing the extraction of intracellular proteoglycans above that observed in 4 M guanidine HCl alone and only moderately successful at extracting cell-associated proteins. This was presumably due to their low intrinsic hydrophobicity (as compared to the other Zwittergents) which prevented complete disruption of the chondrocyte membrane. Zw 3-10 and Zw 3-12 were more effective, extracting >90% of intracellular...
proteoglycans and cell-associated proteins in combination with 4 M guanidine HCl.

The remaining test detergent Chaps, a zwitterionic derivative of cholic acid combining features of both the bile salts and the N-alkylsulfo betaines (i.e. Zwittergents), also has been shown to be extremely effective at solubilizing biological molecules in other systems (15–18). This detergent proved to be just as effective at extracting intracellular molecules as Zw 3-10 and Zw 3-12, with >90% of proteoglycans and proteins solubilized in combination with 4 M guanidine HCl.

These extraction studies on rat chondrosarcoma monolayer cultures demonstrated that three of the test detergents (Zw 3-10, Zw 3-12, and Chaps) could markedly enhance the solubilization of intracellular proteoglycans when used in conjunction with 4 M guanidine HCl. When the functional characteristics of aggregate components were subsequently examined in the presence of the test detergents, different effects were observed which could potentially prove useful in answering different questions about the aggregation process.

Of the three detergents which increased the level of extraction, the binding characteristics of the proteoglycan aggregates remain unaltered only in the presence of Chaps. Chaps had no effect on the stability of intact aggregates or on the ability of components to reform aggregates after dissociation with 4 M guanidine HCl. Likewise, Chaps had no effect on the stability or formation of a monomer-link complex or on its ability to form link-stabilized aggregates with hyaluronic acid. Binding therefore proceeded as would be expected in the absence of added detergent. These characteristics make Chaps an extremely useful detergent in studies concerned with the functional properties of extracted molecules.

The two Zwittergents which enhanced the extraction rate, Zw 3-10 and Zw 3-12, did not cause the destabilization of either intact aggregates or preformed monomer-link protein complexes. However, unlike Chaps these two detergents did prevent both aggregation and the formation of monomer-link complex from disassociated components. Zw 3-10 could be removed (at least partially) by dialysis to allow a significant proportion of monomer and link protein to reaggregate with hyaluronic acid. Zw 3-12, on the other hand had to be dialyzed against Chaps before significant reaggregation, particularly of the link protein, was observed. These results suggested that the detergents were interacting with both the hyaluronic acid binding region of the proteoglycan and, to an even greater extent, with link protein to prevent the binding of each component to hyaluronic acid and to each other. This inhibition could be nearly completely reversed, indicating that no permanent denaturation of the components had taken place. Both Zw 3-10 and Zw 3-12 could prevent the formation of a monomer-link protein complex but, once formed, they could not prevent the complex from binding to hyaluronic acid to form a stable ternary complex. This result suggested that although, as indicated in Fig. 5, d and f, Zwittergent interfered with the binding of proteoglycan to hyaluronate in the absence of link protein, the combined affinity of hyaluronate binding sites in monomer and link protein were sufficiently strong to overcome the detergent interference. These results taken together indicate that the major mechanism for the inhibition of aggregation from individual components by Zw 3-10 and Zw 3-12 was on the initial interaction of link protein and proteoglycan and only to a lesser degree on the interaction of the hyaluronic acid binding sites in the two molecules with hyaluronic acid.

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