CONNECTIVE TISSUE ACTIVATION

XXV. Regulation of Proteoglycan Synthesis in Human Synovial Cells

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In this study, virtually all sulfated glycosaminoglycan (GAG) synthesized and secreted by human synovial cells, both normal and rheumatoid, was detected in the form of proteoglycans of monomeric size. Enzyme hydrolysis studies that were performed demonstrated dermatan sulfate to be the dominant GAG in the proteoglycan, with lesser amounts of chondroitin 4/6 sulfate. Exposure to β-xyloside, used as a false "core protein," resulted in marked enhancement of GAG chain formation, suggesting that the synthesis of the sulfated carbohydrate chain itself was not rate limiting. Proteoglycan synthesis and secretion were stimulated by several types of connective tissue activating peptides (CTAP); CTAP-III stimulation of incremental core protein and glycosaminoglycan was shown to be of a similar magnitude. Since chain synthesis was not rate limiting, it is suggested that stimulated proteoglycan formation caused by the CTAP peptides may be primarily modulated through increased formation of core protein.

We have shown that human connective tissue cells may be activated by low molecular weight proteins found in leukocytes, platelets, and other nucleated cells (1–6). Defined mediators, termed connective tissue activating peptides (CTAP), are found in lymphoid tissue (CTAP-I, CTAP-I_b), tumor cells (CTAP-II), platelets (CTAP-III, CTAP-P_2), and polymorphonuclear cells (CTAP-PMN). Placing these peptides in contact with human fibroblasts results in increased glycolysis, increased formation of glycosaminoglycans (GAG), and sometimes increased DNA synthesis. Earlier studies showed that CTAP-stimulated hyaluronic acid formation in human synovial cultures resulted, in large measure, from increased hyaluronic acid synthetase activity in forming incremental hyaluronate chains (7).

Previous studies (8,9) have documented that human synovial cell cultures synthesize sulfated glycosaminoglycan (SO_4-GAG); no data have clearly defined this material as proteoglycan. This report focuses on increased formation of SO_4-GAG in activated synovial cultures, and presents evidence indicating that SO_4-GAG synthesized in vitro is a proteoglycan (PG) monomer. The increased formation of proteoglycan under circumstances of activation may be modulated by increased formation of core protein.

MATERIALS AND METHODS

Culture and isotopic methods. Human synovial fibroblasts were isolated by an explant method and subsequently subcultured in plastic or glass flasks as previously reported (10). Cultures derived in this way were propagated using medium 1066 (80%), 10% fetal calf serum, and 10% human serum. Complete medium changes were carried out 3 times a week and trypsin dispersal was performed as required for propagation or study. Freezing procedures were as previously reported (10). DNA synthesis was measured by determining the incorporation of ^{3}H-methylthymidine into cells in microtiter well cultures, using 10^4 cells per culture (3). Formation of ^{14}C-glycosaminoglycan and ^{35}S-glycosaminoglycan was measured in microtiter well cultures using 2×10^4 cells per well (11).

In these experiments, uniformly labeled ^{14}C-gluco-

samine (254 mCi/mmole) was used at a concentration of 0.5 μCi/ml, and ^{35}SO$_4$ at 50 μCi/ml. Measurement of incorporation of ^{14}C-glucosamine and ^{35}SO$_4$ into GAG was quantitated by a method reported recently (11). In brief, 100 μl of media was placed on 3 MM Whatman chromatography paper, and the GAG fixed to the paper by immersing it in a 0.1% solution of cetylpyridinium chloride (CPC). Unincorporated label was removed by sequential washes with 0.05M NaCl containing 0.1% CPC in the case of ^{14}C-labeled media, or with 0.3M NaCl for ^{35}SO$_4$ labeled media.

Analytic methods. Fractionation of labeled GAG was carried out on 2.5 × 25 cm columns packed with Sephacryl S-300, Sepharose 4B, or Sepharose 2B in 0.5M acetate buffer, pH 6.9. Columns were calibrated with high molecular weight Dextran blue or proteoglycan aggregate to define the void volume (Vo) and with chondroitin sulfate deproteinized by alkaline borohydride reduction (12) to define the elution position of "free" GAG chains. In addition, the columns were calibrated for the elution position of albumin, cytochrome C, and ^{35}SO$_4$. Radiolabeled media was lyophilized on removal from cultures, taken up in a small volume of column buffer, and dialyzed at 6°C against the same buffer before gel filtration studies.

Identification of sulfated GAG species was accomplished enzymatically using chondroitinases AC and ABC. Fifty microliters of radiolabeled GAG was incubated with 40 μl of the appropriate buffer and 20 μl containing appropriate amounts of enzyme (for 2 hours) in Costar 96-well plates. After incubation, 100 μl was removed, spotted, and subjected to the CPC precipitation and washing procedure. With this procedure, enzymatic hydrolysis leads to loss of specific GAGs, and their identify may be inferred.

Chemical methods included determination of protein by the method of Oyama and Eagle (13), and measurement of uronic acid by a modified carbazole procedure (14).

CTAP-I$_b$ was isolated from human spleen as reported earlier (2); CTAP-III and CTAP-P$_2$ were isolated from outdated human platelets as previously reported (4,5).

RESULTS

Evidence for synovial proteoglycan synthesis. Gel filtration of ^{35}SO$_4$-containing media from human synovial cultures on a Sephacryl S-300 column showed most of the ^{35}SO$_4$-GAG eluting in the Vo, suggesting a molecular weight in excess of 0.75 × 10^6 daltons (Figure 1). Duplicate cultures incubated with a β-xyloside (o-nitrophenyl-β-D-xyloside) exhibited markedly increased synthesis (>6-fold) of sulfated GAGs; these had an elution volume corresponding to that of protein-free GAG chains. Detection of most of the ^{35}SO$_4$-GAG from control cultures in the Vo indicated a molecular volume consistent with proteoglycan monomer and implied the presence of "core protein" in this material. Gel filtration of ^{35}S-PG on a Sepharose 2B column showed the labeled product to be in the retarded volume, indicating that it was proteoglycan monomer, not aggregate. The exuberant synthesis of
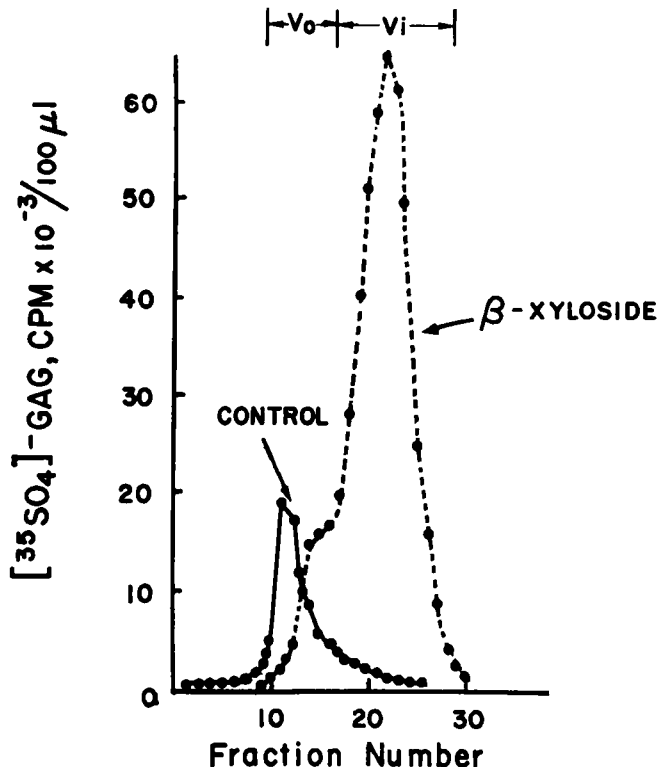

Figure 1. Cultures incubated with o-nitrophenyl-β-D-xyloside (100 μg/ml) had enhanced incorporation of ^{35}SO$_4$ into glycosaminoglycans. Gel filtration of labeled media on Sephacryl S-300 showed most counts from control cultures in the void volume, while β-xyloside treated cultures formed large amounts of "chain" size glycosaminoglycans.

chain-sized GAG when cultures were supplemented with β-xyloside as a false "core protein" (15) might suggest that factors controlling the rate of core protein formation (or linkage sites) may be more critical in limiting the overall rate of SO$_4$-GAG synthesis than are considerations restricted to carbohydrate chain synthesis alone.

Additional evidence for a protein component in the Vo fraction from the Sephacryl S-300 column was provided by studies in which alkaline borohydride hydrolysis removed protein from PG, thus reducing the molecular weight of the labeled material to that of GAG "chain." Data plotted in Figure 2 show the elution position of ^{35}SO$_4$-PG in the Vo, whereas a duplicate aliquot of labeled PG rendered protein-free by β-elimination (alkaline borohydride hydrolysis) became smaller and eluted from the GAG "chain" volume of the calibrated column.
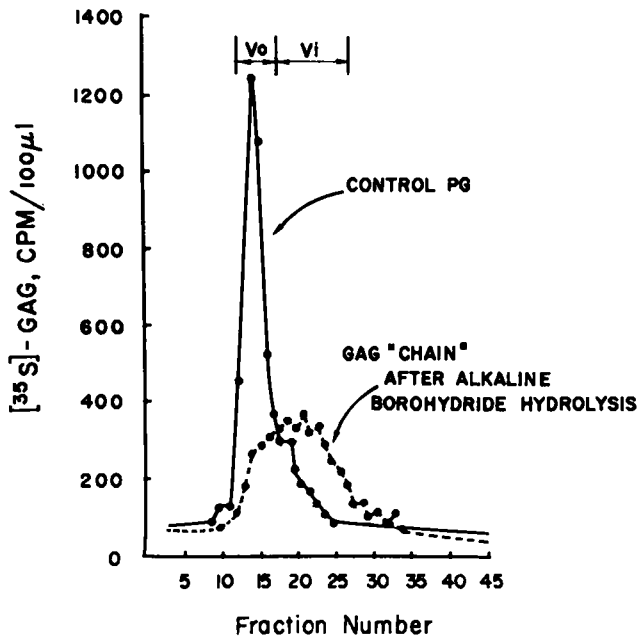
Direct evidence for newly formed protein in the

Figure 2. Gel filtration of ^{35}S-proteoglycan on Sephacryl S-300 showed that removal of protein by alkaline borohydride hydrolysis altered the presumptive molecular weight from $>0.75 \times 10^6$ daltons to approximately 25×10^3 daltons.

PG fraction came from experiments in which duplicate cultures were incubated with either ^{14}C-amino acids or ^{35}SO$_4$, and the media subjected to gel filtration on a Sephacryl S-300 column. As shown in Figure 3, both the ^{35}SO$_4$-PG and ^{14}C-macromolecular material were found in the Vo. The putative ^{14}C-PG was insoluble in 5% trichloroacetic acid (TCA). Incubation of the putative ^{14}C-PG with chondroitinase AC and ABC rendered little (0–3%) of the labeled material soluble in TCA, whereas the proteolytic enzyme pronase rendered most (77–86%) of the ^{14}C-PG soluble in TCA. In addition, inclusion of 10 μg/ml cycloheximide in synovial cultures markedly (by 90%) reduced ^{14}C-amino acid incorporation into TCA insoluble protein. We therefore concluded that the ^{14}C-amino acids were primarily incorporated into protein, with virtually none detected in the GAG side chains of the proteoglycan monomer.

The GAG chain composition of synovial PG from 2 normal and 2 rheumatoid cell strains is shown in Table 1. Cells cultivated under basal conditions (no additives) synthesized ^{35}SO$_4$-GAG chains which were predominantly (48–64%) dermatan sulfate associated with a lesser amount (14–26%) of chondroitin 4/6 sulfate. The enhanced formation of ^{35}SO$_4$-GAG induced by CTAP-I, CTAP-III, and CTAP-P$_2$ included

both dermatan sulfate (34–53%) and chondroitin 4/6 sulfate (17–34%). Data from the CTAP stimulated cultures suggested that synthesis of chondroitin 4/6 sulfate might be disproportionately stimulated; this trend was even more strongly suggested in cultures in which SO$_4$-GAG synthesis was enhanced by including β-xylosides in the media.

Another way to demonstrate that a portion of the ^{14}C-macromolecular Vo material was actually ^{14}C "core protein" would be to digest such material with chondroitinase ABC and show that removal of unlabeled GAG chains from the monomer allowed the ^{14}C core protein to elute from a calibrated gel filtration column in the 200–250,000 dalton zone. To this end, cultures were incubated with either ^{35}SO$_4$ (to label GAG chains) or ^{14}C-amino acids (to label "core protein"), and media was subjected to gel filtration on Sephacryl S-300. Material eluting in the Vo (^{35}S-PG and ^{14}C-PG) was incubated with either buffer or chondroitinase ABC (2.5 units/ml) for 18 hours at 37°C. As shown in Figure 4, when the incubated materials were subjected to repeat gel filtration, ^{35}SO$_4$-PG incubated

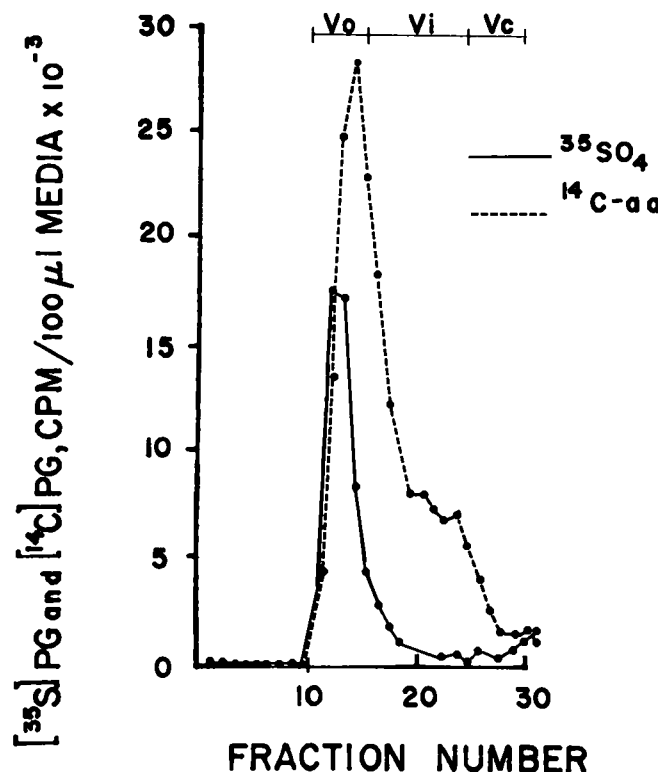Figure 3. Gel filtration on a Sephacryl S-300 column of ^{14}C-amino acid and ^{35}SO$_4$ labeled macromolecules from synovial culture media showed that most incorporated isotope was in the void volume.

Table 1. Carbohydrate composition of synovial sulfated GAG and proteoglycans*

Synovial cell strain	Additive	% digested by:	
		Chondroit-inase AC	Chondroit-inase ABC
Normal 1	0	26	90
	β-xyloside	38	93
RA 1	0	23	83
	β-xyloside	45	96
Normal 2	0	14	62
	CTAP-III	34	68
	CTAP-P₂	27	70
	CTAP-I	26	63
	Cortisol	11	73
RA 2	0	19	76
	CTAP-III	20	73
	CTAP-P₂	17	77
	CTAP-I	33	78
	Cortisol	11	76

* GAG = glycosaminoglycan; RA = rheumatoid arthritis; CTAP = connective tissue activating peptides.

with buffer still eluted in the Vo of a Sephacryl S-300 column, while chondroitinase ABC treated material was markedly reduced in molecular weight and eluted near the end of the column volume. This clearly demonstrated that the enzymatic digestion condition

FRACTION NUMBER

Figure 4. Gel filtration on a Sephacryl S-300 column showed that ³⁵S-proteoglycan digested with chondroitinase ABC was rendered much smaller, eluting near the end of the column volume.

had facilitated removal of the bulk of the GAG chain material from putative "core protein."

Data from the parallel experiment in which ^{14}C-PG was subjected to digestion with chondroitinase ABC are shown in Figure 5. In this experiment, the buffer treated ^{14}C-PG was gel filtered on Sepharose 4B, and a major radioactive peak was found in the Vo. Digestion with chondroitinase ABC markedly reduced the Vo peak, leading to a substantial increase in ^{14}C protein in an internal volume peak with an elution position which would include core protein (16,17). The polydispersity of the ^{14}C-proteins retarded in the Sepharose 4B column may relate to incomplete removal of GAG chains from the protein core as well as the presence of undefined molecular species; the lesser
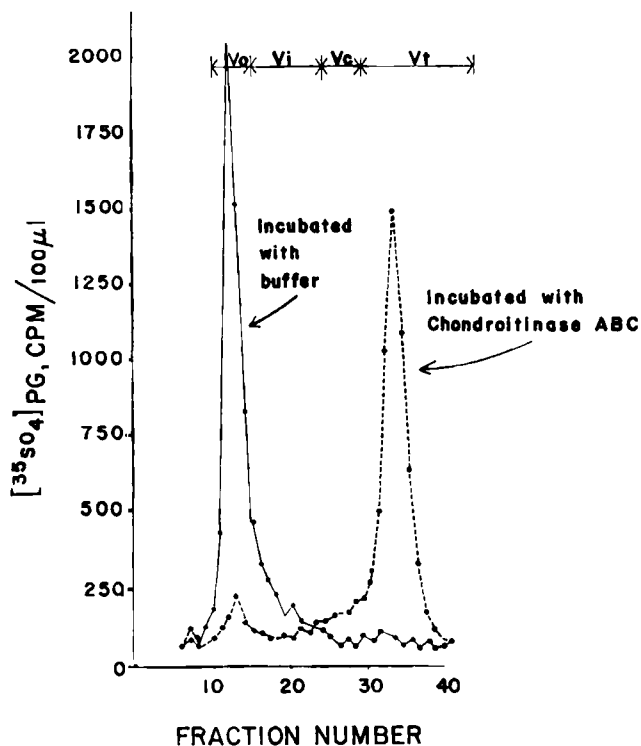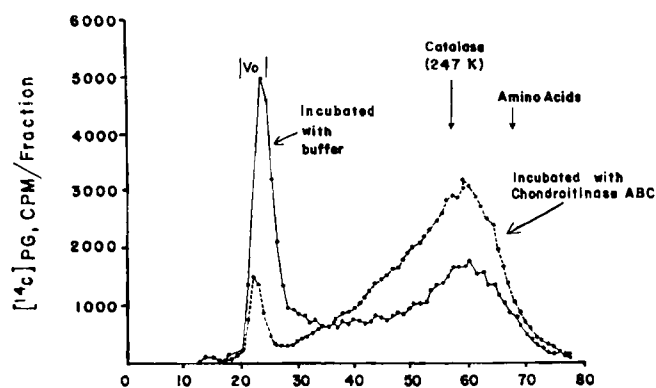

Figure 5. Gel filtration on a Sepharose 4B column showed that digestion of ^{14}C-proteoglycan with chondroitinase ABC to remove unlabeled glycosaminoglycan chains reduced the size of the labeled component so that it eluted near the elution volume of bovine catalase.

peak in the 200,000 dalton region following gel filtration of control ^{14}C-PG may reflect the inclusion of lower molecular weight species from the original Sephacryl S-300 Vo fraction used as starting material.

Stimulation of synovial PG synthesis by autacoid mediators. The stimulatory effect of CTAP-I, CTAP-III, and CTAP-P$_2$ on ^{35}SO$_4$-PG formation is illustrated in Figure 6, which shows that all 3 mediators caused increased synovial cell synthesis of PG eluting in the Vo of a Sephacryl of S-300 column. In this experiment, cortisol modestly depressed PG formation. In a parallel experiment, replicate synovial cultures were incubated with ^{14}C-amino acids in the presence of CTAP-III, buffer vehicle, or cycloheximide. As shown in Figure 7, CTAP-III markedly increased ^{14}C-protein formation which eluted in the Vo of a Sephacryl S-300 column, while cycloheximide markedly depressed protein synthesis in all size categories. A comparison of the effect of CTAP-III labeling of synovial PG by ^{35}SO$_4$ or ^{14}C-amino acids is shown in Table 2. As shown by the similarity in experimental/control ratios, the magnitude of CTAP-III induced increment in ^{35}SO$_4$-GAG side chain was comparable with the increment in ^{14}C-core protein.

DISCUSSION

This report provides evidence that synovial lining cells form one or more sulfated proteoglycan monomers containing chondroitin 4/6 sulfate as the minor component and dermatan sulfate as the major

Figure 7. Gel filtration on a Sephacryl S-300 column showed that connective tissue activating peptide III stimulated incorporation of ^{14}C-amino acids into protein, particularly those eluting in the void volume.
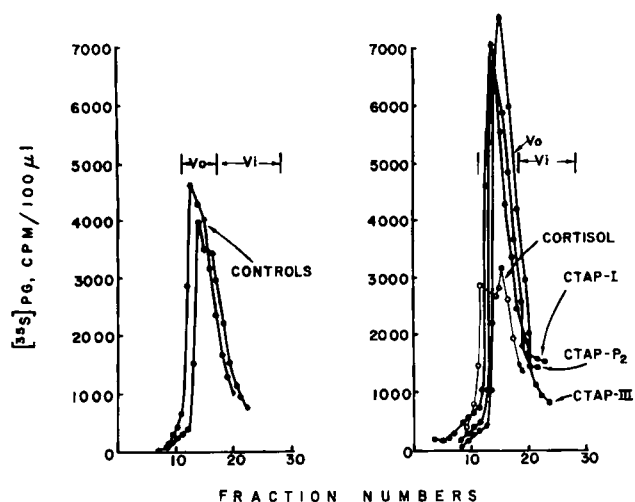

Figure 6. Gel filtration on a Sephacryl S-300 column showed that connective tissue activating peptide mediators stimulated formation of proteoglycan rather than low molecular weight glycosaminoglycan.
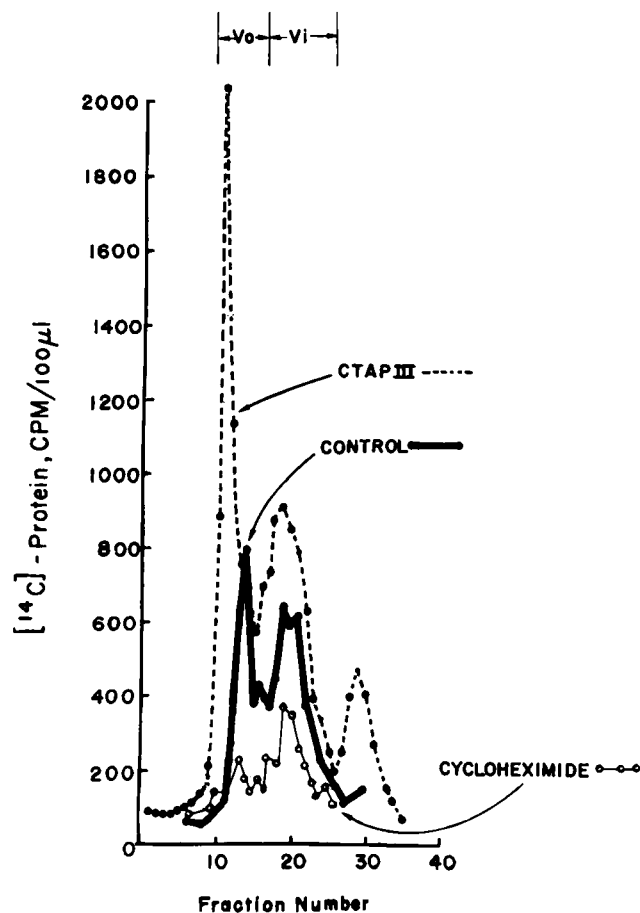
component. This contrasts with recent studies using skin fibroblasts, in which chondroitin 4/6 sulfate was the major component (18). Data supporting formation of PG include gel filtration data showing the ^{35}S-PG is large (>0.75 × 10^6 daltons), and data indicating the presence of "core protein." The presence of core protein was supported by: 1) alkaline borohydride hydrolysis studies showing that removal of protein by β-elimination reduced large PG molecules to chain-sized GAG, 2) ^{14}C-amino acid labeling of protein which elutes in the Vo of Sephacryl S-300 and Sepharose 4B columns, and 3) studies in which chondroitinase ABC digestion of PG labeled in the protein portion lead to gel filtration evidence that ^{14}C-protein denuded of GAG eluted at an elution position compatible with the molecular weight of core protein.

All 3 CTAPs tested stimulated formation of ^{35}S-

Table 2. Comparison of ^{14}C-amino acid and ^{35}SO$_4$ incorporation into synovial proteoglycan*

| | Total radioactivity in Sephacryl S-300 Vo (cpm) | | |
Isotope	Control cultures	CTAP-III treated cultures	Experimental/ control
^{14}C-amino acid	99,180	187,500	1.89
^{35}SO$_4$	486,840	806,820	1.66

* cpm = counts per minute; CTAP = connective tissue activating peptides.

PG; CTAP-III also stimulated formation of high molecular weight ^{14}C-protein (probably partly core protein) to the same extent as it did ^{35}S-PG. Since adding β-xyloside as a "false core protein" led to marked enhancement of GAG chain formation, synthesis of the latter does not appear to be rate limiting. The data, in summary, suggest that autacoid mediator stimulation of PG formation may be primarily dependent on the accelerated synthesis of PG core protein.

These studies, in agreement with others, show no difference between normal and rheumatoid cells with respect to sulfated GAG metabolism. The physiologic significance of synovial cell synthesis of sulfated PG remains an enigma.

ACKNOWLEDGMENTS

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