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EFFECTS OF ANTI-INFLAMMATORY DRUGS ON PROTEOGLYCAN DEGRADATION AS STUDIED IN RABBIT ARTICULAR CARTILAGE IN ORGAN CULTURE

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Abstract—The effects of anti-inflammatory drugs and other agents on *in vitro* degradation of cartilage proteoglycan of rabbit articular cartilage in organ culture were measured by three techniques: (1) the release of ^{35}S-labeled proteoglycan from pre-labeled rabbit articular cartilage into organ culture media, (2) the distribution of radioactivity of such released material on Sepharose 2BCL columns under associative conditions, and (3) the elution profiles of the associated and dissociated forms of ^{3}H-labeled proteoglycans dissociatively extracted from tissue after various times of incubation with ^{3}H-acetate. The anti-inflammatory drugs studied were salicylate (0.5 and 3 mM), hydrocortisone (0.2 mM), indomethacin (0.2 and 1.0 mM), phenylbutazone (0.2 and 1.0 mM), D-penicillamine (0.2 and 1.0 mM), and colchicine (1 mM). These drugs, with the exception of D-penicillamine, inhibited proteoglycan degradation to varying degrees, depending on drug concentration. Inhibition of proteoglycan degradation, as measured by the quantity of released material from pre-labeled tissue, was paralleled by the inhibition of the breakdown of aggregates to smaller units that were included on Sepharose 2BCL. The autocatalytic degradation of proteoglycans in tissue culture and its inhibition by salicylate (3 mM) were also demonstrated in analysis of [^{3}H]proteoglycans synthesized *in vitro*. These studies did not distinguish between inhibitory activities of the anti-inflammatory drugs at the levels of enzyme production and enzyme activity. Significant inhibitory activity, however, was recorded for cycloheximide (0.35 mM), puromycin (0.1 mM), and the antimicrotubular agent colchicine (1.0 mM), which suggests that activation of proteoglycan degradative processes in organ culture requires *de novo* synthesis of protein and associated transport mechanics.

It is clear that normal functioning of articular cartilage requires homeostatic maintenance of its structural integrity and balance of the macromolecular components that comprise it, namely proteoglycan, collagen, and glycoproteins. Our main concern in this study has been the status of the proteoglycan constituent in normal and pathological states of the tissues. Under normal conditions, proteoglycans exist *in vivo* as macromolecular aggregates, in association with hyaluronic acid [1, 2]. These macromolecular aggregates, immobilized in the extracellular matrix, represent hydration determinants for the tissue and therefore control, in part, the influence of mechanical forces on the tissue. Under pathological conditions, such as those in rheumatoid arthritis, the proper mechanical functioning of the joint is impeded, accompanied by degradation of the tissue. It is anticipated from earlier studies that one of the earliest phases of degradation is concerned with the degradation of the proteoglycan component of the cartilage matrix.

Although the actual mechanisms of degradation are not known, recent findings on the normal pathway of proteoglycan degradation in cartilage are of importance. Essentially, two approaches have been employed to study this problem. First, a number of investigators have isolated both acid tissue proteinases from various tissue sources [3, 4] including human articular cartilage [5, 6] and neutral tissue proteinases from human articular cartilage [5] which have been shown to cleave the proteoglycan sub-unit core-protein. The second approach has been directed to the establishment of proteoglycanase activity *in situ* in either cell culture or organ culture. Organ culture of rat costal cartilage at physiological pH [7] demonstrated that degradation is initiated by a limited proteolysis of sub-unit core-protein; it was suggested that the readily diffusible products may be directly eliminated from the tissue or may undergo endocytosis by chondrocytes for more extensive degradation. The mechanism of degradation has been studied more recently in terms of pinocytosis and degradation of labeled proteoglycans and glycosaminoglycans in cultured skin fibroblasts [8].

More recently, Sandy *et al.* [9] have shown that proteoglycan degradation in rabbit articular cartilage is activated by organ culture conditions in which initial proteolysis at neutral pH is directed at or near the hyaluronate binding region of the core-protein. These conclusions were based upon the observation that the product was similar in size to the native sub-unit but was unable to form aggregate with hyaluronate and therefore was rapidly leached from the tissue with organ culture media. Similar non-aggregating material has been identified in the extracellular matrix of embryonic chick chondrocyte cultures together with normal aggregate and a fraction of small molecular weight [10].

There is a paucity of information concerning the

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effects of anti-inflammatory drugs on the degradation pathways of tissue constituents, particularly proteoglycans. Lysosomal hyaluronidases are inhibited by various anti-inflammatory agents [11, 12]. There has been circumstantial evidence for the inhibition of "proteoglycanases" from cartilage by chloroquine [13] and salicylate [14]. There is also some evidence to suggest that damage to articular cartilage by either carrageenin treatment [15] or scarification [16] can be prevented by administration of salicylate, whereas prostaglandin-induced degeneration can not be prevented [17].

The province of this study has been the measurement of the effects of anti-inflammatory drugs on the previously established [9] breakdown of proteoglycan that occurs in rabbit articular cartilage slices that have been maintained in organ culture. Under normal conditions for pre-labeled ^{35}S-whole, cultural tissue slices, the occurrence of proteolytic activity directed at the hyaluronate acid binding site has been postulated on the basis of the time-dependent appearance of non-aggregatable proteoglycan subunit-like material within the culture medium. In contrast, only small quantities of such material were initially present in the tissue, as determined by dissociative extraction and reassociation with hyaluronate.

METHODS

Materials. All chemicals used were AnalaR grade unless otherwise mentioned. Carrier-free sodium [^{35}S]sulfate in isotonic saline (sp. act. <5 mCi/μS) and sodium [^{3}H]acetate (sp. act. 26 Ci/mmol) were obtained from the Radiochemical Centre, Amersham, Bucks, U.K.

D-Penicillamine, phenylbutazone, indomethacin, cycloheximide, benzimidine HCl, phenylmethylsulphonyl fluoride, iodoacetic acid, 6-aminohexanoic acid, soybean trypsin inhibitor, hydrocortisone, puromycin, colchicine, and rooster comb hyaluronic acid were all supplied by the Sigma Chemical Co., St. Louis, MO, U.S.A. Salicylate was supplied by Hopkin & Williams Ltd., England. Fenclofenac was a gift from Prof. A. Boura (Monash University, Clayton, Victoria, Australia).

Culture medium containing indomethacin was adjusted to pH 8.0 to dissolve the drug and, then, returned to pH 7.2 with addition of acid. Hydrocortisone was dissolved in a small volume of ethanol (0.5 ml) and subsequently added slowly to the medium (25 ml). All other additives used were prepared by adding them, as solids, directly to the medium. Media with additives were sterilized by filtration.

An enzyme inhibitor mixture contained 10 mM NaEDTA, 5 mM benzimidine HCl, 0.1 mM phenylmethyl sulphonyl fluoride, 1.0 mM sodium iodoacetic acid, 0.1 M 6-aminohexanoic acid and 1 μg/ml soybean trypsin inhibitor.

The incubation medium used in this work consisted of Dulbecco's modified Eagle's medium supplemented with 0.1 mM ascorbic acid, L-alanine, L-asparagine, L-aspartate, L-proline, L-serine, and glycine, and buffered at pH 7.2 with 15 mM HEPES [2-(*N*-2-hydroxyethylpiperazin-N′-yl)-ethanesulfonic acid], 10 mM BES (2-[bis-(2-hydroxyethyl)-amino]-ethanesulfonic acid), 10 mM TES (2-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl-amino]-ethanesulfonic acid), 2 mM NaH_2PO_4, 3.7 mM $NaHCO_3$, 1 mg/l sodium pyruvate, 1000 units/l penicillin G, and 10,000 μg/l streptomycin sulfate, and contained 20% (v/v) fetal calf serum (GIBCO, New York, NY).

Release of labeled proteoglycans from rabbit articular cartilage in organ culture. The method described by Sandy *et al.* [9] was used. Laboratory rabbits, 6- to 12-months-old, were injected in the hind knee joint with 500 μCi [^{35}S]sulfate in isotonic saline. At 48 hr the rabbits were killed by cervical dislocation, and the hind legs were removed. The articular cartilage was removed and placed in culture medium. One rabbit hind knee joint yielded approximately 60 mg of cartilage; about 15–20 mg was used for each release experiment. The medium was renewed every 48 hrs and spent medium was stored at $-20°$ for subsequent analysis. Release of ^{35}S-labeled material from cartilage into the medium was followed by the analysis of radioactivity in 25 μl aliquots taken from the medium at successive times. At the end of the 4-day incubation, the quantity of ^{35}S-labeled material remaining in the tissue was estimated by papain digestion of the tissue and subsequent radioactive counting.

Organ culture medium samples were fractionated on a Sepharose 2BCL (Pharmacia, Sweden) column (approximately 34 cm $\times$ 1.7 cm) in 0.5 M NaAc (pH 6.8) with 1.5 to 2 g fractions collected.

Analysis of pre-labeled ^{35}S-tissue proteoglycan at zero time under associative conditions. The hind legs were obtained from 48-hr ^{35}S-pre-labeled rabbits, as described in the previous section. The articular cartilage was removed and placed in a dissociative extractant (4 M GuHCl) plus enzyme inhibitor mixture. The tissue was extracted for 24 hr at 4° and resulted in solubilization of 87 per cent of the [^{35}S]proteoglycan from the tissue. The supernatant fraction was then dialyzed against 3×200 vol. of associative solvent (0.5 M sodium acetate, pH 6.8) for 24 hr. The solution was fractionated on an associative CsCl density gradient ($\rho_{init} = 1.5$ g/ml) at 100,000 *g* and the bottom third of the gradient was obtained. This fraction represented 85 per cent of the total [^{35}S]proteoglycan. The sample was then analyzed on Sepharose 2BCL under associative conditions as described in the previous section.

Organ culture and incubation of rabbit articular cartilage with [^{3}H]acetate. Laboratory rabbits, 6- to 12-months-old, were killed by cervical dislocation, and the front and hind legs removed. The leg joints (hind knee and front shoulder) were opened under sterile conditions, and the cartilage was dissected from all legs, pooled, sliced then divided into sterile glass bottles containing 5 ml of growth medium together with the appropriate concentration of salicylate, and incubated at 37°. Samples of approximately 50 mg of the cartilage were removed and placed into vials containing 0.87 ml of fresh medium and the appropriate amount of salicylate, together with 25 μl of 20 mM sodium acetate and 50 μl of 20 mM glutamine. The tissue was then incubated at 37° in an atmosphere of 95% O_2 and 5% CO_2 for

30 min with continuous slow shaking. After 30 min, 500 μCi of [^{3}H]-acetate (sp. act. 2.6 Ci/mmol) was added to the incubation. The incubations were allowed to proceed for an additional 3 hr or 24 hr before being terminated by chilling.

The tissue slices were separated from the medium and extracted with 5 ml of 4 M GuHCl and 0.5 M NaAc (pH 6.8), plus enzyme inhibitor mixture for 24 hr at 4° with gentle stirring. The supernatant extract was then combined with the original medium. It was found that 35–42 per cent of the total ^{3}H-non-dialyzable material formed in the tissue was solubilized during isotope incorporation and dissociative extraction. This yield was found to be essentially independent of isotope incubation time (either 3 hr or 24 hr) and of the presence of salicylate in the incubation medium. To prepare the associative form of [^{3}H]proteoglycan, the combined tissue extracts were dialyzed against 2 × 1000 vol. of 0.5 M NaAc (pH 6.8) at 4° for 48 hr, and then CsCl was added to give an initial density of 1.5 ml/g. Equilibrium density gradient centrifugation was performed in a 10 × 10 Titanium rotor in an MSE 65 ultracentrifuge at 100,000 $g(r_{average})$ for 48 hr at 5°. The tubes were fractionated, and the bottom one-third (A1,2-fraction), which corresponded to approximately 80 per cent of the total ^{3}H-labeled non-dialyzable material in the tube for all preparations, was dialyzed against 2 × 200 vol. of 0.5 M NaAc (pH 6.8) plus enzyme inhibitor mixture at 4° for 48 hr. This fraction was then analyzed on a Sepharose 2BCL column with 0.5 M NaAc (pH 6.8) as the solvent.

Radioactivity counting. Radioactivity was assayed in a Phillips Scintillation Analyzer using a scintillation mixture described by Fox [18].

RESULTS

Kinetic-release studies. Adult rabbit articular cartilage was labeled *in vivo* over 48 hr with [^{35}S]sulfate and then incubated in organ culture at pH 7.2. The distribution of [^{35}S]proteoglycan between the tissue and the medium was followed over 4 days of incubation. The results in Fig. 1 show the time course of release of labeled material in two different experiments: (1) normal organ culture conditions without additives such as drugs (referred to as the "control"), and (2) organ culture with the enzyme inhibitor mixture added. In both cases, a non-linear release profile was obtained.

In normal organ culture, between 50 and 60 per cent of the total content of ^{35}S-labeled material in the tissue was released in the first 2 days, increasing to 65–70 per cent total release after 4 days. This is in agreement with an earlier study [9] in which it was proposed that the relatively rapid release of [^{35}S]proteoglycan from rabbit articular cartilage is initiated by proteolytic action, at neutral pH being directed at or near the hyaluronate binding region. This reaction degrades proteoglycan aggregates present in the tissue to yield ^{35}S-labeled material, similar in size to the native sub-unit, which can rapidly diffuse from the tissue into the incubation medium. Figure 1 demonstrates that, in the presence of the enzyme inhibitor mixture, the rate of release of ^{35}S-material from the tissue was retarded. Similar experiments on the time dependence of release of [^{35}S]proteoglycan in the presence of various anti-inflammatory drugs or other additives are reported in Tables 1 and 2.

In view of the variation in the rates of release of

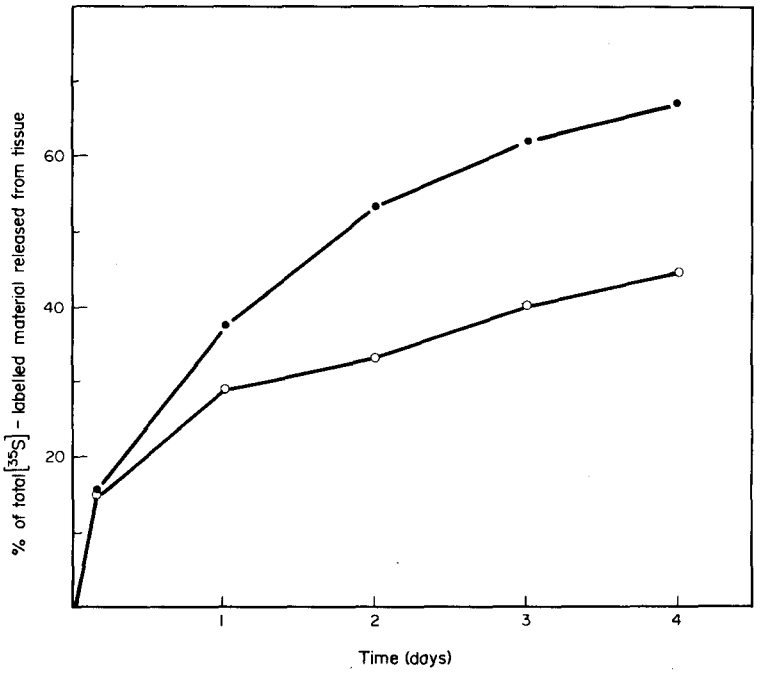

Fig. 1. Normalized release profiles of ^{35}S-labeled material from pre-labeled rabbit articular cartilage in organ culture. Key: (●——●) normal culture conditions, and (○——○) in the presence of the enzyme inhibitor mixture.

Table 1. Effects of anti-inflammatory drugs on ^{35}S-material release from rabbit articular cartilage in organ culture

Medium additive	Additive concn (mM)	Number of release experiments	Total number of time points studied	Per cent of ^{35}S-labeled material released with respect to medium with no additives* (mean ±S.D.)	Observed t value†	% Inhibition
Colchicine	1.0	1	3	60 ± 1	9.8 (4)	40
Fenclofenac	0.2	1	3	97 ± 3	0.68‡ (4)	
	1.0	1	3	79 ± 9	3.2 (4)	21
Hydrocortisone	0.2	2	8	61 ± 7§	12.6 (14)	39
Indomethacin	0.2	4	10	89 ± 9	3.1 (18)	11
	1.0	4	13	82 ± 11	5.0 (24)	18
D-Penicillamine	0.2	1	4	94 ± 7	0.64‡ (6)	
	1.0	2	6	95 ± 15	0.52‡ (10)	
Phenylbutazone	0.2	4	10	98 ± 13	0.42‡ (18)	
	1.0	4	10	87 ± 8	3.9 (18)	13
Salicylate	0.5	4	10	83 ± 7	6.0 (18)	17
	3.0	4	12	76 ± 11	6.4 (22)	24

* This value is $R_A(t)/\bar{R}_C(t)$ and where $R_C(t)/\bar{R}_C(t) = 100 \pm 7$ (N = 21).

† Values in parentheses represent degrees of freedom. The number of control samples was the same as the number of treated samples.

‡ Not statistically significant ($P > 0.05$).

§ Hydrocortisone was dissolved in ethanol prior to dissolution in media. Data in Table 2 would indicate that the ethanol (0.33 M) may have some inhibitory effect.

labeled material from tissues of different rabbits (for example, the percentage of ^{35}S-labeled material released with respect to total labeled material initially present, for culture media with no additives measured at 96 hr, varies from 53 to 72 per cent among five rabbits), it was necessary to perform control experiments (i.e. with no additives), in conjunction with experiments with additives, on tissue samples from pooled tissue isolated from a single rabbit. This protocol may be described as a "within subject" experimental design. The control experiments were performed in duplicate.

The percentage of ^{35}S-labeled material released into the culture medium containing a particular additive [$R_A(t)$] with respect to that of the control [mean of duplicate experiments, $\bar{R}_C(t)$] has been calculated from the ratio of these quantities, $R_A(t)/\bar{R}_C(t) \times 100$ at each time interval, namely 24, 48, 72 and 96 hr. (In certain instances, values of R_A at 72 hr have been omitted). These values are given in column 5 of Tables 1 and 2. The mean and standard deviation of the control experiments were calculated from the variation of $R_C(t)/\bar{R}_C(t) \times 100$ over all duplicate experiments performed. This gave a value for control experiments of 100 ± 7 (N = 21). Student's t-test has been employed (column 6, Tables 1 and 2) to compare means of each treatment and control condition.

The release experiment technique monitored inhibition of release; this was demonstrated when the enzyme inhibitor mixture was present in the medium. A statistically significant inhibition (33 per cent $P < 0.001$) of release (Table 2) was obtained, indicating that some inhibition of enzyme action by the inhibitor mixture was directed either at enzyme activity or enzyme production or both. The results also demonstrate that there was a high background of material release in this inhibited system. Other additives that showed equal, or better, effectiveness at inhibiting release, as compared to the inhibitor mixture, were 1 mM colchicine ($P < 0.001$), 0.2 mM hydrocortisone ($P < 0.001$) (Table 1), 0.35 mM cycloheximide ($P < 0.001$), and 0.1 mM puromycin ($P < 0.001$) (Table 2). In all cases, however, maximum inhibition of release only reached values of 30–40 per cent and a high background of labeled material was leached from the tissue. Such high basal levels may underscore the magnitude of the inhibition value recorded in column 7 of Tables 1 and 2 providing that proteolytic activity is minimized and that the background level represents a purely physicochemical phenomenon. Studies with heat-denatured organ culture medium demonstrated rates of release of material identical to that of the control, suggesting that there was little, if any, proteolytic activity being derived from the organ culture medium (Table 2, J. D. Sandy and H. L. G. Brown, unpublished observations).

Table 1 lists the effects of anti-inflammatory drugs on release. In several instances, namely with the additives 0.2 mM fenclofenac, 0.2 mM phenylbutazone and D-penicillamine at 0.2 and 1 mM, no statistically significant inhibition of release was recorded. In all other cases, inhibition of release was observed, with maximum effects being recorded for 1 mM colchicine (40 per cent, $N = 3$, $P < 0.001$), 0.2 mM hydrocortisone (39 per cent, $N = 8$, $P < 0.001$), and 3 mM salicylate (24 per cent, $N = 12$, $P < 0.001$). A statistically significant concentration dependence of the drug effect (i.e. comparison of means of $R_A(t)/\bar{R}_C \times 100$ at different drug concentrations) was observed in the range of drug concentrations studied for fenclofenac at 0.2 and 1.0 mM ($P < 0.05$, $N_1 = 3$, $N_2 = 3$) and for phenylbutazone at 0.2 and 1 mM

Table 2. Effects of various agents on ^{35}S material release from rabbit articular cartilage in organ culture

Medium additive	Additive concn	Number of release experiments	Total number of time points studied	Percent of ^{35}S-labeled material released with respect to medium with no additives* (mean ±S.D.)	Observed t value†	% Inhibition
Ethanol	0.3 M	2	6	77 ± 4	7.0 (10)	23
Cycloheximide	0.35 mM	3	10	79 ± 25 ‡	2.6 (18)	21
			8	67 ± 10	7.7 (14)	33
Puromycin	0.1 mM	1	4	69 ± 1	8.8 (6)	31
Enzyme inhibitor mixture	(see Methods)	2	4	67 ± 4	8.2 (6)	33
←Temperature 4°→ Heat-inactivated medium (56°, 30 min)		1	2	17 ± 3	15.4 (2)	83
		2	8	98 ± 12	0.41 (14)	

* This value is $R_A(t)/\bar{R}_C(t)$ and where $R_C(t)/\bar{R}_C(t) = 100 \pm 7$ (N = 21).
† Values in parentheses represent degrees of freedom. The number of control samples was the same as the number of treated samples.
‡ Two values in the first calculation (N = 10) were spurious and have been omitted in the second calculation (N = 8).

($P < 0.05$, $N_1 = 10$, $N_2 = 10$) but was not observed for D-penicillamine or indomethacin at 0.2 and 1.0 mM ($t = 1.67$, $N_1 = 10$, $N_2 = 13$) and salicylate at 0.5 and 3.0 mM ($t = 1.81$, $N_1 = 10$, $N_2 = 12$). The concentration dependence of the drug effect relative to the control was, however, observed with indomethacin and salicylate (Table 1).

Profiles of released material on Sepharose 2BCL. The proteoglycan sub-unit exists in equilibrium with hyaluronic acid to form a proteoglycan complex [1, 2]. Fractionation by gel chromatography of these components elutes the proteoglycan complex generally at the void volume of the Sepharose 2BCL column, whereas the proteoglycan sub-unit is included into the column.

The elution profile of a reconstituted complex of endogenous proteoglycan sub-unit and hyaluronic acid extracted by 4 M GuHCl from ^{35}S-prelabeled cartilage prior to incubation *in vitro* is shown in Fig. 2. This fraction represented 70 per cent of the total [^{35}S]proteoglycan formed in the 48 hr *in vivo* labeling period and eluted at the void volume of the column. It is evident that this fraction existed mainly as a proteoglycan complex in the tissue.

The elution profile of material released into the medium from pre-labeled cartilage incubated for 2 days in the presence of inhibitor mixture (Fig. 3a) again showed that the major portion of radioactivity eluted at the void volume of the Sepharose 2BCL column. (The profile has been normalized in terms of per cent of material, released from the tissue, distributed over the total volume of the column, as described in the legend of Fig. 3.) However, when the pre-labeled cartilage was incubated for 2 days in the absence of enzyme inhibitor mixture and the ^{35}S-labeled material released into the medium was again chromatographed on Sepharose 2BCL, the radioactive profile (Fig. 3b) had two distinct peaks.

The radioactivity profiles of ^{35}S-material released into the medium by cartilage cultured in the presence of anti-inflammatory drugs were distinctly different from that obtained in Fig. 3b. In general, as seen in Fig. 4 (panels a–c), the elution profiles had broad, continuous profiles skewed positively toward aggregate material. It appears that when a greater inhibition of release of ^{35}S-material occurred, e.g. in the presence of 1 mM colchicine (Table 1), then relatively more aggregate material appeared in the medium (Fig. 4c). The smaller degree of inhibition of the release of ^{35}S-material in the presence of 1 mM indomethacin and 3 mM salicylate (Table 1) is reflected in decreased amounts of aggregate shown in the Sepharose 2BCL profiles (Fig. 4, panels a and b).

Dialysis prior to chromatography did not appear to alter the position or shape of the major elution peaks of any of the samples shown in Figs. 2–4.

Profiles of dissociatively extracted ^{3}H-labeled proteoglycans at various times of incubation with [^{3}H]acetate in vitro. In this experiment, cartilage slices were incubated for either 3 hr or 24 hr with [^{3}H] acetate. At the end of these incubation periods, the cartilage was dissociatively extracted with 4 M GuHCl with enzyme inhibitor mixture, and

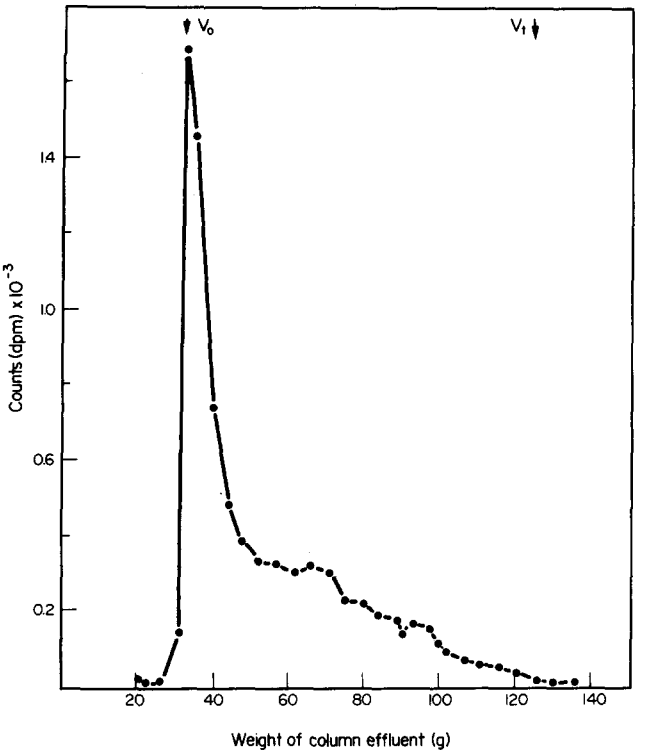

Fig. 2. Elution profile of a dissociatively extracted associative fraction of pre-labeled cartilage at zero time obtained by CsCl equilibrium density gradient centrifugation (see Methods) on Sepharose 2BCL, with 0.5 M NaAc (pH 6.8) as solvent. Column dimensions were 43 cm × 2 cm.

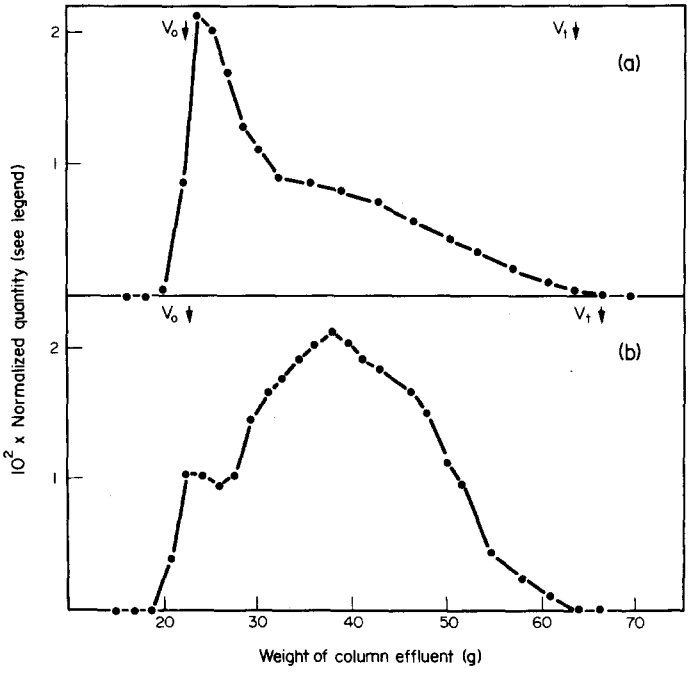

Fig. 3. Normalized elution profiles of the distribution of ^{35}S-labeled material on Sepharose 2BCL, with 0.5 M NaAc (pH 6.8) as solvent, of media samples taken from 2-day-old cultures of pre-labelled cartilage. The ordinate represents the following quantity:

$$\frac{\text{counts/column fraction}}{\text{counts/column}} \times \% \text{ of total } ^{35}S \text{ released into medium.}$$

The abscissa indicates interval units of 10 g solvent. Key: (a) organ culture with enzyme inhibitor mixture present; and (b) normal organ culture conditions.

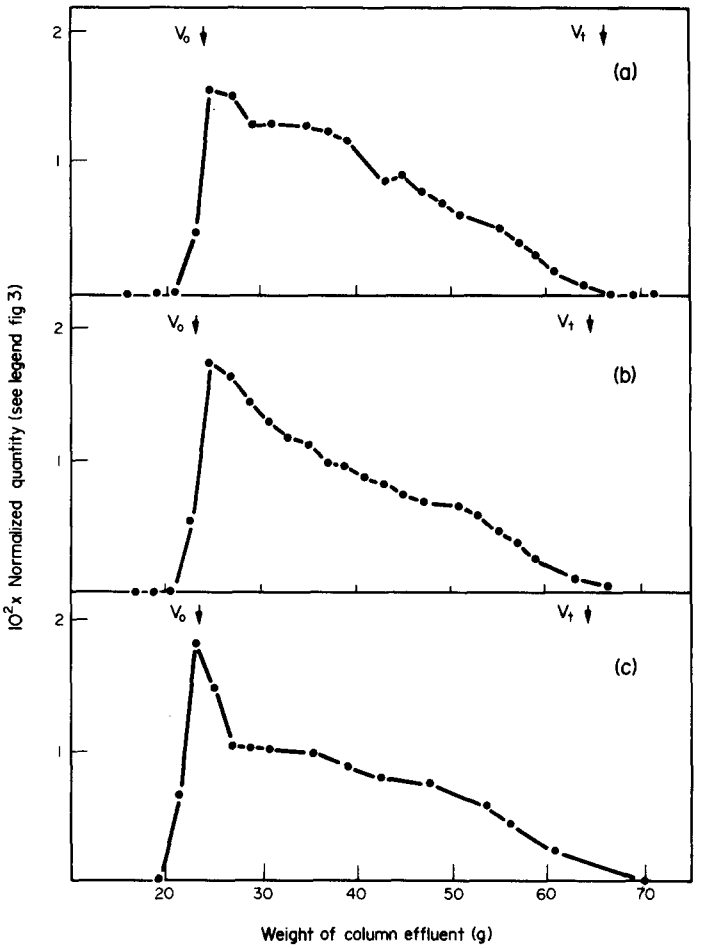

Fig. 4. Normalized elution profiles of the distribution of ^{35}S-labeled material on Sepharose 2BCL, with 0.5 M NaAc (pH 6.8) as solvent, of media samples taken from 2-day-old cultures of pre-labeled cartilage. The following media samples were studied after dialysis against 0.5 M NaAc (pH 6.8): (a) 3 mM salicylate, (b) 1 mM indomethacin, and (c) 1 mM colchicine.

[^{3}H]proteoglycans of the associated form (i.e. reconstituted with endogenous hyaluronic acid) were obtained by equilibrium CsCl gradient centrifugation as described in Methods. Under the same conditions, the [^{3}H]proteoglycans formed in the presence of 3 mM salicylate were also isolated. Striking differences were found when the elution profiles of the associated [^{3}H]proteoglycans were studied, as shown in Fig. 5. The proteoglycan formed during a 3-hr incubation of cartilage slices with [^{3}H]acetate *in vitro*, when chromatographed on Sepharose 2BCL, showed that most of the isotope was present in proteoglycan complex eluting essentially at the void volume (Fig. 5a). However, the ^{3}H-labeled material formed after 24 hr of incubation with [^{3}H]acetate *in vitro* when chromatographed on Sepharose 2BCL showed that a major portion of the radioactivity was now included in the column (Fig. 5a).

When 3 mM salicylate was included in the incubation medium, it had little effect on the radioactivity profile after incubation for 3 hr in the presence of [^{3}H]acetate (Fig. 5b); however, when the incubation was continued for 24 hr *in vitro*, the salicylate appeared to have inhibited the appearance of an included peak (Fig. 5b). The addition of exogenous hyaluronic acid to the dissociated form of the 24-hr [^{3}H]proteoglycan from normal culture had a marginal effect on its elution profile. For experiments where the articular cartilage was preincubated for 24 hr in organ culture prior to the addition of [^{3}H]acetate, identical results were obtained (not shown).

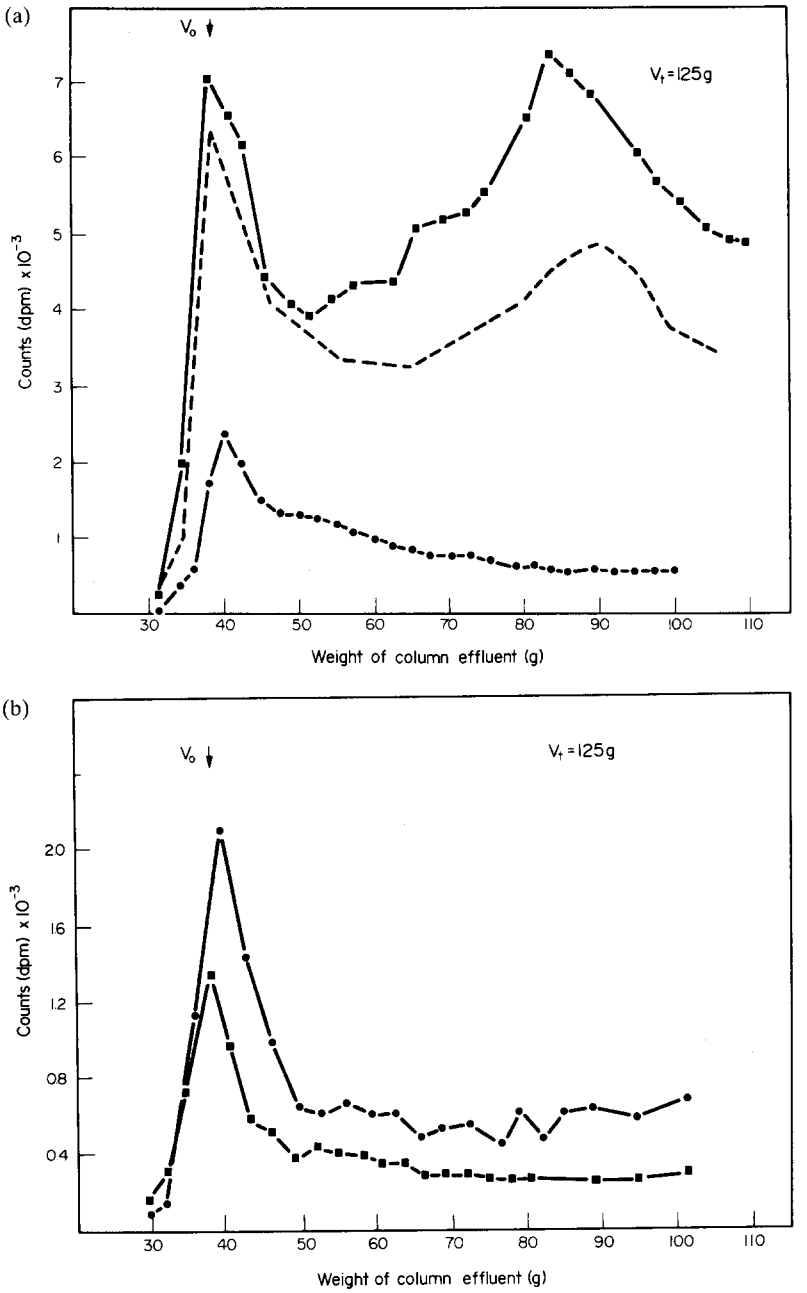

Fig. 5. Elution profiles of (A1 + A2) fraction obtained from incubations of rabbit articular cartilage slices with [^{3}H]acetate for (a) normal culture for 3 hr (●——●) or 24 hr (■——■) and (b) in the presence of 3 mM salicylate for 3 hr (■——■) or 24 hr (●——●). The profiles were performed on a Sepharose 2BCL column (43 cm × 2 cm) with 0.5 M NaAc (pH 6.8) plus enzyme inhibitors as solvent. The dashed line in panel (a) represents a sample of 24-hr [^{3}H]proteoglycan (D1 + D2) fraction from normal culture (3 ml) plus 1 mg of exogenous rooster comb hyaluronic acid.

DISCUSSION

This study demonstrates statistically significant inhibitory effects of some anti-inflammatory drugs on labeled proteoglycan release from rabbit articular cartilage, which have hitherto been unreported and are presumably important in degenerative disease. The inhibition of release of labeled proteoglycan was paralleled by significant changes in the elution profiles on Sepharose 2BCL of media-released material after incubation for 2 days. The inhibition of release of proteoglycan resulted in a shift of material toward aggregate-like material, as demonstrated by gel chromatography.

Interpretation of the elution profiles of released proteoglycan is based on a number of factors. We have shown that approximately 85 per cent of the labeled proteoglycan in the cartilage can be disso-

ciatively extracted with 4 M GuHCl at zero time and so probably exists in an extracellular environment [2]. Further, we have demonstrated that 80 per cent of this extracted material, when isolated as an associative fraction on CsCl density gradients, exists essentially as aggregates with endogenous hyaluronic acid, as demonstrated by gel chromatography (Fig. 2). It is then likely that a major portion of the [^{35}S]proteoglycan can exist in an aggregate form with hyaluronic acid in the tissue.

When pre-labeled cartilage was incubated in medium under normal conditions, it is clear that the system responded in a manner suggesting high degrading activity directed toward proteoglycan. This is evident from the rate of release measurements together with the large proportion of material that was included well into the column. Previous work [9] has shown that this proteoglycan monomer like material is unable for form aggregates with exogenous hyaluronic acid, and it was therefore suggested that proteolytic activity is directed at the hyaluronic acid binding site of the sub-unit core-protein. In any case, it is apparent that some degradation had occurred in this system as compared to proteoglycans obtained by dissociative extraction at zero time and in the media containing enzyme inhibitor mixture. Inhibition of this degrading activity by anti-inflammatory drugs was seen not only in inhibition of release but also in a shift of material toward aggregate-like material on Sepharose 2BCL (Fig. 3).

On incubation of the pre-labeled tissue with media containing the enzyme inhibitor mixture it is evident that the proteoglycan released into the media existed also in an aggregate form (Fig. 3a). Although there was a relatively large amount of material released in this system (approximately 40–45 per cent of the total label in tissue), there appears to have been negligible breakdown of aggregates to smaller units that would be included on Sepharose 2BCL. Therefore, with the enzyme inhibitor mixture we had a high background release, 33 per cent inhibition of release with respect to medium with no additives (Table 2), and no apparent breakdown of aggregates. These results suggest that the high background of release may have been due to destruction of the structural integrity of the cartilage by removing it from an *in situ* environment and, thereby allowing more porous surfaces of the cartilage to be exposed to the medium. Furthermore, although the results with the enzyme inhibitor mixture suggest that the inhibition of enzyme activity was directed at the formation of column-included material, enzyme activity directed at aggregates in the tissue is a possibility. In this case, the formation of smaller aggregates might not have been detected by the column method but might have been released from the tissue to yield high background levels. The registration of almost total inhibition of material release when the tissue was maintained at 4° suggests that either all enzyme activity was inhibited at this temperature and/or proteoglycans underwent a temperature-dependent interaction with other tissue components, with the interaction being most stable at lower temperatures.

The high auto-catalytic degradative activity of the articular cartilage slices on proteoglycan complexes was also confirmed by analysis of *in vitro* labeled [^{3}H]proteoglycan complexes. This system exhibited a degradative response similar to that of pre-labeled cartilage, in that proteoglycan complexes appeared to be degraded. The inhibition of this degradative activity was evident when salicylate (3 mM) was present in the culture medium.

The mechanism of inhibition of enzyme degradative activity is unclear. It is obvious that the additives may exert either a toxic effect on cell viability, and therefore affect enzyme production, or may inhibit the enzymes directly, or both. It is only important to note at this stage that the anti-inflammatory drugs, in general, do appear to inhibit a degradation process. The fact that the various additives used, including the anti-inflammatory drugs, may have exerted their effects by some direct physicochemical interaction with the proteoglycan or may have altered its interaction with the tissue and thereby manifested a particular release effect cannot be discounted entirely. Although proteoglycan–tissue interactions are difficult to monitor at this stage, it is evident from our results (unpublished observations) that dialysis of the release medium against 0.5 M NaAc (p h 6.8) does not result in any significant change in the distribution of macromolecular compounds on the column. We assume that any interaction of the additives is reversible and dissociated by dilution so that their effects, mediated through such a physicochemical interaction, appears negligible.

To distinguish between possible intracellular and extracellular sources by which proteoglycan release occurs, three intracellular agents were used, namely the protein synthesis inhibitors puromycin (0.1 mM) and cycloheximide (0.35 mM) and an antimicrotubular agent colchicine (1 mM). The inhibitor action of cycloheximide was variable but, taken together with the result for puromycin (Table 2), it is anticipated that activation of proteolytic enzymes in organ culture may require *de novo* synthesis of protein (perhaps the enzyme itself). The inhibitory action of colchicine (Table 1) on proteoglycan release also suggests that transport of enzymes by an intracellular microfilament mechanism is also required for their action. It has been postulated that microtubules participate in the control of secretion and endocytosis of lysosomal enzymes and in the endocytosis and degradation of lysosomal substrates such as sulfated proteoglycans in culture skin fibroblasts [19]. The antimicrotubular drug, colchicine, was shown to inhibit endocytosis of sulfated proteoglycan and its subsequent intracellular degradation. As the anti-inflammatory drugs appear to generate a concentration-dependent inhibition of proteoglycan release in organ culture, the questions of practical importance are (1) whether the effects observed at the concentration studied are applicable to active concentration of drug administered clinically, and (2) whether endogenous proteolytic activity of cartilage (the actual cell source of the enzyme is unknown) is important in the degradative arthritic conditions of the tissue. It is of interest in this regard that active plasma concentrations of salicylate may range from 0.1 to 0.6 mM for normal doses in the treatment of rheumatoid arthritis [20]. We, therefore, may predict

that the drug will have an effect on proteoglycan breakdown by endogenous cartilage enzymes.

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