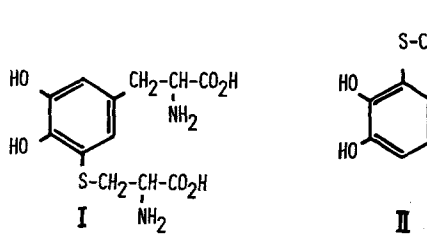
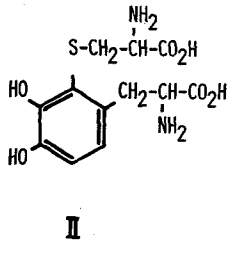
acetic acid–water (60:20:20, v/V) afforded 10 mg of 2-S-cysteinyldopa (**II**)[8] as an amorphous hygroscopic powder, λ_{max} (in 0.1 M HCl) 293 and 255 nm; NMR (D_2O) δ 3.14–3.70 (4H, m, $-CH_2-$), 4.06–4.36 (2H, m, –CH–), 6.89 and 6.99 (each 1H, ABq, J 8.3 Hz, ortho-coupled ArH)[9]. The structure of 2-S-cysteinyldopa was confirmed by a direct comparison of the spectral and chromatographic properties of the isolated product with those of an authentic sample.

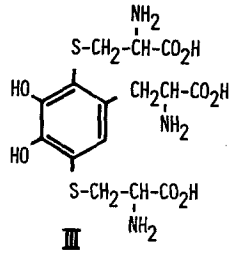
Fractions 14–25 contained a third catechol amino-acid. The fractions were evaporated to dryness and the residue was chromatographed on Whatman 3 MM paper using n-propanol-1 M HCl (3:2) as the eluent. Extraction with 0.1 M HCl of the major UV-quenching band (R_f 0.28) and evaporation of the extract gave 10 mg of the crude compound, which was further purified on a column (1.5 × 77 cm) of Sephadex G 25, developed with 0.1 M HCl. Fractions of 5 ml were collected and monitored by their E_{273}. Fractions 21–25 were combined and evaporated to dryness to give 3.6 mg of an amorphous colourless powder, λ_{max} 303 and 273 nm at pH 1 and 320 nm at pH 6.8 (phosphate buffer), NMR (D_2O)[9] δ 3.07–3.80 (6H, m, $-CH_2-$), 4.08–4.43 (3H, m, –CH–), and 7.10 (1H, s, isolated ArH), which was identified as 2,5-S,S-dicysteinyldopa (**III**) by comparison of the spectral and chromatographic properties with those of an authentic sample.



I



II



III

Indirect evidence for the natural occurrence of 2-S-cysteinyldopa (**II**) has recently been provided[10] by the isolation of trichochrome B from a melanoma urine, while the dicysteinyldopa (**III**) has previously been isolated from the reflecting spheres of the eyes of the alligator gar, Lepisosteus spatula[7].

Although a quantitative assessment of the excretory level of 2-S-cysteinyldopa and 2,5-S,S-dicysteinyldopa with respect to 5-S-cysteinyldopa in melanoma urine is out of the scope of the present work, the data so far available suggest that the relative ratio of **I**, **II** and **III** is approximately 15:3:1. This is consistent with the suggested metabolic origin of the cysteinyldopas and with recent biosynthetic experiments showing that in vitro the addition of cysteine to dopaquinone also leads to a mixture of **I**, **II** and **III** in a ratio very similar to that mentioned above[11,12]. If the same relative ratios of 5-S- and 2-S-cysteinyldopa and of 2,5-S,S-dicysteinyldopa can be demonstrated in quantitative experiments on melanoma urine, it will provide evidence that the dopaquinone-cysteine reaction taking place in the melanocytes proceeds more or less in the same way as in model experiments.

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12 Actually, the reaction leads also to the formation of a trace amount of 6-S-cysteinyldopa. Evidence for the presence of this compound in melanoma urine has previously been obtained by GLC-MS analysis (G. Agrup, C. Hansson, B.-M. Kennedy, K. Persson, H. Rorsman, A.-M. Rosengren and E. Rosengren, Acta derm.-vener., Stockh. *56*, 491 (1976).

Hyaluronic acid in elastic cartilage

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20 September 1976

Summary. Bovine ear cartilage contains more hyaluronic acid than do hyaline cartilages of the same animal. Most of it is in the elastin-rich residue not extractable by 4 M guanidinium chloride where it is associated with chondroitin sulphate in low relative concentration and of lower molecular weight than in non-elastic cartilage residue.

The recent report[2] that cartilage proteoglycans can stimulate the fibrillation of elastin suggests an additional role for the glycosaminoglycans in elastic cartilages such as that of bovine ear. The glycosaminoglycan composition of ear cartilage differs only slightly from that of non-elastic cartilages of the same animal but the chondroitin sulphate chains are shorter[3] and there is a small but definite increase in the non-sulphated fraction[4]. This fraction includes hyaluronic acid which, following the initial report of Hardingham and Muir[5], is now known to cause the aggregation of cartilage proteoglycans even though present in very small quantities in these tissues[6]. In the present work these distinct features of ear cartilage glycosaminoglycans have been studied further to see whether they are associated with the presence of elastic fibres.

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6 V. C. Hascall and D. Heinegard, J. biol. Chem. *249*, 4232 (1975).

Materials and methods. Cartilage from 1–2-year-old steers was freed of adhering tissue, blood vessels and any opaque regions. Portions were finely sliced and extracted with 4 M guanidinium chloride[6] yielding an extract from which crude proteoglycan was precipitated at 4 °C by addition of 4 vol. of 95% aq. ethanol. The glycosaminoglycans were released by papain digestion[6] for 20 h at 50 °C using 1 mg enzyme per g cartilage (or 100 mg proteoglycan or solid residue) and precipitated at 4 °C after addition of 4 vol. of 95% aq. ethanol. The product was dissolved in 0.8 M NaCl and passed through a column (1 ml bed volume per 5 mg glycosaminoglycan) of Dowex 1 × 2 (Cl^-) which was then washed with further 0.8 M NaCl to a total of 5 bed volumes. The chondroitin sulphates were obtained by eluting this column with 2.5 bed volumes of 2 M NaCl while the 0.8 M NaCl effluent was diluted to 0.2 M by addition of water and loaded on a second column (1 ml bed volume per 30 mg original glycosaminoglycan). After washing the column with 0.2 M NaCl is was eluted with 0.8 M NaCl. This eluate, containing the unsulphated glycosaminoglycans, was applied in 2 ml portions to a column (58 × 1 cm) of Sephadex G-100 which was eluted at 10 ml/h with 0.5 M NaCl. All fractions were assayed for uronic acid[7] and the glycosaminoglycan excluded by the gel (giving a peak at 10–16 ml effluent volume) was isolated for further study by precipitation with ethanol as before.

Hexosamine was assayed by amino acid autoanalyser. Cellulose acetate electrophoresis was performed at 22 V/cm for 40 min in a pyridine-acetic acid-water mixture, pH 3.0 (5:100:895, by vol) followed by staining with 0.5% aq. toluidine blue. Digestion with streptococcal hyaluronidase (10 units/ml; Calbiochem. Ltd.) was carried out with 0.5 mg/ml hyaluronic acid in 0.1 M sodium acetate, pH 5.0. After incubation at 50 °C for 6 h the unsaturated oligosaccharides released were measured spectrophotometrically at 232 nm. Both the Dowex 1 column procedure and streptococcal hyaluronidase digests were calibrated for analytical purposes using standard mixtures of hyaluronic acid and chondroitin sulphate previously isolated from ear cartilage.

Results. Hyaluronic acid was isolated from proteolytic digests of cartilage or extracts by ion exchange chromatography followed by gel chromatography on Sephadex G-100 of the fraction eluted from Dowex 1 × 2 by 0.8 M NaCl. The peak excluded by the gel contained equimolar glucosamine and uronic acid together with small amounts of galactosamine (3.2% total hexosamine) and yielded a single band on cellulose acetate electrophoresis of identical mobility to that of umbilical cord hyaluronic acid (Seravac Ltd.) which had been subjected to papain digestion. It was susceptible to digestion by streptococcal hyaluronidase giving 15% more unsaturated oligosaccharide than an umbilical cord hyaluronic acid under the same conditions.

Hyaluronic acid and chondroitin sulphate were estimated in ear and other cartilages from the same animal and in the proteoglycan obtained by extraction of the cartilage with 4 M guanidinium chloride. The results (table) show that ear cartilage contains 2–3 times as much hyaluronic acid as the hyaline cartilages when the results are expressed relative to the total glycosaminoglycan content. Most of the extra hyaluronic acid is not extracted by 4 M guanidinium chloride but remains in the insoluble residue where, in the ear, it amounts to 9.2 μM uronic acid per g dry wt. Of the 3 non-elastic cartilages studied, cricoid was found to contain the most residual hyaluronic acid (8.0 μM uronic acid per g dry wt).

The condroitin sulphates from the insoluble residues of ear and cricoid cartilages were obtained in single chain form by papain digestion and compared by gel chromatography with those extractable by 4M guanidinium chloride. The elution profiles of chondroitin sulphates of extract and residue are superimposable but distinct for ear and cricoid (figure) excluding the possibility of the selective retention of especially short chondroitin sulphate chains in the elastin-rich residue.

Discussion. The hyaluronic acid of ear cartilage is present in a ratio to chondroitin sulphate much in excess of that required for optimal aggregation of cartilage proteoglycans[5]. After extraction of the bulk of proteoglycan the elastin-rich residue should contain any glycosaminoglycan fraction particularly associated with the fibres. Though further enriched, the hyaluronic acid concentration (on a basis of dry weight) was not notably greater in elastic than non-elastic cartilage residues. A specific association between hyaluronic acid and the mature fibres of elastic tissue seems unlikely also because mild procedures which extract collagen selectively and solubilise 70–90% of the residual glycosaminoglycans (such as digestion by tryp-

Hyaluronic acid content of bovine cartilages and of proteoglycan extracted from them by 4 M guanidinium chloride

Source	Hyaluronic acid content expressed as: μmoles uronic acid per g wet wt	% total uronic acid* in whole cartilage	extracted proteoglycan	unextractable** residue
Ear	2.5	3.1	0.80	12.7 (14.0)
Nasal septum	1.3	1.0	0.30	4.1
Cricoid	1.8	1.3	0.41	7.1 (7.3)
Trachea	2.0	1.6	0.49	4.8

*Total uronic acid refers to the sum of sulphated + unsulphated glycosaminoglycans in the preparation. **Figures in this column estimated from the other data together with the percentage of total uronic acid extracted by 4 M guanidinium chloride, 81% (ear), 82% (nasal septum), 86% (cricoid) and 75% (trachea). Figures in parentheses are estimated directly on total glycosaminoglycans using streptococcal hyaluronidase.

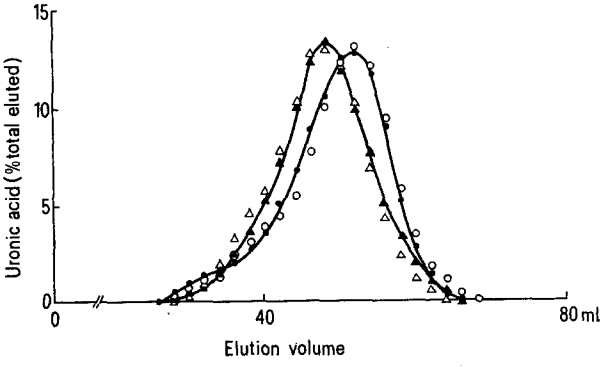

Fig. 1. Gel chromatography on Sephadex G-200 of chondroitin sulphates from bovine ear and cricoid cartilages after extraction by 4 M guanidinium chloride. The column (575 mm × 13 mm, void volume 19 ml) was eluted at 3 ml/h with 0.5 M sodium acetate, pH 6.8. The elution profiles of 2 mg chondroitin sulphates from ear extract (○), ear residue (●), cricoid extract (△) and cricoid residue (▲), all loaded in 1 ml, are shown.

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sin, or collagenase, or pepsin followed by 4 M guanidinium chloride) all fail to show any selective enrichment of hyaluronic acid in the residue (F. S. Wusteman, unpublished work).

The major difference in the unextractable glycosaminoglycans which interpenetrate the network of fibrous protein in elastic cartilage lies in the relatively lower concentration of chondroitin sulphate chains (even after due allowance has been made for their lower mol. wt[3]). No way has yet been found to extract undegraded proteoglycan from these residues but their state of aggregation must be influenced by this difference in composition. Even if this were not so, the steric exclusion effect of hyaluronic acid, which is known to influence in vitro both the nucleation and fibre growth processes of collagen[8], is likely to affect the developing elastic fibre. Hyaluronic acid has been found in high concentration in another elastic cartilage, that of human epiglottis[9], and is far more abundant in elastic ligamentum nuchae than in collagenous tendon[10]. An unsulphated glycosaminoglycan has been implicated, from histochemical studies, in the formation of elastic fibres in arterial walls[11] and it may be that an involvement of hyaluronic acid in elastin fibrillogenesis has been obscured by the presence of other glycosaminoglycans with different functions in the tissue.

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Chemical and field studies on the sex pheromones of the cone and seed moths Barbara colfaxiana and Laspeyresia youngana[1,2]

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Summary. Field studies have shown that mixtures of trans-7-dodecen-1-ol and up to 10% of the cis isomer are effective attractants for male Laspeyresia youngana. Similarly male Barbara colfaxiana have been shown to be attracted to traps containing cis-9-dodecen-1-ol, and preliminary analyses indicate that the natural pheromone of this species contains a dodecen-1-ol.

The Douglas-fir cone moth Barbara colfaxiana and the spruce coneworm Laspeyresia youngana are 2 of the most serious cone ans seed pests indigenous to British Columbia. Damage is caused by larvae feeding mainly on seeds. The species overwinter as pupae (B. colfaxiana) and larvae (L. youngana) and emerge as adult moths in late spring and early summer.

Although neither species pose a serious threat to established forests, they are a problem, and the cause of economic loss in seed orchards. Since seed orchards are generally of small area (e.g. 10–20 ha), contain uniformly sized trees, usually of a single species, we believe they afford a forestry situation in which sex pehromones could be successfully used for population management. This paper presents the results of some preliminary work aimed at the isolation and identification of the sex pheromones of B. colfaxiana and L. youngana.

Table 1. Compounds screened for attractancy against B. colfaxiana and L. youngana

Alcohols	Alcohol acetates	Expoxides
trans-4-dodecen-1-ol	cis-7-dodecen-1-yl acetate	disparlure*
cis-4-dodecen-1-ol	cis-8-dodecen-1-yl acetate	
trans-5-dodecen-1-ol	trans-9-dodecen-1-yl acetate	
cis-6-dodecen-1-ol	cis-9-dodecen-1-yl acetate	
trans-7-dodecen-1-ol	trans-11-tetradecen-1-yl acetate	
cis-7-dodecen-1-ol	cis -11-tetradecen-1-yl acetate	
cis-9-dodecen-1-ol	cis-7-hexadecen-1-yl acetate*	
cis-7-tetradecen-1-ol	cis-11-hexadecen-1-yl acetate*	
trans-11-tetradecen-1-ol*		
cis-11-tetradecen-1-ol*		
cis-7-hexadecen-1-ol		
cis-9-hexadecen-1-ol*		

*Compounds not tested against B. colfaxiana

Materials and methods. The synthetic compounds field screened for attractancy (table 1) were dispensed in polyethylene vial caps (100 µg/cap) and suspended in Pherocon 2 traps. Each compound was replicated six times. For the chemical studies on B. colfaxiana pheromone, insects were reared on a 18/6 h light/dark cycle. The insects were removed from the cones at the late larval and prepupal stage, the sexes thereafter were maintained separately. After emergence the males were maintained for bioassay, while the females were sacrificed by dropping them into hexane when they were 2 days old. Bioassays were carried out using 2-day-old males in petri dishes in subdued light. The males were acclimatized in the dishes for several hours before 1 cm² pieces of filter paper impregnated with the test materials were introduced into the petri dishes. Intense 'buzzing' by the males was considered a positive response.

Extracts of B. colfaxiana were prepared by grinding whole, virgin female insects with hexane in a tissue homogenizer. This process was repeated twice, and the combined hexane extracts dried over magnesium sulphate prior to removal of the solvent at room temperature under reduced pressure. Gas chromatography was performed on a Perkin-Elmer 3920 gas chromatograph fitted with flame ionization detectors. All columns used were 6 × cm

1 Barbara colfaxiana (Kft.) and Laspeyresia youngana (Kft.) (Lepidoptera: Olethreutidae).
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