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# Circulating hyaluronan, chondroitin sulphate and dextran sulphate bind to a liver receptor that does not recognize heparin

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**Chondroitin sulphate, injected intravenously into rats and given prior to intravenous $^{125}$I-labelled hyaluronan with a mean Mw of about 400 kDa, was shown to inhibit the rapid receptor-mediated uptake of hyaluronan by the liver. The labelled hyaluronan that remained in the circulation was shown, by size exclusion chromatography of serum and urine, to be rapidly degraded down to fragments of lower Mw and filtered out into the urine and tissues. When the uptake of $^{125}$I-hyaluronan was inhibited by unlabelled hyaluronan, only very low degradation and urinary excretion were found. Liver uptake could also be inhibited by dextran sulphate but not by heparin. Unlabelled hyaluronan could inhibit the liver uptake of labelled chondroitin sulphate but not labelled heparin. Unlabelled chondroitin sulphate and dextran sulphate inhibited cell association of labelled hyaluronan to liver endothelial cells in culture more effectively than unlabelled hyaluronan. Our data show that the liver hyaluronan receptors also recognize and effectively bind chondroitin sulphate and dextran sulphate but not heparin and that a hyaluronan-specific saturable degradative mechanism exists in the circulation. Such a mechanism could explain why hyaluronan in the general circulation has a much lower Mw than the hyaluronan in lymph. The results also indicate that increased hyaluronan levels in serum, and increased urinary excretion of hyaluronan, may be secondary to increased outflow of chondroitin sulphate from the tissues during some pathological conditions.**

***Keywords*: Polysaccharides, labelling, turnover, biodistribution, metabolism, receptors, liver endothelial cells, size exclusion chromatography.**

## Introduction

Hyaluronan (hyaluronic acid; HA) is a high molecular weight polysaccharide consisting of repeating units of glucuronic acid and N-acetylglucosamine. It is found in high concentrations in connective tissues such as skin and cartilage, in the vitreous body of the eye and in synovial fluid [1]. The polysaccharide can associate with several proteins in the extracellular matrix and also with some cell-surface HA-binding proteins [2].

The serum level of HA is normally very low (10–50 $\mu g l^{-1}$), but elevated in certain disease states such as rheumatoid arthritis, liver cirrhosis, and various malignancies [3]. Circulating hyaluronan comes from the peripheral tissues where most is associated with cells or binding proteins, but some exists in freely mobilized compartments. The polysaccharide enters the general circulation via the lymph [4] after 80–90% is removed in lymph nodes before reaching the bloodstream [5]. The Mw in serum is in the order of $1.5 \times 10^5$ while the Mw of HA is lymph is about $2 \times 10^6$ [6].

The major site for elimination of HA from the bloodstream, under normal circumstances, is via receptor mediated endocytosis by the liver [1, 7]. The $t_{0.5}$ of intravenously administered hyaluronan to experimental animals and man is in the order of a few minutes, and already after 15–20 min the degradation products start to appear in the circulation [7–9]. The uptake is via coated pits and coated vesicles in liver endothelial cells (LEC), while Kupffer cells and hepatocytes are essentially negative for uptake both *in vivo* and *in vitro* [9–11].

The HA taken up by LEC is transported to lysosomes where it is degraded to monosaccharides that ultimateley are broken down to carbon dioxide, urea and water in the hepatocytes [12].

Inhibition studies with LEC in culture show that the receptors recognize other ligands besides HA, such as chondroitin sulphate (CS), dextran sulphate (DxS) and desulphated CS [13–15]. Heparin does not seem to inhibit the binding of high Mw HA to LEC [12, 13], but has been reported to inhibit low Mw HA binding at high concentrations [14]. However, as the binding affinity of HA increases with chain length [1, 12], this discrepancy has been explained by a low affinity interaction between heparin and

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HA receptors able to suppress low Mw HA binding at high concentrations of heparin [12].

We have now performed inhibition studies with polysaccharides that might bind to the HA receptor on LEC *in vivo*, and can in the present paper show that CS and DxS, but not heparin, inhibit the clearance of HA by the liver. The HA that remains in the circulation is broken down to smaller fragments by what seems to be a specific and saturable mechanism, as high concentrations of HA inhibit this degradation. This mechanism could explain why circulating HA has a much lower Mw than the HA entering the general circulation via the lymph. Saturation of this mechanism could also explain why extremely high circulating levels of HA can give rise to increased plasma viscosity from high Mw HA.

## Materials and methods

### Polysaccharides

The HA used for labelling and uptake- and turnover-studies was supplied by Hyal Pharmaceutical Corporation (HPC), Toronto, Canada. The molecular weight distribution of the HA was determined by chromatography on a calibrated column of Sephacryl HR with porosities noted as 400, 1000 and 2000 (Pharmacia, Uppsala, Sweden) in 0.25 M NaCl, 0.05% chlorbutanol [16]. The HA content in each fraction was monitored by determination of the absorbance at 214 nm. Radioactivity was measured by gamma-counting on a Packard auto-gamma gamma-counter.

Chondroitin sulphate A from bovine trachea was from Sigma chemical company, St Louis, USA (product number 8529). This batch contained 1.9 ng HA $\mu g^{-1}$ CS as determined by a specific radioassay for HA (HA-50, Pharmacia, Uppsala, Sweden). The mean Mw was, by gel filtration chromatography on Sephacryl S-1000 and S-300 calibrated with HA standards, found to be approximately 30 000 Da.

Dextran sulphate with a Mw of $\sim$ 500 000 Da was from Pharmacia Biotech, Uppsala, Sweden (Code No. 17-0340-01).

Heparin from intestinal mucosa and purified by repeated precipitation with cetylpyridinium chloride (17) was a kind gift from Professor Ulf Lindahl, University of Uppsala, Sweden.

### Labelling of HA, CS and heparin

The HA, CS and heparin were labelled with DL-tyrosine (Sigma Chemical Company St Louis, USA) as previously described [18], after CNBr-activation of the polysaccharide. Briefly, 15 mg HA, CS or heparin was activated at pH 11 by 8 mg CNBr for 5 min. The activated polysaccharide was separated from the reaction mixture on a small column of Sephadex G25 (PD 10, Pharmacia, Uppsala, Sweden) equilibrated with 0.2 M borate buffer (pH 8.0). The activated polysaccharide was incubated over night with 1 mg tyrosine (T) (Sigma Chemical Company, St Louis, USA). The T bound to HA (T-HA), CS (T-CS) or heparin (T-Hep) was separated from unbound T on a PD 10 column equilibrated with phosphate buffered saline (pH 7.5) (PBS), containing NaCl (8 $g l^{-1}$), KCl (0.2 $g l^{-1}$), $KH_2PO_4$ (0.2 $g l^{-1}$) and $Na_2HPO_4$ (1.15 $g l^{-1}$).

A part of the T-HA, T-CS or T-Hep was iodinated with $^{125}I$ by placing 100 μg of T-labelled polysaccharide together with 0.5 mCi $^{125}I$ in a small glass tube covered with a film of 10 μg 1,3,4,6-tetrachloro-3α,6α-diphenylglycouril (Sigma Chemical Company, St Louis, USA). Unincorporated $^{125}I$ was removed on a PD 10 column equilibrated with PBS and the iodinated T-HA ($^{125}I$-T-HA), T-CS ($^{125}I$-T-CS) or heparin ($^{125}I$-T-Hep) stored at 5 °C. The specific radioactivity was usually 1500–5000 dpm $ng^{-1}$.

The $^{125}I$-T-HA kept a high molecular weight-profile upon gel filtration chromatography with a mean Mw of around $0.5 \times 10^6$ Da, and was found to be cleared from the circulation with the kinetics and organ distribution reported for biosynthetically labelled HA of high Mw. The $^{125}I$-labelled T-HA was also taken up by isolated rat liver endothelial cells both *in vivo* and *in vitro*, indicating that the labelling does not interfere with the binding to specific cell-surface receptors found on these cells [1, 2, 9–11].

The $^{125}$-T-Hep was, by gel filtration chromatography on Sephacryl S-1000 and S-300 calibrated with HA standards, found to have the same mean Mw as the unlabelled heparin ($\sim$20 000 Da) and showed a similar size distribution pattern.

### Cells

A single cell suspension was prepared from the liver of Sprague Dawley rats, weighing 200–300 g, by collagenase perfusion for 10 min at 37 °C. Liver endothelial cells, Kupffer cells and parenchymal cells were purified by Percoll®-centrifugation and selective adherance as described by Pertoft and Smedsrød [19], giving approximately 95% pure cells [10, 19]. Monolayer cultures were maintained under standard culturing conditions in RPMI medium supplemented with L-glutamine (2 mM), gentamicin (50 $\mu g ml^{-1}$) and, in the case of parenchymal cells, 10% (v/v) foetal calf serum. Liver endothelial cells were cultured entirely without serum. All cells were cultivated over night before the start of the experiments.

### Uptake studies with cells in culture

$^{125}I$-T-hyaluronan, and in competition experiments unlabelled polysaccharides, were added to cold RPMI medium containing L-glutamine (2 mM) and gentamicin (50 $\mu g ml^{-1}$), and given to cultures of 100 000–250 000 liver endothelial cell $cm^{-2}$ in fibronectin-coated dishes with a diameter of 16 mm. The cultures were kept under standard culturing conditions in 300 μl medium.

After the termination of the incubations, the medium was removed and analysed for radioactivity, thereafter it was in some experiments subjected to gel chromatography on a 24 ml Sephacryl 5300 column to separate degraded from undegraded polysaccharide. The cells washed three times in phosphate buffered saline (pH 7.5) (PBS), containing NaCl (8 $g l^{-1}$), KCl (0.2 $g l^{-1}$), $KH_2PO_4$ (0.2 $g l^{-1}$) and $Na_2HPO_4$ (1.15 $g l^{-1}$), analysed for radioactivity, or homogenized and fractionated as described earlier [18]. Unspecific binding was corrected for by measurement of radioactivity associated to dishes without cells, which generally was just above background levels.

### In vivo studies

Sprague Dawley rates, weighing 200–300 g, were anaesthetized with pentobarbital (45 mg $kg^{-1}$ body weight). They received an injection in the tail vein of 5 μg $^{125}$I-T-HA, $^{125}$I-T-CS or $^{125}$I-T-Hep (8–15 × $10^6$ cpm), in 0.8–1.0 ml 0.15 M NaCl, 10 mM $NaH_2PO_4$ (pH 7.4). In some studies the rats received 1–5 mg unlabelled polysaccharides 30 s prior to the labelled polysaccharide. Blood samples were repeatedly collected from the distal part of the tail during the circulation period. Total blood volume was estimated as 6% of the body weight. In some cases serum was subjected to size exclusion chromatography on a Sephacryl S 300 and the radioactivity of the eluate analysed.

After 10 min–22 h the rat was killed. Liver, lungs, kidneys, heart, spleen and in some instances urine were assayed for radioactivity. The data were processed using a Macintosh SE/30®, Macintosh IIsi® or Macintosh 7200 computer (Apple Computer Inc., Cupertino, CA, USA). The graphs were constructed using the Cricket Graph® program (version 1.3, Cricket software, Malvern, PA, USA) and Canvas (version 3.5, Deneba Systems Inc., Miami, FL, USA). Statistical analysis was performed using Statworks® (version 1.1, Cricket Software, Malvern, PA, USA).

### Scintigraphic studies

The rats were anaesthetized and injected as described above. In dynamic studies the injections were made with the rats placed on a Fuji phosphoimager screen with a high resolution brass collimator between rat and screen. The screen was exposed for 10 min and the image developed and analysed on a Fuji 2000 phospho imager. Some image analysis studies were performed using the NIH Image software.

## Results

A tracer dose of intravenously administered $^{125}$I-T-HA was by phosphoimaging shown to be rapidly cleared by the rat liver (Figure 1). A small amount of radioactivity, attributable to small amounts of $^{125}$I-T lost from the labelled polysaccharide, could be visualized in the urinary bladder (Figure 1). During 22 h, ~20% of the radioactivity disappeared from the liver and could be found in the urine (bedding in the cage).

When CS, at a dose of about 20 mg $kg^{-1}$ body weight, was administered prior to $^{125}$I-T-HA the rapid clearance was lost and the major part of radioactivity could be visualized scattered over the entire animal for hours (Figure 1). The radioactivity after CS blocking was mainly found in the blood with some uptake in liver, spleen and kidney (Figure 2a) and was found to rapidly decrease in Mw (Figure 3). Some labelled polysaccharides with Mw of about 10 000–40 000 Da were found in urine (Figures 2a & 3). Only minute amounts of labelled HA could be found in the urine when liver uptake was blocked by unlabelled HA (20 mg $kg^{-1}$ b.w.) (Figure 2a). The rapid decrease in Mw of circulating $^{125}$I-T-HA seen after CS blocking was not seen with HA blocking and more radioactivity stayed within the general circulation after HA blocking than after CS blocking (Figures 2b & 4). However, trace amounts of radioactivity could be found in the urine after 70 min, this material had similar Mw as the radioactivity found in urine after CS blocking (Figures 2a, 3 & 4).

Dextran sulphate was found to effectively block liver uptake at a dose of 200 mg $kg^{-1}$ b.w., and result in increased outflow of labelled HA from the general circulation as seen by low liver uptake and low recovery of injected HA (Figure 5). When a dose of 20 mg $kg^{-1}$ b.w. was tested it was found that the liver uptake was inhibited by 30–40% (Figure 5). When we tried to label DxS with the same method as for HA, virtually no labelling was achieved (not shown), probably due to the high degree of sulphation of this artificial polysaccharide.

Heparin at a dose of 20 mg $kg^{-1}$ b.w. did not significantly affect the clearance of $^{125}$I-T-HA (Figure 2a), and biodistribution of label was as previously reported for HA with similar labels and Mw:s [18, 21, 22], nor could HA at a dose of 20 mg $kg^{-1}$ bw. inhibit the clearance of $^{125}$I-T-Hep at a tracer dose (Figure 6). Chondroitin sulphate could partially inhibit the liver uptake of $^{125}$I-T-Hep (Figure 6).

The binding of $^{125}$I-T-HA to LEC in culture was effectively inhibited by HA, CS and DxS (Figure 7).

When the biodistribution of intravenous $^{125}$I-T-CS was studied it was found that the liver uptake was lower than for $^{125}$I-T-HA, as was the total recovery of radioactivity, while the urinary excretion was high (Figure 8). The liver uptake could effectively be inhibited by unlabelled CS and HA, resulting in increased urinary clearance (Figure 8).

## Discussion

That the receptor mediated endocytosis of HA by LEC is not specific for HA has earlier been shown by experiments with isolated LEC in culture [13–15]. In such studies the receptors also recognize ligands such as CS and DxS. The present investigation was performed in order to see if some negatively charged polysaccharides will influence the

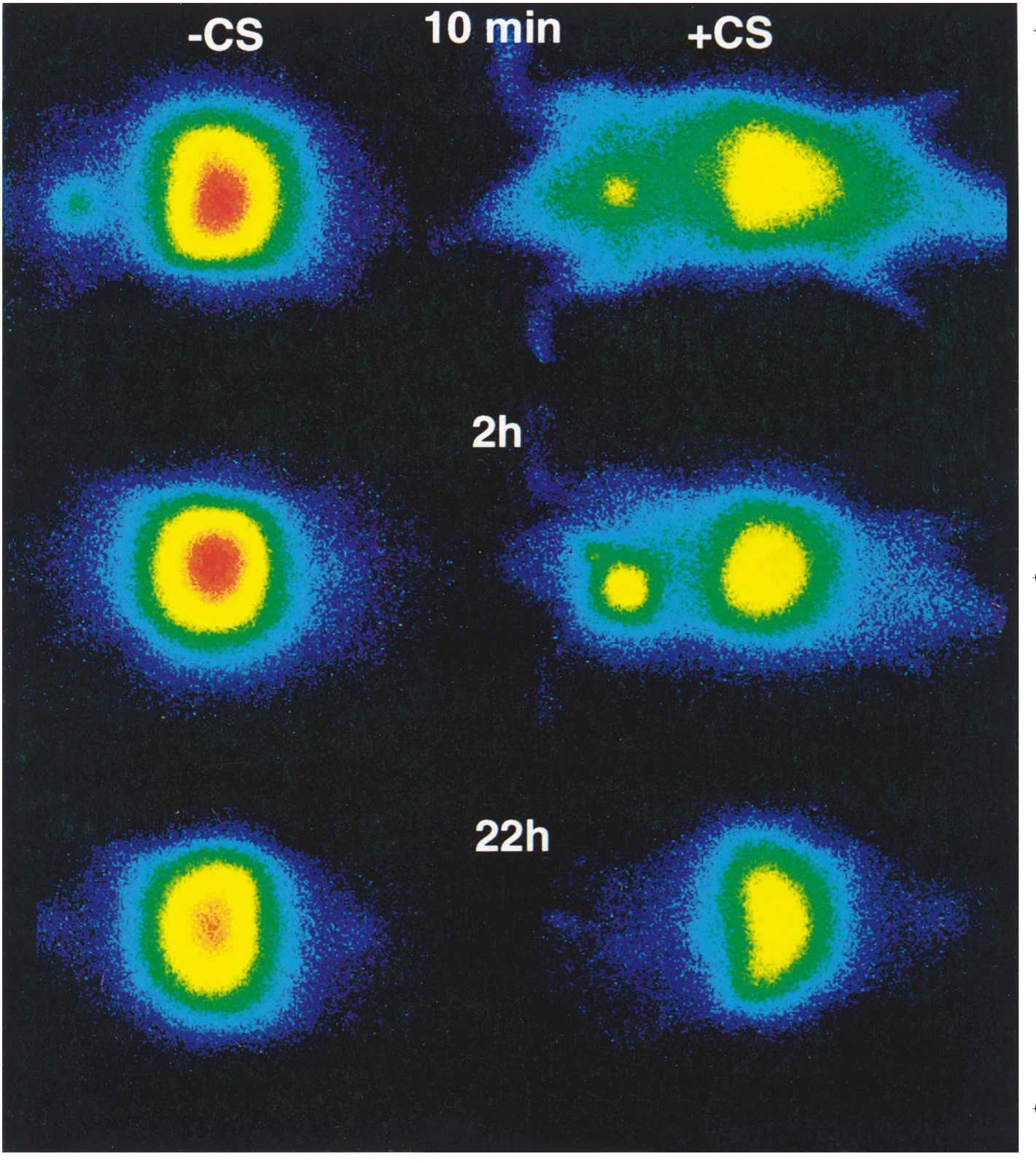


**Figure 1.** Phosphoimages of rats receiving 5 μg $^{125}$I-T-HA with or without CS pretreatment (5 mg 30 s prior to labelled HA). The two rats were injected with $^{125}$I-T-HA at the same time, placed side by side on the screen and the screen exposed for 10 min at the indicated mean time points. Radioactivity is indicated by colour, red > orange > yellow > green > blue > violet. See Materials and Methods for details.

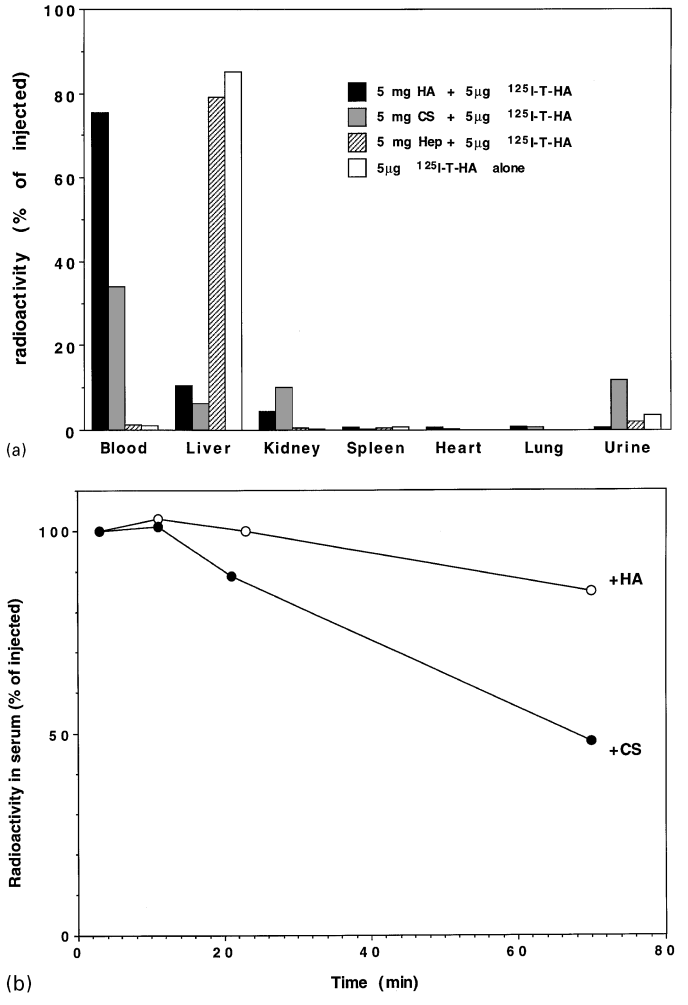


**Figure 2.** (a) The recovery of radioactivity in rat blood, liver, kidney, spleen, heart, lung and urine, 70 min after an intravenous injection of the indicated unlabelled polysaccharides and 5 μg $^{125}$I-labelled hyaluronan (mean Mw approximately 400 000 Da). Details are to be found in the Materials and Methods section. (b) Loss of radioactivity from serum of rats receiving intravenous injections of 5 mg chondroitin sulphate (+CS) or 5 μg hyaluronan (+HA), 30 s prior to 5 μg $^{125}$I-labelled hyaluronan.

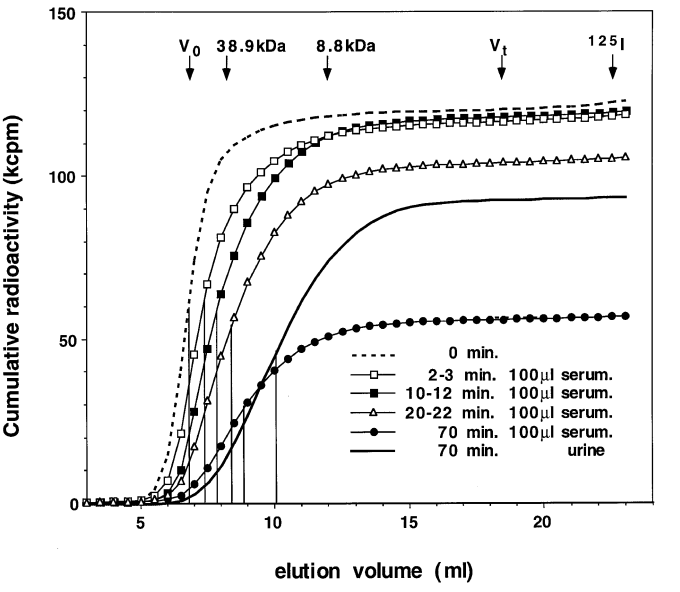


**Figure 3.** Size-exclusion chromatography on a 24 ml Sephacryl S-300 column of rat serum produced from blood drawn at the indicated times after an intravenous injection of 5 mg unlabelled CS and 5 μg $^{125}$I-labelled hyaluronan. A sample of the urine ( ~ 4–5% of total), collected at 70 min after injection, was also analysed. Chromatography of $^{125}$I-T-HA was used to determine the $V_0$ position. The total volume of the column ($V_t$) was determined by $^3$H-$H_2O$. The half heights of the curves are indicated and give Mw values of approximately 37 kDa, 33 kDa and 24 kDa for the 20–22 min serum sample, 70 min serum sample and 70 min urine sample, respectively. The position of HA standards of defined mean Mw:s, as well as the position of free $^{125}$I are also indicated. See Materials and Methods for details.

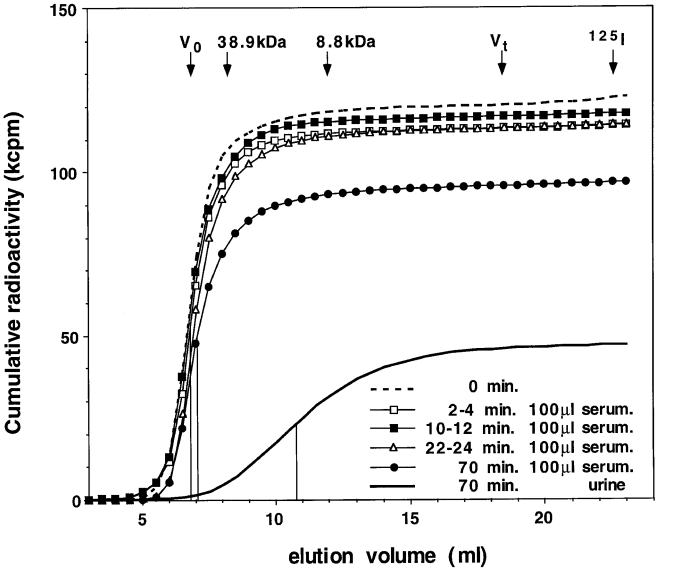


**Figure 4.** Size-exclusion chromatography on a 24 ml Sephacryl S-300 column of rat serum produced from blood drawn at the indicated times after an intravenous injection of 5 mg unlabelled HA and 5 μg $^{125}$I-labelled hyaluronan. A sample of the urine (total amount recovered), collected at 70 min after injection, was also analysed and the half height of the curve indicated a Mw of approximately 18 kDa. See legend for Figure 3 and Materials and Methods for more details.

turnover of circulating HA *in vivo*. We have used a labelling technique that does not alter the Mw nor interfere with the cell binding properties of polysaccharides and results in a derivative with $\gamma$-radiation of high specific activity [18, 20–23]. Such a polysaccharide is advantageous in many respects e.g. it is easy to detect in low amounts and the distribution can be recorded in the live animal using scintigraphic or phosphoimaging techniques. We choose the rat for these studies as many turnover and uptake studies have earlier been performed with this species and normal blood levels and estimated turnover rates are similar to the ones found in man [1, 3, 9, 10, 24].

Our studies show that CS and DxS, but not heparin, inhibit the clearance of HA from the bloodstream via inhibition of the receptor mediated endocytosis by the liver

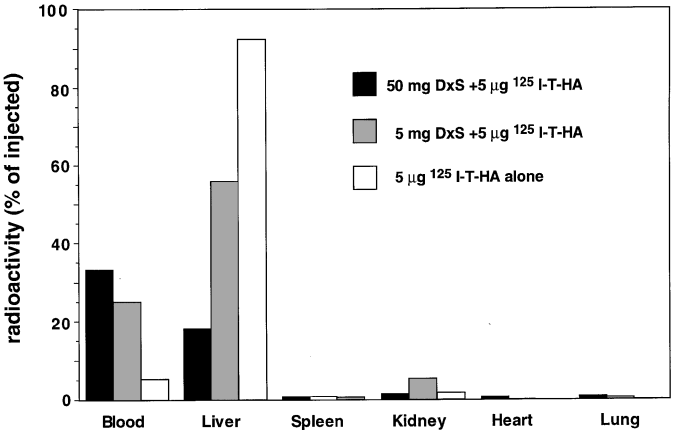


**Figure 5.** The recovery of radioactivity in rat blood, liver, kidney, spleen, heart and lung, 15 min after an intravenous injection of the indicated amount of unlabelled DxS and 5 μg $^{125}$I-labelled HA. Further details are found in the Materials and Methods section.

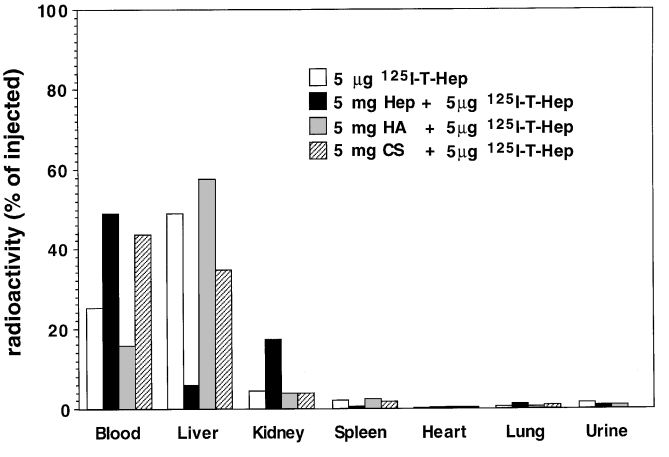


**Figure 6.** The recovery of radioactivity in rat blood, liver, kidney, spleen, heart and lung, 15 min after an intravenous injection of the indicated unlabelled polysaccharides and 5 μg $^{125}$I-labelled heparin. See the Materials and Methods section for details.

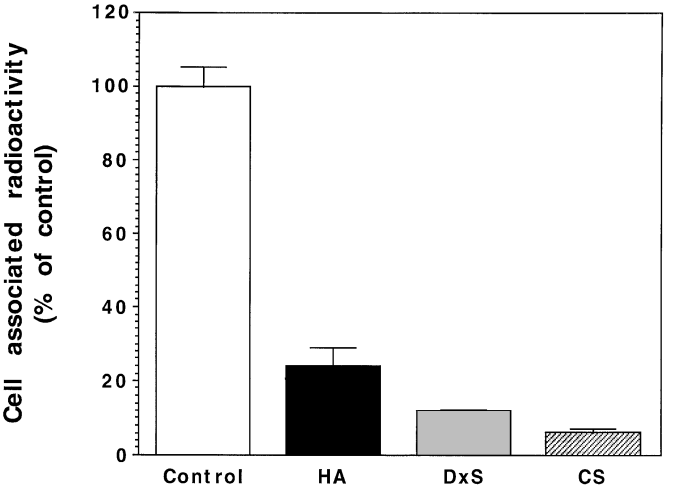


**Figure 7.** Cell association of $^{125}$I-T-HA to liver endothelial cells in culture. Approximately 300 000 cells were incubated for 60 min at 37 °C with 1 μg ml$^{-1}$ labelled hyaluronan after a preincubation for 15 min with 50 μg ml$^{-1}$ unlabelled HA, 50 μg ml$^{-1}$ DxS, 50 μg ml$^{-1}$ CS or medium alone (Control). Results are mean ± SD of three determinations. The labelling, recovery of radioactivity and liver cell separations are described in the Materials and Methods section.

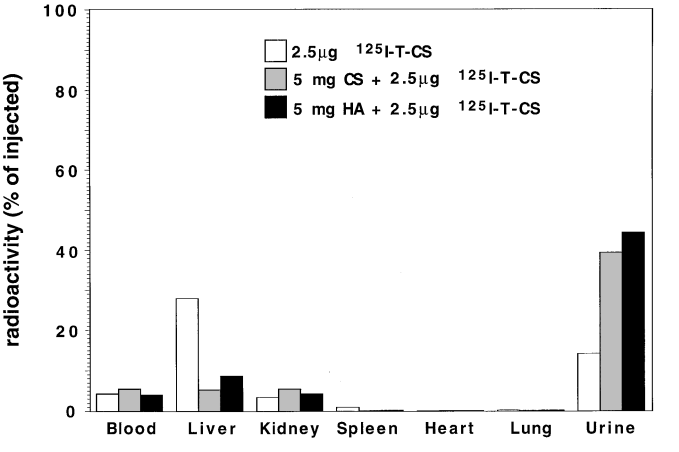


**Figure 8.** The recovery of radioactivity in rat blood, liver, kidney, spleen, heart, lung and urine, 70 min after an intravenous injection of the indicated unlabelled polysaccharides and 2.5 μg $^{125}$I-labelled chondroitin sulphate. See Materials and Methods for details.

(Figures 1, 2a & 5). Heparin seems to be cleared by mechanisms not affected by HA, while CS seems to partially reduce the liver uptake of labelled heparin (Figure 6), but the inhibition is only partial and not as effective as the blocking of labelled HA. As a result of CS or DxS blocking of liver HA-receptors, the labelled HA that remains in the circulation is rapidly broken down to smaller fragments by what seems to be a specific and saturable mechanism, as high concentrations of HA inhibits this degradation (Figures 3 & 4). The fragmentation results in low recovery of injected dose (Figures 2a, b & 5) and the low Mw HA is filtered out into the tissues and via the kidneys out into the urine (Figures 1, 2a, 3 & 4). We have done the size determination of circulating material by size exclusion chromatography of serum or plasma on a column of Sephacryl S-300. This gel will not separate HA above 50 kDa very well so it is possible that the breakdown of the injected material with a Mw of about 400 kDa is broken down by shear forces to a Mw about 50 kDa when unlabelled HA is given to reduce liver uptake. However, the breakdown to smaller fragments, resulting in the appearance of material chromatographing at an included position, occurs early after injection only in the case of CS blocking and not to any great extent when unlabelled HA is used as blocking agent (Figures 3 & 4).

That CS and HA are recognized by the same receptors in the liver is also shown by the fact that not only unlabelled CS but also HA can inhibit the liver uptake of $^{125}$I-T-CS (Figure 8). That the liver uptake of CS is not as high as that of HA probably depends on the fact that the CS used only has a Mw of around 30 kDa compared to about 400 kDa for the HA, and some is therefore rapidly removed from the circulation by filtration so that only a fraction of that injected remains long enough in the general circulation to be taken up by the liver.

Dextran sulphate seems also to bind to the same receptors as CS and HA but with lower affinity as a higher dose is needed to inhibit liver uptake of $^{125}$I-T-HA using DxS than using CS or HA (Figure 5), despite the fact that the Mw is about ten times higher for DxS than for CS.

Our results indicate that the turnovers of the naturally occuring polysaccharides HA and CS are partially an effect of liver uptake of the circulating polysaccharides via a common receptor on LEC. It is therefore possible, that high levels of circulating HA in some conditions can be secondary to increased outflow of CS into the general circulation from the tissues, and vice versa. The present results also argue, due to the presence of a more specific degradative mechanism of HA in the circulation, that if the LEC are blocked by CS, the urinary excretion of HA should be grossly increased in relation to the effect on serum levels, while increases in serum levels of HA due to increased outflow of HA into the circulation or decreased clearance by the liver, would result in only moderate increases in urinary excretion. Such a lack of correlation between serum levels and urinary excretion has been described earlier in a study of rheumatoid arthritis (RA), primary biliary cirrhosis (PBC) and Werners syndrome [25]. All three diseases cause increased serum levels of HA. However, the urinary excretion in RA and PBC is only slightly increased, whereas a tenfold increase in excretion is seen in Werners syndrome, despite the fact that the serum level in this disease was lower than for PBC and RA.

The presence of a saturable degradative mechanism for HA in the circulation could also explain why circulating HA, under normal conditions, has a much lower Mw than the HA entering the general circulation via the lymph [6, 26]. Saturation of the mechanism, as well as differences in activity, could also explain the presence of high Mw HA in the serum of patients with Wilms tumour as well as serum HA of oligosaccharide size in patients with bone metastasizing renal tumours [27].

Size reduction of the polymer in the circulation by mechanical shearing has been suggested as a mechanism by Fraser [26]. However, the Mw and concentration of the unlabelled HA used to block liver uptake in the present study makes it unlikely that the viscosity was influenced to such an extent that breakdown by shearing would be inhibited. Fraser has stated that degradation by serum hyaluronidase does not occur under physiological conditions, that free radical activity in serum would be too low to cause the size reduction and that the degradation of high Mw HA is not inhibited by high doses of low Mw HA [26]. The present study also argues against free radical attack as the degradation occurs in the presence of a high dose of CS that probably would scavenge any active free radicals. The site of degradation could be via a hyaluronidase, fixed on a cell surface and with a Mw-dependent binding of HA necessary for activity. Further studies are needed to characterize this mechanism that might prove to be an important part of HA metabolism.

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## References

1 Laurent TC, Fraser JRE (1992) *FASEB J* **6**: 2396–404.
2 Knudson CB, Knudson W (1993) *FASEB J* **7**: 1233–41.
3 Engström-Laurent A (1989) In *The Biology of Hyaluronan*, *Ciba Foundation Symposium*, *143* (Evered D, Whelan J, eds) pp. 233–47 Chichester: Wiley.
4 Laurent UBG, Laurent TC (1981) *Biochem Int* **2**: 195–9.
5 Fraser JRE, Kimpton WG, Laurent TC, Cahill RNP, Vakakis N (1988) *Biochem J* **256**: 153–8.
6 Tengblad A, Laurent UBG, Lilja K, Cahill RN, Engström-Laurent A, Fraser JRE, Hansson HE, Laurent TC (1986) *Biochem J* **236**: 521–5.
7 Fraser JRE, Laurent TC, Pertoft H, Baxter E (1981) *Biochem J* **200**: 415–24.
8 Fraser JRE, Engström-Laurent A, Nyberg A, Laurent TC (1986) *J Lab Clin Med* **107**: 79–85.
9 Fraser JRE, Alcorn D, Laurent TC, Robinson AD, Ryan GB (1985) *Cell Tiss Res* **242**: 505–10.
10 Smedsröd B, Pertoft H, Gustafson S, Laurent TC (1990) *Biochem J*. **266**: 313–27.
11 Eriksson S, Fraser JRE, Laurent TC, Pertoft H, Smedsröd B (1983) *Exp Cell Res* **144**: 223–8.
12 Smedsröd B (1991) *Adv Drug Deliv Rev* **7**: 265–78.
13 Smedsröd B, Pertoft H, Eriksson S, Fraser JRE, Laurent TC (1984) *Biochem J* **223**: 617–26.
14 Raja RH, McGary CT, Weigel PH (1988) *J Biol Chem* **263**: 16661–8.
15 McGary CT, Raja RH, Weigel PH (1989) *Biochem J* **257**: 875–84.
16 Lebel L, Smith L, Risberg B, Laurent TC, Gerdin B (1989) *Am J Physiol* **256**: (*Heart Circ Physiol* **25**): H1524–31.
17 Lindahl U, Cifonelli JA, Lindahl B, Rodén L (1965) *J Biol Chem* **240**: 2817–20.
18 Gustafson S, Björkman T, Westlin J-E (1994) *Glycoconjugate J* **11**: 608–13.

19 Pertoft H, Smedsröd B (1987) In *Cell Separation: Methods and Selected Applications* (Pretlow TG and Pretlow TP, eds) pp. 1–24. New York: Academic Press.
20 Glabe CG, Harty PK, Rosen SD (1983) *Anal Biochem* **130**: 287–94.
21 Gustafson S, Forsberg N, McCourt P, Wikström T, Björkman T, Lilja K (1994) In *First International Workshop on Hyaluronan in Drug Delivery* (Willoughby DA, ed.) pp. 43–59. London: Royal Society of Medicine Press Ltd.
22 Westerberg G, Bergström M, Gustafson S, Lindqvist U, Sundin A, Långström B (1995) *Nucl Med Biol* **22**: 251–6.
23 Gustafson S., Björkman T, Forsberg N, Lind T, Wikström T, Lidholt K (1995) *Glycoconjugate J* **12**: 350–5.
24 Fraser JRE, Laurent TC (1989) In *The Biology of Hyaluronan, Ciba Foundation Symposium, 143*. (Evered D, Whelen J, eds) pp. 41–52. Chichester: Wiley.
25 Laurent TC, Lilja K, Brunnberg L, Engstöm-Laurent A, Laurent UBG, Lindqvist U, Murata K, Ytterberg D (1987) *Scand J Clin Lab Invest* **47**: 793–9.
26 Fraser JRE (1995) In *Second International Workshop on Hyaluronan in Drug Delivery* (Willoughby DA, ed.) pp. 8–10. London: Royal Society of Medicine Press Ltd.
27 Kumar S, West DC, Ponting JM, Gattamaneni HR (1989) *Int J Cancer* **44**: 445–8.

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