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Studies *in vitro* on the uptake and degradation of sodium hyaluronate in rat liver endothelial cells

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Rat liver endothelial cells in primary cultures at 7°C bind radioactively labelled sodium hyaluronate (HA; M_r 400000) specifically and with high affinity ($K_d = 6 \times 10^{-11}$ M). Maximal binding capacity is approx. 10^4 molecules per cell. Inhibition experiments with unlabelled HA and oligosaccharides from HA indicate that each molecule is bound by several receptors acting co-operatively and that the single receptor recognizes a tetra- or hexa-saccharide sequence of the polysaccharide. At 37°C the liver endothelial cells endocytose the HA. The process combines the features of a receptor-mediated and a fluid-phase endocytosis. The rate of internalization does not show any saturation with increasing HA concentration, but is approximately proportional to the polysaccharide concentration at and above the physiological concentration. At 50 μg of free HA/l each liver endothelial cell accumulates 0.1 fg of the polysaccharide/min. Fluorescent HA accumulates in perinuclear granules, presumably lysosomes. Degradation products from HA appear in the medium about 30 min after addition of the polysaccharide to the cultures. The radioactivity from HA containing *N*-[^{3}H]acetyl groups or ^{14}C in the sugar rings is recovered mainly as [^{3}H]acetate and [^{14}C]lactate respectively. Estimations of the capacity of liver endothelial cells to internalize and degrade HA *in vitro* indicate that these cells may be primarily responsible for the clearance of HA from human blood *in vivo*.

Sodium hyaluronate (HA) injected intravenously into rabbit and man is rapidly cleared from the bloodstream (Fraser *et al.*, 1981, 1984). It is mainly taken up by the liver, as shown by analysis of rabbit tissues (Fraser *et al.*, 1981) or by autoradiography in mice (Fraser *et al.*, 1983). The uptake occurs in the non-parenchymal-cell fraction (Fraser *et al.*, 1981).

Primary cultures of non-parenchymal rat liver cells, seeded on fibronectin-coated dishes, contain mainly liver endothelial cells (Eriksson *et al.*, 1983; Smedsrød *et al.*, 1982; B. Smedsrød & H. Pertoft, unpublished work). A detailed characterization of such cultures (B. Smedsrød, H. Pertoft, G. Eggertsen & C. Sundström, unpublished work) showed at least 90–95% of the cells with the morphology characteristic of liver endothelium. A useful tool for identifying these cells is the uptake of fluorescent ovalbumin, which seems to be specific for the liver endothelial cells (Eriksson *et al.*, 1983; Smedsrød *et al.*, 1982).

A preliminary study showed that cultured liver endothelial cells efficiently degrade HA into low-M_r products, in contrast with cultures of hepatocytes or Kupffer cells produced by well-established techniques (Eriksson *et al.*, 1983). In the present paper we describe a more detailed study of the uptake and degradation of labelled HA in primary cultures of liver endothelial cells.

Abbreviation used: HA, hyaluronic acid (sodium hyaluronate).

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Materials and methods

Polysaccharides

HA, one fraction with M_r of several millions (Healon) and another fraction with M_r 400000 were obtained from Pharmacia AB, Uppsala, Sweden. Oligosaccharides of HA were prepared essentially as described by Hascall & Heinegård

(1974). HA (900mg) dissolved in 100ml of 0.5M-sodium acetate buffer, pH5.2, was digested at 37°C for 22h with 2500 units of bovine testicular hyaluronidase (Leo, Hälsingborg, Sweden). The reaction was terminated by boiling. The oligosaccharides were separated on a column (3cm × 250cm) of Sephadex G-50 (superfine grade) (Pharmacia) equilibrated in 1M-NaCl. The oligosaccharide fractions, identified by their elution positions, were desalted by chromatography on Sephadex G-15, and freeze-dried.

Samples of chondroitin sulphate (Wasteson, 1971), dermatan sulphate (Teien *et al.*, 1976) and heparan sulphate (Iverius, 1971) were as described in the references. Heparin (stage 14, isolated from pig intestinal mucosa) obtained from Inolex Pharmaceutical Division, Park Forest South, IL, U.S.A., was purified by repeated precipitation with cetylpyridinium chloride from 1.2M-NaCl (Lindahl *et al.*, 1965). The samples of chondroitin sulphate and dermatan sulphate were digested with hyaluronidase from *Streptomyces hyalurolyticus* (Ohya & Kaneko, 1970; Seikagaku Kogyo Ltd., Tokyo, Japan) to remove traces of HA: 1mg of polysaccharide in 1ml of phosphate-buffered saline (NaCl, 8000mg/l; KCl, 200mg/l; KH_2PO_4, 200mg/l; Na_2HPO_4, 1150mg/l) was incubated with 2 turbidity-reducing units of enzyme for 18h at 37°C. The digestion was terminated by boiling for 10min.

Proteins

Bovine serum albumin (fraction V, A-4503), ovalbumin (A-5503), alanine aminotransferase from pig heart (EC 2.6.1.2; G-9880; 74 units/mg of protein) and lactate dehydrogenase from the same tissue (EC 1.1.1.27; type XVIII; L-2881; 525 units/mg of protein) were all from Sigma (St. Louis, MO, U.S.A.). According to the manufacturer, the glycoprotein ovalbumin contained 9 mannose residues per molecule. Human fibronectin was kindly prepared by Dr. Staffan Johansson by the method of Vuento & Vaheri (1979).

Labelled compounds

Radioactive HA was prepared by biosynthetic labelling of cultured synovial cells as described by Fraser *et al.* (1981). HA with ^{3}H-labelled *N*-acetyl groups, obtained by incubating the cells in the presence of [^{3}H]acetate, had a specific radioactivity of about 5×10^5d.p.m./μg. ^{14}C-labelled polymer was prepared with D-[U-^{14}C]glucose or D-[U-^{14}C]glucosamine as precursors, and had a specific radioactivity of approx. 2.5×10^4d.p.m./μg. More than 99% of the labelled preparation was susceptible to bacterial hyaluronidase. The radioactive sugars, [^{3}H]acetic acid (300Ci/mol), [U-^{14}C]acetic acid (58Ci/mol), L-[U-^{14}C]lactic acid (161Ci/mol) and L-[2,3-^{3}H]-alanine (36Ci/mol) were from Amersham International, Amersham, Bucks., U.K.

The weight-average and number-average molecular weights of the main batch of [^{3}H]HA, which was used to study uptake by liver endothelial cells, were 4×10^5 and 1×10^5 respectively, as determined by gel chromatography (Laurent & Granath, 1983).

HA (Healon) was labelled with fluorescein amine as described by de Belder & Wik (1975). The labelling was kindly performed by Dr. A. N. de Belder, Pharmacia AB. The polymer had M_r 2×10^6–3×10^6 and a degree of substitution of 5.5×10^{-3} fluorescein groups per sugar residue.

Cell cultures

The preparation of liver endothelial cells, Kupffer cells and hepatocytes has been described (Eriksson *et al.*, 1983; Smedsrød *et al.*, 1982). The cells obtained are viable and separated from each other with less than 5% cross-contamination. The cells were grown on plastic dishes with surface areas of 2.0, 9.6 or 28.2cm^2 (Linbro; Flow Laboratories, Irvine, Scotland, U.K.). They were incubated in 0.2, 0.6 and 1.5ml, respectively, of RPMI medium (Flow Laboratories) containing 100 units of penicillin/ml, 50μg of streptomycin/ml and 1% bovine serum albumin or, in prolonged incubations, 20% (v/v) newborn-calf serum. The calf serum contained about 600ng of endogenous HA/ml as determined by a radiosorbent assay (Laurent & Tengblad, 1980; Engström-Laurent *et al.*, 1983).

Liver endothelial cells and hepatocytes were grown on dishes coated with fibronectin (10μg/cm^2). After seeding of 0.5×10^6 cells (suspended in 150μl) per cm^2, the final endothelial-cell density was approx. 200000/cm^2. The hepatocytes were cultivated at a density of about 100000 cells/cm^2. Pure Kupffer cells were grown directly on plastic at densities of approx. 70000 cells/cm^2. In experiments designed to compare different types of cells, 5μg of fibronectin/ml was added to the final medium of the Kupffer-cell cultures to equalize the conditions as far as possible.

Other growth conditions, microscopy, cell counting, cytochemical methods etc. have been described (Eriksson *et al.*, 1983; Smedsrød *et al.*, 1982).

Determination of radioactivity

Radioactivity was measured by liquid scintillation in a Searle/Nuclear-Chicago Isocap/300 liquid scintillation counter. To 0.5 and 1 ml samples were added 5 or 10ml, respectively, of scintillation fluid (Instagel; Packard–Becker B.V., Groningen, The

Netherlands). The efficiency was determined with commercially available standards.

Determination of binding of [^{3}H]HA to liver cells at 7°C

The cells were seeded on 9.6cm^2 or 28.2cm^2 dishes and were then incubated in medium that contained various concentrations of [^{3}H]HA and bovine serum albumin (no calf serum). In competition experiments, unlabelled HA of M_r 400000, oligosaccharides from HA, chondroitin sulphate, dermatan sulphate, heparan sulphate, heparin or ovalbumin were added to a final concentration of 50μg/ml.

After incubation overnight at 7°C the medium was removed and the cell layer was washed four or five times with phosphate-buffered saline. The cells were dissolved in 1ml of 0.3M-NaOH/1% sodium dodecyl sulphate and the samples were then neutralized with 2M-HCl and analysed for radioactivity.

Accumulation and degradation of [^{3}H]HA in liver cells at 37°C

Cells were incubated with [^{3}H]HA as described above (except for the temperature) on plastic dishes with a growth area of 2cm^2 for chosen time periods up to 8h. Unlabelled saccharides or ovalbumin were added to the cultures as specified in the Results section.

After termination of the experiments, the cell fractions were recovered as described above. The culture medium and washings were combined and chromatographed on a 9ml column of Sephadex G-25 (PD-10, Pharmacia) equilibrated with phosphate-buffered saline. The radioactivity separated into two distinct peaks, one in the void volume and one in the low-M_r region. The proportion of degraded HA in the culture was calculated from the relative proportions of the two peaks.

Isolation of degradation products

Liver endothelial cell cultures in 9.6cm^2 dishes were incubated under standard conditions with 2–3μg of radioactive HA for 20h at 37°C. The medium from the cultures was chromatographed on a column (1cm × 100cm) of Sephadex G-15 (Pharmacia) equilibrated with distilled water. The flow rate was 0.3ml/min, and 0.6ml fractions were collected. Both ^{3}H- and ^{14}C-labelled products emerged as sharp peaks at $K_{av.}$ approx. 0.25 (i.e. less retarded than the same components eluted with a physiological salt solution; Eriksson *et al.*, 1983). The isolated products were used directly for identification studies.

Accumulation of fluorescein-amine-labelled HA into liver endothelial cells

Liver endothelial cells were cultured under the conditions described above, with 1mg of fluorescent HA/ml in the medium. The experiment was terminated after 20h. The cells were washed five times before fixation and mounted for examination by fluorescence microscopy as described by Smedsrød *et al.* (1982).

Electrophoresis and chromatography

High-voltage electrophoresis was performed in veronal buffer, pH8.6, *I*0.075, on 40cm paper strips (Whatman 3MM). Samples (100μl) containing 1000c.p.m. of ^{14}C or 8000c.p.m. of ^{3}H were separated at 100V/cm for 30min. The paper was then dried with a hair-drier and cut into 1cm-wide strips, which were soaked with 0.5ml of water, mixed with scintillation fluid and counted for radioactivity.

Paper chromatography was done at room temperature in butanol/ethanol/water (10:3:5, by vol.) or ethyl acetate/acetic acid/water (3:1:1, by vol.) for 24 or 16h respectively. The papers were dried and analysed for radioactivity as described above.

High-performance liquid chromatography was performed on an Aminex HPX-87H organic acid column (Bio-Rad Laboratories, Richmond, CA, U.S.A.) in an instrument from Constametric Laboratory Data Control (Division of Milton Roy, Riviera Beach, FL, U.S.A.). Aminex HPX-87H is a cation exchanger and separates the acids by ion exclusion and partition chromatography. The column was eluted with 5mM-H_2SO_4 according to the description provided by the manufacturer.

Conversion of lactate into alanine

The enzymic conversion of ^{14}C-labelled degradation products into alanine by the combined action of lactate dehydrogenase and alanine aminotransferase was studied as follows. To three 1ml incubation mixtures containing 0.04M-Tris/HCl buffer, pH7.4, 0.01M-sodium glutamate, 1.7mg of NAD^+ and 5000c.p.m. of ^{14}C-labelled product were added, respectively: (1) 25 units of lactate dehydrogenase and 25 units of alanine aminotransferase; (2) 25 units of alanine aminotransferase; (3) 25 units of lactate dehydrogenase. A fourth sample was identical with incubation mixture (1), except that 7500c.p.m. of standard L-[^{14}C]lactate was added. The samples were incubated for 1h at room temperature and were then acidified to pH2.5 with 3M-acetic acid. Each incubation mixture was applied to a 5ml column of Dowex 50W-X2 (200–400 mesh; Dow Chemical Co., Midland, MI, U.S.A.), which was then washed with 5ml of 0.04M-Tris/HCl buffer, pH7.4, followed by 5ml of water. Adsorbed material was eluted with 0.25M-ammonia.

Results

Binding of [^{3}H]HA to liver endothelial cells at 7°C

Cultures of liver endothelial cells (9.6 or 28.2cm^2) were incubated with various concentra-

tions of [^{3}H]HA overnight at 7°C, and cell-associated radioactivity was measured as described in the Materials and methods section. In parallel experiments the same amounts of radioactive HA were mixed with 50 μg of unlabelled HA (M_r 400000)/ml. At [^{3}H]HA concentrations of less than 1 μg/ml, the excess of unlabelled HA decreased the binding of labelled polymer to the cells by over 95%, i.e. unspecific background binding could be neglected. When the concentrations of free [^{3}H]HA exceeded 1 μg/ml, a considerable unspecific binding occurred that could not be prevented by the addition of unlabelled HA. No HA bound to fibronectin-coated dishes without cells in the presence of 1% bovine serum albumin.

Four series of experiments all gave similar types of binding curves. However, the absolute extent of binding varied in the different series (from 0.55 to 1.35 ng/cm^2 of cell culture at a concentration of 0.1 μg of free HA/ml), presumably owing to variation in cell density in the cultures and to biological variation in the number of binding sites per cell. All curves were therefore adjusted to the average binding capacity (0.93 ng/cm^2 at 0.1 μg of HA/ml) by multiplication by suitable factors. The adjusted binding curve (Fig. 1) shows increasing binding of HA with increasing concentration up to 0.1 μg/ml and then saturation. The high extent of binding, 40% of the added radioactivity, at the lowest concentrations applied excludes the possibility of any major contribution by labelled trace impurities in the HA preparation.

Maximal binding capacity was calculated by plotting the reciprocal value of material bound versus the reciprocal of free HA concentration (not shown). Extrapolation to infinite concentration gave 1.16 ng of [^{3}H]HA bound per cm^2 of culture.

A Scatchard (1949) plot was constructed (Fig. 2) on the basis of a M_r value for HA of 4×10^5 and a cell density of 2×10^5 per cm^2. The slope of the plot gives a dissociation constant of approx. 6×10^{-11} M, and the intercept on the abscissa gives approx. 10^4 binding sites per cell.

Experiments were carried out to investigate whether other polysaccharides or oligosaccharides of HA (at 50 μg/ml) could inhibit the binding of [^{3}H]HA (0.4 μg/ml) (Table 1). Unlabelled HA inhibited 95% of the binding. Oligosaccharides of HA could, however, only partly block the binding, the effect increasing with increasing chain length from 20–30% inhibition by HA-4 or HA-6 to 40% inhibition for HA-20 (the oligosaccharides are denoted by the number of monosaccharide units/molecule). Neither ovalbumin nor the polysaccharides heparin or heparan sulphate had any significant effect. Dermatan sulphate was slightly inhibitory, and chondroitin sulphate was as effective as the longer HA oligosaccharides.

Comparative studies with other liver cells showed more binding of [^{3}H]HA to liver endothelial cells than to hepatocytes or Kupffer cells

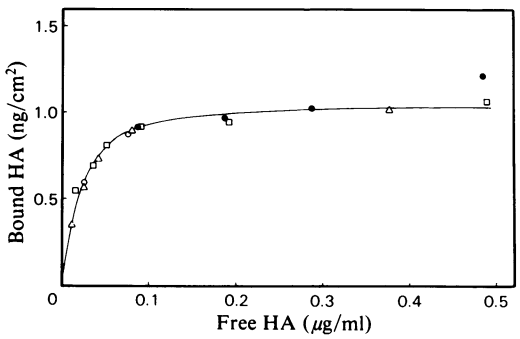

Fig. 1. *Binding of [^{3}H]HA (M_r 400000) to liver endothelial cells at 7°C*

Four experimental series were performed (different symbols) with cultures containing approx. 2×10^5 cells/cm^2. The binding capacity per cm^2 of culture varied in the four series, and each series was therefore adjusted to the average binding at 0.1 μg of free HA/ml by multiplication by a suitable factor (see the text).

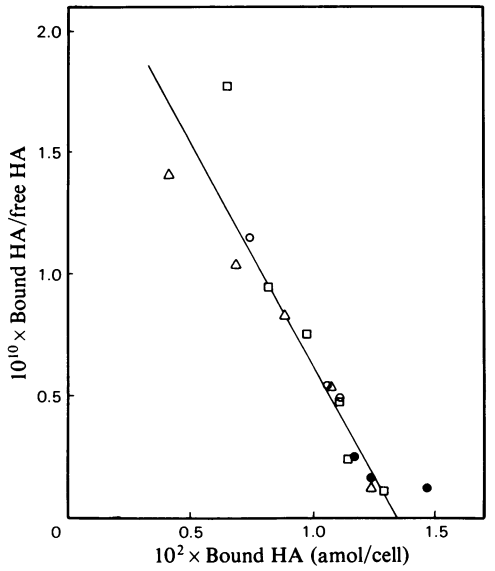

Fig. 2. *Scatchard plot of the HA binding to liver endothelial cells*

The data from Fig. 1 were replotted and the same symbols were used in the two Figures. The intercept on the abscissa gives 8100 binding sites per cell (mean of four experiments; range 4800–11800). The slope gives a dissociation constant of approx. 6×10^{-11} M.

(Table 2). This difference is especially apparent if the small size of liver endothelial cells is taken into account.

Accumulation of [^3H]HA in liver endothelial cells at 37°C

The accumulation of labelled HA by the cells was followed at 37°C during a 135min period (Fig. 3). Three concentrations were chosen, 0.05, 0.2 and 1.0μg/ml [see our preliminary note for similar studies at higher concentrations of HA (Eriksson *et al.*, 1983)]. The extrapolated values for cell-associated HA at zero time corresponded approximately to the amounts of HA bound to the cells in the cold. During the first 1h the accumulation of label by the cells occurred in a linear fashion, reaching values severalfold higher than the maximal binding at 7°C. After 1h the accumulation

Table 1. *Inhibition of binding of [^3H]HA to liver endothelial cells by various polysaccharides, oligosaccharides from HA and ovalbumin*

Endothelial cells were seeded on 9.6cm² dishes as described in the Materials and methods section and incubated with 0.41μg of [^{3}H]HA/ml and 50μg of inhibitor/ml for 18h at 7°C. The amount of cell-associated radioactivity was recorded.

Inhibitor	Cell-associated radioactivity (%)
None	100
HA (M_r 400000)	5
HA oligosaccharides*	
HA-4	80
HA-6	71
HA-8	72
HA-14	66
HA-16	64
HA-20	62
Chondroitin sulphate	60
Dermatan sulphate	79
Heparan sulphate	90
Heparin	93
Ovalbumin	95

* The oligosaccharides are denoted by the number of monosaccharide units/molecule.

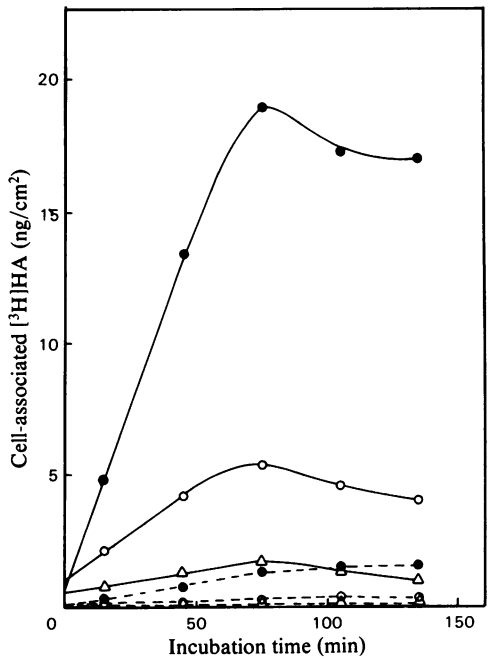

Fig. 3. *Accumulation of [^3H]HA (M_r 400000) by liver endothelial cells at 37°C*

The concentrations of radioactive HA in the medium were 50ng/ml (△), 200ng/ml (○) and 1μg/ml (●) respectively. The experiments were performed in the absence (——) or in the presence (----) of 50μg of unlabelled HA (M_r 400000)/ml.

Table 2. *Binding, accumulation and degradation of [^3H]HA by cultured hepatocytes, Kupffer cells and liver endothelial cells*

The cells were cultured in 9.6cm² dishes in the presence of 1μg of [^{3}H]HA/ml and 5μg of fibronectin/ml as described in the Materials and methods section.

	Hepatocytes	Kupffer cells	Liver endothelial cells
Binding of [^{3}H]HA after 18h at 7°C			
ng/10^6 cells	2.0	1.6	4.4
ng/mg of cell protein*	1.6	14	95
Accumulation of [^{3}H]HA during 75min at 37°C			
ng/10^6 cells	0.2	8.8	36
ng/mg of cell protein*	0.1	77	780
Degradation of [^{3}H]HA after 5h at 37°C			
ng/10^6 cells	1.2	8.7	119
ng/mg of cell protein*	0.9	76	2580

* Calculated by assuming that hepatocytes contain 1280, Kupffer cells 114 and liver endothelial cells 46μg of protein per 10^6 cells (Knook & Sleyster, 1980).

ceased and yielded place to a slow decrease in cell-associated radioactivity. The uptake was now compensated by release of degradation products to the medium and also fell owing to the depletion of high-M_r HA in the medium.

No saturation was observed in the capacity of liver endothelial cells to accumulate HA. The accumulation rate was nearly proportional to the concentration of [^{3}H]HA in the medium. The initial accumulation rates in the experiments shown in Fig. 3 are 19.4, 72 and 285 pg/min per cm^2 of culture area. By dividing these values by the concentrations of free HA in the medium, one obtains values for the 'clearance of HA' (volume of medium cleared of HA per unit time) of 0.39, 0.36 and 0.29 μl/min per cm^2, corresponding to HA concentrations of 50, 200 and 1000 ng/ml respectively.

At very high concentrations of HA the rate of accumulation did not remain proportional to polysaccharide concentration. Addition of 50 μg of unlabelled HA/ml to liver-endothelial-cell cultures containing 1 μg of [^{3}H]HA/ml thus dramatically decreased the rate of accumulation of label by the cells (Fig. 3). However, even under these conditions saturation was not achieved, since significant uptake of label continued also at exceedingly high polysaccharide concentration. This finding is illustrated in Table 3, which summarizes a series of experiments aimed at determining the inhibitory effect of various polysaccharides or oligosaccharides from HA on the uptake of [^{3}H]HA. HA itself was the most effective inhibitor, although it only partly impeded the uptake. The inhibitory power of the oligosaccharides was modest, but increased with increasing chain length. Chondroitin sulphate and dermatan sulphate showed slight inhibitory capacity, whereas heparan sulphate and heparin were inactive.

There was no appreciable accumulation of radioactive HA in hepatocytes and Kupffer cells compared with that in liver endothelial cells (Table 2). The uptake in the Kupffer-cell cultures could be partly due to contamination by liver endothelial cells (B. Smedsrød & H. Pertoft, unpublished work).

Localization of HA in the cell

When fluorescein amine-labelled HA was taken up by liver endothelial cells, the fluorescent material was localized in perinuclear vacuoles (Fig. 4).

Degradation of [^{3}H]HA in liver endothelial cells

Incubation of liver-endothelial-cell cultures with [^{3}H]HA resulted in the appearance in the medium of low-M_r products, which can be separated from the polysaccharide by gel chromatography. The kinetics of the formation of these products is shown in Fig. 5. Low-M_r material, first detected 30–40 min after the addition of HA to the cultures, rapidly accumulated in the medium, and after 8 h 60–70% of the polysaccharide added was present in degraded form. The rate of formation of low-M_r material, after the lag phase, corresponded closely to the initial rate of uptake of [^{3}H]HA exhibited in

Table 3. *Accumulation of [^{3}H]HA by liver endothelial cells in the presence of other polysaccharides or oligosaccharides from HA*

Endothelial cells were seeded in 2 cm^2 dishes as described in the Materials and methods section and incubated with 0.8 μg of [^{3}H]HA/ml and the polysaccharide or oligosaccharide indicated for 2 h at 37°C. The amount of radioactivity in the cell layer was measured.

Saccharide added	Accumulation (% of control) in the presence of unlabelled polysaccharide or oligosaccharide at 25 μg/ml	125 μg/ml
None	100	100
HA (M_r 2×10^6–3×10^6)	32	18
HA oligosaccharides*		
HA-4	82	80
HA-6	71	54
HA-8	65	41
HA-10	67	43
HA-14	51	37
HA-16	51	30
Chondroitin sulphate	70	68
Dermatan sulphate	86	75
Heparan sulphate	96	105
Heparin	90	103

* The oligosaccharides are denoted by the number of monosaccharide units/molecule.

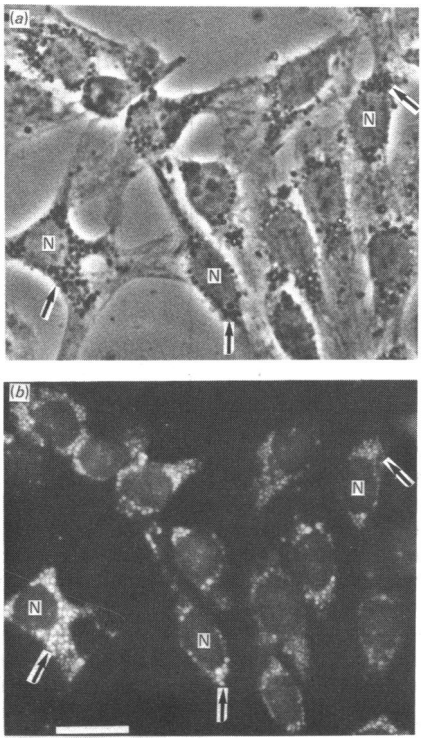

Fig. 4. *Accumulation of fluorescein amine-labelled HA in liver endothelial cells*

Freshly prepared monolayers of liver endothelial cells were incubated with fluorescein amine-labelled HA for 20h and prepared for microscopic examination. When the optics of the microscope were changed from phase contrast (*a*) to fluorescence of appropriate wavelength (*b*), the accumulation of material into perinuclear vesicles (arrows) was clearly revealed in most of the cultured cells. Note that the white fluorescent vesicles in (*b*) appear as identical with the black phase-dense vesicles in (*a*). Abbreviation: N, nucleus. Bar: 25 μm.

Fig. 3. The amount of cell-associated HA at the termination of the experiment depicted in Fig. 5 corresponded to 20%, 15%, 13% and 8%, respectively, of the total (degraded and undegraded) HA in the cultures incubated with 50 ng/ml, 200 ng/ml, 1 μg/ml and 5 μg/ml respectively. The major part of the HA taken up by the liver endothelial cells is thus degraded into low-M_r compounds and released into the medium. The total radioactivity in the cultures remained constant during the experiment, i.e. no volatile products were formed.

The efficiency of other compounds to inhibit the formation of degradation products is shown in Table 4. Unlabelled high M_r HA is most efficient, but oligosaccharides from HA larger than decasaccharides are almost as efficient. Heparin and heparan sulphate have negligible effects, but chondroitin sulphate and dermatan sulphate show some inhibitory power.

Hepatocytes and Kupffer cells produce negligible amounts of extracellular low-M_r products when incubated with [^{3}H]HA (Table 2).

Identification of the degradation products

The labelled degradation product derived from *N*-[^{3}H]acetyl-labelled HA in liver-endothelial-cell cultures was identified mainly as acetate by the following criteria. It migrated in high-voltage electrophoresis and in paper chromatography in butanol/ethanol/water as a sample of standard [^{14}C]acetate. Paper chromatograms developed in ethyl acetate/acetic acid/water contained no detectable radioactivity, presumably owing to evaporation of acetic acid during drying. In high-performance liquid chromatography the degradation product was largely eluted as a sharp peak with the retention time of acetate (Fig. 6), but 22% was recovered as a separate, more retarded, unidentified peak.

Degradation by liver endothelial cells of HA labelled with ^{14}C in the pyranose carbon atoms yielded a radioactive product that behaved like lactate in high-voltage electrophoresis and in the two paper-chromatography systems. Moreover, the material coincided with standard lactate on high-performance liquid chromatography (Fig. 6). [^{14}C]Lactate was conclusively identified by use of the highly specific enzymes lactate dehydrogenase and alanine aminotransferase. Under appropriate conditions these enzymes convert anionic lactate into alanine, which is a cation at low pH. After incubation to equilibrium of the ^{14}C-labelled HA degradation product with lactate dehydrogenase and alanine aminotransferase, 47% of the radioactivity was adsorbed to the cation exchanger. Similar treatment of a sample containing an excess of standard L-[^{14}C]lactate yielded 48% cationic material. When alanine aminotransferase or lactate dehydrogenase was excluded from the incubation mixtures, 13% and 14%, respectively, of the radioactivity was adsorbed to the ion exchanger. This small conversion could be explained by minute cross-contamination of the two enzymes.

Discussion

The present data confirm our previous conclusion (Eriksson *et al.*, 1983) that the endothelial cells are responsible for the uptake and degradation of HA in the liver. No appreciable accumulation or degradation of [^{3}H]HA occurred in hepatocytes or Kupffer cells (Table 2). The accumulation of fluorescent HA in liver endothelial cells (Fig. 4), but not in hepatocytes, Kupffer cells or fat-storing

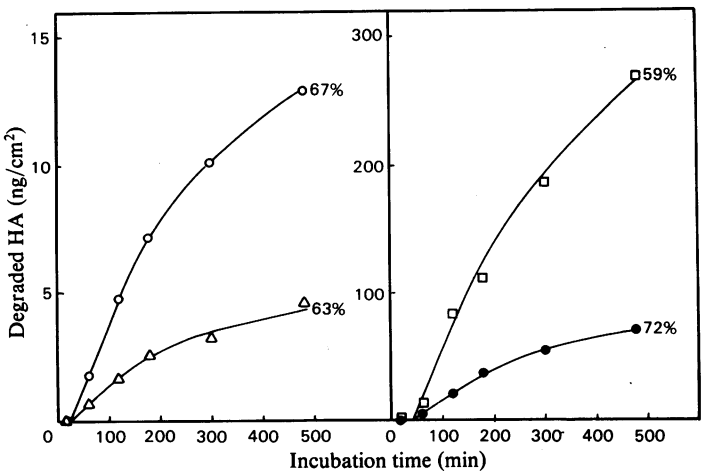

Fig. 5. *Formation of low-M_r degradation products from [^{3}H]HA (M_r 6 × 10^5) in cultures of liver endothelial cells* [^{3}H]HA was added in concentrations of 50 ng/ml (△), 200 ng/ml (○), 1 μg/ml (●) and 5 μg/ml (□) respectively to 2 cm^2 cultures of liver endothelial cells and incubated at 37°C. The medium was recovered at the indicated times and the labelled material separated into high- and low-M_r fractions as described in the Materials and methods section. The percentages shown denote the proportions of the added HA that were recovered as low-M_r degradation products at the termination of the experiment (8 h).

Table 4. *Degradation of [^{3}H]HA in liver endothelial cells in the presence of unlabelled poly- and oligo-saccharides*
Endothelial cells were seeded in 2 cm^2 dishes as described in the Materials and methods section and incubated with 0.8 μg of [^{3}H]HA/ml and 25 or 125 μg of unlabelled polysaccharide or oligosaccharide/ml for 8 h at 37°C. The amount of low-M_r labelled products in the medium was measured.

	Degradation products (% of control) produced in the presence of unlabelled poly- or oligo-saccharide	
Saccharide added	25 μg/ml	125 μg/ml
None	100	100
HA (M_r 2 × 10^6–3 × 10^6)	22	8
HA oligosaccharides*		
HA-4	86	77
HA-6	72	42
HA-8	60	23
HA-10	51	18
HA-14	39	11
HA-16	36	8
Chondroitin sulphate	64	40
Dermatan sulphate	76	52
Heparan sulphate	96	100
Heparin	92	80

* The oligosaccharides are denoted by the number of monosaccharide units/molecule.

cells, seems to rule out the possibility that a minor fraction of the cells in the cultures are responsible for the process. The report by Truppe *et al.* (1977) that HA is taken up by hepatocytes can presumably be explained by a contamination of their cell preparation with liver endothelial cells. It is our experience that most hepatocyte preparations obtained by standard techniques contain endothelial cells (B. Smedsrød & H. Pertoft, unpublished work). Further evidence that liver endothelial cells accumulate HA *in vivo* has been obtained by autoradiography (J. R. E. Fraser, unpublished work).

A HA receptor on liver endothelial cells

For the [^{3}H]HA to associate with the liver-endothelial-cell surface only, without being subsequently internalized, the incubations were performed in the cold. The demonstration of saturable binding that could be inhibited by excess unlabelled HA is evidence for a receptor-mediated reaction. Underhill and Toole have previously demonstrated binding of HA to 3T3 cells and BHK cells (Underhill, 1982; Underhill & Toole, 1979, 1980, 1981; Underhill *et al.*, 1983), and the interaction of HA with liver endothelial cells

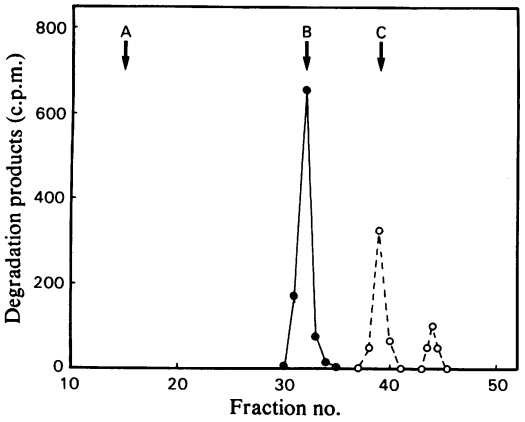

Fig. 6. *High-performance liquid chromatography of degradation products derived from radioactive HA in liver-endothelial-cell cultures*

Liver endothelial cells were incubated with HA labelled with ^{14}C in the pyranose carbon atoms or with ^{3}H in the *N*-acetyl groups. The degradation products were chromatographed as described in the Materials and methods section. The elution position of undegraded HA is marked with an arrow (A). The ^{14}C-labelled product (●) was recovered in one peak (yield 73%) at the site of standard [^{14}C]lactate (B). The ^{3}H-labelled material (○) was recovered quantitatively, 75% being eluted at the position of standard [^{14}C]acetate (C) and 22% in a peak of unidentified material.

described here has several similarities to that described for 3T3 cells. Firstly, the number of molecules bound per cell is of the same order of magnitude. Secondly, although the dissociation constant for liver endothelial cells was found to be about 6×10^{-11} M, whereas that of 3T3 cells was 1×10^{-9}–2×10^{-9} M, the difference can be rationalized in terms of multiple site attachments, as discussed by Underhill & Toole (1980). The multiple-site attachment theory is supported by the finding (Table 1) that high-M_r HA inhibited the binding of the radioactive polymer to the cells much more strongly than did the oligosaccharides. Thirdly, both the receptor found by Underhill & Toole (1979) and the accumulation of HA in liver endothelial cells seem to function in the absence of extracellular Ca^{2+}. Finally, the relative inhibitory potency of the various oligosaccharides in Table 1 is similar to that shown by Underhill & Toole (1979). In view of the multiple-attachment hypothesis, 10^4 binding sites per liver endothelial cell is a minimum number.

Chondroitin sulphate and dermatan sulphate may compete with HA binding, whereas heparin, heparan sulphate and ovalbumin do not. The lack of activity of ovalbumin rules out uptake of HA by the mannose/*N*-acetylglucosamine receptor (Neufeld & Ashwell, 1980).

Internalization of HA

The endocytosis of HA by liver endothelial cells is very efficient and is not saturable in the same way as the binding to the cell surface. Both the results presented in Fig. 3 and those outlined in a previous communication (Eriksson *et al.*, 1983) demonstrate that the accumulation rate increases with increasing HA concentration, reaching no upper limit. At the concentrations indicated in Fig. 3 the uptake is nearly proportional to the concentration of free HA in the medium. Such a relationship points to a fluid-phase endocytosis and can be expressed as a clearance of HA contained in a defined volume of the medium per unit time (2 pl/min per cell). However, fluid-phase endocytosis measured by uptake of polyvinylpyrrolidone in liver endothelial cells is one order of magnitude less than the above value (Munniksma *et al.*, 1980). Moreover, excess unlabelled HA does decrease the uptake of label (Table 3), and the internalization process thus has features of both receptor-mediated and unspecific endocytosis.

Metabolism of HA in liver endothelial cells

The liver endothelial cells have a high capacity for degrading HA, and almost all polysaccharide that has been internalized is secreted into the medium as low-M_r material. The first degradation products are detected after about 30 min, which should thus approximate to the time required for the overall internalization–degradation–secretion process.

The longer oligosaccharides from HA inhibit degradation of [^{3}H]HA as effectively as does high-M_r HA (Table 4). This is expected if they are competing substrates for the degrading enzymes. The finding that the longer oligosaccharides are more effective than the short ones could indicate that hyaluronidase action is the rate-limiting step in the degradation rather than the activity of an exoenzyme. Also chondroitin sulphate and dermatan sulphate could, in view of their similarity to HA, inhibit hyaluronidase (Aronson & Davidson, 1967; Gold, 1982).

It was surprising to find acetate and lactate as major degradation products. It was previously reported (Eriksson *et al.*, 1983) that the products were eluted similarly to monosaccharides when chromatographed on a column of Sephadex G-15 and that standard acetate was eluted later. This result has proved to be a calibration artefact. When standard [^{14}C]acetate was chromatographed in trace amounts on a Sephadex G-15 column, it was eluted at the position of the degradation product.

The observation that acetate and lactate are produced by the cells raises the question whether the liver endothelial cells have an essentially anaerobic metabolism. These cells line the sinu-

soids that conduct the portal blood from the intestinal tract. This blood has a low oxygen partial pressure and a high glucose content, which should favour an energy metabolism based on glycolysis. Nevertheless, electron microscopy of liver endothelial cells (Wisse, 1970) demonstrated mitochondria. When medium from cultures of these cells, which contains the degradation products from HA, is added to cultures of hepatocytes, the products are rapidly converted into CO_2 and water (B. Smedsrød, H. Pertoft, S. Eriksson, J. R. E. Fraser & T. C. Laurent, unpublished work). Liver endothelial cells and hepatocytes may thus act in concert.

Degradation products formed *in vivo* from intravenously injected labelled HA and isolated from the liver showed the presence of *N*-acetylglucosamine and a mixture of glucuronic acid and gulonic acid (J. R. E. Fraser, unpublished work).

Physiological considerations

The binding curve in Fig. 1 shows 50% saturation at approx. 20ng of free HA/ml. This value is within the range of the physiological plasma concentration of HA in both humans and rats (Engström-Laurent *et al.*, 1983). It is thus tempting to propose that binding and internalization of HA by liver endothelial cells may be essential in regulating the blood concentration of HA. This proposal is further strengthened by a comparison of the rates of HA turnover *in vitro* and *in vivo*. The rate *in vitro* at a concentration of 50ng of HA/ml in the medium is approx. 0.1fg of HA/min per cell. The rat liver contains 42×10^6 liver endothelial cells (Knook & Sleyster, 1980). Assuming the same content per g of human liver (1500g) we can calculate the number in an adult human to approx. 6.3×10^{10}. According to the experiments *in vitro*, they should turn over 6.3μg of HA/min. Our studies on HA in human plasma showed an average turnover time of 3.8–7.9min (Fraser *et al.*, 1984). A person with a plasma volume of 2400ml and a plasma concentration of 50ng of HA/ml should therefore turn over 15–32μg/min. In view of the uncertainty of the assumptions made in the calculations, there is a good correlation between uptake of HA in liver endothelial cells *in vivo* and *in vitro*. The same lag time recorded *in vitro* for appearance of degradation products was observed *in vivo* in both rabbit and man (Fraser *et al.*, 1981, 1984). We may thus conclude that the uptake and degradation of HA in liver endothelial cells *in vitro* has many features in common with the process *in vivo*, and, furthermore, that these cells may be primarily responsible for the rapid clearance of HA from plasma.

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