Biochem. J. (1981) **200**, 415–424
Printed in Great Britain

Plasma clearance, tissue distribution and metabolism of hyaluronic acid injected intravenously in the rabbit

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(*Received 26 June 1981/Accepted 17 July 1981*)

The plasma clearance, tissue distribution and metabolism of hyaluronic acid were studied with a high average molecular weight [^{3}H]acetyl-labelled hyaluronic acid synthesized in synovial cell cultures. After intravenous injection in the rabbit the label disappeared from the plasma with a half-life of 2.5–4.5 min, which corresponds to a normal hyaluronic acid clearance of approx. 10 mg/day per kg body wt. Injection of unlabelled hyaluronic acid 15 min after the tracer failed to reverse its absorption. Clearance of labelled polymer was retarded by prior injection of excess unlabelled hyaluronic acid. The maximum clearance capacity was estimated in these circumstances to be about 30 mg/day per kg body wt. The injected material was concentrated in the liver and spleen. As much as 88% of the label was absorbed by the liver, where it was found almost entirely in non-parenchymal cells. Degradation was rapid and complete, since volatile material, presumably ^3H_2O, appeared in the plasma within 20 min. Undegraded [^{3}H]hyaluronic acid, small labelled residues and ^3H_2O were detected in the liver, but there was little evidence of intermediate oligosaccharides. No metabolite except ^3H_2O was recognized in plasma or urine. Two-thirds of the radioactivity was retained in the body water 24 h later, and small amounts were found in liver lipids. Radioactivity did not decline in the spleen as rapidly as in the liver. The upper molecular weight limit for renal excretion was about 25000. Renal excretion played a negligible part in clearance. It is concluded that hyaluronic acid is removed from the plasma and degraded quickly by an efficient extrarenal system with a high reserve capacity, sited mainly in the liver.

Although hyaluronic acid is widely distributed in the connective tissues and various organs, little is known of its movements and disposal elsewhere in the body. Its half-life in skin is about 4 days compared with 7 days for chondroitin sulphates (Schiller *et al.*, 1962). After injection of ^{14}C-labelled hyaluronic acid into the synovial cavity of joints, radioactivity was detected in the regional lymph nodes within 15 min and appeared briefly in the plasma (Antonas *et al.*, 1973). The plasma half-life after direct intravenous injection was only 10 min. In studies with sulphated glycosaminoglycans (Kaplan & Meyer, 1962) and with ^{35}S-labelled proteoglycan and its derivative chondroitin 4-sulphate (Revell & Muir, 1972), the plasma was cleared within a few hours but only small amounts of the saccharide chains were found in the urine. Wood *et al.* (1973) have shown that chondroitin sulphate is taken up mainly in the liver.

Hyaluronic acid is less abundant than other glycosaminoglycans in urine (Wessler, 1971) or in plasma (Murata & Horiuchi, 1977). A specific radioactivity binding assay (Laurent & Tengblad, 1980) has defined a normal plasma concentration of about 300 ng/ml in man (Laurent & Laurent, 1981). The latter investigators have also shown in sheep and rat that lymph contains about ten times more hyaluronate than does blood plasma. The low plasma levels might therefore reflect rapid clearance rather than a low input of hyaluronic acid. In the present study, the removal of hyaluronic acid from plasma has been examined with a polydisperse

0306-3283/81/110415-10$01.50/1

hyaluronic acid preparation of high average molecular weight, labelled biosynthetically with [^{3}H]acetate.

Materials and methods

Materials

Sodium [^{3}H]acetate (3.8 Ci/mmol) was obtained from The Radiochemical Centre, Amersham. Cholera enterotoxin was acquired from Schwarz–Mann, Inc. Bovine testicular hyaluronidase (EC 3.2.1.36; 20000 i.u./mg) was obtained from AB Leo, Helsingborg, Sweden; *Streptomyces hyalurolyticus* hyaluronidase (EC 4.2.2.1; 2000 turbidity-reducing units/mg) was from Seikagaku Kogyo Co., Tokyo, Japan; and staphylococcal hyaluronidase (EC 4.2.99.1; 23000 i.u./mg) was kindly given by G. Jacobs of N.V. Organon, Oss, Holland. Rabbit albumin, collagenase (type 1; EC 3.4.24.3) and deoxyribonuclease (DN-25; EC 3.1.21.1) were from Sigma. Sephadex G-50 (fine) and G-25 (superfine), Sephacryl S-300 (superfine) and Percoll were supplied by Pharmacia. Hyaluronic acid preparations of defined molecular weight, including a preparation of Healon (lot DL 10492), were the gift of Dr. K. Granath of Pharmacia. CsCl and guanidinium chloride were from BDH. Other chemical reagents were of analytical grade.

Preparation of [^{3}H]hyaluronic acid

Stock cultures of human synovial cells (Fraser & McCall, 1965) were treated with cholera enterotoxin to enhance hyaluronic acid synthesis (Fraser *et al.*, 1979), and cultured for 72 h in Eagle's basal medium (Eagle *et al.*, 1956) with 20% (v/v) heat-inactivated human serum, and sodium [^{3}H]acetate, 50 μCi/ml. Hyaluronic acid was prepared from the culture medium by density gradient ultracentrifugation in a Beckman 60 Ti rotor for three periods of 60 h each at 4°C and 135000 ***g***. In the first step, $CsCl_2$ was added to an initial density of 1.505 g/ml. The bottom 8-ml fractions from each 30 ml tube were recovered and pooled. Guanidinium chloride was added to a concentration of 4 mol/l with adjustment of density to 1.455 g/ml for the second and third steps, in which the middle 10 ml were retained from each tube. The final fraction was dialysed at 4°C for three periods of 48 h, first against 4 litres of 0.15 M-NaCl, and then against two changes of phosphate-buffered saline with 80 μg of gentamycin sulphate/ml. The preparation was sterile in aerobic and anaerobic culture for 96 h and pyrogen-free when tested in rabbits. The ^{3}H activity was 15.8×10^6 d.p.m./ml. Specific activity of an identical sample prepared for other purposes was 500000 d.p.m./μg of hyaluronic acid as measured by a specific binding assay (Laurent & Tengblad, 1980). Chromatography in Sephadex G-25 before and after treatment with proteinase-free staphylococcal hyaluronidase (4500 i.u. in 0.5 ml of 0.1 M-sodium acetate buffer, pH 5.0, with 0.15 M-NaCl and 1.5×10^5 d.p.m. of [^{3}H]hyaluronic acid, at 34°C for 6 h) showed that the labelled material was completely susceptible to the enzyme (>99.5%; results not shown).

Table 1. *Molecular weight distributions of ^{3}H-labelled and unlabelled hyaluronic acids used in the animal studies*
The unlabelled material was a sterile solution (10 mg/ml) of sodium hyaluronate prepared from roosters' combs in 0.145 M-NaCl/0.002 M-sodium phosphate, pH 7.3 (Healon, lot DL 10492; Pharmacia). Samples were kindly analysed by Dr. K. Granath with the technique described by Wik *et al.* (1979).

	$10^{-3} \times$ Mol.wt.	
Cumulative fraction (%)	^{3}H-labelled	Unlabelled
2.5	25400	27400
7.5	16800	20400
12.5	10900	15800
17.5	7060	12200
22.5	5170	9510
27.5	3820	7320
32.5	2850	5890
37.5	2130	4790
42.5	1610	3820
47.5	1220	3120
52.5	937	2520
57.5	749	2030
62.5	606	1630
67.5	504	1290
72.5	412	994
77.5	328	781
82.5	247	602
87.5	181	460
92.5	119	309
97.5	55	139
Weight average mol.wt. ($\bar{M}_w$)	4050	6060
Number average mol.wt. ($\bar{M}_n$)	393	987
$\bar{M}_w/\bar{M}_n$	10.3	6.1

Molecular weight analysis of hyaluronic acid used in animal studies

The molecular weight distributions of the ^{3}H-labelled hyaluronate and the unlabelled sample of Healon were kindly determined by Dr. K. Granath, Pharmacia AB, Uppsala. The distributions are displayed in Table 1. All studies reported in this paper were complete 9 weeks after the molecular weight analysis.

Animal studies

Fully mature outbred rabbits of Chinchilla (Willi Gassner GmbH, Sulzfeld, Germany) and Sandeloup (Biomedical Centre, Uppsala) strains were used. Weights were 2.76–4.02 kg. Food and water were unrestricted until the time of study or during longer

experiments. Marginal ear veins were used for injections and blood sampling. About 15×10^6–16×10^6 d.p.m. (30–32 μg) of the labelled material was injected in each animal. The unlabelled material was injected in a concentration of 5 mg/ml. Amounts given were determined by weight. Plasma was immediately prepared from heparinized blood samples by centrifugation at 1900 *g* for 30 min. After barbiturate anaesthesia and exsanguination, tissues and organs were dissected free from extraneous tissue, weighed and frozen in liquid N_2 or at −70°C. Urine was aspirated aseptically from the bladder and cleared by centrifugation.

Estimation of plasma blood volumes

The haematocrit of heparinized blood was determined in a Coulter Model S Plus analyser. The regression of plasma radioactivity with time (t) was calculated in the usual manner and extrapolated to derive the notional plasma ^3H activity when $t = 0$. Then:

$$\text{Plasma volume (ml)} = \frac{\text{total } ^3H \text{ injected (d.p.m.)}}{[\text{plasma } ^3H]_{t=0} \text{ (d.p.m./ml)}}$$

Measurement of radioactivity

Packard Tri-carb 3380 and Searle Nuclear Chicago Isocap/300 scintillation counters were used. Plasma and urine samples (0.1 ml) diluted with 0.5 ml of water were counted in 14 ml of scintillant 299 (Packard–Becker B.V., Breda, The Netherlands). The coefficient of variation in triplicate estimations from 37 experimental samples ranged from 0.7 to 8.7% (median 2.2%). Duplicate tissue samples of 39.6–112.6 mg wet wt. were heated in 1 ml of 1 M-NaOH at 52°C in sealed vials until dissolved, and decolorized with 0.2 ml of 30% (w/w) H_2O_2 when cool. Dimilume (Packard) (15 ml) was then added and the vials were kept in darkness for at least 48 h before counting. Lipid extracts were dissolved in 15 ml of Dimilume. Quench corrections and counting efficiencies were determined with internal [^3H]toluene standards and [^3H]hyaluronic acid diluted to a range of 500–500000 d.p.m./ml in normal rabbit plasma and in aqueous buffers. Other counting conditions are given with the experimental data.

Buffers

For extraction of liver, Clarke and Lubs' borate/KCl/NaOH buffer, pH 8.25, (Documenta Geigy, 1960) was diluted with 0.15 M-NaCl to give a final molarity of about 0.16 (0.16 M-NaCl/borate). The buffer used for chromatography of plasma and urine consisted of 0.15 M-Na_2HPO_4 titrated to pH 7.25 with 0.15 M-NaH_2PO_4 and diluted with 2 vol. of 0.15 M-NaCl (0.15 M-NaCl/phosphate).

Gel chromatography

Samples (1 ml) were applied to columns (1 cm × 106 cm) of Sephadex G-50 and Sephacryl S-300, eluted at a flow rate of 12 ml/h at 23°C; 2 ml fractions were recovered. Recovery of radioactivity (mean 95%, S.E.M. 1.9, $n = 32$) was calculated from fractions with radioactivity greater than 3 S.D. above the average background in each elution.

Separation of cells from liver

The method devised for rat liver (Pertoft *et al.*, 1977) was modified for the more fragile rabbit liver cells. The viscera were perfused until bloodless (2 min) with a respiratory medium (Berglindh & Öbrink, 1976) containing (12 mM-$NaHCO_3$ and aerated at 37°C with air/CO_2 (95:5). Liver tissue (9.7 g) was finely chopped and shaken for 30 min at 37°C in 30 ml of Hanks' balanced salt solution with 10 mg of albumin/ml and 2 mg of glucose/ml, and 24 mg of collagenase. After 2 min, the cells in the supernatant were pelleted at 400 *g* for 5 min and resuspended in 5 ml of 40% (v/v) saline/Percoll (see below) with 25 μl of deoxyribonuclease (10 μg/ml), the final volume being 7.5 ml. A stock solution of saline/Percoll (1.5 M-NaCl/Percoll; 1:9, v/v) was diluted (v/v) with Hanks' salt solution containing albumin and glucose as above and a gradient was formed in a 50 ml polycarbonate tube from the following amounts of diluted saline/Percoll: 7.5 ml of 75%, 5 ml of 60%, 5 ml of 50%, 5 ml of 45% and 5 ml of 40% with the cell suspension on top. After 20 min at 800 *g*, fractions were collected by upward displacement with 75% saline/Percoll. Densities were measured with marker beads (Pharmacia). The cells in each fraction were counted and their viability was ascertained by exclusion of Trypan Blue. Cells were classed as non-parenchymal and parenchymal by size (20 or 40 μm diameter) and appearance in cell culture (Pertoft *et al.*, 1977). Radioactivity in 0.1 ml of each fraction was measured after mixing with 5 ml of Instagel (Packard).

Results

Tolerance of intravenous hyaluronic acid

Two rabbits were given 20 mg of unlabelled material by rapid injection. After 30 min the haematocrit showed no evidence of plasma volume expansion, and there was only a slight reduction in mean erythrocyte volume (about 5%). No circulatory impairment was seen in these or the other experimental animals.

Plasma clearance

After injection of [^3H]hyaluronic acid either alone or before the unlabelled substance, plasma radioactivity decreased exponentially, without deflection until at least 95% had disappeared (Figs. 1*a* and 1*b*).

When large amounts of unlabelled material were injected beforehand (Figs. 1*c* and 1*d*), the initial fall in plasma ^3H was much slower and could be described equally well by logarithmic or linear regression, but the subsequent pattern of clearance could not be easily defined. The calculated half-life times and other data are shown in Table 2.

Nature of ^3H-labelled substances in plasma

Selected samples were analysed in Sephadex G-50 (Table 3). The ^3H activity was found in only two zones, centred round V_0 and V_t, respectively. The former was confirmed as glycosaminoglycan by degradation with testicular hyaluronidase. The ^3H activity in the peak fractions at V_t was completely volatile. Since acetate does not volatilize readily in the same conditions, it was concluded that the [^3H]acetyl radical in the hyaluronic acid was degraded *in vivo* to ^3H_2O. Injection of excess unlabelled hyaluronic acid 15 min after the labelled substance (Fig. 1*b*) arrested the clearance but displaced less than 1% of the amount removed from plasma at that stage. The later rise in plasma ^3H in this and other studies (Fig. 1*a*) was primarily due to generation of ^3H_2O. The delayed detection of plasma ^3H_2O in expt. 6 of Table 3 can be attributed to the

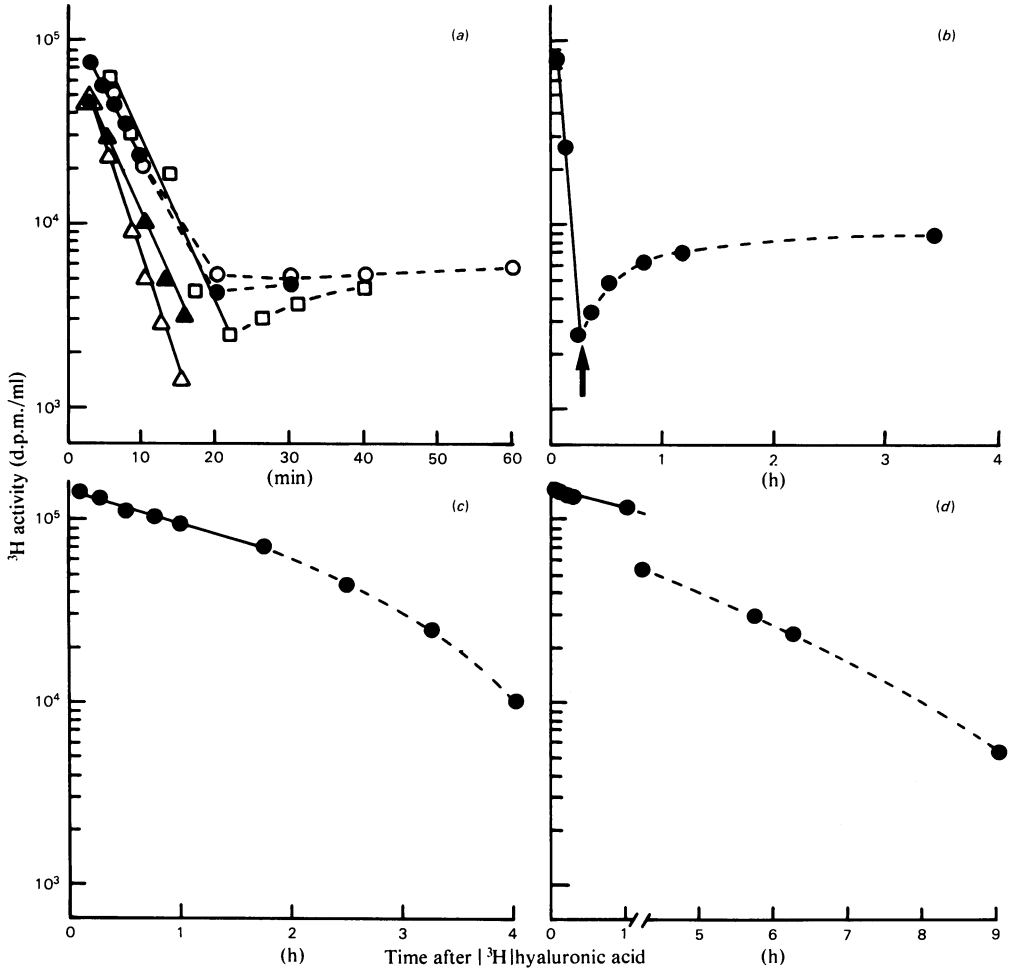

Fig. 1. *Decay of plasma radioactivity after intravenous injection of [^3H]hyaluronic acid*

The amount of tracer injected was approx. 30–32 μg, corresponding to 15×10^6–16×10^6 d.p.m. (*a*) Unmodified clearance. ○, expt. 1; ●, expt. 2; △, expt. 3; □, expt. 4; ▲, expt. 5. (*b*) Unlabelled material (20 mg) was injected 15 min (↑) after the tracer when 98% of the latter had been cleared. Plasma ^3H was 6900 d.p.m./ml at 24 h (expt. 8). (*c*) Unlabelled material (9.7 mg) was injected 12 min before the tracer (expt. 6). (*d*) Unlabelled material (19 mg) was injected 18 min before the tracer. Plasma ^3H was 9400 d.p.m./ml at 24 h. Continuous lines represent intervals for which regressions were calculated. The horizontal and rising deflections are due to release of metabolite (see Table 3).

Table 2. *Decay of plasma radioactivity after intravenous injection of [^{3}H]hyaluronic acid (compare Fig. 1)*
Plasma radioactivity was measured as described in the Materials and methods section. The derived values in expt. 1 are approximate, since too few early samples were obtained for regression analysis. In expt. 8, 20mg of unlabelled material was injected immediately after the 15min blood sample. In expt. 6, 9.7mg of unlabelled material was injected 12min before the tracer and in expt. 7, 19mg was injected 18min before. For correlations between plasma ^{3}H levels and time, $r = -0.93$ to -0.99, $P < 0.01$. Plasma volumes fell within the normal range determined by standard methods (Armin *et al.*, 1952).

Expt. no.	Weight of rabbit (kg)	Plasma vol. (ml)	10^{-3} × Notional activity at $t = 0$ (d.p.m./ml)	$t_{\frac{1}{2}}$ (min)
[^{3}H]Hyaluronic acid alone				
1	3.1	approx. 125	approx. 122	approx. 4.5
2	4.0	137	121	4.2
3	3.9	141	121	2.4
4	2.8	78	213	3.4
5	3.7	188	88	3.3
[^{3}H]Hyaluronic acid followed by unlabelled material				
8	2.6	94	178	2.5
[^{3}H]Hyaluronic acid preceded by unlabelled material				
6	3.3	114	137	103
7	3.0	109	144	195

Table 3. *Fractionation of plasma radioactivity by gel chromatography*
Approx. 1-ml samples of known ^{3}H content were analysed on Sephadex G-50 as described in the Materials and methods section. The ^{3}H-labelled material in the peak V_0 fractions marked * was incubated at 37°C with testicular hyaluronidase, 200i.u./ml, for 4h, and another 100i.u. for 12h. Repeated chromatography in Sephadex showed 94 to 98% to be degraded, mainly to tetrasaccharide ($K_{av.}$ approx. 0.7; Hascall & Heinegård, 1974). Aliquots (1ml) of peak V_t fractions marked † were freeze-dried at $<10^2$Pa for 24h and redissolved in 1ml of water. ^{3}H activity was reduced to background from prior counts of 370–1380d.p.m./ml.

Expt. no.	Time after injection of [^{3}H]hyaluronic acid (min)	Percentage of plasma ^{3}H recovered at: V_0	Percentage of plasma ^{3}H recovered at: V_t	10^{-3} × Plasma ^{3}H content (d.p.m./ml) represented by fractions at: V_0	10^{-3} × Plasma ^{3}H content (d.p.m./ml) represented by fractions at: V_t
[^{3}H]Hyaluronic acid given alone					
1	10	98*	2	20.6	0.4
	20	27	73†	1.5	4.0
	60	11	89	0.6	5.2
2	4	100	0	62.0	0
	10	98*	2	22.5	0.5
	20	29	71†	1.2	3.1
	30	9	91	0.4	4.4
3	16	92	8	1.3	0.1
[^{3}H]Hyaluronic acid preceded by 9.7mg of unlabelled material					
6	30	100	0	108.0	0
	60	100*	0	92.0	0
	240	78	22†	10.0	3.0
[^{3}H]Hyaluronic acid followed 15min later by 20mg of unlabelled material					
8	8	99	1	25.7	0.3
	15	90	10	2.3	0.2
	30	52	48	2.6	2.4
	70	44	56†	3.2	4.1
	24h	12	88†	0.8	6.1

reduced fractional turnover rate caused by dilution with unlabelled material (approx. 1:3000). However, the entire sequence of absorption and degradation of circulating hyaluronic acid can clearly occur within 20min.

Distribution of ^{3}H activity in specimens of whole tissue

The concentration of ^{3}H was much higher in liver and spleen than in other tissues examined (Table 4). The liver, by virtue of its bulk, had absorbed most of

Table 4. *Distribution of radioactivity in tissues after intravenous injection of [^{3}H]hyaluronic acid*

Weighed amounts of frozen tissue were dissolved for determination of radioactivity as described in the Materials and methods section. The data are presented in order of the time elapsed between injection and circulatory arrest. The tissues were immediately frozen after excision and stored at −70°C until analysis. Activity in kidney, lung, suprarenal glands, skeletal and cardiac muscle ranged from 4.8×10^3 to 9.4×10^3 d.p.m./g. The amounts of unlabelled hyaluronic acid injected in expts. 6 to 8 are given with other details in Table 2.

		Liver			Spleen		
Time from injection to circulatory arrest	Expt. no.	Organ wt. (g)	10^{-3} × Activity (d.p.m./g)	Fraction of injected ^{3}H retained in organ (%)	Organ wt. (g)	10^{-3} × Activity (d.p.m./g)	Fraction of injected ^{3}H retained in organ (%)
[^{3}H]Hyaluronic acid alone							
(min)							
19	3	118.1	124.1	88	1.2	91.6	0.6
30	5	171.1	76.3	78	2.0	63.8	0.8
40	4	93.1	85.6	48	1.2	159.3	1.1
47	2	85.9	90.4	47	1.6	90.4	0.9
90	1	72.7	45.4	22	1.0	80.5	0.5
[^{3}H]Hyaluronic acid preceded by unlabelled material							
4.5 h	6	128.9	24.2	20	0.9	101.7	0.6
22 h	7	167.0	7.3	8			
[^{3}H]Hyaluronic acid followed by unlabelled material							
24 h	8	135.7	9.8	8	1.8	30.7	0.3

the ^{3}H activity in the early stages. In the studies with tracer alone, there was a negative correlation between the fraction of the dose retained in the liver and the time elapsed after injection ($r = -0.927$, $t = 4.29$; $P < 0.025$). This was not so in the case of the spleen. The relatively longer retention of radioactivity in the liver in expt. 6 was attributed to the slower uptake of [^{3}H]hyaluronic acid. The low hepatic ^{3}H at 24 h reflects the extent of degradation (see below).

Extraction of radioactivity in the liver

Extraction in aqueous buffer, 0.16 M-NaCl/borate, pH 8.25. Four specimens were analysed to examine variation with time and with modification by unlabelled hyaluronic acid. The pH was chosen to arrest the activity of endogenous hyaluronidase (Aronson & Davidson, 1967). The recovery of ^{3}H (Table 5) was close to that in the whole tissue, indicating that the buffer had extracted any membrane-bound material, and that little was selectively retained in the particulate or lipid fractions. Gel chromatographic analyses are shown in Fig. 2. In the first few hours, material in V_0 was of high molecular weight (Fig. 4) and was almost completely susceptible to testicular hyaluronidase. The changes in the V_0 peak with time can be attributed in the first three studies to degradation of absorbed hyaluronic acid. The resistance of the small 24-h V_0 residue to *Streptomyces* hyaluronidase may represent metabolic transfer of label from hyaluronic acid to other water-soluble macromolecules. The proportions recovered at V_t corresponded well with the volatile fractions in the original extracts (Table 5). Except in expt. 1, the rest was eluted entirely in a zone between tetrasaccharide and V_t, consistent with smaller saccharides or other intermediates.

Extraction of lipids. Incorporation of ^{3}H in lipid was small, whether calculated in terms of the total injected, or the total in the liver (Table 5), but the significant specific activity provided further evidence for the disposal of ^{3}H-labelled hyaluronic acid through acetyl-CoA.

Distribution of radioactivity in cells separated from liver

This was found almost entirely in the non-parenchymal cell fraction (Fig. 3) which includes the Kupffer cells.

Radioactivity in urine

The samples collected represented excretion for the whole of expts. 1 and 3, most of expt. 6, and an unknown period in expt. 8. Volumes were uncertain, but urinary excretion of polysaccharide could account for no more than 1% in expts. 1 and 3 and 5% in the modified conditions of expt. 6. In expt. 8, the urinary radioactivity at 24 h was mainly ^3H_2O. Chromatography and volatilization (Table 6) gave results similar to those in plasma.

Table 5. *Extraction of radioactivity in liver*

Aqueous extracts: approx. 20g of frozen tissue was disrupted in a blade homogenizer for 2.5min with 20ml 0.16M-NaCl/borate, pH8.25. After 36h at 5°C, the homogenate was centrifuged in a Beckman 60 Ti rotor at 113000 $g_{av.}$ and 5°C for 120min, and the aqueous phase between the fat and solid layers was aspirated. Radioactivity was counted in two aliquots of 0.1ml as described for plasma, and in two aliquots of 0.2ml treated with 0.7ml of 1M-NaOH and 0.2ml of propan-2-ol at 37°C for 90min, decolorized with 0.2ml of 30% (w/w) H_2O_2 and dispersed in 15ml of Dimilume. The results are expressed in terms of original wet tissue wt. Volatility was determined by lyophilization for 48h as described in the legend to Table 3. Chloroform/methanol extracts: approx. 2g of tissue was finely minced, weighed and extracted in the dark for 48h in 20ml of (2:1, v/v). The extracts were mixed with 0.4vol. of 0.15M-NaCl and left overnight. Chloroform recovery (88–91%) was estimated from weight, density and fractional solubility in water. The chloroform phase was evaporated to dryness under N_2, and the residue was taken up in 15ml of Dimilume.

		Aqueous buffer extracts		Lipid extracts			
				10^{-3} × Activity (d.p.m./g) in lipid expressed relative to:			
Time from injection to circulatory arrest	Expt. no.	10^{-3} × Activity (d.p.m./g) (mean ± S.D., $n = 4$)	Volatile fraction (%)	Tissue	Lipid	Fraction of dose in hepatic lipids (%)	Concentration of lipid in liver (mg/g wet wt.)
19min	3	122.6 ± 3.5	0	0.2	3.4	0.2	40
40min	4			1.1	14.2	1.2	42
1.5h	1	44.7 ± 1.1	8	0.9	11.1	2.0	45
4.5h	6	26.1 ± 1.2	7	0.2	3.0	0.9	38
24h	8	9.1 ± 0.3	55	1.9	2.9	2.1	42

Molecular size-limit in renal excretion

The [^{3}H]hyaluronic acid in the urine had an average molecular weight of 25000 (Fig. 4). In view of the wide range of higher polymers injected, this appears to be the upper limit for renal excretion of hyaluronic acid in the rabbit. The corresponding molecular weight for proteins with a similar $K_{av.}$ in Sephacryl S-300 is about 400000 (manufacturer's data).

Discussion

Preparation of radioactive hyaluronic acid with a molecular size comparable to that naturally occurring in the tissues (Laurent, 1970) has allowed an approach to the study of its metabolism under physiological conditions. This problem has acquired fresh significance with the introduction of high-molecular weight hyaluronic acid in clinical treatment. The radioactive polymer should be primarily labelled in the *N*-acetyl group on the hexosamine and it is therefore the fate of this residue which has been followed.

Plasma clearance

It is now clear that there is a rapid turnover of plasma hyaluronic acid. The molecular weight distribution of plasma hyaluronic acid is not known. However, the tracer was quite polydisperse and there was no change in the kinetics of its clearance until most had disappeared, indicating that the clearance rate is not highly molecular-weight-dependent. The amount of labelled hyaluronic acid injected added about 30% to the normal amount in rabbit plasma. The clearance of the tracer can therefore be considered to be representative. The average turnover time of hyaluronic acid in the plasma ($t_{\frac{1}{2}}/\ln 2$) ranged from 3.5 to 6min. The amount cleared, assuming a normal level of 0.9μg/ml (U. B. G. Laurent, personal communication), was between 20 and 50mg/day, or about 10mg/day per kg wt. (mean 9.8, S.D. 2.2, $n = 5$).

The clearance of the tracer in the presence of a large excess of hyaluronic acid (Table 2) gave estimates of the maximum removal capacity equivalent to 95mg/day in expt. 6 and 100mg/day in expt. 7; that is, about 30mg/day per kg wt. The total capacity is therefore at least three times the normal rate of clearance. If there is preferential uptake of the largest polymers with saturation of binding, as occurs in virus-transformed mouse fibroblasts (Underhill & Toole, 1979), this figure will be conservative, since the mean mol.wt. of the tracer was lower than that of the unlabelled material. Thus, there is an efficient mechanism for the clearance of plasma hyaluronic acid, with a relatively high reserve capacity.

Tissue distribution

The liver is the main site of uptake as was shown to be the case for chondroitin sulphate (Wood *et al.*, 1973). The clearance rate in terms of plasma volume was far too high, with or without the added load, to be explained by known rates of fluid endocytosis in liver or spleen (Munniksma *et al.*, 1980) and must therefore depend on specific cellular binding. The high levels in liver and spleen indicate that the macrophage class of cell is primarily responsible for

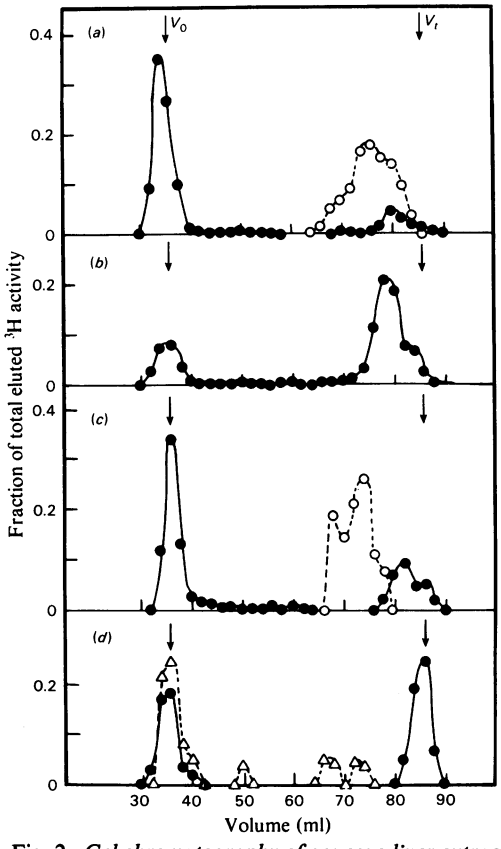

Fig. 2. *Gel chromatography of aqueous liver extracts*
Samples (1 ml) of the liver extracts described in Table 5 were fractionated on Sephadex G-50 equilibrated with 0.16 M-NaCl/borate (see the Materials and methods section). The chromatograms (●) represent the following times from injection to circulatory arrest: (*a*) 19 min (expt. 3); (*b*) 90 min (expt. 1); (*c*) 4.5 h (expt. 6); (*d*) 24 h (expt. 8). Aliquots (1 ml) of peak V_0 fractions (expts. 3 and 6) were brought to pH 5.8 with 0.1 ml of 1 M-acetic acid and incubated at 37°C with 200 i.u. of testicular hyaluronidase. After 6 h another 100 i.u. of enzyme was added and incubation was continued for 18 h. The reaction mixture was centrifuged at 1850 *g* for 40 min, and 1 ml of the supernatant was applied to the same column equilibrated in 0.15 M-NaCl/phosphate (○). The peak V_0 fraction from expt. 8 was incubated at 37°C for 18 h with 100 turbidity-reducing units of *Streptomyces* hyaluronidase in a final volume of 1.5 ml of 0.16 M-NaCl/borate; 1 ml of this was applied to the column (△). Labelled hyaluronic acid was completely degraded to oligosaccharides under the same conditions (results not shown).

uptake of circulating hyaluronic acid. This conclusion is supported by the concentration of radioactivity in the nonparenchymal group of liver cells. These might include some vascular endothelial cells as well as Kupffer cells, but the poor concentration of radioactivity in other vascular tissues indicates that endothelial binding must be relatively minor. The low uptake in hepatic parenchymal cells seems to conflict with the specific binding of [^{14}C]hyaluronic acid by cultured hepatocytes (Truppe *et al.*, 1977), which were shown to generate ethanol-soluble labelled oligosaccharides at the same time. The present findings suggest that the phagocytes dominate the hepatic uptake of hyaluronic acid *in vivo*. The difference may depend upon relative affinity, which has not been directly compared, and on circulatory factors. Other regions in the monocyte–macrophage system have not been considered in this study, but their part in plasma clearance must be quantitatively small. In some tissues, such as bone marrow, the blood flow rate is low; in others, such as the lung, the macrophages are mainly extravascular. Other evidence (Antonas *et al.*, 1973) suggests that the phagocytes in lymph node sinuses might serve to reduce the amount of hyaluronic acid entering the circulation through lymph.

Metabolism

The detection of ^3H in lipid and water is consistent with metabolism of the acetyl group via acetyl-CoA. Unless deacetylation occurs early in degradation, for which no evidence exists at present (Riesenfeld *et al.*, 1980) the scarcity of intermediate oligosaccharides and the appearance of ^3H_2O within 20 min imply that the degradation proceeds rapidly to completion in the liver. Hyaluronidase activity does not appear to be the rate-limiting step in the hepatic catabolism of hyaluronic acid. Small non-volatile labelled metabolites other than ^3H_2O were found in the liver, but not in plasma or urine. These could include acetyl-CoA and other acetyl-derived intermediates, but were not characterized. The decreasing proportion of injected material retained within the liver at successively later intervals can be attributed mainly to degradation and release as water. The ultimate degree of conversion to ^3H_2O was calculated from plasma levels, and the body water fraction (Cizek, 1954). Approx. 45% had been converted to free ^3H_2O after 4.5 h in expt. 6 and approx. 67% was still retained as ^3H_2O at 24 h in expt. 8. Some may be incorporated in organic material by hydrogen exchange and a minor amount converted through acetyl-CoA to lipids and other conjugates.

The spleen retained a relatively high activity as late as 24 h, when most label could be accounted for as water elsewhere. The avidity of uptake is, therefore, not necessarily coupled to the rate of degradation. The speed at which breakdown can occur might nevertheless make the kinetics of cellular binding difficult to ascertain, at least in macrophages, unless catabolism is naturally slow or deliberately arrested.

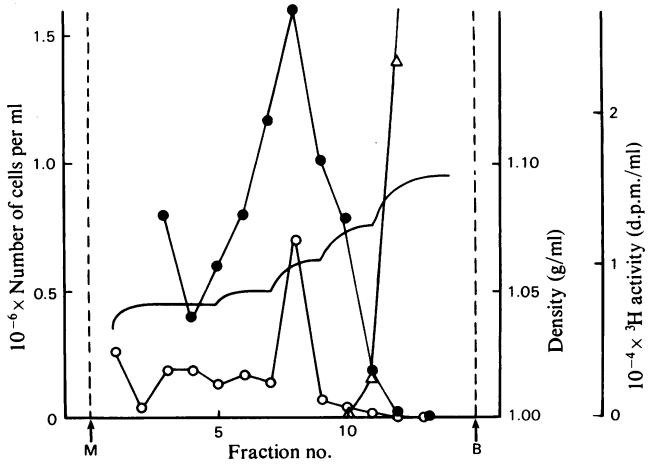

Fig. 3. *Separation of rabbit liver cells in a Percoll gradient*

[^{3}H]Hyaluronic acid was injected intravenously 20min before circulatory arrest (expt. 5). Liver cells were prepared and separated in a stepwise Percoll gradient as described in the Materials and methods section. Fractions (2.5ml) were taken from the gradient and analysed for density (——), radioactivity (○), number of non-parenchymal cells (●) and number of parenchymal cells (△). M and B denote the meniscus and the bottom of the gradient respectively.

Table 6. *Analysis of radioactivity in urine*

The analyses were performed as for plasma (see the Materials and methods section and Table 3). Expt. 6 was modified by prior injection of unlabelled hyaluronic acid.

Time from injection to circulatory arrest	Expt. no.	10^{-3} × Activity in whole urine (d.p.m./ml)	Distribution (%) on Sephadex G-50		Volatile fraction (%) in	
			V_0	V_t	Whole urine	V_t from Sephadex G-50
19min	3	3.4	94	5	11	—
1.5h	1	14.4	62	30	—	97
4.5h	6	70.7	90	6	—	83
24h	8	8.6	2	95	89	—

Physiological aspects

Renal excretion was clearly limited to the lower-molecular-weight fractions of hyaluronic acid. Plasma hyaluronic acid is excluded from Sephadex G-100 (Murata & Horiuchi, 1977) so that its mean molecular weight is likely to be above the critical limit for renal excretion. This observation, taken with the normally low urinary elimination, emphasizes the importance of extrarenal clearance. The limit to the renal excretion of hyaluronic acid must be considered in the light of its exceptionally large hydrodynamic volume in dilute solution (Laurent, 1970; also illustrated here by Sephacryl S-300 chromatography), rather than in terms of its molecular weight. The size of the excreted polymer suggests that its passage into the nephron does not only depend on a simple molecular sieving mechanism in the glomerulus, since many plasma proteins of smaller size do not appear in the urine.

The sources of circulating hyaluronic acid are still not certain, but the levels can increase with disease and its presence in lymph (Laurent & Laurent, 1981) indicates that it may increase significantly, for example with postural changes, and with vigorous physical activity that could accelerate the movement of hyaluronic acid from soft tissues and joints in the limbs into the loosely structured peripheral lymph vessels. The haemodynamic consequences of excessive hyaluronic acid in the circulation are self-evident. The possibility of more subtle effects on the function of macrophages (Forrester & Balazs, 1980; Shannon *et al.*, 1980), other inflammatory cells (Håkansson *et al.*, 1980), cellular immune response (Balazs & Darzynkiewicz, 1973) and virus infectivity (Clarris *et al.*, 1974) suggests that the

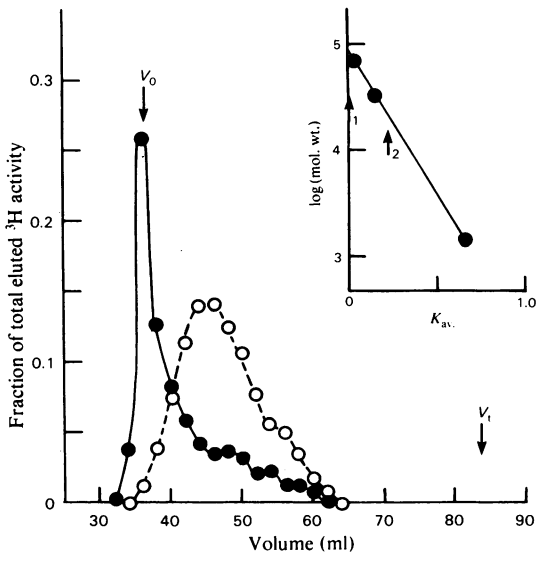

Fig. 4. *Comparison by chromatography on Sephacryl S-300 of [³H]hyaluronic acid extracted from liver and urine*

The material was from expt. 6. After elution from Sephadex G-50, 1ml samples of the peak V_0 fractions from liver extract and from urine were applied to Sephacryl S-300 and analysed as described in the Materials and methods section. ●, Sample from liver extract; ○, sample from urine. The column was calibrated (inset) with defined hyaluronic acid fractions of mol.wt. 67000, 32000 and 3000. Samples applied to the column contained 1mg of each fraction in 1ml of 0.15M-NaCl/phosphate. The contents of the eluted fractions were analysed by the modified carbazole reaction (Bitter & Muir, 1962). $K_{av.}$ for the sample from liver extract is indicated by 1; that for the urine sample by 2.

capacity for clearance of circulating hyaluronic acid may also be important for other reasons.

This work was supported by grants from the Swedish Medical Research Council (project no. 13X-4), and the National Health and Medical Research Council of Australia. We thank Mr. M. Holmes for technical assistance. J. R. E. F. thanks his colleagues in Uppsala for their collaboration in this study, and for their generous provision of facilities.

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