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Labelling of high molecular weight hyaluronan with ^{125}I-tyrosine: studies *in vitro* and *in vivo* in the rat

STEFAN GUSTAFSON[1]*, TOMAS BJÖRKMAN[1] and JAN-ERIK WESTLIN[2]

[1]*Department of Medical and Physiological Chemistry, University of Uppsala, POB 575, S-751 23 Uppsala, Sweden*

[2]*Department of Oncology, Uppsala Academic Hospital, 751 85 Uppsala, Sweden*

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Studies on the metabolism of the polysaccharide hyaluronan has previously been hampered by the lack of radioactive hyaluronan of high molecular weight (MW) and high specific activity. In the present study ^{125}I-tyrosine (T)-labelled hyaluronan was produced after CNBr-activation of the polysaccharide. A specific activity of approximately 0.1 MBq μg^{-1} was achieved using 100 μg of 0.5×10^6 Da hyaluronan labelled for 2 h with 18 MBq ^{125}I. The ^{125}I-T-hyaluronan kept a high MW-profile upon gel filtration chromatography and was found to be cleared from the circulation with the kinetics and organ distribution reported for biosynthetically labelled hyaluronan of high MW. The ^{125}I-labelled polysaccharide is also taken up by 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. The intracellular degradation is slower than that earlier reported for biosynthetically labelled hyaluronan and seems to be halted at the level of low MW oligo- or mono-saccharides that eventually leave the organism via the urine. Scintigraphic images of rats after intravenous injection of ^{125}I-T-hyaluronan showed rapid uptake in the liver and a redistribution of radioactivity from liver to urine with time. Our results indicate that the ^{125}I-T-hyaluronan is suitable for studies of hyaluronan-metabolism in a number of ways. The gamma emitters ^{125}I and ^{131}I are easy to monitor and can be used also for *in vivo* 3D-imaging using single photon emission computer tomography.

Keywords: hyaluronan, hyaluronic acid, radioactive, scintigraphy, liver cells.

Abbreviations: CNBr, cyanogen bromide; T-HA, tyrosine-labelled hyaluronan.

Introduction

The polysaccharide hyaluronan is rapidly cleared from the circulation, primarily by endothelial cells of the liver via receptor-mediated endocytosis [1, 2]. The receptor for hyaluronan on these cells has been characterized and purified from rat liver endothelial cells [3]. Other cell-surface 'receptors' for hyaluronan have been described [4] and include the lymphocyte homing receptor CD44 and a receptor for hyaluronan-mediated motility of fibroblasts. The study of hyaluronan-metabolism has been hampered by the lack of radioactive hyaluronan of high molecular weight (MW) and high specific activity. Recently ^{11}C-labelled hyaluronan has been produced and studied *in vivo* as well as *in vitro*. However, the production and analysis of this tracer needs highly specialized equipment and the half-life of the tracer (20.3 min) makes long-time studies impossible. ^{3}H and ^{14}C-labelled hyaluronan has been widely used. Their primary advantage is that they can be made biosynthetically and therefore are 'true' hyaluronan in the sense that no additional groups have been introduced in the polysaccharide chain. They do, however, have the disadvantages of low specific activity and emission of only low energy beta radiation.

The gamma emitters ^{125}I and ^{131}I have the advantage of medium long half lives making them suitable for studies of hyaluronan-metabolism *in vivo* as well as *in vitro*. Previously, ^{125}I-labelled hyaluronan has been made by chemical modification at the reducing sugar [5] and by coupling tyramine-cellobiose to *N*-deacetylated hyaluronan [6]. Hyaluronan labelled by these procedures generally gives products of low MW and/or low specific activity, which make them unsuitable for many studies of hyaluronan-metabolism. The tyramine-cellobiose can also not be lysosomally metabolized once it is taken up by cells and will therefore accumulate inside the cells. Although this

* To whom correspondence should be addressed.

is a beneficial feature of the compound when determining sites of uptake and degradation, it is potentially harmful in long-term studies *in vivo*.

Recently, we demonstrated that CNBr activation of hyaluronan with a mean MW of 0.5×10^6 Da, followed by coupling with fluoresceinamine and iodination with ^{125}I, does not alter the MW of the polysaccharide nor interfere with the binding to specific cell-surface receptors [7]. The fate of the labelled polysaccharide *in vivo* correlates well with previous studies using biosynthetically labelled hyaluronan of high MW and may be studied by both scintigraphic and fluorometric techniques. However, due to the chemical nature of the added group, the labelled fluoresceinamine will accumulate in cells that take up the polysaccharide and this may cause damage to cells and tissues.

Therefore we have labelled hyaluronan with ^{125}I-tyrosine in order to get a metabolizable tracer with low toxicity that can be used *in vitro* as well as *in vivo* for scintigraphic studies of hyaluronan metabolism in healthy and diseased states.

Materials and methods

Polysaccharides

The hyaluronan used for labelling and uptake- and turnover-studies was supplied by Hyal Pharmaceutical Corporation, Toronto, Canada. Hyaluronan-standards were from Pharmacia, Uppsala, Sweden. All hyaluronans were extracted from avian tissues. The molecular weights of the polysaccharides were 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 [8]. The hyaluronan content in each fraction was monitored by determination of the absorbance at 214 nm of the unlabelled hyaluronan. Radioactivity was measured by gamma-counting on a Packard auto-gamma gamma-counter.

Cells

A single cell suspension was prepared from the liver of male Sprague Dawley rats or female nude (Nu/Nu) 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 adherence as described by Pertoft and Smedsrød [9], giving approximately 95% pure cells [9, 10]. 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) fetal calf serum. Liver endothelial cells were cultured entirely without serum. All cells were cultivated over night before the start of the experiments. The parenchymal cells were cultured for 2–3 h in the absence of fetal calf serum before the experiments started.

Labelling of hyaluronan

The hyaluronan was labelled with DL-tyrosine after CNBr-activation of the polysaccharide by the method of Glabe *et al.* [11]. Briefly, 15 mg hyaluronan 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 hyaluronan was incubated over night with 1 mg tyrosine (T) (Sigma Chemical Company, St Louis, USA). The T bound to hyaluronan (T-hyaluronan) 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-hyaluronan was iodinated with ^{125}I by placing 100 µg of T-hyaluronan together with 0.3–1.0 mCi ^{125}I in a small glass tube covered with a film of 10 µg 1,3,4,6-tetrachloro-3a,6a-diphenylglycouril (Sigma Chemical Company, St Louis, USA). Unincorporated ^{125}I was removed on a PD 10 column equilibrated with PBS and the iodinated T-hyaluronan (^{125}I-T-hyaluronan) stored at 5°C. The specific radioactivity was usually 1500–8000 dpm ng^{-1}. Decay correction (half-life 60 days) was done when quantifying administered or recovered ^{125}I-T-hyaluronan.

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 100 000–200 000 liver endothelial cells 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 and was then, in some experiments, subjected to gel chromatography on a 24 ml Sephacryl 300 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 ($1.15\ g\ l^{-1}$) and analysed for radioactivity as previously described [12], or homogenized and fractionated as described earlier [3]. Unspedific binding was corrected by measurement of radioactivity bound to dishes without cells, which generally was just above background levels.

In vivo *studies*

Male Sprague Dawley rats or female Nu/Nu rats, weighing 200–300 g, were anaesthetized with pentobarbital (45 mg kg^{-1} body weight). They received an injection in the tail vein of 7–8 µg ^{125}I-T-hyaluronan ($5–15 \times 10^6$ cpm), and in some cases 1–2 mg of unlabelled hyaluronan, in 0.8–1.0 ml 0.15 M NaCl, 10 mM NaH_2PO_4, pH 7.4. In kinetic studies,

blood samples (25 µl) were repeatedly collected from the distal part of the tail during the circulation period. The amount of radioactivity present in the blood at 0 min was calculated by extrapolating backwards from the first three determinations. In some cases, at the end of the experiment, a larger blood sample (300–400 µl) was collected.

After 10 min to 24 h the rat was killed. Liver, lungs, kidneys, heart, spleen and in some instances skin, fat, intestines and urine were assayed for radioactivity. In some cases the liver was perfused with collagenase and the different liver cells separated as described above. The calculation of liver uptake, and the distribution between different liver cells, are based on the fact [13] that a normal rat liver contains about 400×10^6 non-parenchymal cells and 1200×10^6 parenchymal cells. Total liver activity was calculated by gamma-counting a portion of the single cell suspension after collagenase perfusion, multiplying to get the activity of the whole volume and adding the activity found in the non-collagenase degradable leftovers. The activity found in pure parenchymal cells (activity per 10^6 cells multiplied by 1200) and non-parenchymal cells (activity per 10^6 cells multiplied by 400) were added to give 100% (this value was generally within ±10% of the total liver value calculated as described above), and the distribution between non-parenchymal cells and parenchymal cells calculated as a percentage of the total liver uptake. Cell number in suspension was determined by counting using a Bürker-chamber in a phase contrast microscope. On dishes the cell number was determined using a microscope equipped with a grid.

Scintigraphic studies

The rats were anaesthetized and injected as described above. In dynamic studies the injections were made with the rats placed on the standard medium resolution collimator of the gamma-camera in order to study rapid uptake in the liver. Images were collected in a 64 × 64 pixel matrix in word mode with a 34 keV 80% window setting in a Gamma 11 system (Philips). The dynamic sequence was preset at one image per min for 15 min. After 15 min, static images were collected at different times after injection in order to study the disappearance of radioactivity from the liver. Images were then transferred to the Hermes® system (Nuclear Diagnostics, Hägersten, Sweden and London, UK) and regions of interest were drawn. When measuring liver uptake it was necessary to draw a region of interest covering an area larger than the liver itself because of scattering.

Results

The labelling of hyaluronan with tyrosine after CNBr activation of the polysaccharide resulted in a polysaccharide that could be iodinated to a high specific radioactivity. A specific activity of approximately 0.1 MBq μg^{-1} was achieved using 100 µg of hyaluronan labelled for 2 h with

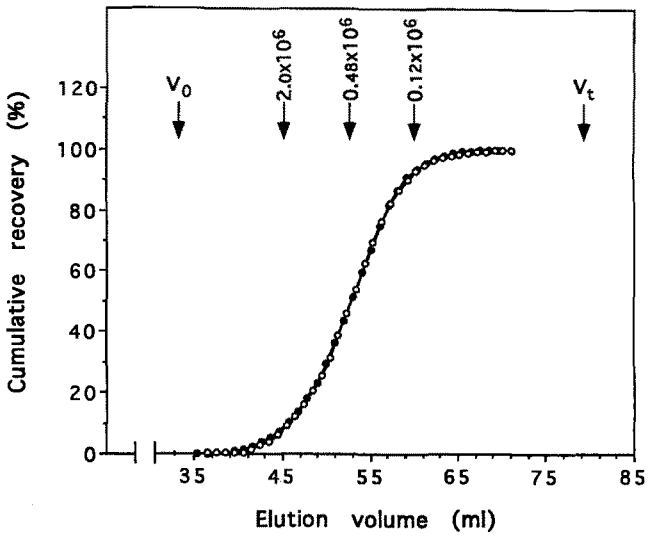

Figure 1. The cumulative recovery of unlabelled hyaluronan (○) and ^{125}I-T-hyaluronan (●) after size exclusion chromatography on a 100 ml calibrated column of Sephacryl HR with porosities noted as 400, 1000 and 2000 (Pharmacia, Uppsala, Sweden) in 0.25 M NaCl, 0.05% chlorbutanol [8]. The void volume (V_0) and the total volume (V_t) of the column as well as the mean elution volume of hyaluronan standards with mean MW of 2.0×10^6, 0.48×10^6 and 0.12×10^6 are indicated.

18 MBq ^{125}I. The radiolabelled hyaluronan chromatographed in an essentially identical manner to the unlabelled material on a calibrated gel filtration column (Fig. 1). The mean MW of the unlabelled, as well as the labelled, polysaccharide was found to be 480 000 Da. The material was polydispersed with approximately 90% of the polymers between 2×10^6 Da and 100 000 Da (Fig. 1).

The ^{125}I-T-hyaluronan was effectively taken up by liver endothelial cells in culture (Fig. 2). The uptake was in the

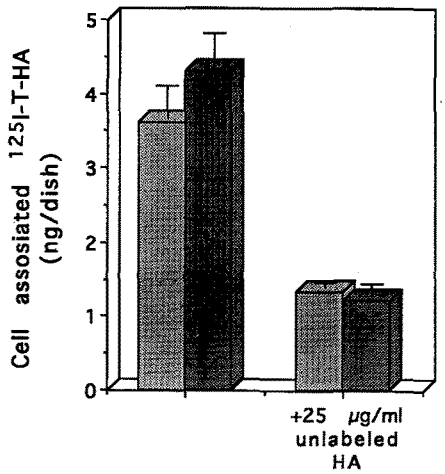

Figure 2. Cell association of ^{125}I-T-labelled hyaluronan (^{125}I-T-HA) to liver endothelial cells in culture. Approximately 100 000 cells were incubated for 75 min at 37°C with 1 µg ml^{-1} ^{125}I-T-HA. Results are mean ± SD of four determinations using two different batches of ^{125}I-T-HA. Bars represent the two different batches. See the Materials and methods section for details.

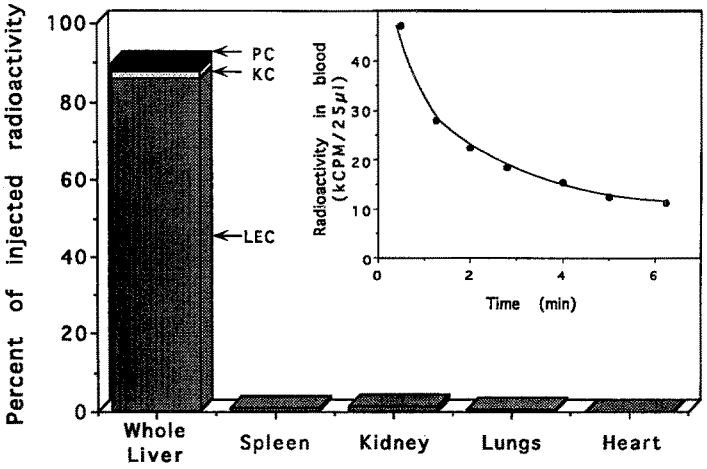

Figure 3. The recovery of radioactivity in different organs of the rat 10 min after an intravenous injection of 7.5 µg ^{125}I-T-hyaluronan. KC denotes Kupffer cells, PC denotes parenchymal cells and LEC denotes liver endothelial cells. *Inset:* Disappearance of radioactivity from blood after intravenous injection in rats of 7.5 µg ^{125}I-T-hyaluronan.

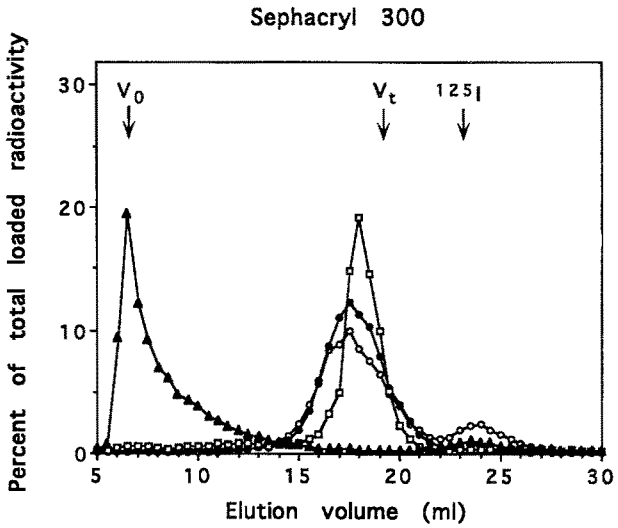

Figure 4. Size-exclusion chromatography on a 24 ml Sephacryl 300 column of the medium and the soluble fraction of liver endothelial cells 24 h after a 75 min 'pulse' of ^{125}I-T-hyaluronan (1 µg ml^{-1}) or ^{3}H-hyaluronan (1 µg ml^{-1}). ^{125}I-labelled material in the medium (○) and in the cytosol (●). ^{3}H-labelled material in the medium (□). ^{125}I-T-hyaluronan before incubation (▲), indicating the V_0 position. The total volume of the column (V_t) was determined by ^{3}H-H_2O. The position of free ^{125}I is also indicated.

same order as that reported for biosynthetically labelled ^{3}H-hyaluronan in the same cells [14] and could effectively be inhibited by unlabelled hyaluronan (Fig. 2). The degree of inhibition was also in the same order as earlier reported for ^{3}H-hyaluronan [14]. No significant uptake was observed in cultured parenchymal cells (not shown).

When a tracer dose of the ^{125}I-T-hyaluronan was injected intravenously in the rat it rapidly disappeared from the circulation in a logarithmic fashion with a $T_{1/2}$ of approximately 2 min (Fig. 3 inset). When the tracer was mixed with a 200-fold excess of unlabelled hyaluronan the disappearance was dramatically slowed down and resembled zero-order kinetics (not shown). Most of the label from the tracer studies was recovered in the liver (Fig. 3). After 10 min of circulation approximately 10% was still remaining in blood while 80–85% of the injected dose was found in the liver. This uptake was inhibited to about 90% by the simultaneous injection of an excess of unlabelled polysaccharide. Some uptake was also found in spleen, kidney and lung (Fig. 3). However, the uptake in these organs was small and could not be inhibited by an excess of unlabelled hyaluronan. When the different liver cells were isolated after uptake of a tracer dose *in vivo*, approximately 90% of cell associated radioactivity was found in liver endothelial cells, 5% in kupffer cells and only 2–3% in parenchymal cells (Fig. 3). The results from the *in vivo* experiments are in accordance with those earlier published using other tracers [7, 15, 16].

Some rats were studied for longer times to see if the tracer could be metabolized and washed out of the organism. Twenty-four hours after the injection of a tracer dose approximately one-third of the radioactivity had disappeared from the liver and left the body via the urine. This rate of elimination from the body is considerably slower than that reported for ^{3}H-hyaluronan [2, 14, 15]. In order to determine if the degradation of the polysaccharide is slowed down by the labelling with ^{125}I-T, the labelled hyaluronan was incubated with purified liver endothelial cells in culture for 75 min. After this incubation the incubation medium was removed, new medium was added and the medium and cells analysed for degradation products on a gel filtration column. It was found that no high MW material was present in the soluble fraction of the cells after 24 h of incubation (Fig. 4). However, there was a substantial amount (about 70%) of low MW material still left inside the cells at this time point. This was not the case using ^{3}H-hyaluronan where all the radioactivity was found as low MW material in the medium. The ^{125}I low MW material in the medium chromatographed in two well separated peaks. One peak came at a position similar to degraded ^{3}H-hyaluronan and was also found inside the cells. Another small peak appeared after the total volume of the column at the same position as free ^{125}I.

Scintigraphic images of rats after intravenous injection of ^{125}I-I-hyaluronan, showed rapid uptake in the liver (Fig. 5) and a redistribution of radioactivity from liver to urine with time. The radioactivity over the liver decreased by approximately 98% over 5 d (Fig. 5).

Discussion

Studies on the metabolism of hyaluronan has previously been hampered by the lack of radioactive hyaluronan of high MW and high specific activity. Recently we reported

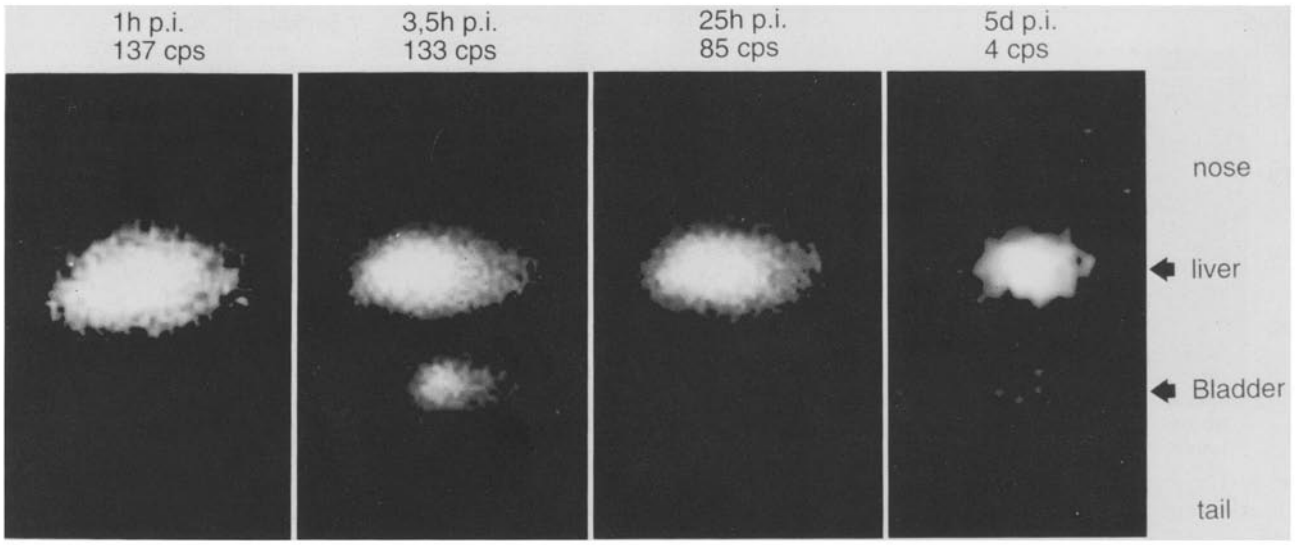

Figure 5. Scintigraphic images of a rat after an intravenous injection of 7.5 μg ^{125}I-T-hyaluronan. 1 h, 3.5 h, 25 h and 5 d after injection. The activities over the liver and the urinary bladder are indicated. The count rate in each image is given in counts per second (cps). Sampling was stopped and the images produced at approximately 12 000 counts.

that labelling of hyaluronan with fluorosceinamine followed by ^{125}I after CNBr activation resulted in a labelled molecule with unaltered size and receptor-binding properties. However, fluorosceinamine is not a suitable molecule to be introduced in humans and would, to a large degree, probably be trapped inside cells that endocytose the polysaccharide. Therefore we have tried to synthesize a degradable hyaluronan with the possibility that it could be used in human studies.

The present study shows that CNBr activation and coupling of tyrosine, followed by iodination with ^{125}I, does not alter the MW of the polysaccharide (Fig. 1) nor interfere with the binding to specific cell-surface receptors (Figs 2 and 3). The fate of the labelled polysaccharide *in vivo* correlates well with previous studies using biosynthetically labelled hyaluronan of high MW. However, after intracellular degradation, the washout of radioactivity from the cells was quite slow (Figs 4 and 5). While 96% of biosynthetically labelled ^{3}H-hyaluronan had left the cells after 24 h, only 29% of the radioactivity from the ^{125}I-T-labelled hyaluronan had left the cultured cells after 24 h. The observed elimination from the liver endothelial cells *in vitro*, also appears to determine the rate of clearance of radioactivity from the body *in vivo* (Fig. 4). After 24 h approximately 35% of the activity initially taken up by the liver had left the body and was predominantly found in the urine. In one experiment the rat was studied for 5 d after injection. After this length of time only 2–3% was still in the animal. This is what could be expected from an exponential disappearance and indicates that approximately 60% of an injected tracer dose leaves the body in the first 2 d. After an additional 2 d 80% of the administered radioactivity will have left the organism. No appreciable accumulation of radioactivity in the thyroid could be observed at any time point indicating that probably very little free iodine is released during degradation of the polysaccharide.

Taken together, our results indicate that ^{125}I-T-hyaluronan is suitable for studies of hyaluronan metabolism in a number of ways. The gamma emitters ^{125}I and ^{131}I are easy to monitor and can be used for *in vivo* imaging using single photon emission computer tomography. The slow washout of radioactivity from sites of uptake is an advantage when studying tissues with relatively low levels of uptake. A week after injection about 99% of the radioactivity would be expected to have left the cells involved in uptake thus allowing a new experiment to be performed. The turnover in nude (Nu/Nu) rats seems to be no different from that found in Sprague Dawley rats, indicating that nude rats, often used in experimental tumour models, can be used in studies of hyaluronan turnover in such models. ^{125}I-T-hyaluronan is also suitable for autoradiographic studies of tissues *in vitro* after administration *in vivo*, so that cells involved in the uptake of hyaluronan can be identified.

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