Plasma clearance of rat bikunin: evidence for receptor-mediated uptake

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Bikunin is a chondroitin sulphate-containing protease inhibitor with a molecular mass of 25 kDa. It is secreted into the blood by hepatocytes, and recent observations indicate that it may have an extravascular function. Here we have studied the plasma clearance of bikunin in rats and mice. On intravenous injection, radiolabelled bikunin was found to have a half-life of 10 min; in rats with ligated renal arteries, the clearance time was twice as long, implying that the kidneys account for half the uptake. As judged by gel filtration, the size of bikunin is similar to that of albumin. Autoradiographic analysis of kidneys removed 2 min

after the injection of radiolabelled bikunin indicated that, despite its size, bikunin is cleared by glomerular filtration. On ligation of the renal arteries, the plasma concentration of bikunin increased linearly to at least four times normal. This finding shows that the non-renal uptake system is saturated and therefore presumably receptor-mediated. Most of the non-renal uptake of injected bikunin was found to occur in non-visceral tissues such as the skin. Analysis of skin samples by autoradiography after injection of radiolabelled bikunin suggested that bikunin had been transferred from the plasma to the interstitial space.

INTRODUCTION

Bikunin was originally described as the major trypsin inhibitor in human urine [1] and was later detected in serum, both in free form [2,3] and as a subunit of inter- α -inhibitor [4,5] (for a review see ref. [6]). It consists of a 16 kDa polypeptide, an 8 kDa chondroitin sulphate chain and an N-linked oligosaccharide of about 1 kDa [7]. The name bikunin, which was recently introduced to replace the various names previously used [8] such as urinary trypsin inhibitor and acid-stable trypsin inhibitor, reflects the fact that it consists of two protease-inhibiting domains of the Kunitz type [5,9]. Bikunin has been shown to inhibit a number of different proteases but binds them less strongly than other more abundant inhibitors in the plasma [10,11] making its physiological role in this compartment unclear. More recently, bikunin was reported to be a mitogen for endothelial cells [12] and fibroblasts [13] as well as inhibiting invasion of metastasing cancer cells [14,15]. These observations suggest that bikunin has a function in the extravascular space.

Information about clearance rate and site of uptake may be useful for the elucidation of the function of a plasma protein. Previous reports on these aspects of bikunin have yielded various results; for example, bikunin isolated from human urine and labelled with ^{125}I was found to have a half-life of 9 min in rats [16] whereas that isolated from rat serum was found to have a half-life of 43 min in mice [17]. In the present study we have determined the half-lives of bikunin isolated from both rat urine and the medium of rat hepatocyte cultures; the two forms of the protein yielded essentially the same result. In two previous studies, in which the tissue uptake of bikunin was assessed from the distribution of radioactivity 15 min after the injection of ^{125}I -labelled bikunin, the kidneys were found to account for 15 and 44% of the total injected amount [16,17]. In the present study we show that redistribution of degradation products may distort the

values obtained with this technique. Therefore we have estimated the contribution of the kidneys to bikunin uptake from the rate at which its plasma concentration increases when the kidneys are excluded from the circulation, together with its plasma half-life. Although it has been known for a long time that bikunin can pass through the kidneys in an undegraded form [18], it has not been clear by what path this occurs. The hydrodynamic size of bikunin, as judged by gel filtration or dialysis, is similar to that of albumin [19–21] and it has a higher negative charge: approximately -27 compared with -17 for albumin [7]. These characteristics suggest that renal uptake does not occur primarily through glomerular filtration [22,23]; an alternative site of uptake is the peritubular capillaries [24]. Using autoradiography we present evidence that bikunin does indeed pass through the glomeruli. Furthermore, we have used microscopic autoradiography to localize bikunin in the skin, a tissue that appears to be a major site of uptake.

MATERIALS AND METHODS

Chemicals

Trypsin was obtained from Boehringer-Mannheim, Mannheim, Germany, $^{35}\text{SO}_4$ (22–40 Ci/mg) from Amersham International, Amersham, Bucks., U.K. and sodium 5-*sec*-butyl-5-ethyl-2-thiobarbiturate (Inactin) from Byk-Gulden, Konstanz, Germany.

Isolation of bikunin from rat urine

Male Sprague–Dawley rats weighing 200–350 g were kept in metabolic cages with free access to water and a standardized diet (R3; Ewos, Södertälje, Sweden). NaN_3 was added to the collection tubes, so that its final concentration in the urine was at

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least 15 mM. The urine was collected once a day and stored at -20°C . Ten rats produced about 0.7 litre in 1 week. After thawing, the urine was filtered first through coarse and then fine filter paper (Munktell no. 3 and OOH respectively). This and all subsequent procedures were carried out at 4°C . The urine was then mixed with an equal volume of 0.1 M sodium citrate (pH 6.5), and 0.1 M EDTA (pH 7.0) was added to a final concentration of 10 mM. Bikunin was then isolated by affinity chromatography on immobilized trypsin essentially as described [25]. Trypsin was coupled to CNBr-Sephacryl CL4B (Pharmacia, Uppsala, Sweden) as described by the manufacturer, yielding 7 mg of trypsin/ml of gel. The urine was applied to a trypsin-Sephacryl column (3 cm $\times$ 2.5 cm) at a flow rate of 300 ml/h. The gel was then washed with 300 ml of 50 mM sodium acetate (pH 4.0). Elution of bound protein was performed with 0.25 M glycine/HCl (pH 2.5) at a flow rate of 40 ml/h, and 4.5 ml fractions were collected into tubes containing 750 μl of 1.0 M Tris/HCl (pH 9.0). Fractions with trypsin-inhibiting activity [26] were pooled, concentrated to 1 ml in a collodium bag and applied to a Sephacryl S-200 column (1 cm $\times$ 78 cm) equilibrated with 50 mM Tris/HCl, pH 8.0, containing 150 mM NaCl. Fractions that yielded a 40 kDa protein band, as judged by SDS/PAGE and silver staining, in addition to exhibiting trypsin-inhibiting activity were pooled and concentrated. The concentration of bikunin was determined from the absorbance at 278 nm using the coefficient $0.79 \text{ litre} \cdot \text{g}^{-1} \cdot \text{cm}^{-1}$ [7]. The yield was 0.4–0.7 mg/l of urine. Protein samples were frozen in liquid nitrogen and stored at -70°C .

Radioactive labelling

Labelling of urinary bikunin with ^{125}I was performed as previously described [7]. For metabolic labelling, rat hepatocytes were isolated and cultured in 60 mm dishes as previously described [27]. The cells, which were used 20–24 h after seeding, were rinsed twice with 1.5 ml of buffer 3 (137 mM NaCl, 5 mM KCl, 1.2 mM CaCl_2 , 0.7 mM MgCl_2 and 25 mM Hepes/NaOH, pH 7.4) [28]. Eagle's minimum essential medium (with Earle's salts) lacking MgSO_4 and NaHCO_3 and supplemented with MgCl_2 , 25 mM Hepes/NaOH (pH 7.4) and 60 MBq $^{35}\text{SO}_4$ was then added (1.5 ml) and the cells were incubated on a rocking device at 37°C for 1 h. At the end of the labelling period the medium was collected and cell debris removed by centrifugation (5 min at 14000 g). The medium samples were subjected to gel filtration on a Sephacryl S-200 column and the fractions analysed by SDS/PAGE followed by fluorography [29]. Fractions containing a 35–45 kDa protein were pooled and concentrated; the total radioactivity of these samples was 1.0×10^6 – 1.9×10^6 c.p.m.

Plasma clearance in rats

Male Sprague–Dawley rats (ALAB, Sollentuna, Sweden) weighing 250–350 g were used. They had free access to water and standardized diet (R3) until 15–18 h before the experiment when the chow was withdrawn. They were anaesthetized with sodium 5-sec-butyl-5-ethyl-2-thiobarbiturate given intraperitoneally at a dose of 120 mg/kg body weight. A servo-controlled heating pad maintained the rectal temperature at 37.5°C . After tracheostomy, the left femoral vein was cannulated and Ringer solution (129 mM NaCl, 2.5 mM KCl, 25 mM NaHCO_3 and 0.75 mM CaCl_2) was continuously infused (5 ml/h per kg body weight). The left femoral artery was cannulated for monitoring blood pressure and blood sampling. The bladder was cannulated through a suprapubic incision and the urine was collected. In

some experiments, the renal veins and arteries were ligated. When the surgery was completed, the rats were given an extra infusion of 1.0 ml of Ringer solution supplemented with 50 units of heparin. After 20–30 min, 1 ml of $^{35}\text{SO}_4$ -labelled or 200 μl of ^{125}I -labelled bikunin in 50 mM Tris/HCl (pH 8.0) and 0.15 M NaCl [$(0.7\text{--}1.9) \times 10^6$ and $(0.6\text{--}9.0) \times 10^6$ c.p.m. respectively] was infused at a rate of approx. 0.5 ml/min. From 2 min after the end of the administration, 100 μl of blood was withdrawn every 5 min; subsequently an extra infusion of 0.2 ml of Ringer solution was given. At the end of the experiment, the rats were killed by an injection of saturated KCl into the heart and placed on ice. They were then dissected, the different organs weighed and their radioactivity was determined.

Whole-body autoradiography

Male Sprague–Dawley rats were anaesthetized as described above and ^{125}I -bikunin (10×10^6 c.p.m.) in 0.8 ml of 0.15 M NaCl was injected. The animals were killed after 30 min and prepared for whole-body autoradiography [30]. Briefly, the rats were frozen by immersion in *n*-hexane, cooled with solid CO_2 (-78°C) and then embedded in a gel of CM-cellulose which was quickly frozen. Sagittal sections (10, 40 and 60 μm thick) were taken on adhesive tape (no. 810; 3M Co., St Paul, MN, U.S.A.) at different levels, comprising all organs through the entire animal. They were then freeze-dried and placed on Hyperfilm ^3H (Amersham International) or Structurix D7 (Agfa-Gevaert). The films were developed after 4–6 weeks.

Microscopic autoradiography

^{125}I -bikunin [$(30\text{--}40) \times 10^6$ c.p.m. in 150 μl of PBS] was injected into the tail vein of female C57 black mice weighing 18–25 g. After 30 min the animals were killed by cervical dislocation and samples of various tissues were excised and fixed with 3% glutaraldehyde. These were then embedded in paraffin, sectioned and immersed in film emulsion (NTB2; Kodak) as described previously [30].

Tissue uptake in mice

^{125}I -bikunin [$(5\text{--}10) \times 10^6$ c.p.m. in 150 μl of 50 mM sodium phosphate buffer (pH 7.0) containing 0.15 M NaCl] was injected into the tail vein of the same strain of mice as described above. The animals were killed at different times by cervical dislocation. They were then placed on ice, dissected and the radioactivity in the organs was determined. In those experiments in which the degradation of bikunin was studied, the back of the dead animal was shaved and a sample of the skin (1 $\times$ 1 cm) was excised. The skin was then cut into smaller pieces (2 $\times$ 2 mm) and homogenized in 3.0 ml of a solution containing 50 mM Tris/HCl (pH 8.0), 0.15 M NaCl, 1 mg/ml BSA, 2 mM EDTA, 1 mM PMSF and 10 mg/ml saponin in a porcelain mortar. After centrifugation, the supernatant, which contained 85–90% of the total radioactivity, was applied to a Superose 12 column (Pharmacia; 1.0 cm $\times$ 30 cm) and eluted with 50 mM Tris/HCl containing 0.15 M NaCl at a flow rate of 0.3 ml/min.

In some experiments, the mice were anaesthetized as described for the rats and the left femoral vein was cannulated. The renal veins and arteries were then ligated and ^{125}I -bikunin was injected into the femoral vein. The animals were killed at different times by the injection of KCl, put on ice and dissected.

RESULTS

Plasma clearance of ^{125}I -bikunin

To determine the time course for the clearance of ^{125}I -labelled bikunin, we injected the radiolabelled protein intravenously into rats. Blood samples were then taken every 5 min and their radioactivity was determined (Figure 1). The rate at which the radiolabelled protein disappeared decreased rapidly during the first 50 min but was then relatively constant. The half-life of the slow phase was estimated by fitting the late data points to an exponential curve; the resulting half-life was 119 ± 20 min (mean $\pm$ S.D.). This phase can be accounted for by contaminating proteins or aggregates of bikunin or it may be due to the protein being taken up and slowly released from a different compartment. Analysis by acid precipitation showed that the relative amount of intact ^{125}I -labelled bikunin in the blood decreased from 92 to 79 % during the course of this experiment. The inset in Figure 1 shows a semilogarithmic plot of the values obtained between 2 and 47 min for one experiment in which the contribution of the slow phase was deducted. This graph for two experiments yielded a half-life for bikunin of 8 ± 1 min.

Autoradiography

To locate the sites at which the radiolabelled bikunin was taken up after intravenous injection, we used whole-body autoradiography. To this end, ^{125}I -labelled bikunin was injected into rats which were processed for autoradiography after 30 min. Figure 2 shows that the kidneys had the highest specific radioactivity followed by the gastric contents. The gastric mucosa, liver, blood, urine (not shown) and thyroid gland (not shown) displayed lower levels. The radioactivity in the liver was unevenly

Figure 1 Plasma clearance of ^{125}I -labelled bikunin

Bikunin was isolated from rat urine, labelled with ^{125}I and injected intravenously into two rats. Blood samples were taken after 2 min and then every 5 min and their radioactivity was determined. The graph shows the radioactivity values obtained in two experiments relative to the respective initial values. The points between 50 and 90 min from one experiment have been fitted to an exponential curve which describes the disappearance of a slowly cleared component. The inset shows the values from one experiment from which the contribution of this component was subtracted.

Figure 2 Whole-body autoradiography of rat injected with ^{125}I -bikunin

Radiolabelled bikunin was injected intravenously and 30 min later the animal was rapidly frozen and sectioned. The sections were put on film and kept at -20°C . The autoradiograph is of a $20\text{ }\mu\text{m}$ -thick section developed after 4 weeks.

Table 1 Tissue distribution of ^{125}I -labelled bikunin in rats and mice

^{125}I -labelled bikunin was injected intravenously. After 30 min, the animals were killed, dissected and the radioactivity in the organs was determined. The radioactivity in the different tissues relative to the total recovered amount is shown. Two animals were used for each experiment and results are means $\pm$ S.D. In the anephric mice the renal arteries were ligated before injection.

Organ	Radioactivity (%)		
	Rat	Mouse	Anephric mouse
Kidneys	23 ± 4	14 ± 2	3 ± 1
Urine + bladder	$3 \pm 3^*$	—	—
Skin	27 ± 3	21 ± 2	16 ± 4
Liver	9 ± 1	4 ± 1	7 ± 1
Stomach	3 ± 1	8 ± 2	3 ± 1
Intestine	6 ± 1	8 ± 1	14 ± 2
Remainder	32 ± 4	47 ± 2	61 ± 8

* For one of the animals used in this experiment, no buffer was infused intravenously resulting in a low production of urine.

distributed, with spots of higher activity; densitometric analysis showed that the specific radioactivity in these spots was at least twice that of the blood. The skin, lungs, myocardium, red pulp of the spleen and bone marrow also showed significant levels of radioactivity. The radioactivity in the muscles and central nervous system was similar to that of the background.

Organ distribution

To complement the semiquantitative information obtained from whole-body autoradiography, we also determined the radioactivity in various organs after the injection of ^{125}I -labelled bikunin into rats. Table 1 shows that 30 min after the injection, the renal system (kidneys, urinary bladder and urine) contained 26 % of that injected. The skin was also a major recipient, accounting for 27 % of the uptake. The specific radioactivity of this tissue 30 min after the injection was two to four times lower than that of the blood (not shown). As the relative volume of blood in the skin is only 2 % [31,32], the former value implies that accumulation had occurred in this tissue. Similarly, the specific radioactivity of the liver was about half of that of the blood; the relative volume of blood in the liver is 15 % [33]. Consistent with the autoradiographic analysis, the stomach and

Figure 3 Appearance of bikunin in urine

^{125}I -bikunin was injected intravenously into a rat and urine was collected during 15 min intervals. Total radioactivity ($\square$) and radioactivity of acid-insoluble material from each fraction ($\blacksquare$) were then determined (a). The acid-insoluble material was analysed by SDS/PAGE followed by fluorography (b). Lanes 1–6 represent material collected during the six 15 min intervals indicated in (a). The arrow indicates the top of the separating gel. The molecular masses in kDa of [^{14}C]methylated standard proteins are indicated on the left.

intestines also contained relatively high amounts of radioactivity. The radioactivity in the gastric contents was apparently associated with degradation products, as it remained in the supernatant after acid precipitation. Several organs such as the lungs, testicles, thymus, pancreas, thyroid, spleen and brain were found to contain less than 1% of the total radioactivity. In animals dissected 90 min after the injection, the organ distribution was similar to that found at the earlier time point, except that the urine contained more radioactivity than the kidneys (not shown). The same analysis, but less extensive, was carried out with mice, and similar results were obtained (Table 1).

Renal uptake

It has previously been shown that a portion of intravenously injected ^{125}I -labelled bikunin appears in intact form in the urine [16,17]. In order to quantify this process, we collected urine continuously at 15 min intervals after the injection of the radiolabelled protein. The total radioactivity and the radioactivity of acid-insoluble material in the fractions were then determined (Figure 3a). This analysis indicated that 5–10% of the radioactivity was in undegraded bikunin. Each fraction was also analysed by SDS/PAGE followed by fluorography. Figure 3(b) shows that a band of the same apparent molecular mass as that of bikunin (40 kDa [7]) appeared in the urine between 15 and 30 min after the injection. From the volume of the urinary catheter and the flow rate, it can be calculated that there was a

Figure 4 Microscopic localization of ^{125}I -bikunin in kidney

^{125}I -bikunin was injected into the tail vein of a mouse. The animal was killed 2 min later and kidney tissue was fixed, dehydrated and paraffin-embedded. Sections 5 μm thick were prepared and processed for autoradiography. A glomerulus is indicated with a G and two proximal tubules with arrowheads.

delay of at least 6 min in the transfer of the radioactive material from the kidneys to the collecting tubes.

In an attempt to reveal the pathway taken by bikunin through the kidneys, we injected ^{125}I -labelled bikunin into mice. The kidneys were removed after different times, fixed, embedded in paraffin, sectioned and processed for autoradiography. Figure 4 shows that, 2 min after injection, the level of radioactivity in the proximal tubules (arrowheads) was higher than in the glomeruli (labelled G). This was also the case after 30 s but the difference was smaller (results not shown). There was no apparent lumen in the tubules presumably because the liquid in this space had been absorbed during sample preparation (not apparent after photographic reproduction). Within the epithelial cells of the proximal tubules, the radioactivity was severalfold higher in the apical than in the basal part suggesting that the radiolabelled protein had been resorbed.

Dermal uptake

At 30 min after the injection of ^{125}I -bikunin into mice, pieces of skin were processed for autoradiography as described for kidney tissue (Figure 5). As low-molecular-mass compounds are extracted in this procedure, the detected radioactivity should represent mainly intact bikunin. The extensive white areas are fat droplets (one of which is marked F) between which capillaries are surrounded by silver grains can be seen; one capillary is indicated with arrowheads. In the lower part of the micrograph there is an arteriole (labelled A). The grain density around the capillaries is

Figure 5 Microscopic localization of bikunin in skin

^{125}I -bikunin was injected into the tail vein of a mouse and the animal was killed 30 min later. A piece of the dorsal skin was removed and processed for autoradiography as described in Figure 4. A muscle fibre (M), an arteriole (A) and a fat droplet (F) are indicated. The arrowheads show a capillary.

at least as high as in the blood (as in the lumen of the arteriole), indicating that the radiolabel had been transferred through the vascular wall. The muscle fibres (dark-grey areas marked M) are virtually devoid of radiolabel. Below the muscle-containing layer, radiolabel could be seen in what appeared to be fibrous structures (not shown).

To characterize further the uptake of ^{125}I -bikunin in the skin, mice were killed at various times after the injection and skin samples were taken. These were homogenized in the presence of saponin, a compound that permeabilizes membranes [34], and centrifuged. The supernatants, which contained 85–90% of the total radioactivity, were then subjected to gel filtration. In a skin sample collected after 30 min, about 40% of the radioactivity (Figure 6a, peak I) was eluted as the injected protein, whereas the remainder was in smaller degradation products (peak II). Figure 6(b) shows the relative amount of degraded bikunin in skin samples taken at different times after the injection. Apparently, the degradation products were mainly formed during the first 10 min after the injection of the labelled bikunin.

Plasma clearance of $^{35}\text{SO}_4$ -labelled bikunin

To determine the clearance characteristics of newly synthesized bikunin, we isolated from rat hepatocyte cultures bikunin that had been metabolically labelled with $^{35}\text{SO}_4$ [29]. Figure 7 (closed circles) shows that, after intravenous injection into rats, this protein was rapidly removed from the bloodstream, with little radioactivity remaining after 50 min. The half-life obtained from a semilogarithmic plot (Figure 7, inset, closed circles) was 11 ± 1 min.

Effects of nephrectomy

The tissue distribution of the radioactivity in anephric mice 30 min after the injection of ^{125}I -bikunin was determined (Table

Figure 6 Extraction of ^{125}I -bikunin from skin

^{125}I -bikunin was injected into mice which were then killed at different times. A piece of dorsal skin was excised and homogenized in a saponin-containing buffer. The homogenates were centrifuged and the supernatants applied to a Superose 12 column. The fractions were collected (500 μl) and their radioactivity was determined. (a) Radioactivity in a skin sample collected 30 min after the injection. Peak I is intact ^{125}I -bikunin with an apparent molecular mass of 70 kDa. Peak II, which was eluted after the total volume, is probably iodotyrosine [23,35]. The elution volumes of Blue Dextran (Pharmacia) and $^3\text{H}_2\text{O}$ were 8 and 22 ml respectively. (b) Amount of radioactivity in peak II relative to that of both peaks in skin samples taken at different times after the injection of bikunin.

1). Of the organs examined, the skin had the highest amount of radioactivity. Analysis by gel filtration showed that 15% of this radioactivity was in degradation products. This result together with those of Figure 6 indicates that, in the normal animal, transfer of degradation products from the kidneys to the skin occurs.

Under steady-state conditions, the rate at which a plasma protein is synthesized is equal to its rate of clearance. Therefore the rate at which a protein is taken up in an organ can be experimentally determined as the initial rate of increase in its plasma level after removal of this organ from the blood circulation. Using a radioimmunoassay, we found that, on ligation of the renal arteries of rats, the bikunin concentration initially doubled in 26 ± 1 min (Figure 8).

The plasma clearance of $^{35}\text{SO}_4$ -labelled bikunin in anephric rats was also determined (Figure 7, open circles). When these values were fitted into an exponential function, a half-life of 44 ± 7 min was obtained (not shown). However, the fact that the specific radioactivity of the plasma bikunin decreased during

Figure 7 Plasma clearance of $^{35}\text{SO}_4$ -labelled bikunin

$^{35}\text{SO}_4$ -labelled bikunin was injected intravenously into two rats. Blood samples were withdrawn after 2 min and then every 5 min, and their radioactivity was measured. The graph shows the radioactivity values relative to the respective initial value (●). The inset shows a semilogarithmic plot of the values from one animal (●). The renal arteries of two rats were ligated and $^{35}\text{SO}_4$ -labelled bikunin was injected intravenously. Blood samples were taken after 2 min and then every 5 min, and their radioactivity was measured. The graph shows the values obtained relative to the respective initial value (○). From the graph describing the increase in the bikunin concentration on ligation of the renal arteries (Figure 8), the bikunin concentration at each time point was obtained. The radioactivity values were divided by these concentration values and normalized. The inset shows a semilogarithmic plot of the resulting values for one experiment (○).

Figure 8 Change in plasma concentration of bikunin on removal of kidneys from circulation

The renal arteries of a rat were ligated and blood samples were taken at different times. The concentration of free bikunin in the plasma was determined by radioimmunoassay [7].

these experiments must be taken into account. The inset of Figure 7 (open circles) shows a plot of the values obtained when the original radioactivity values were divided by the respective

bikunin concentration, as determined in the radioimmunoassay (Figure 8); this graph yielded a half-life of 22 ± 1 min.

DISCUSSION

In this study we have characterized the plasma clearance of bikunin as part of an effort to elucidate the function of this protein. Using radiolabelled bikunin of two different origins (urine and hepatocyte medium), we obtained similar half-lives (8 and 11 min), indicating that they are similar to that of the endogenous protein. To assess the role of the kidneys in the uptake, we determined the time required for the plasma concentration of bikunin to double on ligation of the renal arteries (Figure 8), which is equivalent to the half-life of bikunin if the kidneys alone accounted for the clearance. This value (26 min) together with the estimated half-life of bikunin implies that the kidneys account for about half the plasma uptake. Consistent with this conclusion we found that the half-life of labelled bikunin in anephric animals was 22 min (Figure 7). A determination of the tissue distribution of ^{125}I -labelled bikunin 30 min after injection (Table 1) gave a lower value for the contribution of the kidneys to the uptake than the kinetic analysis. However, chromatographic analysis of the skin of normal and anephric animals indicated that the difference was due to the release of degradation products from the kidneys. Using autoradiography, we could detect radiolabelled bikunin in the tubules of the kidneys shortly after intravenous injection (Figure 4), supporting the idea that, despite its relatively large hydrodynamic size and high negative charge, bikunin is cleared by the kidneys via glomerular filtration [36]. The fact that the 'effective radius' of extended polymers in glomerular filtration is smaller than their hydrodynamic radius [37] could account for this property of bikunin.

In agreement with Sugiki et al. [17], we found that a small portion of the injected bikunin was taken up in the liver. Liver cells have receptors for chondroitin sulphate, and cartilage-derived chondroitin sulphate has been found to be rapidly taken up by the liver on injection [38]. However, the binding affinity is dependent on the size of the molecule [39], and the relatively small size of the chondroitin sulphate chain of bikunin may be the reason why the liver is not a major site of uptake. We have previously detected bikunin in bile [7]. Serum proteins enter the bile either by leakage between the hepatocytes or by being transported through them [40]. To obtain information about the mode of transfer, we collected bile during the clearance of ^{125}I -labelled bikunin and measured the radioactivity (M. Sjöquist and E. Fries, unpublished work). We found that the level of radioactivity increased during the first 30 min after the injection and then remained constant for at least 60 min; 90–95% of the radiolabelled material was in degradation products. These findings indicate that the bikunin taken up by the liver is partially degraded and slowly released into the bile.

We found that, on removal of the kidneys, the bikunin concentration increased linearly to at least four times normal. Similarly, plasma concentrations of bikunin that are up to 21 times higher than normal have been reported for patients on dialysis [41]. These results indicate that the non-renal uptake system is saturated at physiological concentrations, implying that it is a receptor-mediated process and does not occur by leakage between or through vascular cells. These findings and the observation that the plasma level of bikunin, except in renal diseases [36], is relatively constant under various conditions [42] is difficult to reconcile with the fact that bikunin seems to be a mitogen for endothelial cells and fibroblasts [12,13].

Our results indicate that bikunin is taken up to some degree in most tissues of the body. Autoradiographic analysis of skin after the injection of ^{125}I -labelled bikunin showed that a large part of the radioactive label was located around blood capillaries (Figure 5). These results suggest that bikunin may be actively transferred across endothelial cells in a receptor-mediated process as seems to be the case for insulin [43]. Bikunin was also found in subcutaneous fibrous structures. It is possible that bikunin, like certain growth factors [44], is bound to the extracellular matrix and is released during tissue reorganization. The idea that bikunin may act in such a way is supported by the observation that inter- α -inhibitor, of which bikunin is a subunit, appears to be part of the hyaluronan-containing coat that surrounds different cell types [45,46].

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