Degradation of atrial natriuretic factor in the rat

Kishore K. MURTHY, Gaétan THIBAUT,* Raul GARCIA, Jolanta GUTKOWSKA, Jacques GENEST and Marc CANTIN

Clinical Research Institute of Montreal, 110 Pine Avenue West, Montreal, Quebec, Canada H2W 1R7

The biologically active circulating form of atrial natriuretic factor (ANF) in the rat is the 28-amino-acid peptide ANF-(Ser-99–Tyr-126). Degradation of this peptide *in vivo* as well as *in vitro*, in whole blood, in plasma and by the isolated mesenteric artery was investigated. Studies *in vivo* in the rat demonstrated that the elimination and degradation of ANF was extremely fast: within 3 min more than 95% of the injected immunoreactive material was eliminated from circulation. The production of a short C-terminal peptide was detected on injection of ¹²⁵I-ANF-(Ser-99–Tyr-126) into the rat. This peptide increased proportionately with incubation time. Experiments *in vitro* in the presence of whole blood or plasma did not cause any major destruction of ANF even after incubation for 60 min. After this prolonged incubation in plasma, ANF-(Ser-99–Tyr-126) was partially converted into ANF-(Ser-103–Tyr-126), a less potent peptide. Isolated mesenteric-artery preparation appeared to degrade ANF in a manner very similar to the system *in vivo*. These results suggest that degradation of ANF may occur either after internalization in the vascular cells or by a membrane-bound enzyme in the vasculature.

INTRODUCTION

Atrial natriuretic factor (ANF) is a peptide hormone with potent natriuretic and diuretic [1] and vasorelaxant [2,3,4] properties. This is stored in specific granules in mammalian cardiocytes [5–8] as its precursor molecule [9,10]. The biologically active circulating form of this hormone, however, is the 28-amino-acid peptide ANF-(Ser-99–Tyr-126) [11,12], the sequence of which is given below:

Ser-Leu-Arg-Arg-Ser-Ser-Cys-Phe-Gly-Gly-Arg-Ile-Asp-Arg-
Ile-Gly-Ala-Gln-Ser-Gly-Leu-Gly-Cys-Asn-Ser-Phe-Arg-Tyr

Even though extensive work is being done on the biological effects of this hormone (for reviews see [13–15]), not much attention has been paid to its degradation. Our previous study on the disappearance of ¹²⁵I-ANF peptides from circulation of the rat indicated an extremely rapid elimination of radioactive peptide from the system [16]. In an effort to understand the nature of this degradation/elimination process, the present study was undertaken. ANF-(Ser-99–Tyr-126) and its radioiodinated form were injected into the rat, blood samples were withdrawn at different time intervals and the products present in circulation were analysed. The degradation of this peptide hormone in whole blood and plasma as well as in the isolated perfused mesenteric artery was also studied.

MATERIALS AND METHODS

Materials

ANF-(Ser-103–Tyr-126), ANF-(Arg-101–Tyr-126) and ANF-(Ser-99–Tyr-126) were purchased from Armand Frappier (Laval, Quebec, Canada). ANF-(Glu-54–Tyr-126) and ANF-(Asn-1–Tyr-126) were purified from rat atria as reported elsewhere [17,18]. The peptides were iodinated by the lactoperoxidase method and later purified by reverse-phase h.p.l.c. [16]. The preparations

had specific radioactivities of 200–300 $\mu\text{Ci}/\mu\text{g}$ of peptide.

Collection of blood

Blood was withdrawn from the abdominal aorta of male Sprague–Dawley rats anaesthetized with sodium pentobarbital (60 mg/kg, intraperitoneally) and collected into pre-chilled Vacutainer tubes containing either

EDTA or heparin as anticoagulant. Plasma was separated by centrifugation at 3500 rev./min for 20 min at 4 °C and stored on ice until used.

H.p.l.c. separation

Samples dissolved in 10% (v/v) acetonitrile in 0.1% trifluoroacetic acid were loaded on a C₁₈ μ Bondapak column (0.39 mm $\times$ 30 mm) and eluted with a linear gradient of 15–40% acetonitrile in 0.1% trifluoroacetic acid at a flow rate of 1 ml/min and a slope of 0.5%/min. Fractions were collected every 1 min and counted for radioactivity in an LKB gamma counter. For non-radioactive samples, the elution was monitored at 210 nm with an on-line u.v. detector.

Molecular-sieve h.p.l.c. was performed on a BioSil TSK-125 column (0.75 cm $\times$ 60 cm) by using a Varian 5060 liquid-chromatograph system. The peptides were eluted isocratically at 0.5 ml/min with 30% (v/v) CH₃CN containing 0.2 M-NaCl and 0.1% trifluoroacetic acid. The column was calibrated with radioiodinated ANF peptides, ANF-(Ser-99–Tyr-126), ANF-(Glu-54–Tyr-126) and ANF-(Asn-1–Tyr-126). Fractions were collected every 0.3 min and counted for radioactivity in an LKB gamma counter.

Abbreviation used: ANF, atrial natriuretic factor.

* To whom correspondence should be addressed.

Radioimmunoassay

The immunoreactive material in the samples was assayed by using three different antibodies, which recognize different portions of the ANF-(Arg-101-Tyr-126) molecule: anti-(human ANF) antibody (Peninsula Laboratories, Belmont, CA, U.S.A.), which recognizes the C-terminus [19], anti-(rat ANF) antibody, which recognizes the mid portion of the molecule [20], and a monoclonal antibody which recognizes the N-terminal portion [21]. The radioimmunoassays were done as outlined in the respective references.

Degradation studies *in vivo*

Male Sprague-Dawley rats (200–250 g) were anaesthetized with sodium pentobarbital (60 mg/kg, intraperitoneally), and the left carotid artery and right jugular vein were cannulated with PE-50 catheters. ANF-(Ser-99-Tyr-126) (5 and 10 μ g) was injected into the jugular vein, with heparinized (100 units/ml) saline containing 0.01% bovine serum albumin as vehicle. The total volume of each injection was 200 μ l. Blood samples (approx. 500 μ l) were collected through the carotid artery at timed intervals, as indicated in Fig. 1, directly into Eppendorf tubes kept on ice and immediately centrifuged in an Eppendorf centrifuge at 4 °C. Plasma (200 μ l) was added to an equal volume of 8% (v/v) acetic acid, loaded on a C₁₈ Sep-Pak cartridge (Waters Associates), and ANF was extracted by the method of Lang *et al.* [22]. The extraction is based on the principle that ANF and shorter peptides bind to the C₁₈ matrix of the cartridge by hydrophobic interaction. Non-specifically adsorbed material, representing mostly salt (including ¹²⁵I), and free amino acids are eliminated by an initial water wash, which is followed by elution of the adsorbed material with 86% (v/v) ethanol in 4% acetic acid. A known volume of the eluate was evaporated to dryness on a Speed-Vac concentrator (Savant Instruments), reconstituted in the radioimmunoassay buffer and analysed by radioimmunoassay by using the three different anti-ANF antibodies as outlined above.

In another set of experiments, ¹²⁵I-ANF-(Ser-99-Tyr-126) was injected into the jugular vein of the rat, with heparinized saline as vehicle, as mentioned above. Blood samples were collected at timed intervals, and ANF was extracted from plasma by using C₁₈ Sep-Pak cartridges and analysed by reverse-phase h.p.l.c.

In a third set of experiments, ¹²⁵I-ANF-(Ser-99-Tyr-126) (10×10^6 c.p.m.) was injected intrajugularly, and blood (approx. 6 ml) collected between 20 and 25 min into a pre-chilled polystyrene tube. ANF was extracted from the plasma by using C₁₈ Sep-Pak cartridge and analysed by molecular-sieve h.p.l.c. as outlined above.

Degradation in blood

Radiolabelled ANF. ¹²⁵I-ANF-(Ser-99-Tyr-126) was incubated with whole blood or plasma for 5, 10 and 30 min at 37 °C. A portion of the sample was removed at the end of each incubation period, and ANF was extracted from plasma by using C₁₈ Sep-Pak cartridges and analysed by reverse-phase h.p.l.c. As a control for the whole-blood study, ¹²⁵I-ANF-(Ser-99-Tyr-126) was added to pre-chilled blood, centrifuged immediately at 4 °C, and the plasma was treated like the test samples. As a control in the incubation studies with plasma, ¹²⁵I-ANF-(Ser-99-Tyr-126) was added to

plasma after addition of acetic acid and treated just as the test samples.

Unlabelled ANF. ANF-(Ser-99-Tyr-126) (5 μ g) was incubated with 1 ml of plasma (containing EDTA or heparin) for different intervals of time from 15 s to 30 min at 37 °C. A sample (100 μ l) of plasma was removed at the end of each incubation period, and ANF was extracted on a C₁₈ Sep-Pak cartridge and analysed by radioimmunoassay with the three different antibodies as outlined under 'Radioimmunoassay'. As a control, 500 ng of ANF-(Ser-99-Tyr-126) was added to 100 μ l of plasma after addition of acetic acid and further treated just like the test samples.

In another set of incubation studies, 40 μ g of ANF-(Ser-99-Tyr-126) was incubated with 1 ml of plasma for 5, 30 and 60 min at 37 °C. At the end of each incubation period, a sample (250 μ l) was extracted on a C₁₈ Sep-Pak cartridge and subjected to reverse-phase h.p.l.c. The peptide elution was monitored at 210 nm and the individual peaks were collected manually. Immunoreactivity of these peaks was checked by using anti-(rat ANF) antibody and the immunoreactive peaks were re-chromatographed (twice) individually on a C₁₈ μ Bondapak column with a linear gradient of 18–30% CH₃CN (slope 0.25%/min) in 0.1% trifluoroacetic acid. The eluted peptide peaks were monitored at 210 nm and collected manually. Samples collected after

Fig. 1. Degradation of ANF-(Ser-99-Tyr-126) *in vivo*: radioimmunoassay

ANF-(Ser-99-Tyr-126) (5 μ g) was injected intrajugularly, and 500 μ l blood samples were collected at timed intervals as indicated on the abscissa. ANF was extracted from the plasma by using C₁₈ Sep-Pak cartridges and assayed by radioimmunoassay using the different antibodies as outlined in the Materials and methods section. ●, Anti-(human ANF) antibody (C-terminal); □, monoclonal antibody (N-terminal); ○, anti-(rat ANF) antibody (mid-portion).

the final fractionation were subjected to amino acid analysis. Known volumes of the eluate containing the peptides were evaporated to dryness on a Speed-Vac concentrator, hydrolysed in 6 M-HCl in the presence of 2.5 nmol of norleucine as internal standard and analysed on a Beckman 120C amino acid analyser equipped with a model 126 computing integrator.

Studies with isolated perfused mesenteric artery

The isolated mesenteric artery was prepared from male Sprague-Dawley rats (200–250 g) by the method of McGregor [23]. The preparation was perfused with oxygenated Krebs buffer at a constant flow of 2 ml/min and maintained at 37 °C throughout the experiment. ^{125}I -ANF-(Ser-99-Tyr-126) (10×10^6 c.p.m.) was injected into the vascular bed, and the perfusate was collected at timed intervals directly into vials containing an equal volume of 8% acetic acid kept on ice. ANF peptides were extracted with C_{18} Sep-Pak cartridges and analysed by reverse-phase h.p.l.c. as outlined above.

RESULTS

Degradation *in vivo*

Earlier studies on the intravenous injection of ^{125}I -ANF-(Ser-99-Tyr-126) into the rat demonstrated an extremely rapid disappearance, within 1 min, of the labelled peptide from circulation [16]. To see if the

disappearance of immunoreactive material paralleled this pattern, ANF-(Ser-99-Tyr-126) (5 μg) was injected intrajugularly, blood samples were collected at timed intervals and ANF concentrations in plasma assayed by using three different antibodies which recognize different portions of the ANF-(Arg-101-Tyr-126) molecule. The results of this study are given in Fig. 1. The immunoreactive material disappeared very quickly from circulation, reaching values less than 5% of the injected dose in about 3 min. After 5 min the values were undetectable. No difference was observed with the three different antibodies. Similar results were obtained when 10 μg of the peptide was injected into the circulation.

After the injection of ^{125}I -ANF-(Ser-99-Tyr-126) into the rat, the radiolabelled peptide fragments present in circulation at different time intervals were studied. The Sep-Pak-adsorbed radioactivity, represented as a percentage of the total (remaining in the blood) loaded on the cartridge, is given in Table 1. In the first 80 s only 77% of the radioactive material loaded was adsorbed to the Sep-Pak cartridge. This value fell to 37% after 5 min and to 19% by 26 min, an observation consistent with degradation of ANF.

The h.p.l.c. pattern of the radioactive material adsorbed to the Sep-Pak cartridge is given in Fig. 2. In the first sample ($t = 5\text{--}20$ s) only one peak of radioactivity corresponding to the elution position of the 28-amino-acid peptide was observed. However, in the sample collected between 60 and 80 s, two peaks of

Fig. 2. Degradation of ^{125}I -ANF-(Ser-99-Tyr-126) *in vivo*: reverse-phase h.p.l.c.

^{125}I -ANF-(Ser-99-Tyr-126) (10×10^6 c.p.m.) was injected intrajugularly into the rat, and blood samples (approx. 1 ml) were collected at timed intervals as indicated in the Figure. ANF was extracted from plasma by using C_{18} Sep-Pak cartridges and analysed on a reverse-phase h.p.l.c. column as outlined in the Materials and methods section.

Table 1. Degradation of ^{125}I -ANF-(Ser-99-Tyr-126) *in vivo*

^{125}I -ANF-(Ser-99-Tyr-126) (10×10^6 c.p.m.) was injected intrajugularly into a normal rat, and blood samples were collected at timed intervals as indicated in the first column. The first blood sample (5–20 s) had the maximum radioactivity, and that in the subsequent samples was calculated as a percentage of this value. ANF was extracted from plasma by using C_{18} Sep-Pak cartridges and subjected to reverse-phase h.p.l.c. on a C_{18} μ Bondapak column. The radioactive peak eluted at the position of the intact peptide was pooled and its contribution to the total radioactive material loaded on the h.p.l.c. column calculated. The last column represents the percentage of intact peptide present in the circulation after correcting for the decrease with time in radioactivity in blood as shown in column 2 and also the amount of radioactive material not bound to the Sep-Pak cartridge (column 3).

Time of sampling	Radioactivity in blood (% of maximum)	Radioactivity bound to Sep-Pak (%)	Intact peptide (from h.p.l.c. pattern)	
			As % of radioactivity loaded on h.p.l.c. column	As % of radioactivity in blood
5–20 s	100	94	93	88
60–80 s	27	77	61	13
150–180 s	19	55	33	3
5–6 min	20	37	10	1
25–26 min	31	19	0	0

Fig. 3. Degradation of ^{125}I -ANF-(Ser-99-Tyr-126) *in vivo*: molecular-sieve h.p.l.c.

^{125}I -ANF-(Ser-99-Tyr-126) (10×10^6 c.p.m.) was injected intrajugularly into the rat, and blood (approx. 6 ml) was collected between 20 and 25 min. ANF was extracted from the plasma by using C_{18} Sep-Pak cartridges and loaded on a molecular-sieve h.p.l.c. column. Arrows represent elution positions of ANF peptides used to standardize the column. Key: V_0 , void volume; V_t , total volume.

radioactive material were observed, one eluted at 17% CH_3CN and the other eluted at the position of the intact peptide. There was a gradual increase in the percentage of the peptide eluted at 17% CH_3CN with increasing duration of time in circulation and a concomitant decrease in the proportion of the intact peptide (Fig. 2 and Table 1). In the sample collected after 5 min, only 10% of the radioactive material adsorbed to the Sep-Pak cartridge represented the intact peptide. However, when the value was corrected for the radioactive material in blood (percentage of maximum), it constituted only 1% (Table 1). In the sample collected at 25 min no intact peptide was observed. The peak eluted at 17% CH_3CN had no immunoreactivity, whereas the binding of the other peak was similar to that of ^{125}I -ANF-(Ser-99-Tyr-126) when tested with anti-(rat ANF) antibody.

To verify the nature of the peptide molecule eluted at 17% CH_3CN , ^{125}I -ANF-(Ser-99-Tyr-126) was injected intravenously into the rat, blood was collected between 20 and 25 min and the plasma was extracted with a Sep-Pak cartridge. The molecular-sieve h.p.l.c. pattern of the eluate is given in Fig. 3. The radioactive material was eluted very close to the fractionation limit of the column. A M_r of about 1000 was calculated for this molecule, suggesting it to be only a short C-terminal fragment of the 28-amino-acid peptide. Since tyrosine forms the C-terminus of the ANF peptide, this eluted peptide would represent a C-terminal fragment of ANF-(Ser-99-Tyr-126). The formation of any other short peptide from the N-terminus could not be detected under the conditions used in our study.

Degradation *in vitro*

Incubation of ^{125}I -ANF-(Ser-99-Tyr-126) in whole blood and plasma. There was no significant increase in the radioactive material passing through the Sep-Pak cartridge on incubation of the ANF peptide with whole blood. Only 10% of the radioactive material passed through the cartridge after 30 min of incubation. The h.p.l.c. pattern of the material adsorbed to the cartridge after a 30 min incubation in blood is given in Fig. 4.

Fig. 4. Incubation of ^{125}I -ANF-(Ser-99-Tyr-126) with whole blood: reverse-phase h.p.l.c.

^{125}I -ANF-(Ser-99-Tyr-126) (1×10^6 c.p.m.) was incubated at 37°C with EDTA-containing whole blood (2 ml) for different time intervals. A sample (500 μl) was removed at the end of the incubation period, and ANF was extracted from plasma by using a C_{18} Sep-Pak cartridge and analysed by reverse-phase h.p.l.c.

After this incubation period ($t = 30$ min), a second peak of radioactivity that was eluted very close to the ^{125}I -ANF-(Ser-99-Tyr-126) peak was observed. A small amount (approx. 5%) of radioactive material eluted at 17% CH_3CN also became apparent. Similar results were obtained on incubation of ^{125}I -ANF-(Ser-99-Tyr-126) with plasma.

Incubation of ANF-(Ser-99-Tyr-126) with plasma. Incubation of ANF-(Ser-99-Tyr-126) with plasma resulted in a partial loss of immunoreactivity of the samples over a period of 30 min. As measured with the anti-(human ANF) antibody (Peninsula Laboratories), which recognizes the C-terminus [19], and the anti-(rat ANF) antibody, which recognizes the mid-portion of the ANF-(Arg-101-Tyr-126) molecule [20], the loss of immunoreactivity was about 15–20% after 30 min. However, when the samples were assayed with the monoclonal antibody which recognizes the N-terminus of the ANF-(Arg-101-Tyr-126) molecule [21], the loss of immunoreactivity was about 40–50%. This suggests a

partial degradation of the peptide at the N-terminal. Both heparinized plasma and plasma with EDTA gave similar results.

The h.p.l.c. pattern of the material adsorbed to the Sep-Pak cartridge after incubation of ANF-(Ser-99-Tyr-126) with plasma is given in Fig. 5. The first panel ($t = 0$ min), which represents the control sample, gave two peaks of u.v. absorbance, the first being a non-specific absorbance peak from plasma; immunoreactive material was present only in the second peak. After 30 min incubation a third absorbance peak was observed. This peak became pronounced in the 60 min sample, with a proportional decrease in the second peak. No other degradation products were obvious.

The amino acid compositions of the two immunoreactive peaks obtained in plasma degradation studies are given in Table 2. ANF-(Ser-99-Tyr-126), ANF-(Arg-101-Tyr-126) and ANF-(Ser-103-Tyr-126) were also analysed along with the test samples. The amino acid analysis indicated peak II to be ANF-(Ser-99-Tyr-126) and peak III, the newly formed peak, to be ANF-(Ser-103-Tyr-126).

Studies with isolated perfused mesenteric artery

After a bolus injection of ^{125}I -ANF-(Ser-99-Tyr-126), most of the radioactivity (approx. 90%) was obtained in the eluate collected in the first 2 min. However, there was a gradual fall in the eluted radioactive material thereafter, continuing up to 30 min (Table 3). Samples were collected at different time intervals and extracted by using Sep-Pak cartridges. The radioactivity adsorbed to the Sep-Pak (% of injected sample) is given in Table 3. There was a gradual decrease in the amount of Sep-Pak-adsorbed material with increase in incubation time. In the 10–15 min sample, about 50% of the material passed through the Sep-Pak cartridge. The h.p.l.c. elution profile of the Sep-Pak-adsorbed material is given in Fig. 6. The amount of intact peptide in the samples decreased with time after the bolus injection of radiolabelled peptide (Fig. 6 and Table 3). By 15 min, only 37% of the total radioactivity loaded on the h.p.l.c. column represented the intact peptide. After correction for the radioactivity passing through the Sep-Pak cartridge, the amount of intact peptide was just 19%. This was further decreased to 13% after 25 min. The immunoreactivity of this peak with anti-(rat ANF) antibody was similar to that of ^{125}I -ANF-(Ser-99-Tyr-126).

Accompanying this decrease in the intact peptide was a proportionate increase in a radioactive peak eluted at 17% CH_3CN . This peak had no immunoreactivity when tested with anti-(rat ANF) antibody.

DISCUSSION

The results of our studies of degradation *in vivo* by using radiolabelled peptide indicated that ANF-(Ser-99-Tyr-126) was degraded very quickly in the circulation of the rat. By the first 80 s after the peptide injection, less than 50% of the radioactive material remaining in circulation represented the intact peptide. When the value for the percentage of intact peptide in circulation is corrected for the decrease in circulating radioactivity at the time of blood collection, the value is further decreased to 13%. This is comparable with the decrease in immunoreactive material in circulation observed as a

Fig. 5. Incubation of ANF-(Ser-99-Tyr-126) with plasma: reverse-phase h.p.l.c.

ANF-(Ser-99-Tyr-126) (40 µg) was incubated at 37 °C with rat plasma (1 ml) for different time intervals. A sample (250 µl) was removed at the end of each incubation period, and ANF was extracted by using C₁₈ Sep-Pak cartridges and analysed by reverse-phase h.p.l.c.

Table 2. Degradation of ANF-(Ser-99-Tyr-126) in plasma

ANF-(Ser-99-Tyr-126) (40 µg) was incubated with 1 ml of plasma for 60 min. A sample (250 µl) of plasma was removed, passed through a Sep-Pak cartridge and subjected to reverse-phase h.p.l.c. as indicated in the Materials and methods section. The two A₂₁₀ peaks obtained were subjected to amino acid analyses. ANF-(Ser-99-Tyr-126), ANF-(Arg-101-Tyr-126) and ANF-(Ser-103-Tyr-126) were used as standards in the same analysis. Values in parentheses are rounded off to the nearest integer. Cysteine was not measured.

Residue	Amino acid composition (residues/molecule)				
	ANF- (Ser-99-Tyr-126)	ANF- (Arg-101-Tyr-126)	ANF- (Ser-103-Tyr-126)	Peak II	Peak III
Arg	4.43 (5)	4.60 (5)	2.71 (3)	4.60 (5)	3.05 (3)
Asp	1.89 (2)	1.98 (2)	2.05 (2)	2.02 (2)	2.00 (2)
Ser	4.77 (5)	4.01 (4)	4.17 (4)	4.99 (5)	4.12 (4)
Glu	1.00 (1)	1.13 (1)	1.06 (1)	1.09 (1)	1.12 (1)
Gly	4.94 (5)	5.10 (5)	5.41 (5)	5.30 (5)	5.14 (5)
Ala	1.09 (1)	1.11 (1)	1.12 (1)	1.25 (1)	1.18 (1)
Ile	1.77 (2)	1.87 (2)	1.83 (2)	1.87 (2)	1.85 (2)
Leu	1.90 (2)	1.01 (1)	0.95 (1)	1.97 (2)	1.10 (1)
Tyr	1.36 (1)	0.96 (1)	0.67 (1)	1.23 (1)	0.76 (1)
Phe	2.00 (2)	2.00 (2)	2.00 (2)	2.00 (2)	2.00 (2)
					[≡ ANF-(99-126)] [≡ ANF-(103-126)]

function of time. These results suggest that the disappearance of ANF from circulation is due to a combination of binding to receptors in various tissues as well as degradation. The radioactive material observed in blood (20%) after 3 min represents only the degraded

fragment of ANF circulating in the system, and not the biologically active peptide.

This extremely fast disappearance of ANF from circulation, accompanied by the rapid degradation observed *in vivo*, would probably explain the short

Table 3. Degradation of ^{125}I -ANF-(Ser-99-Tyr-126) by the isolated perfused mesenteric artery

^{125}I -ANF-(Ser-99-Tyr-126) (10×10^6 c.p.m.) was injected into an isolated mesenteric-artery preparation perfused with oxygenated Krebs buffer at a flow rate of 2 ml/min. Perfusate samples were collected at timed intervals and the radioactivity was calculated as a percentage of injected dose. ANF was extracted from the perfusate by using C_{18} Sep-Pak cartridges and subjected to reverse-phase h.p.l.c. The radioactive peak eluted at the position of the intact peptide is represented as a percentage of the total radioactivity loaded on the h.p.l.c. column. This value, corrected for the radioactive material passing through the Sep-Pak cartridge (column 3), is presented in the last column.

Time of sampling	Radioactivity (% of that injected)	Radioactivity bound to Sep-Pak (%)	Intact peptide (from h.p.l.c. pattern)	
			As % of radioactivity loaded on h.p.l.c. column	As % of radioactivity loaded on Sep-Pak
0-2 min	90	96	96	92
2-4 min	7	83	70	58
10-15 min	2	51	37	19
20-25 min	1	51	26	13

Fig. 6. Degradation of ^{125}I -ANF-(Ser-99-Tyr-126) by isolated perfused mesenteric-artery preparation: reverse-phase h.p.l.c.

^{125}I -ANF-(Ser-99-Tyr-126) (10×10^6 c.p.m.) was injected into an isolated perfused mesenteric-artery preparation and samples were collected at different time intervals as indicated. ANF was extracted by using C_{18} Sep-Pak cartridges and analysed by reverse-phase h.p.l.c.

duration of the biological response *in vivo* observed by de Bold *et al.* [1], and subsequently by Garcia *et al.* [6] and Ackermann and co-workers [24]. However, from those studies it is difficult to single out any particular organ as being responsible for this rapid disappearance process, since receptors for ANF have been localized in a large number of organs of the rat [25].

There have been several reports in the literature on ANF metabolism in blood. The conversion of the propeptide [ANF-(Asn-1-Tyr-126)] into the active form by rat serum has been reported by Bloch *et al.* [26] and Glembofski & Gibson [27]. Another group [28] has reported the degradation of low- M_r ANF. However, the degradation products have not been characterized. Since

ANF-(Ser-99-Tyr-126) has now been identified in the circulation of the rat [11,12], it was of interest to see if it is degraded in blood. The results of our study indicate that ANF-(Ser-99-Tyr-126) is converted very slowly by a plasma proteinase into a 24-amino-acid form, ANF-(Ser-103-Tyr-126). This would probably account for the presence of ANF-(Ser-103-Tyr-126) as a minor form in the circulation of the rat, as reported by Schwartz *et al.* [12]. In an earlier study Veress *et al.* [28] followed the inactivation of low- M_r ANF in blood by measuring the decrease in diuretic and natriuretic response. They observed a decrease of 50% in the activity after a 60 min incubation with whole blood or a platelet-rich fraction. However, no degradation was observed in either plasma or the red-cell fraction. It is very difficult to compare these results with what we have reported here, since our study was done with ANF-(Ser-99-Tyr-126), the circulating form of ANF, whereas the material used by the above-mentioned workers was a partially purified ANF preparation probably containing more than one peptide species. However, this extremely slow degradation of ANF (about 10–15% in 30 min) would suggest that blood does not contribute to any great extent in the degradation of ANF.

Vascular tissue of the rat is known to contain a large number of high-affinity binding sites for ANF peptides [29,30]. Since the vascular tissue has a very large surface area and ANF-(Ser-99-Tyr-126) has been shown to be a circulating hormone, it was of interest to study its degradation in a vascular bed. Isolated mesenteric-artery preparation was used as a model system to study this process. Preliminary studies indicated an initial rapid wash-out of a major portion of the injected radioactivity. This was followed by a gradual release of radioactive material from the tissue. Since this was a single-passage system, the gradual release of radioactive material may indicate a possible binding followed by internalization and a subsequent release of either the intact peptide or one of its degraded forms. Reverse-phase h.p.l.c. analysis of samples collected at different time intervals after injection of radiolabelled peptide, however, indicated degradation to be taking place. The degradation product was eluted at the same position as in the studies *in vivo*, suggesting that the product formed in both these studies was very similar.

Peptide hormones are known to interact first with membrane receptors on the cell surface and subsequently to be degraded, one of the major pathways being degradation after internalization in the target cell. In this pathway, the peptide hormones are internalized by receptor-mediated endocytosis, followed by degradation in the lysosomes [31,32,33]. Degradation of insulin by adipocytes [34,35] and of epidermal growth factor by either human fibroblasts [36] or GH₄C₁ cells (a clonal strain of rat pituitary tumour cells) [37] are good examples of this process. However, somatostatin degradation in the GH₄C₁ cells does not follow this pathway, and it is degraded by some membrane-bound proteinases after binding to specific receptors on the cell surface [38]. In our study, since there is no recirculation of the sample through the tissue, degradation is likely to be taking place by either of the above-mentioned pathways. However, from our results it is difficult to say which of these processes is important in ANF degradation. In an earlier study on ANF degradation by rat vascular smooth-muscle cells in culture, Hirata *et al.* [38] have

shown internalization of the 28-amino-acid peptide to precede degradation. It is quite likely that this pathway plays a major role in the degradation of this potent natriuretic peptide.

In conclusion, it could be said that ANF is eliminated from the circulation of the rat by a combination of binding and degradation. The biologically active 28-amino-acid peptide is converted into a short C-terminal fragment in the course of its circulation. Incubation studies with whole blood and plasma indicate that blood does not play a major role in ANF degradation. The 28-amino-acid peptide ANF-(Ser-99-Tyr-126) is, however, converted into a 24-amino-acid species, ANF-(Ser-103-Tyr-126), in an extremely slow process. Isolated mesenteric artery appears to degrade ANF in a manner very similar to the degradation observed *in vivo*. This study also suggests that degradation occurs either after internalization of the peptide into the vascular smooth-muscle cells or by some membrane-bound enzyme in the vasculature. Further work is needed to substantiate this hypothesis.

We thank Dr. R. Milne of our Institute for providing monoclonal antibody to ANF and also the laboratories of Dr. N. G. Seidah and Dr. M. Chrétien for help with the amino acid analysis. We thank Ms. C. Jutras for secretarial assistance. This work was supported by grants from the Medical Research Council of Canada, the Canadian Heart Foundation, the National Research Council of Canada, and the Ministère de la Science et de la Technologie du Québec, to the Multidisciplinary Research Group on Hypertension and by the Medical Research Council of Canada (MA-9594) to G.T.

REFERENCES

1. de Bold, A. J., Borenstein, H. B., Veress, A. T. & Sonnenberg, H. (1981) *Life Sci.* **28**, 89–94
2. Currie, M. G., Geller, D. M., Cole, B. R., Boylan, J. G., Sheng, W. Y., Holmberg, S. W. & Needleman, P. (1983) *Science* **221**, 71–73
3. Grammer, R. T., Fukumi, H., Inagami, T. & Misono, K. S. (1983) *Biochem. Biophys. Res. Commun.* **116**, 696–703
4. Garcia, R., Thibault, G., Cantin, M. & Genest, J. (1984) *Am. J. Physiol.* **247**, R34–R39
5. de Bold, A. J. (1982) *Can. J. Physiol. Pharmacol.* **60**, 324–330
6. Garcia, R., Cantin, M., Thibault, G., Ong, H. & Genest, J. (1982) *Experientia* **38**, 1071–1073
7. de Bold, A. J. & Salerno, T. A. (1983) *Can. J. Physiol. Pharmacol.* **61**, 127–130
8. Nemeh, M. N. & Gilmore, J. P. (1983) *Circ. Res.* **53**, 420–423
9. Kangawa, K., Tawaragi, Y., Oikawa, S., Mizuno, A., Sakuragawa, Y., Nakazato, H., Fukuda, A., Minamino, N. & Matsuo, H. (1984) *Nature (London)* **312**, 152–155
10. Flynn, T. G., Davies, P. L., Kennedy, B. P., de Bold, M. C. & de Bold, A. J. (1985) *Science* **228**, 323–325
11. Thibault, G., Lazure, C., Schiffrin, E. L., Gutkowska, J., Chartier, L., Garcia, R., Seidah, N. G., Chrétien, M., Genest, J. & Cantin, M. (1985) *Biochem. Biophys. Res. Commun.* **130**, 981–986
12. Schwartz, D., Geller, D. M., Manning, P. T., Siegel, N. R., Fok, K. F., Smith, C. E. & Needleman, P. (1985) *Science* **229**, 397–400
13. Cantin, M. & Genest, J. (1985) *Endocr. Rev.* **6**, 107–127
14. Maack, T., Camargo, M. J. F., Kleinert, H. D., Laragh, J. H. & Atlas, S. A. (1985) *Kidney Int.* **27**, 607–615
15. Flynn, T. G. & Davies, P. L. (1985) *Biochem. J.* **232**, 313–321

16. Murthy, K. K., Thibault, G., Schiffrin, E. L., Garcia, R., Chartier, L., Gutkowska, J., Genest, J. & Cantin, M. (1986) *Peptides* **7**, 241–246
17. Thibault, G., Garcia, R., Cantin, M., Genest, J., Lazure, C., Seidah, N. G. & Chrétien, M. (1984) *FEBS Lett.* **167**, 352–356
18. Thibault, G., Garcia, R., Gutkowska, J., Bilodeau, J., Lazure, C., Seidah, N. G., Chrétien, M., Genest, J. & Cantin, M. (1986) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **45**, 526
19. Gutkowska, J., Bourassa, M., Roy, D., Thibault, G., Garcia, R., Cantin, M. & Genest, J. (1985) *Biochem. Biophys. Res. Commun.* **128**, 1350–1357
20. Gutkowska, J., Thibault, G., Januszewicz, P., Cantin, M. & Genest, J. (1984) *Biochem. Biophys. Res. Commun.* **122**, 593–601
21. Milne, R., Gutkowska, J., Thibault, G., Schiller, P., Charbonneau, C., Genest, J. & Cantin, M. (1986) *Mol. Immunol.*, in the press
22. Lang, R. E., Tholken, H., Ganten, D., Luft, F. C., Ruskoaho, H. & Unger, Th. (1985) *Nature (London)* **314**, 264–266
23. McGregor, D. D. (1965) *J. Physiol. (London)* **177**, 21–30
24. Ackermann, U., Irizawa, T. G. & Sonnenberg, H. (1984) *Can. J. Physiol. Pharmacol.* **62**, 819–826
25. Bianchi, C., Gutkowska, J., Thibault, G., Genest, J. & Cantin, M. (1985) *Histochemistry* **82**, 441–452
26. Bloch, K. D., Scott, J. A., Zisfein, J. B., Fallon, J. T., Margolies, M. N., Seidman, C. E., Matsueda, G. R., Homcy, C. J., Graham, R. M. & Seidman, J. G. (1985) *Science* **229**, 397–400
27. Glembotski, C. C. & Gibson, T. R. (1985) *Biochem. Biophys. Res. Commun.* **132**, 1008–1017
28. Veress, A. T., Chong, C. K. & Sonnenberg, H. (1985) *Can. J. Physiol. Pharmacol.* **63**, 1615–1617
29. Cohen, M. L. & Schenck, K. W. (1985) *Eur. J. Pharmacol.* **108**, 103–104
30. Schiffrin, E. L., Chartier, L., Thibault, G., St.-Louis, J., Cantin, M. & Genest, J. (1985) *Circ. Res.* **56**, 801–807
31. Kahn, C. R. (1976) *J. Cell Biol.* **70**, 261–286
32. King, A. C. & Cuatrecasas, P. (1981) *New Engl. J. Med.* **305**, 77–88
33. Anderson, R. G. W. & Kaplan, J. (1983) *Mod. Cell Biol.* **1**, 1–52
34. Marshall, S. (1985) *J. Biol. Chem.* **260**, 13517–13523
35. Marshall, S. (1985) *J. Biol. Chem.* **260**, 13524–13531
36. Wiley, H. S., Van Nostrand, W., McKinley, D. N. & Cunningham, D. D. (1985) *J. Biol. Chem.* **260**, 5290–5295
37. Presky, D. H. & Schonbrunn, A. (1986) *J. Cell Biol.* **102**, 878–888
38. Hirata, Y., Takata, S., Tomita, M. & Takaichi, S. (1985) *Biochem. Biophys. Res. Commun.* **132**, 976–984

Received 19 May 1986/18 July 1986; accepted 13 August 1986