A New Plasma Kinin in the Turtle, *Pseudemys scripta elegans*

Although there is much information concerning bradykinin and other plasma kinins in mammals and birds, there is only scanty knowledge of these vasodepressor and smooth-muscle stimulating peptides in other vertebrates[1–3]. Therefore studies were extended to the turtle, *Pseudemys scripta elegans*. Blood from this species was collected from the left aorta in siliconed plastic syringes, lightly heparinized, and centrifuged at 2000 *g* for 30 min at 4 °C. The plasma obtained was diluted 1:1 with turtle saline[4], and incubated with glass beads (surface area, 50 cm^2/ml undiluted plasma) for 40 min at 25 °C, in the presence of the kininase inhibitor, 8-hydroxyquinoline sulfate (1 mg/ml undiluted plasma). Assay on the isolated rat uterus[5,6] against synthetic bradykinin (Sandoz) showed a marked release of kinin, between 0.6 and 3.0 µg/ml undiluted plasma, bradykinin equivalent. During the course of these experiments, other workers[7] reported similar results for the alligator and for other species of turtles. Further studies in our laboratory showed that the enzymic mechanisms for the production of turtle kinin were closely similar to those of rat plasma. However, this did not imply that the kinins produced were identical, and therefore purification of turtle kinin was carried out.

900 ml of plasma was obtained from 30 turtles, and kinin was released by glass activation, as described above. Free kinin was extracted from plasma by the following modified butanol extraction procedure[8]: the plasma was treated with ethanol, and filtered free of precipitate; the filtrate was washed with ether in the presence of glacial acetic acid, evaporated to dryness at 40 °C and 0.1 mm Hg pressure, and taken up in saturated sodium chloride solution; the resulting solution was extracted with butanol at pH 1.5. The butanol extract was reduced to dryness in a flash evaporator, and the residue was dissolved in 0.02*M* ammonium acetate buffer, pH 5.0. This solution was passed through a G-25 Sephadex gel filtration column (90 × 2.5 cm), and the oxytocic activity was found to elute as a single peak. The active eluate was applied to a CM-Sephadex ion exchange resin in the same buffer. A gradient to 1.0*M* ammonium acetate, pH 5.0, eluted the kinin, again as a single peak. The overall purification was 1475-fold, as judged by the increase in the oxytocic activity per mg of total 'peptide' (method of LOWRY, ROSEBROUGH, FARR and RANDALL[9]). A summary of the purifications obtained is given in Table I.

Chemical and pharmacological tests were made on the active eluates at various stages of purification. Turtle kinin showed chromatographic behaviour indistinguishable from that of bradykinin on gel filtration columns (G-25 Sephadex), ion exchange columns (CM-Sephadex), and on paper chromatography in butanol : acetic acid : water, 4:1:5; in contrast, the synthetic analogue lys-bradykinin (Schwarz Bioresearch) was sharply separated by the last two systems. Despite these chromatographical results, multiple pharmacological assays showed marked differences between synthetic bradykinin and turtle kinin when they were tested directly against one another on certain of the following preparations: isolated rat uterus[5], isolated rat uterus sensitized with chymotrypsin[10], rat duodenum (relaxation)[11], guinea-pig ileum[12], rat blood pressure (vasodepressor)[12] and rabbit blood pressure (vasodepressor)[12]. Tests were carried out by the 4-point statistical method[5], using synthetic bradykinin as standard, and the results are given in Table II, with their errors expressed as confidence limits, p 0.05. If turtle kinin and bradykinin were identical, the ratios between assays would always have been unity. In fact, this was true for certain ratios, and the value of rat uterus/rat blood pressure activity for the purest preparation of turtle kinin was only marginally different from unity (1.3 ± 0.2). However, the ratios of rat uterus/guinea-pig ileum activity (2.5 ± 0.4) and of rat uterus/rabbit blood pressure activity (7.0 ± 1.8) showed striking discrepancies

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Table I. The purification of glass released turtle kinin

Stage of purification	Total volume of active preparation (ml)	Total peptide present (mg) LOWRY[9] peptide[a]	Total rat uterus oxytocic activity present (µg) bradykinin equivalent[b]	Recovery[c] (%)	Specific activity (µg) bradykinin equivalent (mg) LOWRY[9] peptide	Factor of purification[d]
Original plasma	900	468.0	2490 ± 250	–	0.53	1
Butanol extract	16	60.0	531 ± 18	21.3	8.9	16.8
G-25 Sephadex pooled eluates	75	1.95	370 ± 40	69.7	189	357
CM-Sephadex pooled eluates	25	0.45	352 ± 26	94.5	782	1475

[a] Peptide determined by the method of LOWRY, ROSEBROUGH, FARR and RANDALL[9]. [b] Oxytocic activity determined by the 4-point method of HOLTON[5], with errors expressed as confidence limits, P 0.05. [c] The recovery represents the percentage recovery obtained by the particular purification procedure indicated in the first column. [d] The factor of purification = specific activity of the preparation/specific activity of the original plasma.

Table II. Pharmacological studies; the ratios of different biological activities of turtle kinin, assayed against synthetic bradykinin

Method of assay	Turtle kinin preparation assayed[a]	Assay value; bradykinin equivalent (μg/ml)	Rat uterus oxytocic activity; bradykinin equivalent (μg/ml)	Ratio Rat uterus activity; biological activity assayed
Rat uterus, sensitized with chymotrypsin	Butanol extract	2.5 ± 0.1[b]	2.3 ± 0.2	0.9 ± 0.1
Rat duodenum	G-25 Sephadex eluate	0.16 ± 0.03[c]	0.17 ± 0.01	1.0 ± 0.1
		0.19 ± 0.03[c]	0.19 ± 0.01	1.0 ± 0.1
Rat blood pressure	CM-Sephadex eluate	10.1 ± 1.5	13.2 ± 1.0	1.3 ± 0.2
Guinea-pig ileum	G-25 Sephadex eluate	0.10 ± 0.04	0.19 ± 0.01	2.0 ± 0.8
	CM-Sephadex eluate	5.2 ± 0.7	13.2 ± 1.0	2.5 ± 0.4
Rabbit blood pressure	CM-Sephadex eluate	0.95 ± 0.15	6.7 ± 0.8	7.0 ± 1.8

[a] The stages of purification, and degrees of purity are as indicated in Table I. [b] All assays were carried out by the 4-point method of HOLTON[5]; errors are expressed as confidence limits, P 0.05. [c] Values obtained during 2 separate runs through G-25 Sephadex.

in this same preparation. This indicated that turtle kinin was less active than bradykinin on the guinea-pig ileum and on the rabbit blood pressure, and this suggested that it was a different kinin. Therefore, turtle kinin was analyzed for its amino acid composition.

145 μg bradykinin-equivalent of the CM-Sephadex purified turtle kinin was hydrolyzed for 36 h at 107 °C in 6N HCl, and a control of 100 μg synthetic bradykinin was treated in a similar manner. Amino acid analyses were carried out on a Biocal 200 analyzer, and the results are given in Table III. Turtle kinin gave molar ratios of arginine (2), proline (3), glycine (1), and phenyl alanine (2) exactly as would have been predicted for bradykinin, but it differed in the presence of one molar ratio of threonine and the absence of one molar ratio of serine. Other amino acids were present in only trace amounts. Synthetic bradykinin gave the expected analysis. The simplest conclusion we can draw from these analyses is that turtle kinin may be an analogue of bradykinin in which threonine has been substituted for serine, and is therefore 6-threonine bradykinin:

H-Arg-Pro-Pro-Gly-Phe-*Ser*-Pro-Phe-Arg-OH
Bradykinin[13].

H-Arg-Pro-Pro-Gly-Phe-*Thr*-Pro-Phe-Arg-OH
6-Thr-bradykinin: proposed turtle kinin.

Support for this hypothesis comes from the pharmacological properties of turtle kinin. Synthetic 6-Thr-bradykinin was studied by SCHRÖDER and HEMPEL[14], and although it was as active as bradykinin on the rat uterus, the ratio of rat uterus/guinea-pig ileum activity was 3, and the ratio of rat uterus/rabbit blood pressure activity was 10. Although these approximate values contrast with those of many other synthetic bradykinin analogues[15], they are in good agreement with the values obtained for turtle kinin (Table II).

Table III. Chemical studies; the molar ratios of amino acid residues from hydrolysates of bradykinin and turtle kinin

Amino acids	Molar ratios of amino acid residues from hydrolysates, Arg 2.00			
	Experimental ratios		Deduced ratios	
	Bradykinin	Turtle kinin	Bradykinin[a]	Turtle kinin
Arginine	2.00	2.00	2	2
Proline	2.93	3.16	3	3
Glycine	1.06	1.15	1	1
Phenyl alanine	2.01	1.82	2	2
Serine	1.07	0.14	1	0
Threonine	0.10	0.99	0	1

Molar ratios of amino acids present in trace amounts only. 1. Bradykinin: Ala, 0.24; Val, 0.18; Lys, 0.16; Asp, Glu, Met, Ile, Leu, Tyr, His, <0.10. 2. Turtle kinin: Glu, 0.18; Ala, 0.14; Val, 0.13; Asp, Met, Ile, Leu, Tyr, His, Lys, <0.10. [a] The values given in this column are in agreement with the known values for bradykinin[13].

Although final conclusions must await sequence analysis of the active peptides concerned, the evidence obtained in this study suggests the presence in turtle plasma of a kallikrein-kinin system very similar to that of mammalian plasma, but based on threonine containing peptides, and culminating in the production of 6-threonine bradykinin as the natural plasma kinin of the turtle, *Pseudemys scripta elegans*. This is the first evidence to suggest that 6-threonine bradykinin occurs in nature, and it is the first example of a naturally occurring analogue of bradykinin in which there has been a replacement within the fundamental bradykinin chain. A mutation which could cause the interchange of serine and threonine is a reasonable possibility, since it involves a single base change in the genetic code[16]. This raises the possibility that other mutations could have produced yet different analogues in other vertebrate plasmas, and it is possible that further work could reveal a pattern of molecular evolution throughout the vertebrate tree[17].

Zusammenfassung. Eine pharmakologische Untersuchung, biologische Studie und chemische Analyse des isolierten und teilweise gereinigten Kinins im Blutplasma der Schidkröte, *Pseudemys scripta elegans*, wird beschrieben. Das Kinin der Schildkröte unterscheidet sich vom Bradykinin und ist vermutlich 6-Thr-Bradykinin.

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