Fate of Circulating Serotonin in the Hemolymph of the Crayfish, *Orconectes limosus*

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The fate of injected [^{14}C]serotonin (0.1 μCi/1.6 nmol per animal) was investigated in the crayfish *Orconectes limosus* after injection. Specific radioactivities reached highest values in the green gland, followed by intestine, gills, nervous tissue and digestive gland. Label accumulation was lowest in the tail muscle.

Cold serotonin injected into the hemolymph (50 nmol/100 g wet wt) was cleared with a mean half life of 12.9 +/− 2.6 min (15 °C; N = 5). By HPLC separation of hemolymph samples taken at intervals after the injection of [^{14}C]serotonin, the formation of a metabolic product could be demonstrated. The formation of the metabolite was not, however, detectable by native fluorescence, suggesting the metabolic breakdown of the indole ring.

The combined amounts of acid soluble radioactivity recovered from hemolymph, green gland, digestive gland and intestine yielded 80–100%, 50–70% and 30–50% of the injected dose after 7 min, 1 h, and 4 h, respectively. Since other tissues did not account for the balance and less than 5% of the injected label had been excreted into the medium after 4 h the results suggest the gradual incorporation of the metabolites into the tissues.

Introduction

The role of biogenic amines as neurohormones in crustaceans is supported by their effect on a variety of physiological functions such as the regulation of hemolymph glucose levels [1], hemolymph clotting, [2], rate and strength of heart muscle contraction and modulation of neuromuscular transmission [2–4]. The pericardial organs have been identified as a source of biogenic amines and have been shown to release serotonin and octopamine after stimulation [5, 6]. In the light of the physiological role of biogenic amines as neurohormones, crustaceans must have developed control mechanisms to regulate their concentration in the hemolymph. In spite of their known release into the hemolymph, little information exists on the fate of circulating biogenic amines or catecholamines and the breakdown and re-uptake mechanisms which seem necessary to avoid their buildup in the hemolymph and to keep their concentrations at appropriate physiological levels.

In a previous paper [7] we reported the clearance rates for different catecholamines in the Chinese crab *(Eriocheir sinensis)*. Dopamine and DOPA showed relatively short half lifes of 6 min (measured at 15 °C), whereas noradrenaline and adrenaline had half lifes between 20 and 40 min at the same temperature. The distinct clearance rates found for the different catecholamines studied suggests a specificity of the uptake system towards different catecholamines. Given the now demonstrated existence of a clearance system, the continuous presence even of low concentrations of biogenic amines and catecholamines in the hemolymph [7, 8] suggests their continuous release. Uptake and removal systems thus represent one possible control point for the regulation of the levels of these active compounds in crustacean hemolymph.

The demonstration of the existence of uptake mechanisms for catecholamines, and most likely, other biogenic amines raised the question of the tissues responsible for uptake and further metabolism of these compounds. This study was undertaken as a first approach to the identification of such tissues. In this paper, the fate of serotonin in the crayfish, *Orconectes limosus,* following injection into the hemolymph was investigated.

Materials and Methods

Animals

Crayfish (*Orconectes limosus,* 20–30 g live weight) were obtained commercially and kept in running lake water at 14–15 °C.

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Determination of hemolymph serotonin

About 0.5 ml of hemolymph was drawn at the base of the cheliped into preweighed 2 ml disposable syringes containing 0.5 ml of anticoagulant solution [9]. After addition of 40 µl (16 pmol) N-methyl-serotonin as an internal standard, the hemolymph cells were removed by brief centrifugation (1500 × *g*). The supernatant was loaded onto a 1 ml extraction column (BondElut C_{18}, Analytichem International). The column was washed with 1 ml water and eluted with 500 µl of methanol saturated with sodium metabisulfite. The eluate was taken to dryness using a vacuum concentrator and redissolved in 100 µl 50% methanol containing 2 mg/ml sodium metabisulfite. This solution was applied to a second 1 ml extraction column containing a weakly acidic cation exchanger (BondElut CBA, Na^+-form). The column was washed with 1 ml of water and then eluted with 500 µl 250 mM formic acid. The eluate was again taken to dryness, dissolved in 100 µl of mobile phase (see below), briefly centrifuged, and an aliquot used for the HPLC determination of serotonin (see below). Recovery was between 65 and 87% judged from the internal standard.

The hemocyte pellet was washed with anticoagulant solution and finally homogenized in 100 µl 0.25 M perchloric acid/0.1%. EDTA/0.2% sodium metabisulfite by applying two 5 sec bursts of an ultrasonic disintegrator. After centrifugation, a 50 µl aliquot of the supernatant was assayed for serotonin by HPLC (see below).

Determination of serotonin degradation rates in the hemolymph

Crayfish were injected with 1 mM solutions of serotonin freshly prepared in 0.2 M NaCl. The injected volume (12.5–15 µl) was adjusted to give a dose of 50 nmol/100 g live weight. Hemolymph sampling was performed as described previously [7]. The weighed samples (80–100 µl) were deproteinized with 100 µl 0.4 M perchloric acid containing 10% methanol, 0.1% EDTA and 0.1% sodium metabisulfide. After centrifugation (6000 × *g*, 2 min), an aliquot of the supernatants was analyzed for serotonin by HPLC as described below. The data were subjected to linear regression analysis after ln transformation and the first order disappearance constant (k_d) was used to calculate the apparent half life ($\ln 2/k_d$) in the hemolymph. The coefficient of determination of the data (r^2) was >0.95.

HPLC determination of serotonin

Serotonin and other indole compounds were separated isocratically by ion pair chromatography on a reverse phase column (Nucleosil $5C_{18}$, 150 × 4.6 mm) and a mobile phase consisting of 100 mM citric acid, 50 mM sodium acetate, 100 mg/l sodium octylsulfate, 25 mg/l EDTA and 8% methanol, pH 3.0. Indole compounds were detected by their native fluorescence at excitation and emission wavelengths of 278 and 335 nm, respectively. In one experiment, the degradation of serotonin in the hemolymph (see above) was also measured electrochemically using a glassy carbon electrode (Bioanalytical systems model LC4) and an oxidation voltage 0.75 V coupled in series with the fluorimetric detector (Perkin-Elmer model LS 5). The detection methods were in close agreement.

Labelling experiments

Crayfish were injected with 0.1 µCi (200,000 cpm) [^{14}C]serotonin in 20 µl 200 mM NaCl into the arthrodial membrane at the base of a walking leg. During the experiment, the animals were kept in a glass bowl containing 200 ml aerated lake water at 15 °C. Animals were divided into four groups kept for different time periods (6–10, 18–25, 60, 250–260 min) after the injection. At the end of each incubation, a hemolymph sample (about 1 ml) was drawn from the base of a cheliped leg and the animals were then decapitated. The different tissues were removed, briefly blotted, divided in two (except for nervous tissue, green gland and intestine), and homogenized with 2 ml 0.2 M perchloric acid containing 0.1% EDTA and 0.2% sodium metabisulfite using a Polytron homogenizer (Brinkman Instruments). After centrifugation (6000 × *g*) for 2 min, 1.5 ml of the supernatants were counted for 5 min in a Beckman LS series liquid szintillation counter using a xylene based scintillation cocktail containing 25% Triton X-114 (Sigma) and 3 g/l 2,5-diphenyloxazol (Packard). An aliquot (20 µl) of the injected [^{14}C]serotonin solution was also counted with every experiment. Quenching of the tissue extracts

was determined by the addition of an internal standard to each sample; the counting efficiency was found between 80 and 107% with respect to the internal standard. Recovery of the label in the perchloric acid extracts was determined in tissue homogenates to which a serotonin standard had been added, and was found to be around 95% for all tissues but gill (80%) and muscle (85%). The tissue radioactivity was expressed in cpm/g tissue and normalized for a 25 g crayfish and a standard dose of 100,000 cpm. Radioactivity released into the medium was determined by counting 1 ml water samples after 4 h of incubation.

To detect metabolic products in the hemolymph, 0.2 μCi [^{14}C]serotonin was injected and subsequent hemolymph samples were taken and processed as described above. For each sample a 100 μl aliquot of the PCA extract was then counted directly for total radioactivity and another was subjected to HPLC separation and fluorescence detection of serotonin as described above except for a higher methanol concentration (15%) in the mobile phase. The column eluate was collected between 0.7–2.5 min (2.1 ml), 2.5–3.5 min (1.2 ml) and 3.5–5.5 min (2.4 ml) and designated fraction I, II and III, respectively. Serotonin eluted in fraction III as determined by external standard. The radioactivity of these fractions was determined (5 min counts) and expressed as cpm/ml hemolymph.

Chemicals

5-Hydroxy[side-chain-2-^{14}C]tryptamine creatinine sulfate (specific activity 54 mCi/mmol) was purchased from Amersham Buchler, Braunschweig, F.R.G. It was purified before use by passage through a weakly acidic cation exchange column (CBA, Na^+-form, Analytichem International) and eluted with 0.25 M formic acid. Aliquots were lyophilized and stored frozen. Before use, they were taken up in 100 μl 0.2 M NaCl. Cold serotonin creatinine sulfate came from Serva (Heidelberg, F.R.G.). All other chemicals were reagent grade.

Results

Serotonin content of the hemolymph

The serotonin content of the hemolymph in *Orconectes limosus* was near the detection limit of the method used here, and below 1 nM (N = 4). The hemocyte pellets (see Materials and Methods) were also investigated for serotonin after perchloric acid extraction but did not contain detectable amounts.

Degradation of serotonin in the hemolymph

Clearance of injected cold serotonin from the hemolymph as monitored by the decrease of native fluorescence followed an exponential time course from which a mean apparent half life of 12.9 +/− 2.6 min was calculated (N = 5). 90% of the injected dose was cleared from the hemolymph after 1 h.

Label accumulation in different tissues

The uptake of acid extractable label in different tissues after injection of [^{14}C]serotonin was followed over 4 h (Fig. 2). The green gland and digestive gland showed a constant acid soluble radioactivity after 1 h, whereas in the other tissues investigated only a transient label accumulation was found with a peak in the second sample (20 min). The uptake of the label was tissue specific. The radioactivity of different tissues (expressed as cpm/g tissue) 1 h after injection is shown in Fig. 1. The tissue radioactivity was highest in the green gland

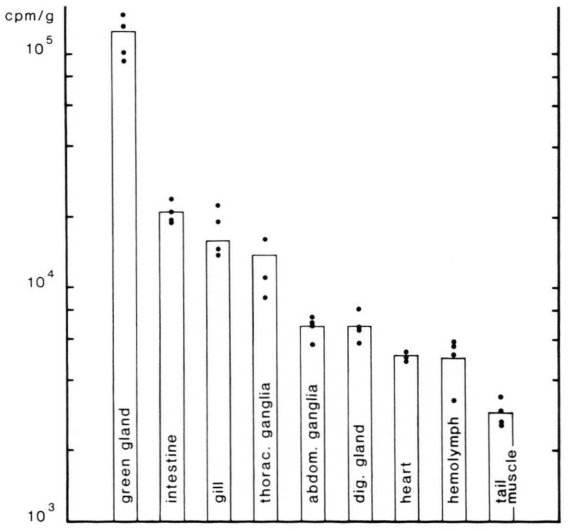

Fig. 1. Radioactivity (counts per min/g tissue) of different tissues of *Orconectes limosus* 1 h after injection of 0.1 μCi (1.6 nmol) [^{14}C]serotonin into the hemolymph. Dots represent individual determinations.

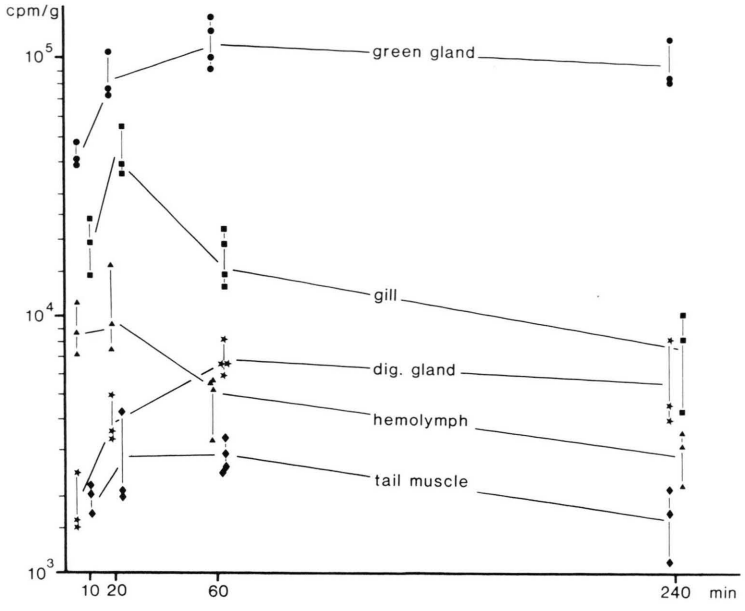

Fig. 2. Time course of label accumulation in some tissues and in the hemolymph (counts per min/g tissue) of *Orconectes limosus* after injection of 0.1 μCi (1.6 nmol) [^{14}C]serotonin into the hemolymph. Individual measurements are connected by bars.

and lowest in the tail muscle in which only one tenth as much accumulated.

The percentage of acid soluble radioactivity recovered from hemolymph, gills, green gland, digestive gland and intestine was 80–100% of the injected dose after 7 and 20 min, 50–70% after 1 h, and 30–50% after 4 h (Table I). The radioactivity present in the hemolymph space was calculated assuming a hemolymph volume of 30% of the live weight (see [7]). The amount of acid soluble radioactivity recovered from the tissues was always less than that present in the hemolymph and reached 21% of the injected dose after 1 h. Only little radioactivity, which accounted for less than 5% of the injected dose, was found in the surrounding water after 4 h. At this time, the radioactivity present in the hemolymph was only 20–30% of the injected dose.

In those hemolymph samples which had been fractionated after HPLC separation (see Materials and Methods) the decrease of serotonin was followed by both native fluorescence detection (data not shown) and by the decrease of specific radioactivity in fraction III. At the same time, the activity in fraction I increased (Fig. 3). Combining fractions I and III yielded values close to those obtained for total hemolymph radioactivity (Fig. 3). No radioactivity was present in fraction II. An ex-

Table I. Recovery of acid soluble radioactivity from different tissues of *Orconectes limosus* at different times after the injection of 0.1 μCi (1.6 nmol) [^{14}C]serotonin. Values (mean/range) are given as percentage of the injected dose.

Tissue	7 min (N = 3)	20 min (N = 3)	60 min (N = 4)	240 min (N = 3)
hemolymph	72 (60–85)	77 (62–93)	41 (28–46)	25 (18–29)
green gland	3.2 (3.0–3.3)	6.7 (4.4–9.5)	10 (8.0–14)	7.5 (6.5–9.3)
dig. gland	1.2 (0.9–1.5)	3.2 (2.2–3.6)	4.5 (3.3–5.5)	3.0 (1.9–5.0)
gill	10.7 (7.8–15.8)	17 (16–19)	6.1 (3.0–7.2)	2.7 (1.8–3.7)
intestine	0.6 (0.4–0.8)	1.5 (0.7–2.1)	1.1 (0.7–1.4)	0.2 (0.2–0.4)
sum (mean)	88	105	63	38

ample of the chromatographic separation of serotonin and the fractionation procedure is shown in Fig. 4.

Discussion

The half life of serotonin in the hemolymph of *Orconectes limosus* was relatively short and similar to those found for serotonin and octopamine in the Chinese crab *Eriocheir sinensis,* which average 12.5 min and 13.5 min, respectively, at 15 °C (Hoeger, in prep.). Since the serotonin clearance followed an exponential time course, the half life is independent of the hemolymph concentration. Therefore, the decay constant was used to judge the clearance time at physiological hormone levels. If we assume a stimulus induced release of serotonin into the circulation to levels that have been shown to elicit significant effects on isolated organs (*i.e.*, 10^{-9} M; [3] and apply the half lifes measured in this study, it would take about 50 min to return to the basal levels of around 1 nM in *Orconectes limosus* (this study) and 0.5 nM in another crayfish, *Pacifastacus leniusculus* [8]. This estimate is consistent with results obtained from the shore crab, *Carcinus maenas,* in which the stress-induced increase in heart rate took about 1 h in returning back to resting values [10].

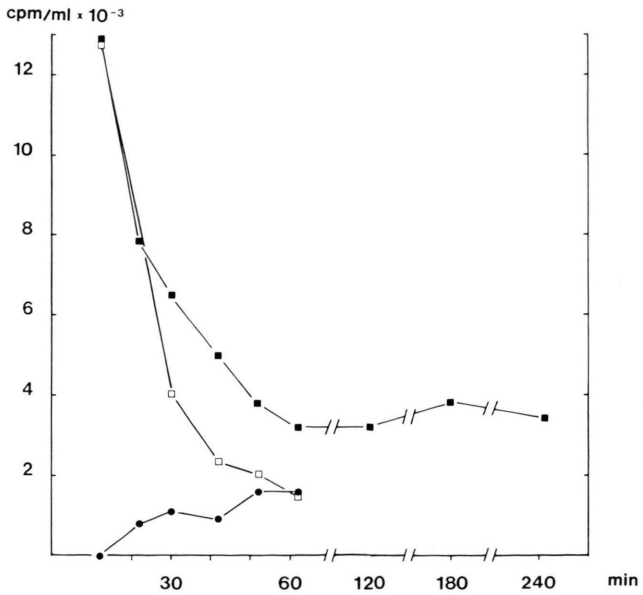

Fig. 3. Changes in the total radioactivity (cpm/ml hemolymph) after injection of 0.2 µCi (3.2 nmol) [^{14}C]serotonin into the hemolymph of *Orconectes limosus*. Total radioactivity of the hemolymph (■) and in fraction I (●) and fraction III (□) of the hemolymph samples after HPLC separation (see Materials and Methods).

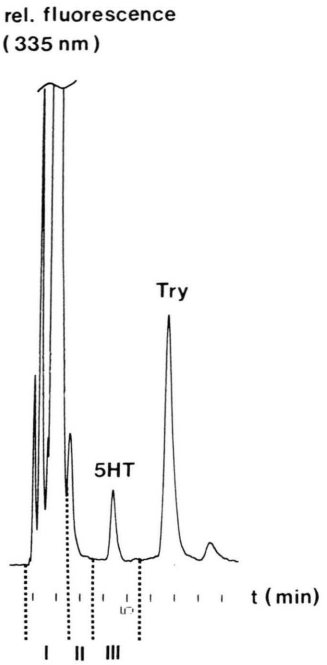

Fig. 4. HPLC determination of serotonin in the hemolymph of *Orconectes limosus* by native fluorescence detection ($\lambda_{exc} = 278$ nm, $\lambda_{em} = 335$ nm). The chromatogram was obtained 12 min after the injection of 3.2 nmol of labelled [^{14}C]serotonin. The eluates were divided into fractions I (I), II (II) and III (III) as indicated. 5HT = serotonin, Tr = tryptophan. For chromatographic conditions, see Materials and Methods.

Although 90% of the hemolymph serotonin was removed after 1 h (see Fig. 3), only 20–25% of the injected label was recovered from the quantitatively important tissues: green gland, digestive gland and gills (see Table I). This was apparently due to the immediate metabolism of serotonin leading to the appearance of at least one metabolite in the hemolymph (see Fig. 3). Following the disappearance of serotonin from the hemolymph as measured fluorimetrically, the major portion of the acid soluble radioactivity which now represents the metabolic product remained in the hemolymph. This suggests that the metabolism of serotonin takes place at the hemolymph/tissue interface or that the

metabolic product is released into the hemolymph immediately after being metabolized in the tissue. The metabolite in turn is most likely incorporated into the tissues; this is suggested by both the progressive decrease in the recovery of the label with increasing incubation time (see table) and the absence of significant label excretion into the medium after 4 h. The percentage of acid soluble label accumulated in the digestive and green gland (see Table) probably represents serotonin which is only slowly metabolized since the values did not further increase between one and 4 h after the label injection. Uptake of significant amounts of the metabolite by these tissues would have resulted in a further increase in their specific radioactivity considering the time course of the appearance of the metabolite in the hemolymph (see Fig. 3).

The high specific radioactivity present in the green gland compared to other tissues was remarkable, since this tissue accumulated about 10% of the injected label in spite of its small size (0.3–0.4% of the body weight). Considered primarily as an organ involved in excretion and ionic regulation [11], it is also involved in active resorption of organic molecules such as glucose and amino acids [12, 13]. The present findings suggest a similar function in the removal of circulating serotonin and perhaps other neurohormones, which could become more significant at physiological serotonin levels. It should be considered that the amount of injected label necessary to study the fate of circulating serotonin in these experiments transiently raised the normal hemolymph concentration of serotonin three orders of magnitude over the physiological levels of around 10^{-9} M present in *Orconectes limosus* (this study) and another crayfish (*Pacifastacus leniusculus*; [8]. This could lead to increased uptake by passive diffusion into other tissues, which would be facilitated by the lipophilic nature of the indole ring. The ability of the isolated rabbit lung to remove different amines from the perfusate was related to their relative lipid solubility [14]. Uptake by passive transport could be significant, especially for those tissues with a large perfused inner surface such as gill and the digestive gland.

The metabolic end product of serotonin released into the hemolymph is a compound more polar than serotonin, since the fraction containing the radioactive metabolite(s) eluted earlier than serotonin from the reverse phase column. The metabolite(s) are not likely to be indole compounds, since no additional peaks showing indole fluorescence were observed on the chromatograms in the corresponding fraction I. The metabolic products of serotonin arising in the hemolymph are therefore different from to the known end products of biogenic amine inactivation found in crustacean and other invertebrate nervous tissue which involves the formation of an N-Acetyl-, beta-alanyl- or gamma-glutamyl conjugate of the amine and/or its sulfation [15–18]. Neither the formation of N-acetyl-serotonin nor the monoaminooxidase metabolite 5-hydroxyindole acetic acid was detected fluorimetrically in the hemolymph after serotonin injection. However, the transient formation of these compounds within a tissue cannot be excluded.

The fate of serotonin found here in *Orconectes limosus,* however, may not represent a general route for the metabolism of circulating biogenic amines. In the shore crab, *Carcinus maenas,* the biogenic amine histamine is rapidly transformed into the pharmacologically inactive beta-alanyl conjugate by an enzyme located in the nervous tissue [18]. After release into the hemolymph, the conjugate is taken up exclusively by the heart. This organ seems to act as a sink for beta-alanyl histidine since its concentration remained unchanged over several days [18]. This is in contrast to the low label uptake found in the heart in this study (see Fig. 1).

Both the mechanism and the site of serotonin degradation remains to be investigated. In mussel gills, an oxidase like activity also active on serotonin was found [19, 20]. However, no evidence for the presence of such an enzyme was obtained by incubating crude enzyme extracts from various crayfish tissues with serotonin.

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