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OCCURRENCE OF 5-HYDROXYTRYPTAMINE-LIKE FLUORESCENCE IN THE BLOOD CLOT OF TAIL-FINS OF GOLDFISH

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Fluorescence spectra of a blood clot formed at the cut edge of the tail-fin of goldfish were examined, with particular attention to the fluorescence of 5-hydroxytryptamine (5-HT). An attempt was made to use HCl and *o*-phthalaldehyde (OPT), which are known to intensify the fluorescence of 5-HT. Prior to the study on blood clot fluorescence, the fluorescence spectra of 5-HT in solution were examined at various HCl concentrations with or without the addition of OPT.

Maximal fluorescence of the blood clot appeared approx. 10 min after cutting the fin, and an emission maximum with a 470 nm peak was obtained by excitation at 360 nm in 0.1 N HCl and 0.005% OPT. This spectral property was similar to that obtained with 5-HT in solution.

Since blood clotting in the fish occurs immediately, bleeding is greatly reduced. However, little is known about the blood clotting process in relation to thrombocytes in the peripheral blood of lower vertebrates. In higher vertebrates, thrombocytes store 5-hydroxytryptamine (5-HT) in their subcellular particles (3, 13). It has also been reported that the release of 5-HT from blood platelets by reserpine does not significantly affect bleeding time (11). In the present report, an attempt was made to study the blood clotting process in fish, with particular reference to the mobilization of 5-HT. The fluorescence of the blood clot was microfluorometrically analyzed by using HCl and *o*-phthalaldehyde (OPT), which are known to intensify the fluorescence of 5-HT (1, 7).

MATERIALS AND METHODS

a) *Analysis of 5-HT fluorescence in solution.*

Measurement in HCl solution (HCl-method): Serotonin creatinine sulfate was obtained from Wako Chemicals Co., Osaka. Based on the method of Bogdanski et al. (1), the fluorescence of 5-HT in solution (2 g/μml) in a 1 cm quartz cuvett was measured with a Hitachi MPA-2A type fluorometer at various HCl concen-

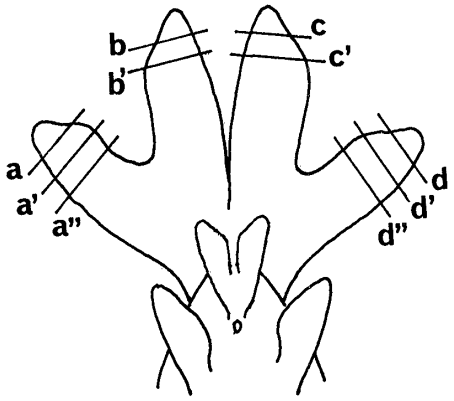

FIG. 1. A diagramatic view of the ventral side of tail-fins. The fin ends are cut off along lines of a, b, c and d. Fin-edges including the blood clots were removed by cutting along lines of a′, b′, c′, d′ and then along lines of a″ and d″ in order to make tissue pieces of 3 mm width.

trations (3 N, 2 N, 1 N and 0.1 N). Emission spectra were obtained by excitation at 290 nm.

Measurement in HCl solution containing *o*-phthalaldehyde (HCl-OPT method): Using the method of Maickel et al. (7), measurements were taken at various HCl concentrations (5.5 N, 1.2 N and less) containing 0.005% OPT (Wako Chemical Co., Osaka). Emission spectra were obtained by excitation at 290 nm and 360 nm. The effects of heating at 100°C for 15 min were also studied.

b) *Microfluorometry of the blood clot.*

Goldfish (*Carassius auratus* L.) bred in a circulating water bath for a one month period were used. The tail of the goldfish is separated into 4 lobar fins. These tail-fins were cut simultaneously at approx. 3 mm from the distal end, as is illustrated in Fig. 1, a, b, c and d. Blood clotting occurred at the proximal cut edges a, b, c and d of the fins. The clotting became visible at the cut edge after only a few minutes. The fin-edge, including the blood clot, was cut along lines a′, b′, c′, d′ and removed, then again along lines a″ and d″ to make 3 mm wide tissue pieces. The tissue pieces were put on a quartz slide and mounted in 0.1 N HCl solution with and without 0.005% *o*-phthalaldehyde. A quartz cover slip was fixed on the quartz slide with a nail-manicure to prevent evaporation of the medium.

Blood clot fluorescence on the fin tissue piece was measured by a microfluorometer combined with a Zeiss UMSP type I. The optical system used was a Zeiss ultrafluar 32 × objective with a measuring field of 20 μm in diameter, with a opening slit of 0.5 mm and 2.0 mm width at the front of the xenon light source. The microfluorometer was equipped with a grating monochromator and an UV-cut filter (Nikon). The emission spectra, which ranged from 400 nm to 650 nm, were automatically recorded.

RESULTS

1. Examination of the fluorescence spectra of 5-HT in HCl solution: The effects of HCl at various concentrations on the excitation and emission spectra are

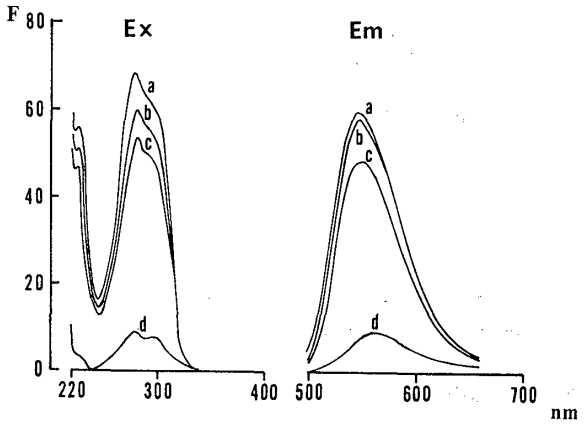

Fig. 2.

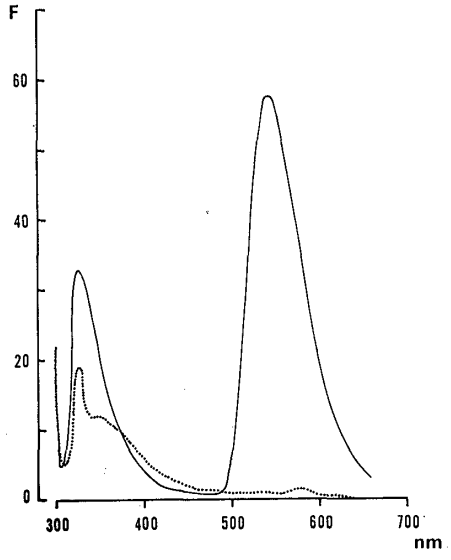

Fig. 3.

FIG. 2. Effects of various HCl concentrations on the emission and excitation spectra of 5-HT creatinine sulfate.

Ex: Excitation spectra emitted at 550 nm with 3 N HCl(a), 2N HCl(b), 1 N HCl(c) and 0.1 N HCl(d).

Em: Emission spectra excited at 290 nm with 3 N HCl(a), 2 N HCl(b), 1 N HCl(c) and 0.1 N HCl(d).

FIG. 3. The emission spectrum of 5-HT (2 μg/ml, solid line) is compared with that of tryptophan (2 μg/ml, dotted line) in the presence of 3 N HCl. Tryptophan does not emit at 550 nm when excitation is fixed at 290 nm.

shown in Fig. 2. An excitation maximum occurred at 290–295 nm when the emission was fixed at 550 nm (F550), and an emission maximum at approx. 550 nm when the excitation was fixed at 290 nm (Ex290). It should be noted that these spectral properties continued even when HCl molarity was lowered to 0.1 (peak d in Fig. 2).

The emission spectrum of 5-HT was then compared to that of tryptophan, which was rich in the tissue (Fig. 3). The emission spectrum of tryptophan showed a peak at approx. 340 nm, but no significant emission occurred at 550 nm.

2. Examination of the fluorescence spectra of 5-HT in HCl solution containing *o*-phthalaldehyde: A small amount of OPT (0.005%) intensifies the fluorescence of 5-HT. As shown in Fig. 4, a high excitation peak at approx. 360 nm and an emission maximum at about 470 nm were markedly induced. The relative intensity (F470) in 5.5 N HCl solution was about 4 times greater than that in the 1.2 N HCl solution. A low excitation spectrum peak of approx. 290 nm (Ex290) was identical to the excitation maximum obtained with HCl solution alone. Accordingly, emission spectra excited at 360 nm were compared with those excited at 290 nm (Fig. 5). After heating at 100°C in 1.2 N HCl solution, the emission maximum (peak b in Fig. 5) was detectable at 570–580 nm, even when excitation was made at 290 nm. Emission spectra excited at 360 nm under various conditions are shown in Fig. 5-B. By heating in 5.5 N HCl solution emission was greatly

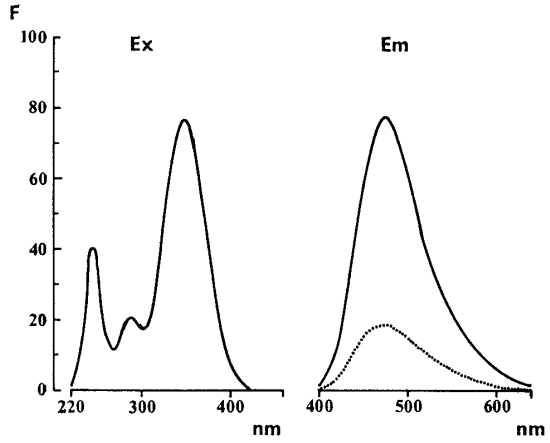

Fig. 4.

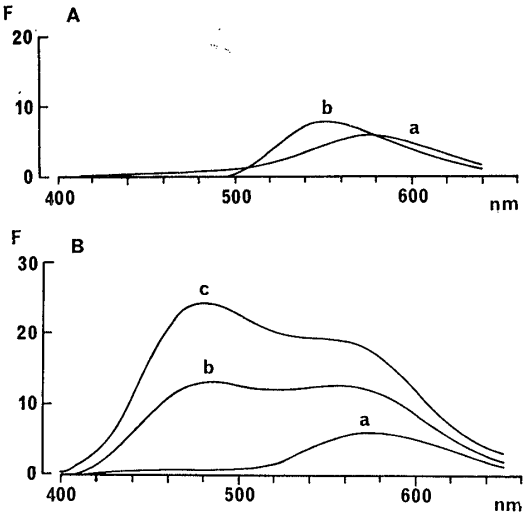

Fig. 5.

FIG. 4. Fluorescence spectra of 5-HT by HCl-OPT method.
Ex: Excitation spectrum emitted at 470 nm.
Em: Emission spectra excited at 360 nm with 5.5 N HCl (solid line) and 1.2 N HCl (dotted line).

FIG. 5. Emission spectra of 5-HT by the HCl-OPT method under various conditions.
A: Emission spectra excited at 360 nm(a) and 290 nm(b) by heating at 100°C for 15 min. An emission maximum is detectable at 570–580 nm with excitation at about 360 nm(a) and at about 550 nm with excitation at 290 nm(b).
B: Emission spectra excited at 360 nm by heating at 100°C for 15 min. with 1.2 N HCl(a), with 5.5 N HCl(c) and by non-heating with 5.5 N HCl(b). The F of (c) becomes higher than that of non-heated(b), showing two moderate peaks at about 470 nm and 570 nm. A peak at a shorter wavelength (470 nm) disappears in (a).

intensified (B-c), with two moderate peaks at about 470 nm and 570 nm. The 470 nm peak disappeared in 1.2 N HCl solution with heating (B-a). This shift in emission spectrum seems to be useful in identification of 5-HT.

3. Microspectrophotometry of the fluorescence of the blood clot in 0.1 N HCl solution: To prevent damage of the tissue, HCl was used at a lower concentration, i.e., 0.1 N. The emission spectra obtained by excitation at 290 nm are shown as in Fig. 6 as dotted lines. The spectra obtained by 1 min., 5 min. and 10 min. excitation are shown by the dotted lines in Fig. 6-A, 6-B and 6-C, respectively As shown by the dotted line of Fig. 6-A, emission spectrum after 1 min. excitation showed several peaks, *a, b, c* and *d*. Point *a* was approximately F470, *b* was F500, *c* was F540 and *d* was F570. It should be noted that the highest emission peak *c* (F540) resembles the emission peak of 5-HT excited at 290 nm in HCl (Fig. 2). By prolonged excitation, the emission spectra developed a broad peak ranging from 460 nm to 560 nm. This peak seems to be formed by the overlapping of peak *b* with peak *c* (Fig. 6-B and Fig. 6-C).

4. Microspectrophotometry of the fluorescence of a blood clot in 0.1 N HCl containing *o*-phthalaldehyde.

Excitation at 290 nm: The fluorescence spectrum was altered by the combined use of HCl solution with OPT, where peaks $b+d$ of the solid line in Fig. 6-A

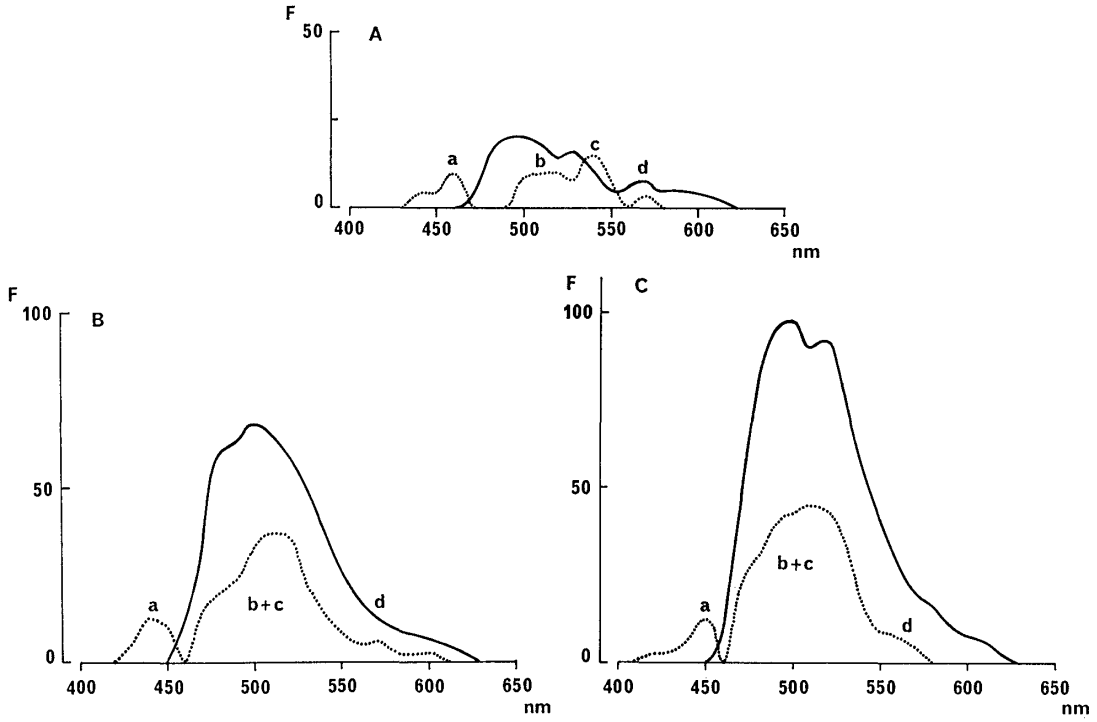

FIG. 6. The emission of the blood clot at the fin-edge consists of several peaks at approximately 470 nm (a), 500 nm(b), 540 nm(c) and 570 nm(d), which are obtained by subtraction of an autofluorescence in distilled water. After 1 min. irradiation with 290 nm (A), emission peaks are obtained with HCl (dotted line) and with HCl-OPT (solid line). Emission peaks of the b and c are enhanced by irradiation at 290 nm for 5 min.(B) or for 10 min.(C).

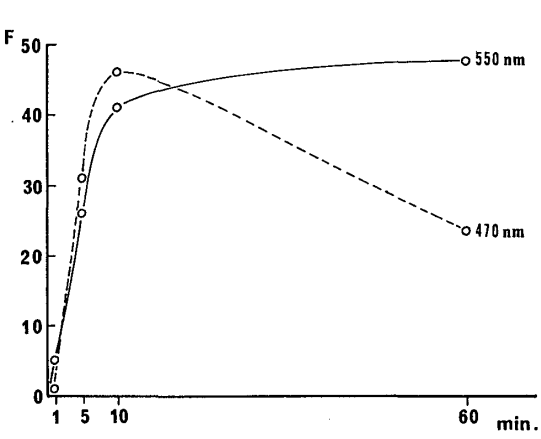

Fig. 7.

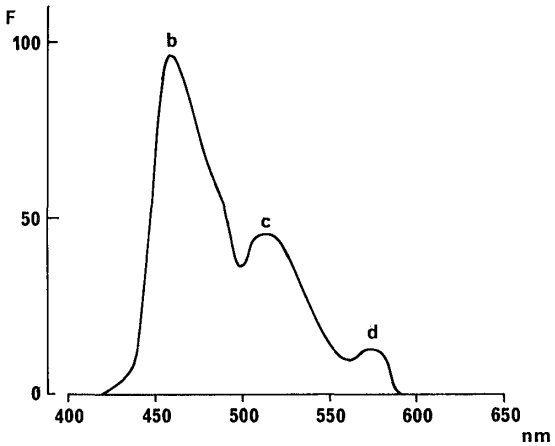

Fig. 8.

FIG. 7. The enhancement of fluorescence is shown along the time course until the 60 min point by continuous irradiation at 290 nm in the presence of 0.1 N HCl and 0.005% OPT. The F550 reaches a maximum at 10 min irradiation and continues steadily thereafter a plateau, whereas F470 declines.

FIG. 8. An emission spectrum obtained with excitation at 360 nm 10 min after bleeding from the fin-edge. There are 3 emission peaks, b, c and d. The maximum emission b is at about 470 nm which resembles the emission of 5-HT.

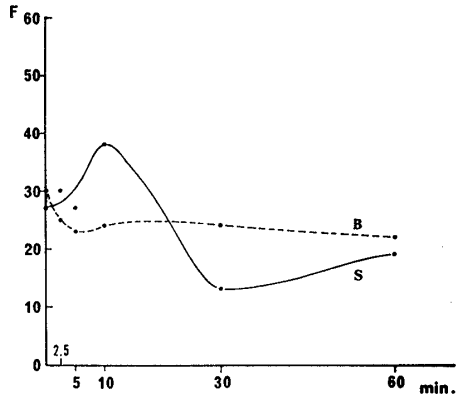

Fig. 9.

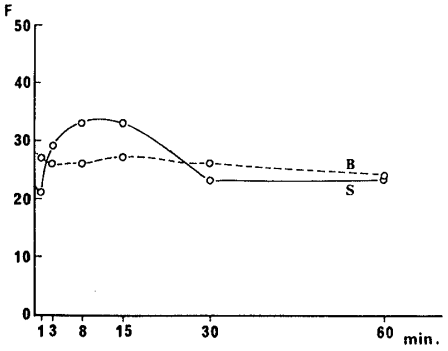

Fig. 10.

FIG. 9. The time course of the occurrence of emission at 470 nm(S) from the fin-edge. The emission excited with 360 nm reaches a maximum 10 min after bleeding, then declines to below the level of background fluorescence(B).

FIG. 10. The emission at 470 nm from the blood clot obtained 1 min after bleeding(S) by heating with continuous irradiation at 700 nm. The emission excited with 360 nm reaches a maximum after 8 min irradiation and decreases to a background level(B).

became higher, and peak *a* disappeared. Considering the emission of 5-HT by excitation at 290 nm in the HCl-OPT method (Fig. 5-A), the significant increase of emission at peak *d* (solid line, Fig. 6-A) may indicate the presence of 5-HT in the blood clot. When the blood clot was irradiated by 290 nm for various periods ranging from 1 min to 60 min, the peak *c* fused with *b* and became higher (Fig. 6-B, C). This fluorescence enhancement is exhibited until the 60 min. point (Fig. 7). F550 reaches a maximum after 10 min. irradiation and continues steadily thereafter, whereas the F470 decreases.

Excitation at 360 nm: An emission spectrum obtained 10 min after cutting the fin is shown in Fig. 8. There are three emission peaks, *b*, *c* and *d*. Peak *b* is quite similar to the emission of 5-HT excited at 360 nm in the presence of OPT (Fig. 4). The emission intensities (F470) from the blood clots were compared at different time intervals ranging from 1 min to 60 min after cutting the fins. The time course of F470 is shown as S in Fig. 9. The F470 reaches a maximum 10 min after cutting the fin, then declines and does not recover again within a 60 min period.

Heating: The specimen was heated continuously with irradiation at 700 nm, and the emission at 470 nm was measured by excitation at 360 nm. As shown in Fig. 10, the emission(S) reaches a maximum after about 8 min irradiation and declines to a background level (B) after a 30 min irradiation.

DISCUSSION

The blood platelet is known to be a storage cell of 5-HT in higher vertebrates (10). According to Tranzer and Miller (13) and Da Prada et al. (3), 5-HT is stored in particular organelles of blood platelets. They have provided strong evidence that 5-HT is bound to the dense osmiophilic bodies (3). This binding is also observed in thrombocytes of fowl by Kuruma et al. (6). The presence of

5-HT in the platelets and polymorphnuclear leucocytes was reported by Humphrey and Jacques (5), and its relation to clotting was studied by Zucker and Bovelli (15). Shore et al. (11) reported that reserpine had little effect on the bleeding period before clotting.

For histochemical demonstration of 5-HT, Petillä (9) used formaldehyde, by which 6-hydroxy-3,4-dehydro-β-carboline was produced. This condensed substance with 5-HT showed an emission at 510–520 nm, excited with 410 nm. Norberg and Hamberger (8) found the emission at 535 nm with a excitation at 405 nm as well as Falck et al. (4), and a microfluorometry of 5-HT was initiated by Caspersson et al. (2). These observations were performed on dried tissues but are not suitable for the present study of blood clotting in water. In our fluorometric study on the wet state of the tail-fin, the specific fluorescence is strongly interfered with in all ranges of the wavelength by the autofluorescence due to formaldehyde. Therefore, it became necessary to use HCl (1) and *o*-phthalaldehyde (7). HCl has previously been used for assay of 5-HT in high concentration (higher than 3 N) (1). Histologically, such a high concentration is not applicable. For histological purposes, modification of the HCl-OPT method (7) for 5-HT as follows provides a suitable method: To enhance the fluorescence of 5-HT, 0.1–1 N is available in combination with 0.005% OPT in distilled water or saline solution. The emission should be measured at 470 nm (F470) by exciting at 360–365 nm within 10 min after HCl-OPT is added. As 5-HT may be released at a high concentration of HCl, the determination should be made quickly as possible. This method seems useful for clarifying the presence of 5-HT in the tissue.

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