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DEMONSTRATION OF MELATONIN IN AMPHIBIA

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The presence of melatonin in the amphibian epiphysis has been ascertained earlier by several indirect methods, demonstrating the synthesizing enzyme or precursors of the compound. This communication describes the presence of melatonin in amphibian brain in a direct way, using dextran gel chromatography as a separation method.

The observation of McCord and Allen (1917) that mammalian pineal extracts possessed a strong pigment aggregating capacity in amphibian melanophores, led to the isolation of the active compound, melatonin (Lerner et al., 1958). Bagnara (1960) suggested that melatonin might also be synthesized by the amphibian epiphysis and might be responsible for the blanching reaction of amphibian larvae in absolute darkness. This hypothesis needs the precise, direct information that melatonin is actually present in amphibian pineal organs. To obtain such localized information proved to be difficult because the amounts present, even in mammalian pineals, is extremely small. This communication describes the direct demonstration of melatonin in amphibians.

A number of indirect methods to demonstrate the presence of melatonin in various organs have been put forward. Charlton (1964) demonstrated an accumulation of injected labelled precursors of melatonin in the epiphysis of larvae of amphibians. Axelrod and Weinsbach (1960) discovered the enzyme that converts N-acetyl-serotonin to melatonin. The demonstration of this methylating enzyme, hydroxyindole-O-methyl transferase (HIOMT), in pineal organs of rats (Wurtman et al., 1963), hens (Axelrod et al., 1964) and amphibians (Axelrod et al., 1965) is considered as proof for the presence of melatonin. Using the same method, Quay (1965) demonstrated the methylating enzyme in the pineal organ and the retina of birds, reptiles, amphibians and fishes.

Other methods, directly demonstrating melatonin, have also been described for mammals. In general, they consist of the preparation of an extract, followed by a separation procedure for melatonin and a suitable test method. By far the most sensitive, though not the most specific assay method for melatonin, is the blanching reaction of larvae of *Xenopus laevis* (Bagnara, 1960). The demand for a specific assay method decreases, however, with an increasing specificity of the separation procedure. Prop and Ariëns Kappers (1961) used a paperchromatographic separation method for the demonstration of melatonin in the pineal organ of the albino rat. The disadvantage of organic solvents used in this method, was overcome by chromatography of an aqueous extract over dextran gel (Van de Veerdonk, 1965). This method was used for the separation of melatonin in the pineal organs of cow, lamb, rat and hen. The present investigation describes the use of the same procedure in the demonstration of melatonin in amphibians.

Three possible sources of amphibian melatonin were investigated: a) the roof of the brain of *Xenopus laevis*' larvae; b) the eyes of larvae of *Xenopus laevis*; and c) the pineal area of adult *Rana temporaria*. With regard to the earlier mentioned hypothesis of Bagnara (1960), the identification of melatonin in the brain of larval *Xenopus* was the most interesting problem.

A number of about 1000 *Xenopus*' larvae, stages 47-49 (Nieuwkoop and Faber, 1956), were killed in cold heptane of -60°C. Regarding the diurnal variations in HIOMT-content, found in birds (Axelrod et al., 1964), the animals were killed at noon in one experiment, and at midnight in another one. The roofs of the brain were removed and freeze-dried. An aqueous extract was prepared and the melatonin content examined, using the procedure already described (Van de Veerdonk, 1965).

A chromatographic column of 40 cm length

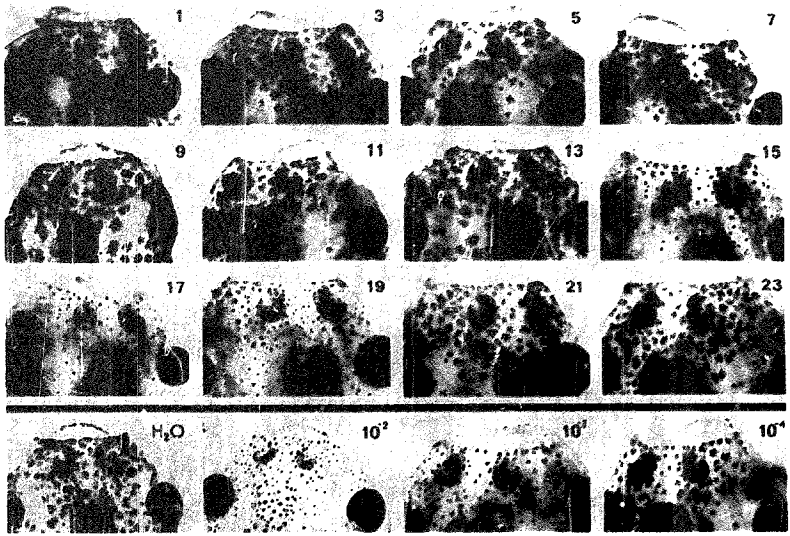

Fig. 1. Upper part: Rostral part of the head of the test larvae used in the bioassay of the various chromatographic fractions (see text). Lower part: Control solutions in the same bioassay: water and melatonin solutions of 10^{-2}, 10^{-3} and 10^{-4} µg/ml.

and a diameter of 18 mm, containing 15 grams of Sephadex (G 25, fine) was prepared in distilled water, and the elution volume of pure melatonin determined fluorimetrically to be between 110 and 140 ml. After washing with distilled water, the same column was used for the separation of the aqueous extract. A number of 24 fractions was collected, the volume of each fraction being 7.5 ml. The fractions were used for the bioassay in the following way: each fraction was diluted with an equal amount of tap water, and three black background adapted *Xenopus* larvae were left swimming in it on an illuminated black background. After 20 minutes these test larvae were fixed in formaldehyde and photographed (only the odd numbers). For the sake of convenience only the rostral parts of the head of these test larvae are brought together in fig. 1. The upper half shows the result of the bioassay: a marked pigment concentration in fractions 15-19 is observed. Since this elution volume is characteristic for melatonin, the result demonstrates the presence of melatonin in the extract.

There was no marked difference in the melatonin content at noon and at midnight. Further investigations on this question are in progress.

The results obtained with eyes of *Xenopus* larvae also show a small melatonin content in the identical elution fractions, although the number of two experiments in this area is too small to draw definite conclusions. The same counts for the few experiments with the excised pineal areas of adult *Rana temporaria*.

Investigations on the exact localization of melatonin in the diencephalic roof of amphibian brain are in progress.

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