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# QUANTITATIVE BIOLOGICAL ASSAY OF MELATONIN

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**Abstract**

A quantitative calibration curve was prepared by measurement of the contraction of the melanophores on the action of commercial melatonin in two test objects, frog and newt. The upper and lower limits of the quantitative reaction were established (20 and 0.5 ng/ml).

The MT-dependent quantitative melanophore reaction was then used to determine the MT content of bovine C. P. extracts.

The data reveal that the method is very suitable for the quantitative determination of MT within the ranges indicated (the linear section of the curve). The method is simple, cheap and rapid.

Melatonin (MT)** is one of the most important hormones of the corpus pineale (C. P.) (AXELROD, 1970a; WOLSTENHOLM et al. 1971). It is known that the histological structure of the C. P. in lower-order vertebrates is similar to that of the retina (OKSCHE et al. 1969; OWMAN et al. 1970; PETIT, 1971). In this group of animals the C. P. is part of the system which (in addition to the hypophysis) controls the accommodation of the animals to light (WOLSTENHOLM et al. 1971).

In higher-oreder vertebrates the C. P. has another function, namely the inhibition and control of the sexual activity during the maturation of the animals. The observation that the skin of frogs (Amphibia) is decolorized by a bovine C. P. extract was reported by LERNER et al. (1959) and they isolated the active substance responsible for this decolorization (KERNER et al. 1960). MT induces the color changes of the skin by aggregating the melanin around the melanophore cell nuclei. The hypophysis melanin-stimulating hormone (MSH) possesses antagonist properties towards MT. MT concentrates melanin not only in Amphibia but also in Pisces (JOSS, 1973;. REED et al. 1969). MT is the most potent mediator causing the contraction of the melanophores in the skin of Amphibia. From such an aspect it is $10^5$ times more active than noradrenaline (AXELROD, 1970b). The secretion of MT follows a diurnal-nocturnal rhythm, which is of importance in the daily rhythm of many physiological functions (AXELROD, 1970b; REITER, 1973). The MT content of the C. P. is very low, and accordingly its quantitative determination demands a sensitive method.

MT can be separated from the other compounds with indole skeleton and measured spectrophotofluoromerically in a very complex method involving repeated purification. Similar limiting factors must also be reckogned with in chromatogra-

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** Abbreviations used: MT = melatonin (5-methoxy-N-acetyl-tryptamine); C. P. = corpus pineale; MSH = melanin-stimulating hormone; TLC = thin-layer chromatography.

phic (paper chromatography and TLC) methods, because of the preliminary purifications (LERNER et al. 1959; LERNER et al. 1960; MCISAACet al. 1964). The MT content of the C. P. can also be followed indirectly by measurement of the hydroxyindole-0-methyl-transferase activity, since this enzyme is responsible for the final step in the synthesis of MT (AXELROD et al. 1961).

## Materials and Methods

Adult animals from the following species were used experimentally to determine the MT activity: Rana ridibunda, Triturus vulgaris, Carassius auratus spec. Japonicus var. bicaudatus.

In animals from each group measurements were first made of the rest size of the melanophores, employing a CYTOPLAST stereomicroscope (PZO, Poland) a Bürker chamber and an ocular micrometer. The shape of the melanophores is extremely similar to that of the multipolar neurocytes in mammals. The areas in which the melanophores were observed were the abdominal skin in the the membrane between the toes in the newt, and the tail fin and abdominal skin in the fish. Chloral hydrate was used to immobilize the test objects.

The MT used for the preparation of the calibration curve was a Calbiochem (USA) product. Ethanolic solutions of various concentrations were prepared. These were administered to the animals by injection, diluted with known amounts of physiological salt solution if necessary. A normal scale is given in the recording of the calibration curve, where the melanophore size is indicated in mm, and the MT concentration in ng/ml (see Fig. 1).

In general 50 melanophores were observed in the visual field. Their average size is given later.

The method described here is a specific biological determination. The decrease in intensity of the colour of the skin can be observed by two methods: (a) photometrically, measuring the transparency of the skin ;(b) the shape of the melanophores and their contraction can be followed directly by microscope, and the change can be measured quantitatively. We selected the latter method for the determination of MT, since we consider it more accurate than the former (MORI et al. 1960; RALPH et al. 1970).

## Results and discussion

The size of the melanophores of the immobilized animals was measured in the rest state.

1. It was found that under normal light conditions the size of the melanophores was largest between 1 and 3 p.m.:

| | |
|---|---|
| *Carassius a.* | 0.1562—0.1704 mm, |
| *Triturus v.* | 0.0852 mm, |
| *Rana* r. | 0.0852 mm. |

2. In strong light the size of the melanophores in the fish did not change, but those in the Amphibia were more dispersed:

| | |
|---|---|
| *Triturus v.* | 0.0994—0.1136 mm |
| *Rana r.* | 0.0994—0.1136 mm |

The melanophore size was measured on maximum contraction after administration of MT. The highest MT concentration used was 30 ng/ml (see calibration curve, Fig. 1). The calibration graph reveals that even 20 ng/ml MT gives rise to a strong contraction, this not being increased further by a concentration of 30 ng/ml.

| | |
|---|---|
| *Carassius a.* | 0.0142 mm, |
| *Triturus v.* | 0.0142 mm, |
| *Rana r.* | 0.0142 mm. |

Following this, a calibration curve was prepared on new experimental objects by measuring the average size of the melanophores at different MT concentrations.

| Melatonin concentration (ng/ml) | Melanophore size (mm) |
|---|---|
| 30.0 | 0.0142 |
| 20.0 | 0.0142 |
| 15.0 | 0.0200 |
| 10.0 | 0.0282 |
| 5.0 | 0.0426 |
| 1.0 | 0.0568 |
| 0.5 | 0.0710 |
| 0.2 | 0.0750 |

The average was calculated from the frog and newt melanophore data in 10 fields of vision. (It did not prove possible to obtain a linear correlation between the MT concentration and the reaction of the pigment cells in the case of the fish species examined.)

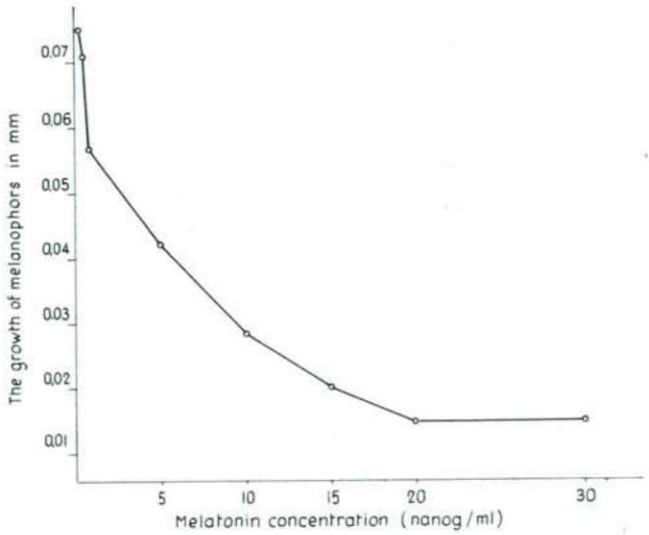


Fig 1

It can be seen from the curve that size of the melanophores does not change in the MT concentration range 20—30 ng/ml. In the range 0.5—20.0 ng/ml the curves were linear. No change in size could be demonstrated at MT concentrations lower than 0.2 ng/ml. From this the conclusion can be drawn that under normal conditions in the frog and newt 0.2—0.5 ng/ml MT maintains equilibrium with the given amount of MSH.

Subsequently, extracts were prepared from 20 bovine C. P. The extraction was performed with petroleum ether at room temperature from the homogenizate

prepared with physiological salt. The petroleum ether phase was separated and discarded (lipid extracts). The homogenizate was then extracted twice with its own volume of ethyl acetate. The ethyl acetate extracts were separated and evaporated to dryness under $N_2$ at 40 °C. The aqueous phase was reextracted in the same manner with ethyl acetate and the ethyl acetate extracts were combined and evaporated to dryness as before. The collected residue was dissolved in 1 ml dry ethanol and the quantitative determination carried out by the above detailed method.

The dry residue of the ethyl acetate extract of the lipid-free C. P. homogenizate gave rise to a melanophore response corresponding to an MT concentration of on average about 10 ng/ml per corpus pineale.

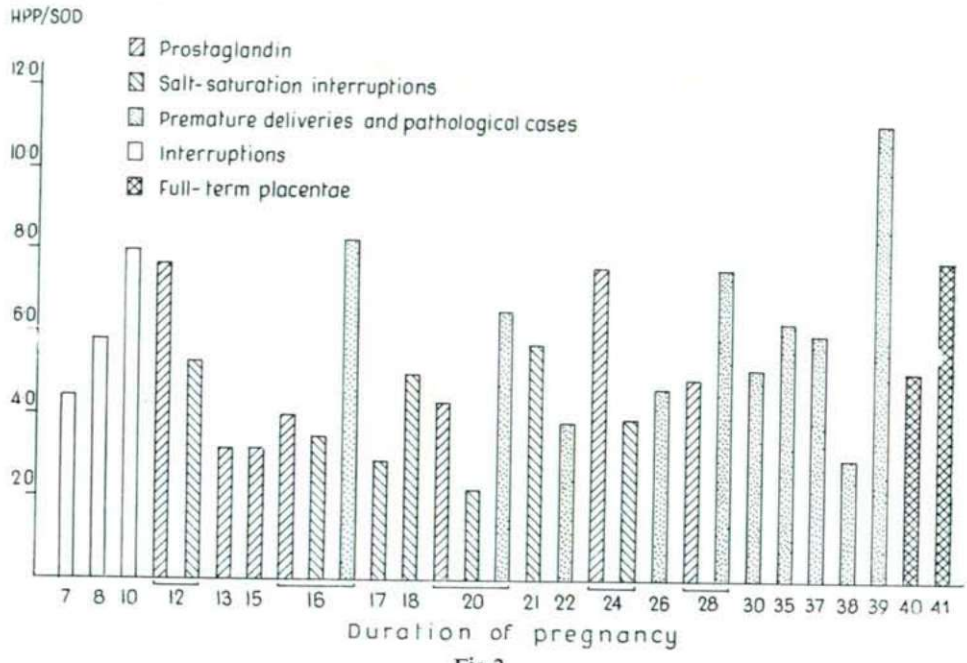


Fig 2

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