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A Chemical and Pharmacological Study on the Role of Catecholamines in the Dispersion Reaction of *Xenopus laevis*

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Chemical analyses have been made of dopamine in the skin of black background-adapted *Xenopus laevis* treated with α-methyl-*p*-tyrosine (α-MPT), an inhibitor of tyrosine hydroxylase, and without such treatment.

Based on the assumption that dopamine is involved in the dispersion reaction induced by MSH, it was expected that the amount of dopamine in treated toads was less than in untreated ones. For several reasons, which will be discussed, the expectation was not realized.

When unphysiological high doses of α-MSH were administered several times during the experiment with α-MPT, the percentage of dopamine in these animals was far less than in control animals without any treatment. In this way a direct relation proved to be present between the dispersion reaction induced by MSH and catecholamines involved in this process.

In another way, by blocking the degradation rather than the synthesis of catecholamines, a similar relation was demonstrated. Inhibition of the enzyme catechol-*O*-methyl transferase in MSH-treated animals resulted in an extension of the dispersion period compared with toads receiving MSH only.

Amphibians, like many other lower vertebrates, are able to change the color of their skin. Generally, the adaptation to a white and black background is studied. This kind of adaptation is caused by migration of a brown pigment, melanin, which is found in cells called melanophores. Dispersion of the pigment in the melanophores is initiated by a melanophore-stimulating hormone (MSH), which is released from the intermediate lobe of the pituitary gland. Apart from MSH, several other substances with a dispersing activity are known.

Dispersion of the melanophores *in vitro* and *in vivo* with catecholamines has been described by Burgers *et al.* (1953) for *Xenopus laevis*. Davey (1960) suggested that the MSH-induced dispersion in amphibians is mediated by biogenic amines. On the basis of their findings, it was assumed that in *Xenopus laevis* catecholamines may be involved in the dispersion reaction. A first indication was found after histochemical identification of a catecholamine in the skin, released upon black background adaptation (Brouwer and van de Veerdonk, 1969). In addition, dopamine was determined in skin extracts of *Xenopus laevis*.

Blockade of the dopamine synthesis in black background-adapted animals resulted in a decreasing dispersion (Brouwer, 1970). It was assumed that aggregation of the melanophores after treatment with α-methyl-*p*-tyrosine (α-MPT), a blocking agent of tyrosine hydroxylase (Spector *et al.*, 1965), was caused by a deficiency of dopamine in the skin reservoirs which are depleted by the MSH-induced dispersion of the melanophores.

An extension of this assumption, presented in this paper, is based on chemical analyses of the percentages of dopamine in black background-adapted animals

treated with and without α-MPT. In addition to studying the effect of blocking the synthesis of catecholamines, the inhibition of the degradation of catecholamines has also been investigated. During black background adaption, dopamine, stored in skin reservoirs, will be released for the dispersion of the melanophores. During the extracellular transport, this catecholamine is inactivated. Several authors (among others Bloom and Giarman, 1968) have mentioned, largely with respect to the peripheral sympathetic nervous system, that catecholamines are metabolized intraneuronally by monoamine oxidase (MAO) and extraneuronally by catecholamine-*O*-methyltransferase (COMT). For this reason, pyrogallol, an inhibitor of COMT (Crout, 1961), was used to determine the influence upon the dispersion reaction.

MATERIALS AND METHODS

Adult *Xenopus laevis* of 20–40 g weight have been used. The toads were adapted to a black background by placing them in illuminated black-painted jars. The animals were selected as to the rate of aggregation and dispersion of the melanophores. The melanophore index (MI) was recorded according to Hogben and Slome (1931).

Pharmacological Experiments

The drugs were injected into the dorsal lymph spaces. α-MPT was dissolved as described by Spector *et al.* (1965) and injected in doses of 80 mg/kg in a volume of about 0.3 ml every 3 hr during the experiment. Control animals received identical volumes without the drug.

Pyrogallol was used as blocking agent of the enzyme COMT. It was dissolved in amphibian Ringer, according to Burgers and van Oordt (1956), in doses of 200 mg/kg in 0.5 ml of solution.

α-MSH was generously supplied by Dr. W. Rittel of CIBA, Ltd., Basle, Switzerland. α-MPT was obtained from Sigma Chem. Co., St. Louis, Missouri. Pyrogallol was obtained from the Chem. Pharm. Groothandel, Dr. Lamers, and Dr. Indemans, 's-Hertogenbosch, the Netherlands.

Dopamine Measurements in Skin Extracts

Preparation of tissue. The toads were killed by plunging them in liquid nitrogen. Pieces of back skin were cut off in the frozen state and stored at approximately −60°C, if further treatment of the material was not immediately feasible.

Subsequently the pieces of skin were pulverized in a mortar in liquid nitrogen. The powder was lyophilized to determine the dry weight of the skin, which was then treated for extraction of dopamine.

Extraction. The dried powder of skin was homogenized in a centrifuge tube, in 3 ml of 0.01 *N* HCl, according to Welch and Welch (1969). The extraction of the homogenate was achieved in an ultrasonic vibrating apparatus, in ice-cold water during 30 min. The extract was centrifuged at 2500*g* for 20 min, and the supernatant was decanted in a test tube. The pH of the extract was adjusted to about 4 with 0.1 *N* NaOH. The dopamine content in the extract was estimated, using the fluorimetric method of Bertler (1958). A modification of the method, described in the same paper, has been applied using a larger column. Contrary to Bertler (1958), a column was used of 60 mm length and 5 mm ϕ, and the effluent was collected in 4.0-ml portions instead of 2.5 ml.

Fluorimetric analysis. Dopamine in the eluates was determined by the method described by Udenfriend (1962). A slight variation has been applied to obtain more reproducible results. To the test tubes was added 0.5 ml of 0.1 *N* phosphate buffer (pH 6.5) and water to bring the total volume to 3.8 ml, and then 0.05 ml of 0.02 *N* iodine solution. After 5 min, 0.5 ml of alkaline sulfite solution was added, and 5 min later, 0.6 ml 5 *N* acetic acid. According to Welch and Welch (1969), 1:1 glacial acid:concentrated HCl reagent was added to the test tubes to adjust the final pH to 3.8–4.2. When tissue extracts were analyzed, one half of the sample was used to determine the quantity of dopamine fluorescence and the other half as a "tissue blank." The reagents were added in the reverse sequence to the test tubes of a "tissue blank."

The fluorescence measured in the "tissue blank" was subtracted from that obtained in the normally treated sample. Fluorescence was measured in a spectrophotofluorometer (ZFM 4C, Zeiss, Germany). Activation and fluorescence peaks were found at 335 nm and 383 nm respectively.

To establish a functional role of dopamine in the dispersion reaction it appeared to be unnecessary to determine exact quantities; comparison of the amount in treated and untreated animals proved to be sufficient for our purpose. For this reason, the measured fluorescence is given in arbitrary units.

Special care was taken to carry out the extraction, the fluorimetric determination of dopamine and the reading of the spectrofluorometer under standardized conditions.

RESULTS

Dopamine Analysis after α-MPT Treatment

In each experiment six animals were injected with α-MPT (80 mg/kg) every 3 hr during 39 hr, whereas four animals in the same way received the solvent only. The toads were kept on an illuminated black background. Each experiment was repeated once. At the end of the experiment the average value of the MI was 2.0–2.5 for the test animals and 4.8–5 for the controls.

Skin (70–100 mg dry weight) was used to determine the amount of fluorescence caused by dopamine in extracts of treated and control animals. The amount of fluorescence was calculated on the basis of 100 mg of skin (dry weight). The fluorescence, estimated in the samples of the test and control animals, showed a considerable variation with the calculated values overlapping each other.

These results suggest the possibility that only a minor part of the dopamine, accumulated in a reservoir in the skin, is used in the dispersion reaction. This might explain why a decrease in the percentage of dopamine in test animals compared to that in control animals has not been found. In addition, the great variability in the quantities of dopamine in different animals must obscure any eventual differences. Taking the view that dopamine is involved in the dispersion reaction induced by MSH, it may be expected that a further release of dopamine will be obtained after administering MSH during treatment with α-MPT in black background-adapted animals. A greater difference in the amount of dopamine in test- and control animals may then probably be the result.

Dopamine Analysis after Treatment with α-MSH and α-MPT

The test animals (six per experiment) were adapted to a black background, receiving every 3 hr, during 42 hr, 80 mg/kg α-MPT. When the MI of these animals dropped below 3.0, they were injected with 5 μg of α-MSH per animal. In control animals (four per experiment) only the solvent

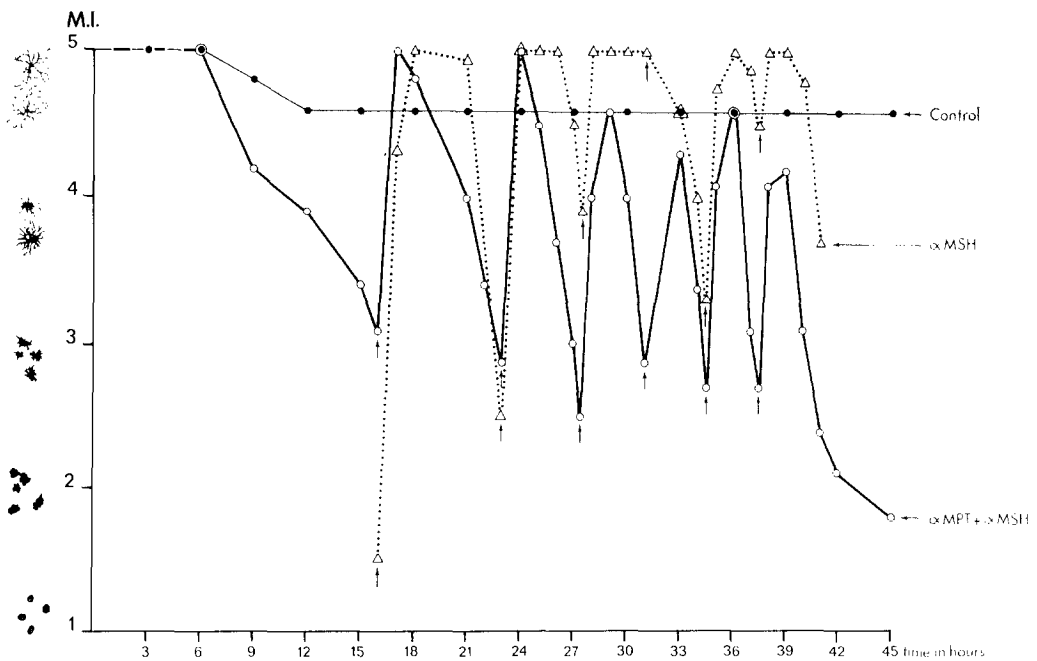

FIG. 1. Effect of repeated injections of α-MSH (5 μg/animal) on black background-adapted toads treated with α-MPT (80 mg/kg) every 3 hr, during 42 hr (○——○), compared with a control treatment without drug (●——●). (△- - - △) White background-adapted toads receiving 5 μg α-MSH at the same time as the test animals. ↑ indicates the time of injection of α-MSH.

was administered every 3 hr, during 42 hours. As control for the MSH-induced dispersion, two animals were placed on a white background, and also received 5 μg MSH per animal at the same time as the test animals. The toads used in this experiment were of similar weight. The experiment has been repeated once.

As shown in Fig. 1, an identical dose of α-MSH in α-MPT treated animals, after being administered repeatedly, has a diminishing effect upon the dispersion reaction compared with the control animals injected with α-MSH only. This applies to the degree of darkening as well as to the duration.

Under these experimental conditions a marked decrease in the amount of dopamine is observed in the α-MPT + α-MSH-treated animals (B) compared with animals receiving α-MPT only (A) (Fig. 2).

MSH-induced Dispersion and Pyrogallol

For a study of the effect of pyrogallol, a COMT inhibitor, on the MSH induced darkening of the skin, the same animals were used as test and control animals. Five toads were injected with 0.26 μg of α-MSH per animal. The MI was recorded during 330 min (Fig. 3). The next day they received 0.26 μg MSH in combination with pyrogallol (200 mg/kg). Blockade of the catabolism of catecholamines appears to have a prolonging effect upon the dispersion induced by MSH (Fig. 3). This experiment has been repeated once.

DISCUSSION

Apart from MSH, which is generally supposed to be the primary substance acting extracellularly on the melanophores in the physiological dispersion reaction, Davey (1960) presumed a possible role of biogenic amines in amphibians. He postulated an indolalkylamine stored in the skin of *Rana pipiens* causing a dispersion of the melanophores induced by MSH.

Previous results confirmed the involvement of biogenic amines in the darkening reaction and suggested catecholamines to be the mediating substance (Brouwer and van de Veerdonk, 1969; van de Veerdonk and Konijn, 1970; Brouwer 1970). In order

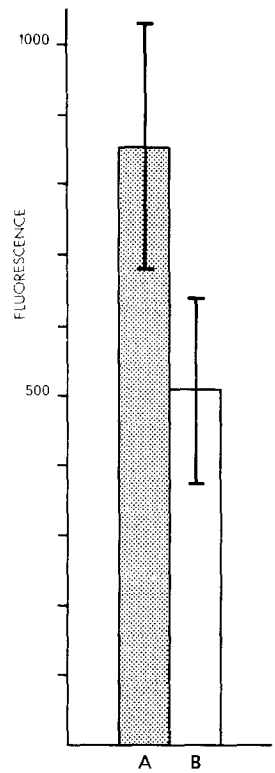

FIG. 2. The effect of repeated injections of α-MSH (5 μg/animal) and α-MPT (80 mg/kg) on the amount of dopamine in skin extracts (B), compared with control animals receiving the solvent without drug (A). Fluorescence is indicated in arbitrary units. The deviation expressed in this figure is based on the individual variation of fluorescence found in the samples.

to confirm these results, the dopamine level of skin was determined in black background-adapted animals in which the synthesis of this compound was blocked. Unfortunately, it proved to be impossible to detect any significant differences between the treated and untreated toads. As already briefly stated (see Results section) several reasons may be presented for this negative result.

First of all, it is not unlikely that only a small quantity of dopamine is used for the dispersion of the melanophores. A similar situation has been described concerning adrenergic neurotransmission in nerve terminals, where a major fraction of monoamines appears to be present in reserve pools without immediate functional importance (Andén *et al.*, 1969).

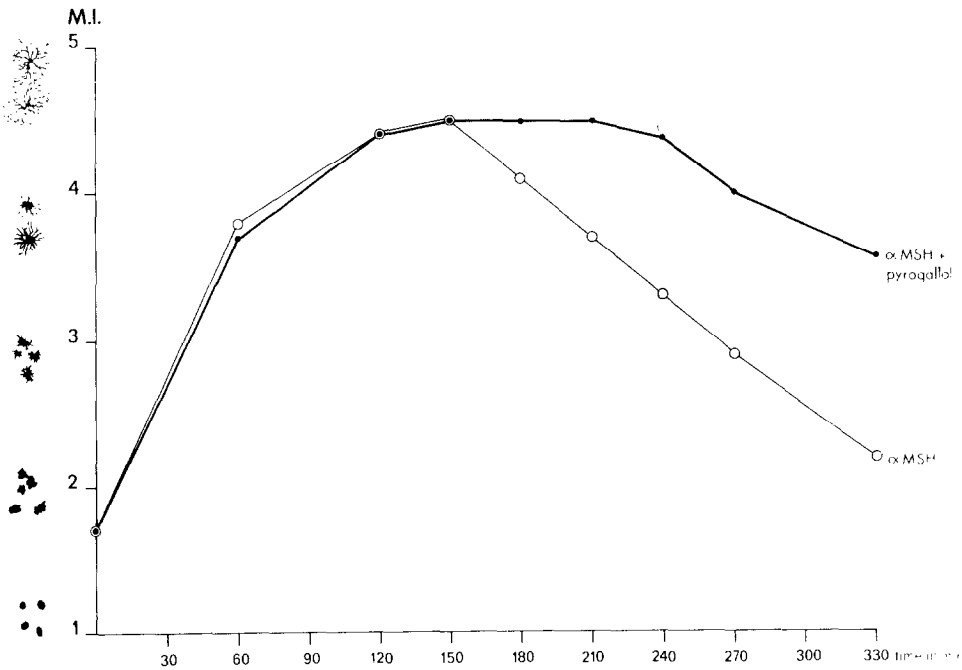

FIG. 3. The effect of pyrogallol (200 mg/kg) on the MSH-induced dispersion in white background-adapted animals (●——●) compared with the effect of an identical dose of α-MSH (0.26 μg/animal) administered the previous day to the same animals (○— ·○).

Second, it was found that the amount of dopamine in skin extracts of individual toads shows considerable differences. This is a disadvantage in comparing percentages of dopamine in treated and untreated animals, particularly when only small quantities of dopamine are involved in the dispersion reaction. For this reason, it was decided to deplete the dopamine reservoirs in the skin with successive nonphysiological high doses of α-MSH, on the assumption that MSH is responsible for the release of dopamine from the skin. Under these experimental conditions a significant decrease in the amount of dopamine was found compared with that in animals receiving the solvent only.

Based on this result, it may be concluded that MSH is involved in the release of dopamine from the reservoir in the skin. Together with a reduction of the amount of dopamine in black background-adapted animals treated with α-MPT and α-MSH, a decrease of the maximum MI values and a shortening of the time period for the dispersion reaction have been observed. This points in a direct way to a cooperation of MSH and catecholamines in the dispersion of the melanophores.

Contrary to inhibition of the synthesis, blockade of the degradation of catecholamines by COMT leads to a prolongation of the MSH induced dispersion. This fits well in the proposed cooperating activity of MSH and catecholamines in the dispersion reaction. Blockade of MAO, the other enzyme involved in the degradation of catecholamines, has not yet been attempted. It is well known from investigations about the peripheral sympathetic nervous system that MAO activity is found intraneuronally and COMT activity extraneuronally. On the other hand, it was not possible to identify any MAO activity in skin sections of *Xenopus laevis* after enzyme histochemical investigations (unpublished data). Consequently, the enzyme COMT was supposed to be involved in the degradation of catecholamines in the skin of *Xenopus laevis*.

Several questions concerning the involvement of catecholamines in the darkening

reaction of *Xenopus laevis* remain unanswered. One of them is the question which catecholamine is actually cooperating in the dispersion reaction with MSH. It is possible that after release of dopamine this substance is converted into norepinephrine or epinephrine before a dispersion of the melanophores is achieved. Van de Veerdonk and Konijn (1970) showed an increase in cAMP in skin extracts of *Xenopus laevis* after incubation with norepinephrine and epinephrine. Recently, it has been observed *in vitro* that norepinephrine has a dispersing activity stronger than dopamine or epinephrine and comparable with MSH (van de Veerdonk and Brouwer, to be published). It may well be that norepinephrine is the substance which is ultimately involved in the skin darkening induced by MSH.

Another question is whether other amphibian species have a dispersion mechanism similar to that described for *Xenopus laevis*. Only *Scaphiopus couchi* behaves like *Xenopus laevis* in dispersing the melanophores with catecholamines (Goldman and Hadley, 1969). In other species catecholamines have an activity contrary to that of MSH. In such animals another dispersion mechanism is likely to be present. The role of catecholamines in the dispersion reaction in different species of amphibians needs further investigation.

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