ACTION OF MAGNESIUM SULPHATE ON MELANOPHORES OF RANA TIGRINA*

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Many a worker has been fascinated by the complex system of camouflage that exists in nature and one is aware that such a system exists in different species of frogs. Light, darkness, temperature and humidity have been shown to be the most important stimuli for colour change in the Hyla cinerea and Hyla arborea (species of tree frogs). However, in constant light they do not show any alteration of the green colour (4).

There are a number of reports describing the effect of substances like mersalyl, caffeine, acetylcholine, adrenaline and also of ions like Na^{++} and Ca^{++} (9,10) both on melanocytes in vivo and on isolated frog's skin. In these studies the colour changes were studied in Rana pipiens. During our routine under-graduate experiments demonstrating the CNS depressant effect of $MgSO_4$ and its reversal by Ca^{++}, it was observed that the frogs (Rana tigrina) changed their colour from brownish black to green and sometimes to a light brown colour. Thus, the subject of this report is the mechanism of action of hypertonic $MgSO_4$ on the melanophores of Rana tigrina.

MATERIALS AND METHODS

Frogs of either sex weighing between 150 *gm* and 250 *gm* were used for the study, which was carried out under the usual laboratory conditions and light. All drugs were injected in the dorsal lymph sac. The colour changes were observed with the naked eye and the web melanophores of the hindlimbs were viewed under the microscope (magnification x100). Each frog was enclosed in a small chamber provided with adequate ventilation. One of the hindlimbs was brought out through a hole in the side of the chamber. The web was splayed out over a circular hole in a small wooden board and the toes were tied in position. The board was then placed on a microscope stage and the web was viewed under the low power lens. The distribution of melanin in the cells was recorded in five grades according to the method of Hogben and Slome (5). All counts were done by a technician who had no knowledge of the nature of drugs that were injected. The study was carried out in four groups which consisted of 33, 25, 20, and 20 frogs respectively. A control melanophore count was done in all the frogs before injecting a drug. Calcium gluconate was injected in all the frogs to counteract the CNS depression produced by $MgSO_4$.

Group I :

20% $MgSO_4$ (1 *ml*/100 *gm* weight) was injected and the frogs were observed. 15-20 minutes later it was seen that there was a marked depression of the CNS which was manifested in a loss of the righting reflex. At this time there was an obvious change in the colour of the

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frogs which turned lighter and some even attained a green hue. The melanophore count was then done as described above. Subsequently, 10% calcium gluconate (2 *ml*/100 *gm*) was injected and the observations continued. Then caffeine (1 *mgm*/100 *gm*) was injected and the web count repeated.

Group II :

In this group after a control melanophore count was done, caffeine in the aforementioned dose was injected first and the count was repeated half an hour later. Then $MgSO_4$ was injected and the same procedure as in group I was repeated (caffeine was not injected again after $MgSO_4$).

Group III :

These frogs were given dibenzyline (25 *mg*/*kg*) half an hour before the injection of $MgSO_4$.

Group IV :

The frogs in the last group were treated with reserpine (5 *mg*/*kg*). The melanophore count was done 24 hours later when $MgSO_4$ was injected and the above procedure repeated. The same was once again repeated after 48 hours.

RESULTS

Group I :

When the CNS depressant effect of $MgSO_4$ was seen (depressed respiration with a loss of righting reflex), there was a visible lightening of the frogs. The melanophore count showed that most of the cells belonged to grades 1 and 2 *i.e.* they looked contracted (Fig. II). The mean percentages are given in Table I. It was then found that 10% calcium gluconate reversed the respiratory and CNS depression but had no effect on the colour as seen by naked eye as well as from the melanophore count. Caffeine given at this stage caused a reversion of the colour to the original hue. The results are recorded in Table I. In two frogs, caffeine produced excessive stimulation leading to death.

TABLE I

Effect of 20% $MgSO_4$ and its Reversal by Caffeine
Mean ± S.D.

	GRADES				
	1	2	3	4	5
1. CONTROL MELANOPHORE COUNT (33)	1 ± 0.55	2.39 ± 2	9.33 ± 3.21	12.18 ± 2.67	75.69 ± 1.51
2. AFTER 20% $MgSO_4$ 1 *ml*/100 *gm*	*29.36 ± 4.0	*23.84 ± 1.88	*25.57 ± 3.60	*14.81 ± 1.76	*5.45 ± 2.53
3. AFTER 10% CALCIUM GLUCONATE 2 *ml*/100 *gm*	27.1 ± 2.23	25.3 ± 1	20.34 ± 3.89	18.0 ± 1.65	8.00 ± 1.89
4. AFTER CAFFEINE 1 *mgm*/100 *gm*	*1.81 ± 2.62	*5.63 ± 3.8	"14.12 ± 2.22	17.45 ± 4.21	*60.42 ± 3.63

*P > 0.001 "P < 0.05

The Figure in Parentheses Indicates the Number of Animals

Group II :

Here it was found that caffeine-induced darkening was also reversed by $MgSO_4$ (Table II). The frogs appeared to be more active after caffeine and the usual CNS depression was also seen after $MgSO_4$. Calcium gluconate, however, was not able to overcome this depression in three of the frogs.

TABLE II

Reversal of Caffeine Darkening by 20% $MgSO_4$

MEAN ± S.D.

	GRADES				
	1	2	3	4	5
1. CONTROL MELANOPHORE COUNT (25)	2.21 ± 1.6	2 ± 2	10.8 ± 2.8	14.11 ± 1.92	72 ± 3.0
2. AFTER CAFFEINE 1 *ml*/100 *gm*	--	--	"1 ± 0.67	"5.5 ± 1.86	*94 ± 3.2
3. AFTER 20% $MgSO_4$ 1 *ml*/100 *gm*	*20.22 ± 3.81	*36.18 ± 2.10	"10.0 ± 4.8	*22.1 ± 3.61	*10 ± 3.02

*P < 0.001 "P < 0.05

The Figure in Parentheess Indicates the Number of Animals

Group III :

These frogs did not show any colour change half an hour after dibenzyline. With $MgSO_4$ the R.R. was lost as usual but there was no colour change, the melanophore count being more or less the same (Table III).

TABLE III

Effect of Dibenzyline on $MgSO_4$ Action

MEAN ± S.D.

	GRADES				
	1	2	3	4	5
1. CONTROL MELANOPHORE COUNT (20)	1 ± 2.4	1 ± 2	4.3 ± 2.28	16.89 ± 4.9	75.2 ± 2.18
2. AFTER DIBENZYLINE 25 *mg/kg*	1.2 ± 3.6	3.8 ± 1.89	2.6 ± 3.06	18.33 ± 4.6	76 ± 3
3. AFTER 20% $MgSO_4$ 1 *ml*/100 *gm*	3.81 ± 4.13	5.88 ± 2.89	"10 ± 1.0	12.1 ± 2.62	68 ± 2.62

*P < 0.001 "P < 0.05

The Figure in Parentheses Indicates the Number of Animals

Group IV :

It was found that reserpine pretreatment for 24 hours had no effect on the melanophore count as seen from Table IV; but at the same time $MgSO_4$ did produce a significant colour change. However, when the same frogs were again given $MgSO_4$ after 24 hours (*i.e.* 48 hours after reserpine), although the CNS depression was seen, there was no appreciable change in the colour (Table IV).

TABLE IV

Effect of Reserpinization

MEAN ± S.D.

	GRADES				
	1	2	3	4	5
1. CONTROL MELANOPHORE COUNT (20)	—	1.08 ± 2	6.64 ± 3.91	11.19 ± 4.22	70.81 ± 1.86
2. 24 HRS AFTER RESERPINE 5 *mg/kg*	—	—	10.6 ± 3.82	2.29 ± 3	87.21 ± 4.92
3. AFTER 20% $MgSO_4$	3.1 ± 2.83	*27.83 ± 3.11	10.28 ± 1.11	*38.1 ± 1.81	*22.2 ± 3.67
4. 48 HRS AFTER RESERPINE (17)	2.22 ± 1.67	12.1 ± 3.9	6.83 ± 1	10.33 ± 2.28	70.20 ± 1.90
5. AFTER 20% $MgSO_4$	1.12 ± 0.68	8.83 ± 2.21	15.22 ± 2.48	16.83 ± 3.30	60.23 ± 1.86

*P <0.001 "P <0.05

The Figures in Parentheses Indicate the Number of Animals

DISCUSSION

Although various pigments may occur in the tissues of some amphibia, most of their colours are produced by different arrangements of three kinds of pigment cells—the melanophores (dermal and epidermal), the lipophores and the guanophores. Black or brown colour is due to a predominance of melanophores in a fully expanded state, yellow or red is due to the lipophores while white is due to the guanophores. Very often one sees shades of blue or green which are produced by various combinations of these cells. The lipophores lie directly under the epidermis while the melanophores are most superficial. The guanophores lie in between.

When the colour lightens, the cell processes are not contracted, but the pigment is withdrawn into the body of the cell. When the melanin is fully dispersed in the processes, the skin in that region appears black (8).

Most amphibia can darken or lighten their body colour but the tree frog is unique in its ability to undergo rapid colour changes (18). Rana tigrina apparently does not change its colour so rapidly when conditions of light and temperature are kept constant. However, the

mechanisms controlling the expansion and contraction of the pigment cells may vary from species to species.

Besides the specific hormones MSH and ACTH which elicit melanophore dispersion, a number of lightening and darkening agents have been studied by various workers. Conventionally, caffeine is used as a darkening agent (10), other such substances being marsalyl, theophylline (9), sodium iodoacetate, $HgCl_2$ (2), progesterone, marsilid and mesantoin. Potent aggregating agents (*i.e.* those producing darkening) are melatonin, NA, A, acetylcholine, hydrocortisone, tri-iodo thyronine, serotonin (7) and histamine liberators (1). Bivalent ions like Na^{++} and Ca^{++} have also been shown to have a dispersing action. Dickstein *et al.* have mentioned that Mg^{++}, another bivalent ion, behaved like Ca^{++} in supporting the action of MSH but not in a concentration upto isotonicity (3).

It has been stated (8), that an insufficient supply of oxygen brings about a contraction of the melanophores and hence respiratory disturbances would have some effect on the colouration. At first we thought that this could explain the action of $MgSO_4$ on the melanophores but it is not likely since the colour did not revert to normal even after the respiratory depression was counteracted by calcium gluconate. Moreover, in preliminary studies it was found that $MgSO_4$ was effective in decapitated frogs as well as on injection into the subcutaneous lymph sacs of isolated limbs of frogs, thus pointing to a direct action on the skin melanophores.

From the present work it is seen that hypertonic $MgSO_4$ produces an aggregation of melanin within the melanocyte thereby producing a lightening, Fig. I and Fig. II. In preliminary studies it was found that both hypotonic and isotonic $MgSO_4$ did not produce any colour change in Rana tigrina. These findings are quite contrary to those of Dickstein (3) and could possibly be a species variation.

Fig. 1 (*Control*)

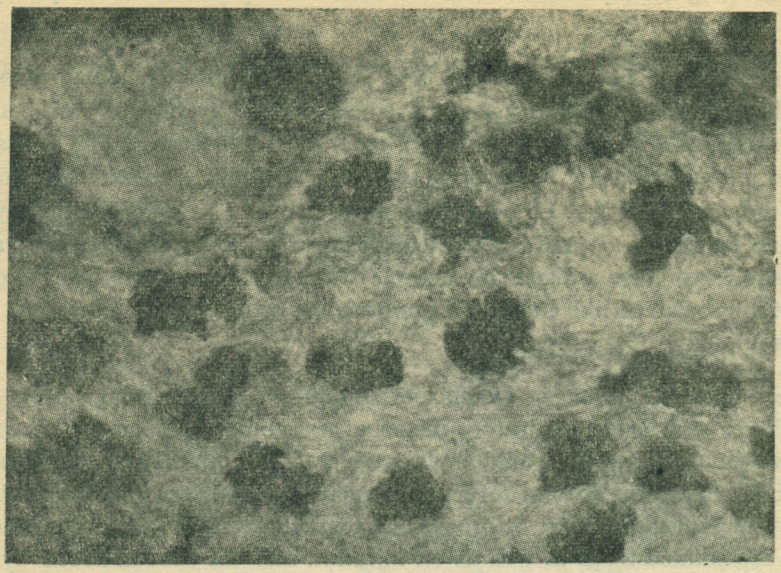

Fig. 2 (*After* $MgSO_4$)

It was subsequently thought that a release of endogenous adrenaline may be implicated in the response of the melanophores to $MgSO_4$ (Bhide, N.K.—personal communication). With this in view, the third group of frogs was treated with dibenzyline which has been shown by Bhide (1) to block the effect of adrenaline and noradrenaline on frog melanophores. After dibenzyline pretreatment, $MgSO_4$ had no effect on the melanophores (Table III), but the CNS depressant effect was well manifested. Further, reserpine pretreatment did not alter the response after 24 hours whereas 48 hours later the effect was blocked. This shows that there was incomplete catecholamine depletion after 24 hours whereas it was almost complete after 24 hours, so that $MgSO_4$ was ineffective then. Thus it seems quite evident that $MgSO_4$ action on the melanophores must be through a release of catecholamines from stores. One possibility may be that Mg^{++} may be displacing cellular Ca^{++} thereby producing a lightening. This can be based on the theory postulated by Dickstein *et al.*(3), who state that the effect of Ca^{++} on melanophores of Hyla arborea (a passive state of melanophore dispersion) is induced by entry of Ca^{++} into the cell and exit of K^{+}. They also postulate that the active stage of melanophore contraction needs an energy pump for removal of Ca^{++} from cells or forcing potassium in. This in fact would lend further support to our thesis. The released adrenaline would cause an accumulation of cyclic 3′ 5′ AMP which could provide the necessary energy for forcing Ca^{++} out of the cell. However, as no studies are available regarding the levels of cyclic AMP of frog melanocytes one cannot rest on this assumption. Moreover, in Rana pipiens the situation is just the reverse—dispersion of melanin granules (darkening) requires energy and a correlation exists between extent of melanocyte dispersion and increase in cyclic AMP in the skin in vitro (6). It would thus be interesting to see if the formation of cyclic AMP is related to any of these processes.

SUMMARY

1. Magnesium sulphate (20%) produces a colour change (lightening) in Rana tigrina.

2. This lightening is reversed by caffeine but not by Ca^{++}.

3. Caffeine induced darkening is also reversed by $MgSO_4$.

4. Dibenzyline pretreatment blocked the effect of $MgSO_4$ on the dermal melanophores.

5. Reserpine pretreatment did not have any effect after 24 hours but 48 hours later it did show blockade of $MgSO_4$ action. Thus it was concluded that $MgSO_4$ acts on the melanophores through a release of catecholamines from stores. Some other possibilities have also been considered.

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