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EFFECT OF SIZE OF DOSE AND PROLONGED ADMINISTRATION OF ALPHA MSH ON THE MELANOPHORES OF THE FROG*

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In amphibian skin, melanin pigmentation is due to the presence of melanophores in the epidermis and dermis. Many factors including hormones, skin moisture and temperature, are known to influence the movement of melanin granules within these pigment cells. The researches of Allen (1), Smith (2) and Hogben (3) showed that pituitary secretions caused a dispersion of melanin granules in the dermal melanophores. Frieden and Bozer (4), Karkun and Mukerji (5) subsequently showed that the prolonged administration of a pituitary extract resulted in an increase in the total amount of melanin in amphibian skin. The object of the present work was to study the effects of different doses of alpha MSH on the movement of melanin granules in the epidermal and dermal melanophores of the frog's web, in order to determine whether these two cell types possessed the same dose responses. In addition the effect of prolonged administration of large doses of the hormone on the pigment cells was observed so as to ascertain whether the degree of sensitivity of the target cells for a given dose of hormone remained constant. A careful morphological study of the pigment cells following the withdrawal of the hormone was also planned.

MATERIALS AND METHODS

Adult male *Rana pipiens* were used in all experiments. Each frog was placed in a plastic chamber provided with adequate ventilation and partially filled with water. The background was grey in color. The lighting and temperature of the room were kept as constant as possible throughout the experiments. The animals were force fed once a day with calves liver. Before each experiment the frogs were left in their container for a week without hormone treatment so that they would become acclimatized to their environment. The pigment cells in the skin of the webs were examined at 9:00 a.m. and 4:00 p.m. daily by placing the web moistened with water on a slide on the stage of a microscope. Transillumination of the web readily revealed the pigment cells in the epidermis and dermis. The degree of dispersion or aggregation of the melanin granules was assessed and a melanophore index shown in Text Figure 1 was used to record the findings.

Experiment I. Fourteen groups of four animals each received a single injection of natural porcine alpha-melanocyte stimulating hormone* (MSH) in 0.5 ml of Ringer solution into the dorsal lymph sac. The first group received 2.5 units of MSH and the remainder received 5, 10, 20, 40, 50, 100, 500, 5,000 50,000 250,000, 500,000, 5 million, and 50 million units of MSH respectively. The melanophore index for the pigment cells was recorded every 15 minutes for three hours. In the groups which received the very high doses of hormone less frequent observations were made over a longer period of time once the skin became black in color.

Experiment II. Three groups of four animals were used. Each frog received 5×10^7 units of alpha MSH in 0.5 ml of Ringer solution daily into the dorsal lymph sac. The melanophore indices for the web were recorded twice daily. Group I received the hormone for 1 week, Group II for 5 weeks and Group III for 12 weeks. On stopping the hormone the melanophore indices were read twice daily until they returned to a constant level after which they were read daily for a further week.

It was noted that the frogs which were receiving hormone in Group III started to return to their normal light color after 7½ weeks of treatment. The dose of alpha MSH was raised to 100 million units per day. Two weeks later the tendency to lighten recurred and the hormone was given as two doses of 50 million units per day. Three days later in order to keep the frogs black in color it was necessary to raise the dose to 100 million units twice daily. One week later it was necessary to raise the dose to 200 million units twice daily.

RESULTS

The morphology of the epidermal and dermal melanophores of frog skin has already been described (6–8). Examples of each type are shown in Figures 1 and 2. The epidermal pig-

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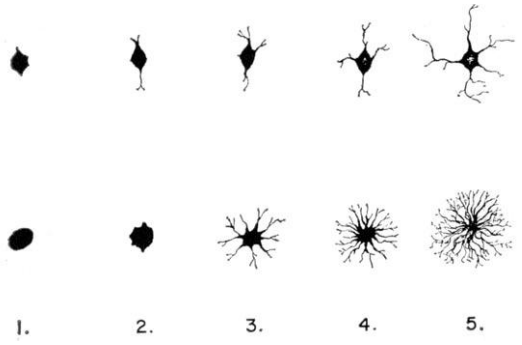
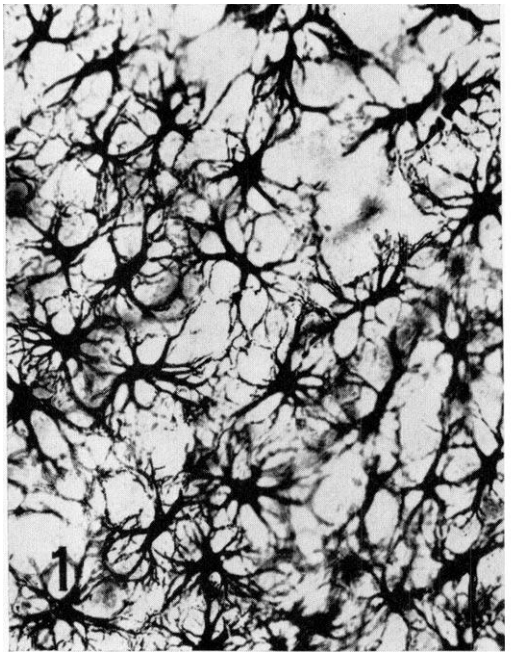
* The hormone was prepared by Drs. A. B. Lerner and S. Lande.

ment cells are situated in the basal layer of the epidermis and donate melanin to adjacent epidermal cells. The much larger dermal pigment cells lie in the dermis and they retain their melanin granules. Observations of the melanophore index levels for a week prior to the hormone treatment showed that for each frog the epidermal and dermal readings did not vary beyond half a point on the index scale and this could be due to slight variations in the handling of the frog. These initial readings constituted the normal basic melanophore index level for each frog.



TEXT FIGURE 1. An artist's impression of the epidermal melanophores (above) and the dermal melanophores (below) of the frogs web at different stages in the dispersion of melanin granules. The cells are not drawn to scale.

Experiment I. The results of six of the groups are shown graphically in Text Figure 2. It was seen that the greater the dose of MSH given the greater the degree of dispersion of the melanin granules in both the epidermal and dermal melanophores. With doses of 5,000 units of hormone and larger doses the dispersion was extreme (Fig. 3) and greater than that represented diagrammatically in Text Figure 1 as melanophore index 5. In these circumstances it was often extremely difficult to recognize the epidermal malanophores. The larger the dose of MSH the greater the period of time that the melanin granules remained in a dispersed state in both sets of pigment cells. In most groups of animals the dermal cells responded more rapidly than the epidermal cells. With doses of MSH of 40 units or greater the granules of the epidermal cells remained in a dispersed state for



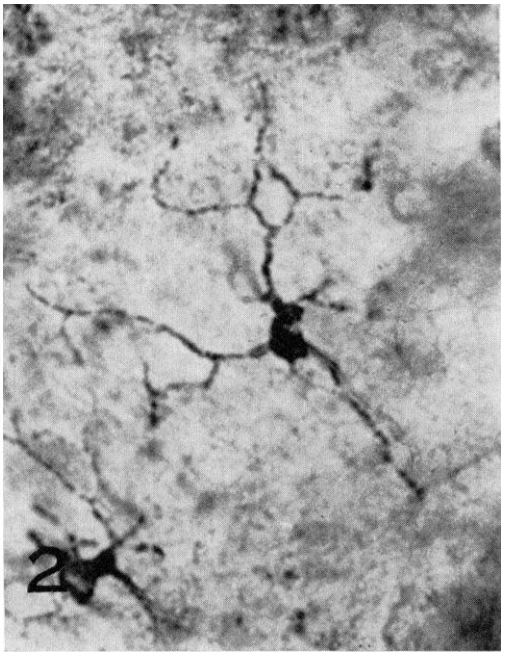


FIGURE 1. Shows a group of dermal melanophores of the frog's web. The melanin granules are in a partial state of dispersion (M.I. 3.5). Note that the dendritic processes of adjacent cells are in contact but there is no evidence that they are in continuity. × 123.

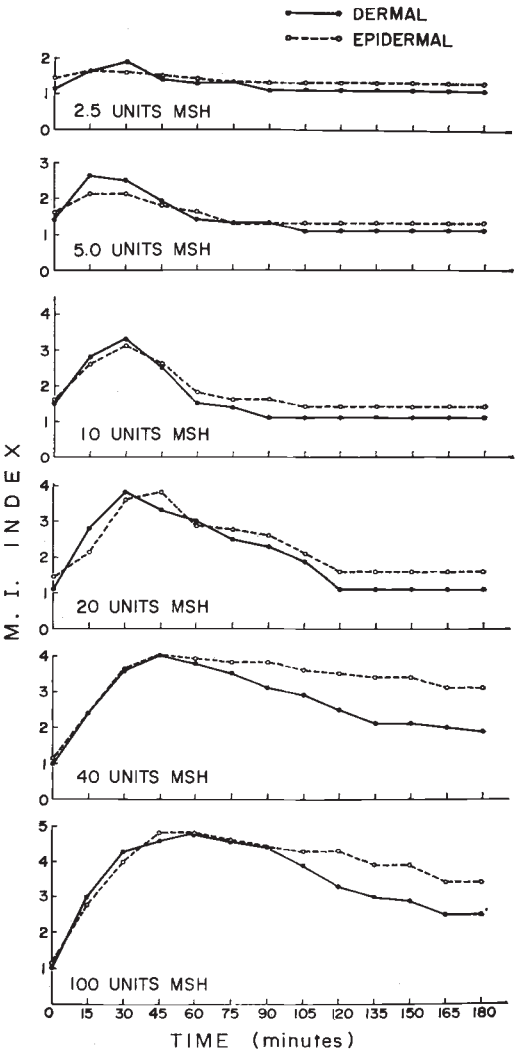
FIGURE 2. Shows two epidermal melanophores of the frog's web. Note the presence of large numbers of melanin granules in the dendritic processes. Scattered fine melanin granules can also be seen in the surrounding epidermal cells. × 331.

a longer period of time than those of the dermal cells (Fig. 4). The rate of dispersion of the melanin granules in the dermal cells was not appreciably affected by the dose of the hormone but the granules in the epidermal cells responded more rapidly with higher doses.

Experiment II. Group I animals received MSH for 1 week. The frogs became black in color within two hours and both the epidermal and dermal cells showed extreme dispersion of melanin. When the hormone injections were stopped, 8 days elapsed before the melanin granules returned to their normal basic position in both groups of cells. There was initially a tendency for the epidermal melanophores to aggregate their granules more slowly. The dermal cells during the early stages of the aggregation process often showed marked fluctuation in their indices at different times during the same day. It was noted also that in a given area the melanin in some dermal cells was much more aggregated than in others (Fig. 5). In order to record a melanophore index at any given time an overall average was taken. On the sixth day following the end of the hormone treatment many of the dermal melanophores displayed a feathery appearance (Fig. 6). This was due to the fact that the rate of aggregation of the melanin granules was unequal in the different dendritic processes. In the epidermal cells groups of melanin granules were often seen to be left behind in the dendrites giving them a fragmented appearance (Fig. 7). At no time did the melanophore index of either the epidermal or dermal cells fall below the normal basic level.

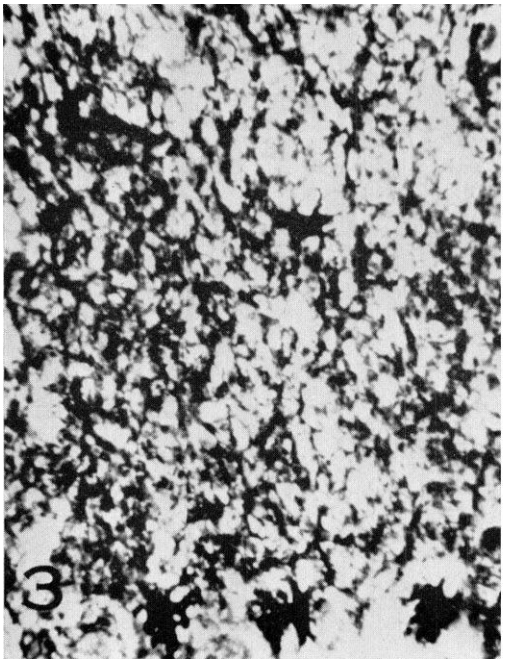
Group II received MSH for 5 weeks. The response of the epidermal and dermal melanophores during the hormone treatment was identical to that seen in Group I. When the hormone was stopped 10 days elapsed before the melanin granules returned to their normal basic level.

Group III received MSH for 12 weeks. Initially the frogs remained dark in color with the maximum degree of dispersion of melanin seen within the epidermal and dermal pigment cells. At 7½ weeks melanin aggregation began in both groups of cells in spite of the continued hormone treatment. In order to maintain the maximum melanin dispersion the dose of MSH had to be repeatedly raised. Two frogs suddenly



TEXT FIGURE 2. Graphs showing the effects of different doses of alpha MSH on the melanophore index of the epidermal and dermal pigment cells of the web of the intact frog. Each graph represents the average results in 4 frogs.

died during the last week of hormone treatment. On stopping the hormone 15 days elapsed before the melanophore index returned to the normal basic level. The irregularity in the speed of aggregation in the different cells and the feathery appearance of the dermal melanophores was more striking in this group as compared with the other two. Once again the index of both the epidermal and dermal cells never fell below the normal basic level. The melanophore indices for Groups II and III are shown in Text Figure 3.



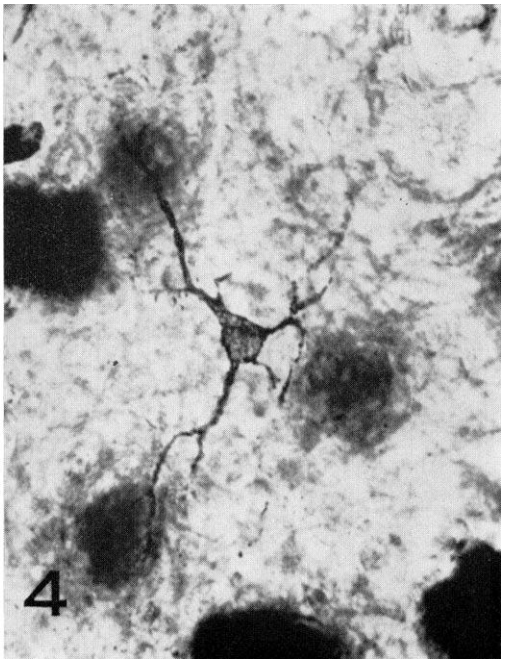


FIGURE 3. Shows a group of dermal melanophores of the frog's web with maximum dispersion of melanin following MSH treatment (M.I. > 5). The finest branches of the dendrites are packed with melanin. × 97.

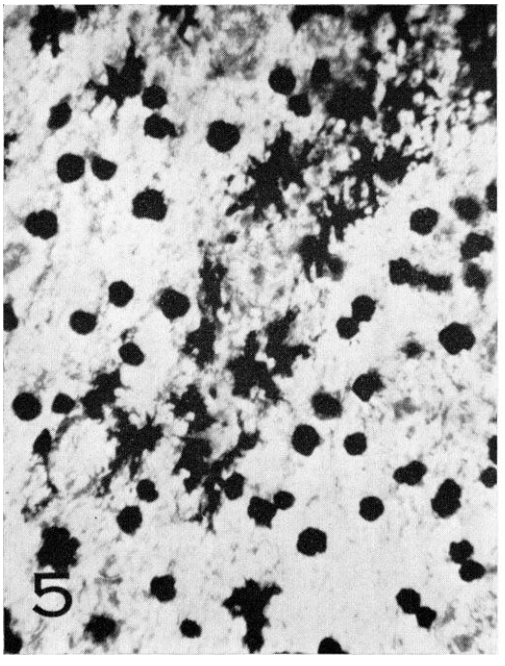
FIGURE 4. High power view of epidermal melanophore of the frog's web following the cessation of MSH treatment. Large numbers of melanin granules are still present in the dendritic processes (M.I. = 4.5). Note that melanin in the dermal melanophores has almost completely returned to the cell bodies (M.I. = 1.5). × 455.

One frog in Group III was studied for a total of 28 days following the end of the hormone treatment. It was then killed and an in vitro study of the effect of 10 units of alpha MSH on the reaction of the melanophores was compared to that of normal control frog skin. The in vitro frog skin assay method was used (9). The results are shown graphically in Text Figure 4. It is seen that the experimental frog skin reacted much less than the normal to MSH but responded well to melatonin ($2 \times 10^{-3}/\mu g$).

DISCUSSION

The frog has been extensively used in investigations into the hormonal control of human skin color. The excised skin of *Rana pipiens* bathed in Ringer has formed the basis of an extremely sensitive *in vitro* assay method (9). To begin with, investigators relied solely on the changes in intensity of reflected light from the skin to determine whether it had become darker or lighter in color. More recently the changes in the morphology of the two types of melanin-containing pigment cells in frog skin in response to different hormone preparations have been studied. McGuire and Möller (7) showed that the epidermal and dermal melanophores both react to MSH by dispersion of melanin granules. The epidermal cells like those of mammals (10) failed to respond to melatonin while the melanin granules in the dermal melanophores were aggregated by this hormone. They also showed that acetylcholine and norepinephrine had the same effects on the two groups of cells as melatonin. Snell and Kulovich (6) investigated the possible connection between the activity of the nervous system and the movement of melanin granules in the frog melanophores. Electrical stimulation failed to produce any response in either of the two pigment cell groups.

Various theories have been put forward to explain the underlying mechanism for the movement of melanin granules in frog pigment cells. Marsland (11) showed that gel-to-sol transformation occurs in the protoplasm of melanophores when the granules are dis-



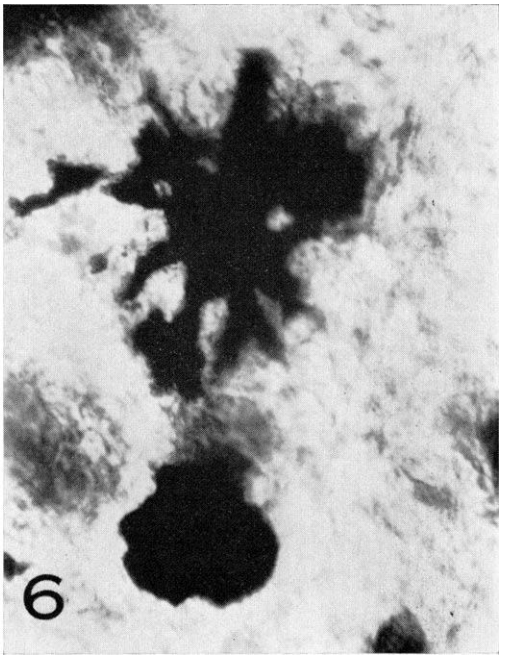


FIGURE 5. Shows dermal melanophores of frog's web following cessation of MSH treatment. Note that the degree of aggregation of melanin differs greatly in different groups of cells in the same area. × 114.

FIGURE 6. High power view of the dermal melanophores of frog's web following cessation of prolonged MSH treatment. The upper cell has a "feather-like" appearance due to the unequal rate of aggregation of melanin granules in the different dendritic processes. The melanin in the lower cell has almost completely returned to the cell body. × 406.

persing. His experiments would suggest that in the dispersed state the protoplasm is in a sol state, but gelled in an aggregated state. Other workers (12) have proposed that melanin granules are attached to a network of protein fibrils situated in the cytoplasm and that MSH causes solation and streaming of cytoplasm towards the cell center; the melanin granules then disperse outwards to fill the void left by the inflow of cytoplasm. Kinositas' experiments (13) suggest that electrophoresis might be responsible for melanin movement with pigment cells. More recently Martin and Snell (14) showed that MSH dispersion of melanin granules in frog dermal melanophores is not accompanied by a change in membrane potential. In fact potassium induced deplorization of the cell membranes has no effect on the ability of MSH to cause melanin dispersion. Washing out of the hormone with Ringer was followed by melanin aggregation under the same depolarized conditions. These experiments show that movement of melanin granules is completely independent of the membrane potential of the dermal melanophore.

The present work using intact male *Rana pipiens* has shown that with a single injection of MSH into the dorsal lymph sac the higher the dose the greater the degree of melanin dispersion occurs in the epidermal and dermal melanophores. The dose response was practically linear until the maximum degree of dispersion was reached with a dose of 5,000 units. Furthermore the higher the dose of hormone the longer the time the melanin granules remained in a dispersed state. This was probably due to the greater saturation of tissues by large amounts of hormone. In doses below 50,000 units the dermal cells responded more rapidly to MSH than the epidermal; with higher doses the response in the two cell groups was practically equal. The closeness of the dermal melanophores to the blood capillaries may in part explain this difference in response. With large doses the tissue concentrations of hormone are built up rapidly and the responses of the

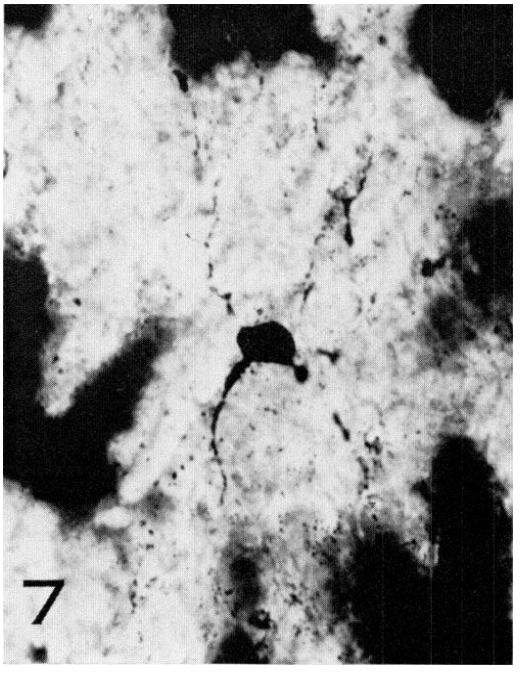


FIGURE 7. High power view of epidermal melanophore following cessation of prolonged MSH treatment. Clumps of melanin granules are seen to be left behind in the dendritic processes giving the dendrites a fragmented appearance. The melanin in the deeper neighboring dermal melanophores can also be seen. × 450.

epidermal and dermal cells are equally rapid. With doses of MSH greater than 40,000 units the melanin in the epidermal cells remained in a dispersed state for a longer time than the dermal cells. This phenomenon had been observed previously in hypophysectomized frogs (6). Two explanations would seem possible: 1) The turnover of tissue fluid within the epidermis is slower than in the dermis so that MSH remains in contact with the epidermal cells longer than the dermal cells and 2) an inherent difference may exist in the response of the two types of pigment cells to stimulation. It would appear that the principal function of the small epidermal melanophore is to provide adjacent epidermal cells with melanin pigment, whereas the much larger dermal melanophores hold on to their melanin. It is the latter cells which enable the frog to quickly change color and so adapt itself to the color of its surroundings.

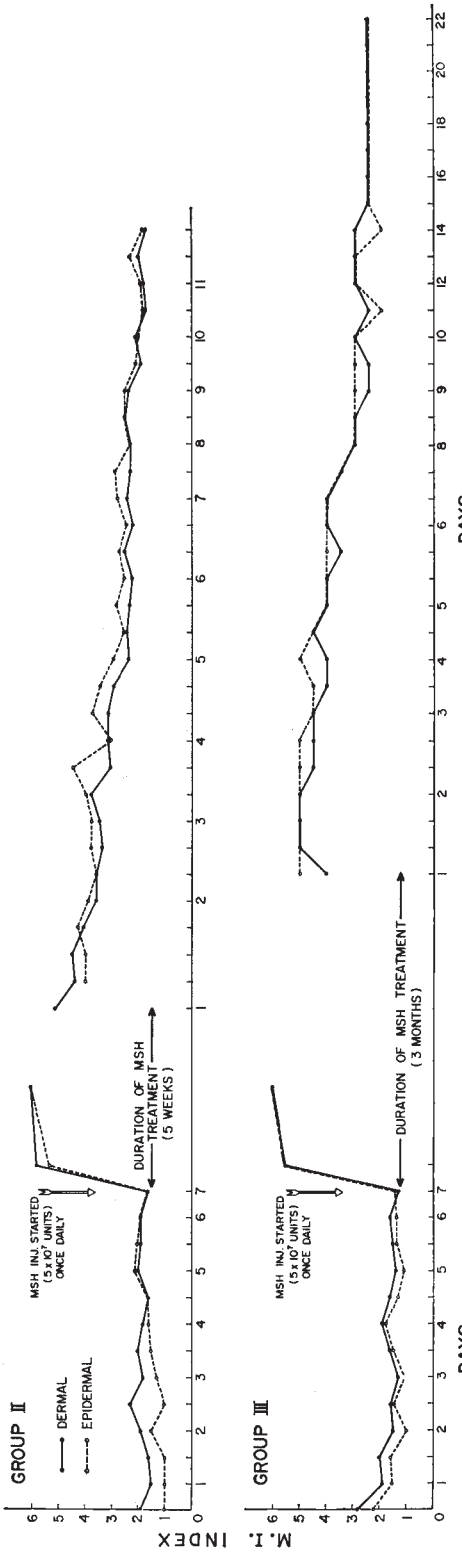
The effect of prolonged administration of large doses of MSH on twelve frogs demonstrated again the effect of saturation of tissues with the hormone and the consequent delay in the aggregation process. The fate of MSH in the body is not exactly known although Shizume and Irie (15) have shown that the liver is an important MSH-inactivating site. Lerner *et al.* (16) showed that 4 to 6% of the activity of intramuscular injected MSH in man can be recovered in the urine. In the present experiments the frog water was changed daily to prevent the influence of accumulated MSH in the bathing fluid.

The variations in the speed of aggregation seen in different groups of epidermal and dermal pigment cells was of interest. This may be due to different turnover rates of tissue fluid in different skin areas. Another possibility is that different pigment cells in a group have different hormonal sensitivities. In human skin it is very rare indeed to find all melanocytes in a group displaying the same melanogenic activity (17).

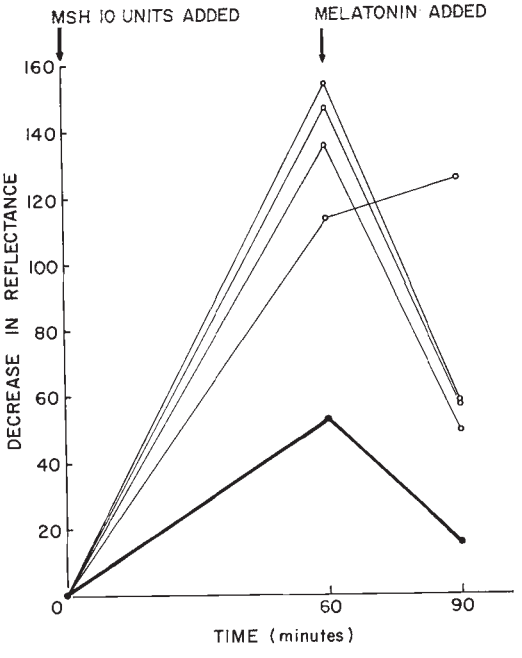
The "feather-like" appearance of some dermal melanophores and the scattered retention of melanin clumps in the dendrites of epidermal melanophores during the aggregation process was only seen following prolonged dispersion of melanin. It would seem that the normal smooth aggregation mechanism has been upset by the prolonged state of melanin dispersion and that the melanin granules become stuck together and their streaming movement is inhibited. Further observations of this phenomenon may give us added insite into the whole mechanism of melanin movement.

The eventual return of the melanin granules to a normal basic position in all the experiments irrespective of the duration and amount of exogenous MSH used showed that the normal delicate balance between the aggregating effect of endogenous melatonin and the dispersing effect of MSH in the frog had been preserved. It conclusively demonstrated that the output of endogenous MSH by the pituitary had not been inhibited by the repeated high doses of porcine MSH, for at no time did the melanophore index fall below the normal basic level for each animal.

The fact that the frog skin began to lighten at 7½ weeks in spite of continued administration of very high doses of MSH was of considerable interest. This could be attributed to two things. (1) After 7½ weeks of treatment the



TEXT FIGURE 3. Graphs showing the melanophore index of the epidermal and dermal pigment cells of the web of the intact frog in Groups II and III. Days 0–7 show the normal basic variation in the indices before the start of daily injections of alpha MSH.



TEXT FIGURE 4. Graphs showing the response of excised experimental frog skin which had received large daily doses of MSH for 3 months to 10 units of MSH (thick line). The response is compared with that of 4 normal control pieces of skin (fine lines). A decrease in reflectance is directly proportional to darkening of skin, i.e. dispersion of melanin granules within melanophores. The response of the experimental skin to melatonin is within normal limits.

rate of breakdown of exogenous MSH may have been accelerated. (2) The pigment cells may have become insensitive to exogenous hormone stimulation possibly as the result of the frog developing antibodies to the porcine MSH. The comparison of the response of excised experimental frog skin (1 month after the cessation of 12 weeks of hormone treatment) with normal skin *in vitro* when exposed to 10 units of porcine MSH showed clearly that the experimental pigment cells no longer responded normally to porcine MSH. This result would support the second explanation for the skin lightening. However, in view of the enormous amounts of MSH required to carry out this research only a small number of animals were used. Until larger amounts of MSH become readily available so that we can confirm these latter findings we would prefer to record them as "interesting observations" only.

SUMMARY

1. The effect of alpha MSH on the movement of melanin granules in the epidermal and dermal melanophores of web skin was studied in 68 intact adult male *Rana pipiens*.
2. Single doses of hormone were injected into the dorsal lymph sac of 56 frogs. The doses of MSH extended from 2.5 units to 50 million units and the dose response was studied using a melanophore index.
   a. The higher the dose of MSH the greater the degree of melanin dispersion occurred. The maximum degree of dispersion was reached with a dose of 5,000 units.
   b. The higher the dose of MSH the longer the time the melanin granules remained in a dispersed state.
   c. The dermal melanophores responded more rapidly for a given dose of hormone (up to 50,000 units) than the epidermal cells. Above 50,000 units both cells responded at the same rate.
   d. With a dose of hormone of 40,000 units or greater the epidermal cells remained in a dispersed state for a longer time than the dermal cells.
3. Prolonged administration of very high doses of MSH was studied in 12 frogs.
   a. After 1 week of hormone 8 days elapsed before the epidermal and dermal melanophores returned to a normal state. Great variations in speed of melanin aggregation were noted in both pigment cell groups.
   b. After 7½ weeks of hormone the frog skin started to lighten in spite of continued hormone injections. Only by raising the dose to an extremely high level and giving the injections twice daily was it possible to maintain the melanin in a fully dispersed state.
   c. Excision of frog skin 1 month after cessation of 12 weeks of high hormone treatment showed that *in vitro* response to MSH was much reduced as compared with normal. A normal melanin aggregation response to melatonin was present.

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