

THE MELANOCYTE MODEL

Colchicine-like Effects of Other Antimitotic Agents

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ABSTRACT

The effect of various agents that cause metaphase arrest in dividing cells was studied on the rapid reversible darkening of frog skin under the influence of melanocyte-stimulating hormone (MSH). Darkening is due to dispersion of melanin granules in melanocytes and is thought to be accompanied by a gel-to-sol cytoplasmic transformation. After subsequent washing the skin lightens, with aggregation of melanin granules and cytoplasmic gelation. As previously shown with colchicine, preincubation of frog skin with vinblastine, vincristine, or colcemid produced an increase in darkening induced by MSH, as compared to control skins, and a dosage-dependent inhibition of subsequent lightening. Preincubation with each drug, without subsequent MSH, produced a gradual, irreversible, dosage-dependent darkening over several hours. On a molar basis, the relative strength of the various agents was vinblastine > vincristine > colcemid > colchicine; vinblastine was about 100 times stronger than colchicine. Preincubation of frog skin with griseofulvin, followed by washing, had no subsequent effects on darkening or lightening. However, effects similar to those of the *Colchicum* and *Vinca* alkaloids were seen if griseofulvin was kept in the ambient media. These effects were rapidly reversible on removal of the drug from the media. These findings support the melanocyte model originally proposed for the action of colchicine, and emphasize certain facts that models of melanin granule movement will have to accommodate.

INTRODUCTION

The effect of colchicine has previously been studied on the rapid reversible darkening of frog skin under the influence of melanocyte-stimulating hormone (MSH) (Wright, 1955; Malawista, 1965). Darkening is due largely to dispersion of melanin granules in dermal melanocytes¹ and has been thought to be accompanied by a gel-to-sol cytoplasmic transformation. After subsequent washing, the skin lightens, with aggregation of melanin granules and cytoplasmic gelation.

¹ Melanocytes that participate in rapid color changes of animals by intracellular displacements of melanin granules are also called melanophores.

In this system, preincubation with colchicine produces an increase in darkening induced by MSH, and a dosage-dependent inhibition of subsequent lightening. Colchicine alone produces a gradual, irreversible, dosage-dependent darkening over several hours.

The melanocyte model has been used to construct a general theory of colchicine action on living cells, an action resulting in decreased protoplasmic viscosity. In this formulation colchicine lowers the potential limit of protoplasmic gelation, and does it rapidly, reversibly, in low concentration, in a dosage-dependent manner, and without killing the cell (Malawista, 1965).

In the unfertilized oocyte of the marine polychaete, *Pectinaria gouldi*, the mitotic spindle, itself a highly organized protoplasmic gel, undergoes diminution in size and in birefringence and eventual dissolution, under the influence not only of colchicine analogues (Inoué and Sato, 1967), but also of certain other agents, including the chemotherapeutic *Vinca* alkaloids, vinblastine and vincristine, and the fungistatic antibiotic, griseofulvin (Malawista et al., 1968). In the present studies the effects of colchicine in the frog skin system are compared to those of several of the agents with colchicine-like effects on the mitotic apparatus, including colcemid, vinblastine, vincristine, and griseofulvin. The results support this system as a general model. They also emphasize certain questions regarding the mechanism of movement of melanin granules in melanocytes, especially applicable to the possible role of microtubules.

METHODS

Preparation of Frog Skin

This procedure has been described previously in detail (Malawista, 1965). In brief, four samples of skin from each of four frogs (*Rana pipiens*) were mounted on rings (Shizume et al., 1954) and arranged in four groups so that each group contained one skin specimen from each frog and one from each area sampled (right or left, thigh or leg) (Wright and Lerner, 1960). Thus, for any single experiment one control group and three experimental groups were available.

All skin specimens were soaked in several changes of frog Ringer's solution over a period greater than 1 hr before any observations were made; this maneuver results in maximal aggregation of melanin granules which persists for several hours. (A change of Ringer's solution is also referred to as a "wash".)

Measurement of Melanin Granule Dispersion

Changes in the state of dispersion of melanin granules within melanocytes were measured as changes in reflectance when the whole skin specimen, immersed in approximately 20 ml of solution in a 50 ml beaker, was placed over a search unit attached to a photoelectric meter (Shizume et al., 1954; Malawista, 1965). The reading of a given skin specimen after initial soaking in Ringer's solution was taken as its base line, or zero value; subsequent increase or decrease in reflectance units was measured from base line. 16 specimens could be read in a 5 min period. The graphs were prepared by totaling the changes in reflectance within each treatment group and plotting these values against time.

Preincubation with Drugs

Each group of skins treated with a given drug received it after the base line reading. After a 30 min preincubation, each group was washed twice unless noted otherwise, and a second set of readings was taken.

Incubation with or without MSH

After the second set of readings, in some experiments MSH was added to all groups for 90 min, during which time three more sets of readings were made at intervals of 30 min. Then all groups were washed twice, and readings were taken at several additional intervals thereafter. In other experiments, no MSH was added after the preincubation; readings were taken at several intervals thereafter.

Solutions Used

FROG RINGER'S SOLUTION: The suspending medium was prepared as previously described (Wright and Lerner, 1960; Malawista, 1965). All test substances were dissolved in Ringer's solution before use.

DRUGS: Agents used included colchicine USP (Sigma Chemical Co., St. Louis, Mo.), deacetyl-methylcolchicine (colcemid, Ciba Pharmaceutical Co., Summit, N.J.), vinblastine sulfate (Velban, Eli Lilly & Co., Indianapolis, Ind.), vincristine sulfate (Oncovin, Eli Lilly & Co.), and griseofulvin (Ayerst Laboratories, New York, N.Y.). The last drug, because of its insolubility in aqueous media, was first dissolved in *N,N*-dimethylformamide at a concentration of 2×10^{-2} M, and diluted in Ringer's solution before use. The formamide, diluted to the concentration at which it accompanied the highest concentration of griseofulvin used, was employed as an additional control. All drugs were freshly diluted for each experiment and protected from light before use.

MSH was prepared from a water extract of beef posterior pituitary powder (Armour Pharmaceutical Co., Kankakee, Ill.) as described previously (Shizume et al., 1954). 1 mg of the lyophilized product was diluted so that each beaker received 0.2 ml of the final product containing 10 units of MSH (Shizume et al., 1954).

RESULTS

The Comparative Effects of Preincubations with Colcemid, Colchicine, Vinblastine, and Vincristine

WITH MSH (FIGS. 1 A AND B, 2 A AND B): The addition of a standard amount of MSH to a

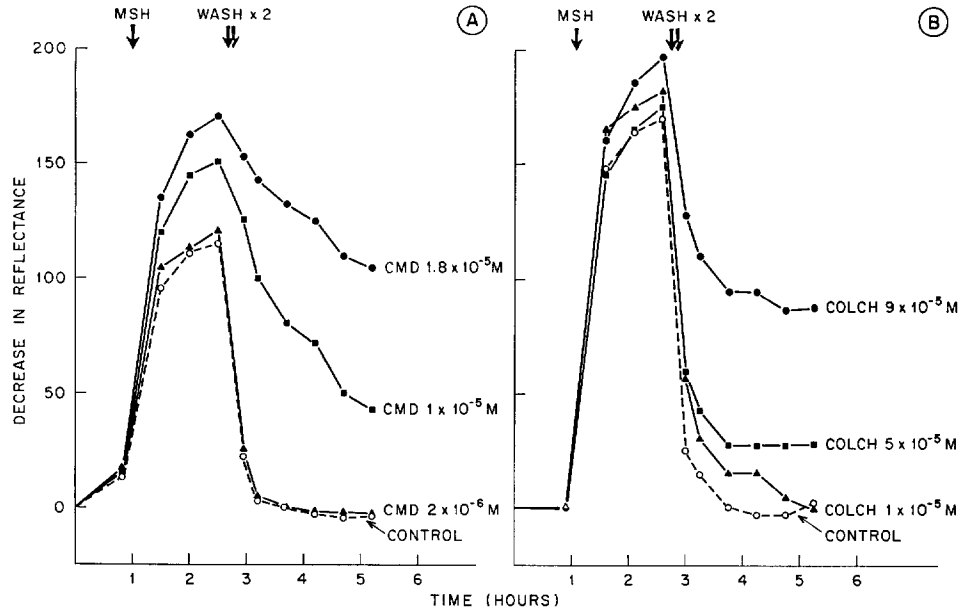


FIGURE 1 The effect of preincubation with (A) colcemid (CMD) and (B) colchicine (COLCH). MSH: melanocyte-stimulating hormone (see text).

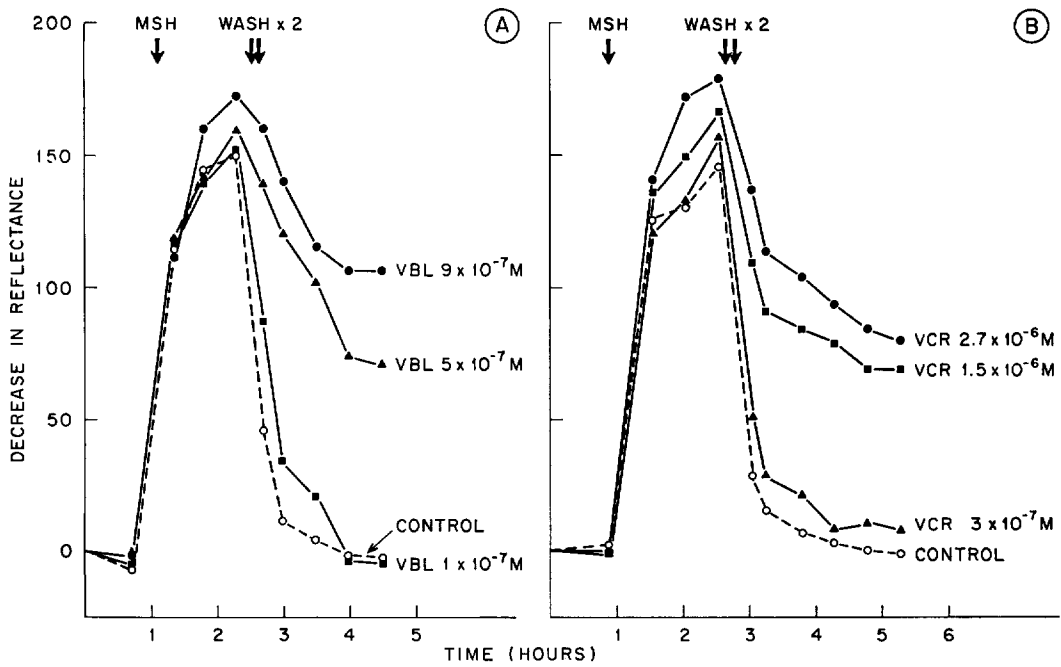


FIGURE 2 The effect of preincubation with (A) vinblastine (VBL) and (B) vincristine (VCR). MSH: melanocyte-stimulating hormone (see text).

control group of skins results in a rapid decrease in reflectance, maximal at 60–90 min (Shizume et al., 1954). The effect is reversed by a double wash in Ringer's solution, whereupon the reflectance returns to base line (Figs. 1 and 2).

After preincubation with colcemid (Fig. 1 A), the results were similar to those previously reported for colchicine (Malawista, 1965) and presently repeated for comparison in Fig. 1 B. When MSH was added, groups preincubated with colcemid

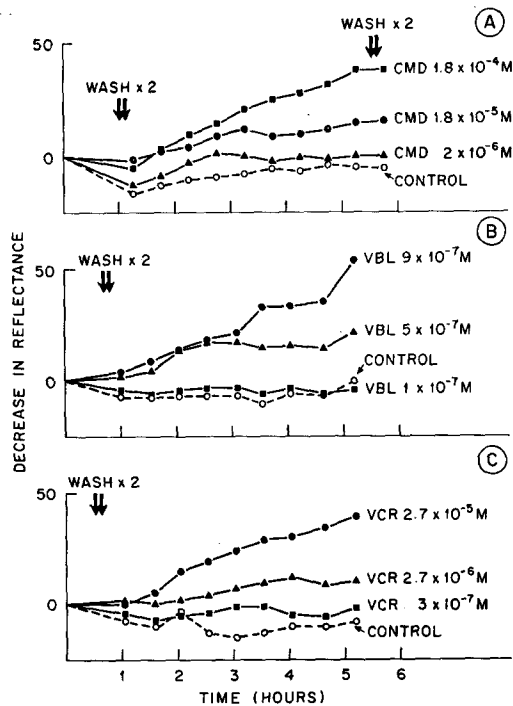


FIGURE 3 The effect of preincubation with (A) colcemid (CMD), (B) vinblastine (VBL), and (C) vincristine (VCR). No darkening agent was added (see text).

darkened more than did the control group. After subsequent washing, inhibition of lightening was apparent, and this inhibition depended on the concentration of colcemid used during the preincubation. On a molar basis the effect of colcemid was greater than that of colchicine; only about one-fifth the concentrations of colchicine was required to approximate, with colcemid, the effects of colchicine.

After preincubation with vinblastine or vincristine, the results were again similar to those with colchicine (Figs. 2 A and B): when MSH was added, preincubated groups darkened more than did control groups, and after washing, there was a dosage-dependent inhibition of lightening. The concentrations necessary to produce these effects were low; on a molar basis vinblastine was about 3 times stronger than vincristine and about 100 times stronger than colchicine.

WITHOUT MSH (FIGS. 3 A, B, AND C): Preincubation with colchicine alone produces a gradual, irreversible, dosage-dependent darkening over several hours (Malawista, 1965). Colcemid, vinblastine, and vincristine had similar effects, as

shown in Fig. 3. Repeated washing, as shown for example in Fig. 3 A, did not return the preincubated groups to base line. Compared to experiments in which MSH was used, preincubations that employed greater differences in concentration of a given drug were often required to see subsequent differences in darkening. The relative strength of the various agents remained the same as when MSH was used.

The Effect of Griseofulvin (Figs. 4 A and B)

An advantage of the melanocyte model in analyzing the action of colchicine was that after the period of preincubation, colchicine was no longer required in the ambient Ringer's solution; thus, the effects of subsequent agents, such as MSH, could be separated from that of colchicine (Malawista, 1965). This property also held for colcemid and for the *Vinca* alkaloids (above), but not for griseofulvin. If the skins were washed after an initial preincubation with griseofulvin, and no further griseofulvin was applied, no subsequent differences with respect to control skins were seen.

To see if a rapidly reversible effect of griseofulvin might be present, the following experiment was done (Fig. 4 A). After the initial preincubation with griseofulvin, the skins were not washed; MSH was added to groups of skins in Ringer's solution that still contained griseofulvin. After the skins had been allowed to darken for 90 min, they were not washed with plain Ringer's solution; instead, the media containing MSH and griseofulvin were replaced by media containing griseofulvin. Finally, after a suitable interval, all groups of skins were washed twice.

Under these conditions, incubation with griseofulvin produced an increase in darkening induced by MSH as compared to the control group, and a dosage-dependent inhibition of subsequent lightening. However, on washing the skins, all groups returned rapidly to base line. *N,N*-dimethylformamide alone, in the same concentration at which it was present with the higher concentration of griseofulvin, did not inhibit lightening of frog skin.

In a similar experiment without MSH (Fig. 4 B), incubation with griseofulvin produced a gradual, dosage-dependent darkening over several hours, which again was rapidly reversible when the skins were washed.

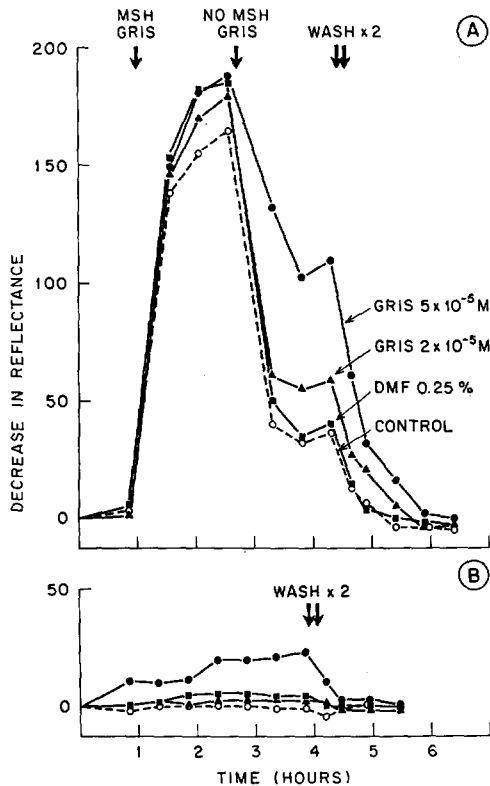


FIGURE 4 The effect of griseofulvin (GRIS) (A) with melanocyte-stimulating hormone (MSH), and (B) without MSH. DMF: *N,N*-dimethylformamide (see text).

The Effects of Drugs, Shown in Photomicrographs (Figs. 5 a and b)

Four samples of skin from a single frog were treated with colcemid, vinblastine, vincristine, or griseofulvin at the highest concentrations shown in Figs. 1, 2, and 4. 30 min later, the first three samples were washed twice; then MSH was added to all four samples, to induce dispersion of melanin granules in melanocytes and gross darkening of the skin. All the samples were washed twice 90 min later, and photographed after two additional hours. Photomicrographs were taken of spotted areas, where melanocytes are most dense.

The sample incubated with griseofulvin is shown in Fig. 5 a. Each black dot represents the melanin granules of an individual melanocyte which have returned toward their maximally aggregated condition, with gross lightening of the frog skin. In this unstained specimen, the borders of the melanocytes themselves are not visible.

This picture is similar to that of control skin not treated with drug (Malawista, 1965).

The sample preincubated with vincristine is shown in Fig. 5 b. Melanin granules have remained dispersed, outlining the dendritic processes of melanocytes, with persistence grossly of the dark appearance of the frog skin. This picture is similar to that seen in the samples preincubated with vinblastine or colcemid, and shown previously for colchicine (Malawista, 1965).

DISCUSSION

The Melanocyte Model

Colcemid and the *Vinca* alkaloids behaved like colchicine in the melanocyte model (Figs. 1, 2, and 3), and did so at concentrations lower than those necessary for comparable effects with colchicine—100 times lower in the case of vinblastine. In comparison, when oocytes of *Pectinaria* were the target cells, vinblastine and colcemid were roughly equivalent on a molar basis, and were about 10 times stronger than either vincristine or colchicine (Malawista et al., 1968).

In *Pectinaria*, griseofulvin was the most rapidly reversible spindle disrupter found (Malawista et al., 1968). Mitotic spindles of oocytes treated with griseofulvin, 1×10^{-6} M, disappeared in 3–6 min and, on perfusion with fresh media, reappeared and grew back to normal size and birefringence within 5–10 min. It was this rapid reversibility in *Pectinaria* oocytes that suggested that the lack of a preincubation effect of griseofulvin in frog skin might be due to washing out a griseofulvin effect at the end of the period of preincubation. Indeed, when griseofulvin was left in the ambient Ringer's solution, subsequent effects were similar to those seen with the *Colchicum* and *Vinca* alkaloids, but when the skins were washed the effects were rapidly reversible (Fig. 4).

Microtubules and the Movement of Melanin Granules

When the drugs used in the present study were applied to *Pectinaria* oocytes, as the mitotic spindle underwent diminution in size and in birefringence and eventual dissolution, the microtubules of the spindle correspondingly became shorter and fewer and finally disappeared (Malawista et al., 1968; Inoué and Sato, 1967). All these agents have the ability to bind the cytoplasmic protein that appears to form microtubules (Borisy and Taylor, 1967 a

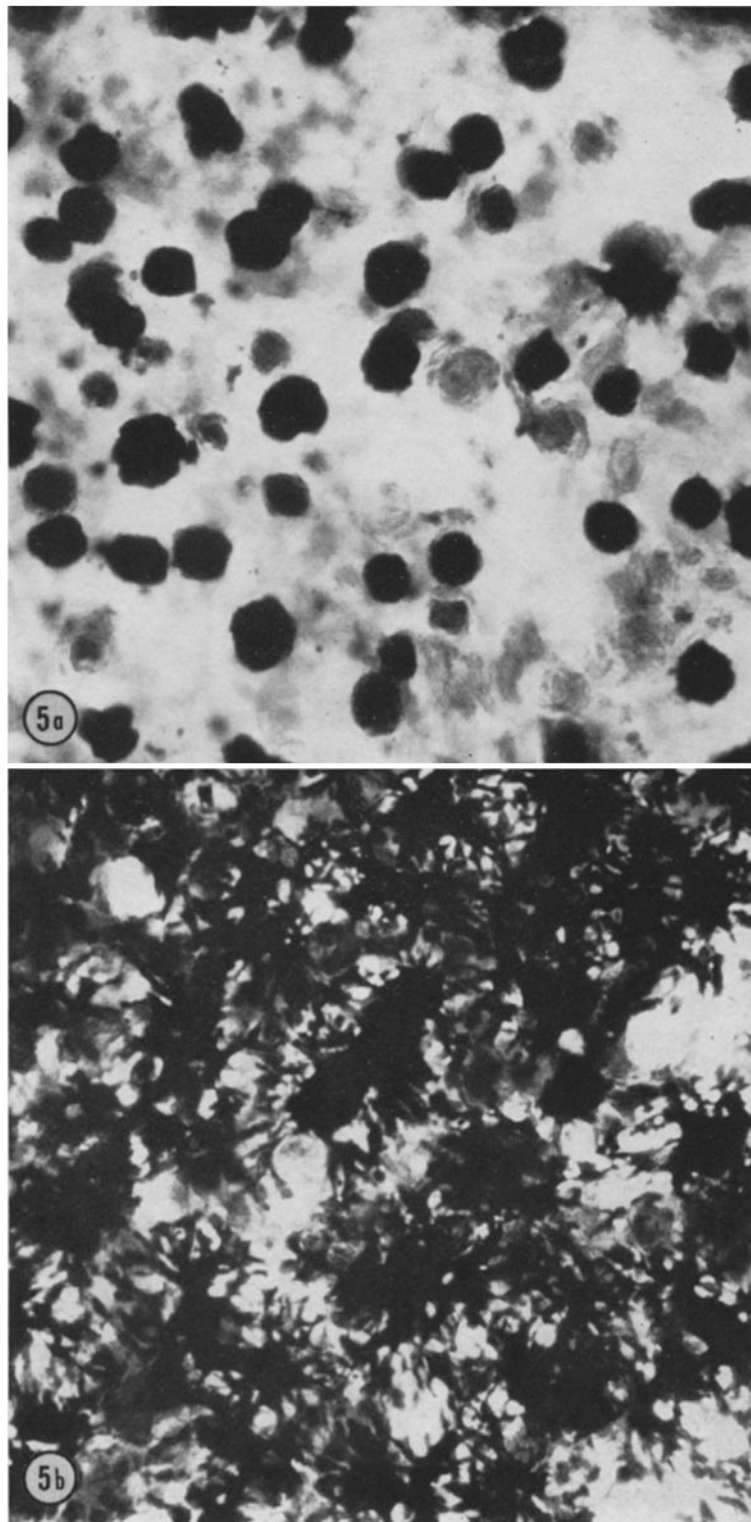


FIGURE 5 The effect of treatment with (a) griseofulvin and (b) vincristine. The frog skin was treated subsequently with MSH and then washed (see text). Scale: $100 \mu = 32 \text{ mm}$.

and *b*; Shelanski and Taylor, 1967; Wilson and Friedkin, 1967; Creasey, 1967; Weisenberg et al., 1968). Microtubules may perform mechanical work in cells (Ledbetter and Porter, 1963); they may contribute to protoplasmic gel strength (Tilney et al., 1966). In melanophores of the teleost *Fundulus heteroclitus*, microtubules are aligned parallel to the direction of pigment movement, and persist whether the melanin granules are dispersed or aggregated (Bikle et al., 1966; Novales and Novales, 1966; Green, 1968). Microtubules are also present in frog dermal melanocytes (S. E. Malawista and W. R. Adams. Unpublished observations.), though by no means in the abundance found in pigment cells from *Fundulus*. Besides defining the channels in which the pigment moves, the microtubules are thought possibly to exert the motive force for pigment migration (Bikle et al., 1966; Green, 1968), but how they may do it is obscure.

In considering the possible role of microtubules in such a motive force, the agents used in the melanocyte model are useful because their effects are unidirectional; they interfere with lightening of frog skin but not with darkening. (A similar effect has been found for colchicine with *Fundulus* melanophores [Wikswo and Novales, 1969].) Thus, after the initial preincubation, when the *Colchicum* or *Vinca* alkaloids are no longer in the ambient Ringer's solution, the darkening response to hormonal stimulation is at least as great as in control skins (Figs. 1 and 2). Only on subsequent washing do we recognize a dosage-dependent inhibition of lightening, an inhibition whose potential was established *before* addition of the darkening hormone. Without the hormone, the skin will nevertheless gradually darken and exhibit a dosage-dependent inhibition of lightening (Fig. 3), but without the magnification and resultant rapid recognition that are provided by the use of MSH.

These findings suggest that if a single network of microtubules is responsible for the movement of melanin granules in melanocytes and is disrupted by these agents, it would have to be involved in aggregation and not dispersion. An alternative suggested by Bikle et al. (1966) for *Fundulus* melanophores is that there are two interdigitating sets of microtubules with separate activation for aggregation and dispersion. In that case, however, under the conditions of the present studies, one would have to assume that the microtubules involved in lightening are sensitive to antimototic

agents while those involved in darkening are insensitive. Another alternative is that some as yet unconsidered constituents of melanocytes, as, for example, microfilaments (Buckley and Porter, 1967), are important for aspects of the movement of melanin granules in relation to microtubules, and suffice for dispersion but not for aggregation. All these possibilities are subject to ultrastructural investigation.

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Note Added in Proof: In support of the third possibility above, I have shown recently that cytochalasin B produces a reversible, dosage-dependent inhibition of darkening of frog skin (unpublished observations).

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