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Effects of Sulfhydryl Inhibitors on Migration of Pigment Granules in the Melanophore of *Oryzias latipes*

Tetsuro IGA
Department of Biology, Shimane University, Matsue 690
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Abstract

The effects of two sulfhydryl inhibitors, Igrosin (containing mersalyl as an effective agent) and N-ethylmaleimide (NEM), were studied on pigment migration in melanophores of *Oryzias latipes*. Activities of melanophores were measured photoelectrically and simultaneously confirmed by microscopic observation. Pretreatment with each of these inhibitors at a concentration of 10^{-3} M blocked completely pigment aggregation within the melanophores to KCl or adrenaline. The effects of these agents could not be removed by washing with the physiological solution alone. Cysteine reversed the effects of these inhibitors on the response of melanophores to KCl or adrenaline. These inhibitors produced dispersion of pigment in punctate adrenaline-treated melanophores which was markedly rapid as compaired with that produced by the saline alone. If one of the inhibitors was applied in combination with adrenaline, following temporary pigment aggregation within the melanophores rapid pigment dispersion was induced. Based on these results, possible involvement of the elements of protein –SH groups on pigment aggregation mechanism was discussed.

It is well known that rapid color changes of teleost fishes are generally due to the activity of the chromatophores in the integument. Among chromatophores those which contain melanin pigment are called the melanophores, and play the major role on the color changes. The activity of the melanophores is caused by the intracellular aggregation or dispersion of the pigment granules, melanosomes, within a fixed shape, but not by the amoeboid movement of the cell.

Various mechanisms have been proposed to explain melanosome migration (for reviews see Fujii, 1969; Fujii and Novales, 1969; Bagnara and Hadley, 1973). However, at present none of the theories are satisfactory in accounting for all aspects of pigment migration. On the basis of measurement of the membrane potentials in the scale melanophores of *Oryzias*, Kinosita (1953, 1963) proposed that migration of the melanosomes, which were negatively charged, occurred by electrophoretic mechanism. However, some data recently reported are rather inconsistent with this electrophoretic theory (Martin and Snell, 1968; Fujii and Novales, 1969). Bikle et al. (1966) have shown that an ordered array of microtubules is present in the processes of the melanophores of the killifish, *Fundulus heteroclitus*, and have suggested that these

microtubules may be involved in melanosome migration. These observations have been confirmed by Novales and Novales (1966), Green (1968) in the same kind of fish and also in some other fishes (Fujii and Novales, 1969; Murphy and Tilney, 1974). Marsland (1944) on the basis of high hydrostatic pressure experiments came to the conclusion that pigment migration depends on the sol-gel transformation of the cytoplasm. High hydrostatic pressure caused a reversible breakdown of microtubules and also caused a dispersion of pigment (Marsland and Meisner, 1967; Murphy and Tilney, 1974). Colchicine decreased the rate of melanosome aggregation in the melanophores of *Fundulus heteroclitus* in response to adrenaline (Wikswo and Novales, 1968, 1969). Electronmicroscopically, treatment of the melanophores with colchicine resulted in a marked loss of microtubules in these cells (Wikswo and Novales, 1972; Murphy and Tilney, 1974). Thus, microtubules are more and more becoming implicated in melanosome migration. Moreover, Wikswo and Novales (1969) observed that cysteine reverses the effect of colchicine on the response of melanophores to adrenaline. This may indicate that the action of colchicine involves a reaction with sulfhydryl groups.

Present investigations, therefore, were designed to examine the effects of some sulfhydryl inhibitors on pigment migration in *Oryzias* melanophores. In addition, to gain further confirmation in the mechanism of action of these agents, experiments were tried to see if these effects could be reversed by cysteine.

Materials and Methods

Experiments were performed with isolated scales of *Oryzias latipes*. A scale carefully removed from the anterior dorsolateral region of the fish with the fine forceps was attached epidermal side down under a cover glass which was mounted on the perfusion chamber for microscopic observation, which was filled with physiological saline solution, which had the following composition: 128 mM NaCl, 2.6 mM KCl, 1.8 mM $CaCl_2$, pH 7.2 by $NaHCO_3$. The perfusion chamber in which the preparation was mounted was placed on the stage of a microscope (Fig. 1). The experimental solution was changed by means of a suction pipette operated by a stream pump. The activities of the melanophores were measured by a photoelectric method and simultaneously confirmed by microscopic observation. A cadmium sulphide was employed as a photoconductive cell, which was attached behind one of the eyepieces of the trinocular microscope. The activities of a single melanophore were exclusively measured. For this purpose, a diaphragm was placed at the plane of the real image inside the eyepiece, so that light transmitted through the restricted area of the scale could be projected on the photoelectric transducer. The photoelectric current was recorded on a paper chart recorder (Yokogawa Electric Works, 3051) at a driving speed of 20 cm per hour.

As pigment aggregating agents, isotonic KCl (pH 7.2 by $KHCO_3$) or 10^{-5} M adrenaline made up in the physiological solution was used. Igrosin (containing 0.1

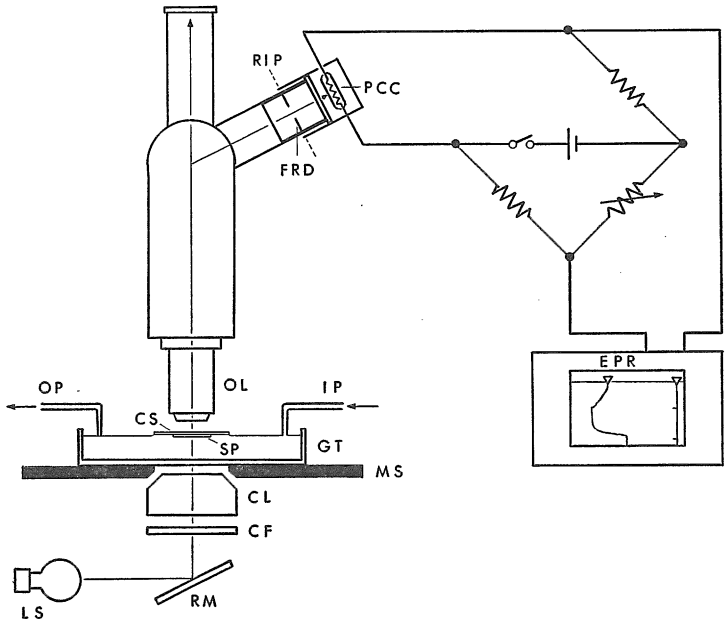


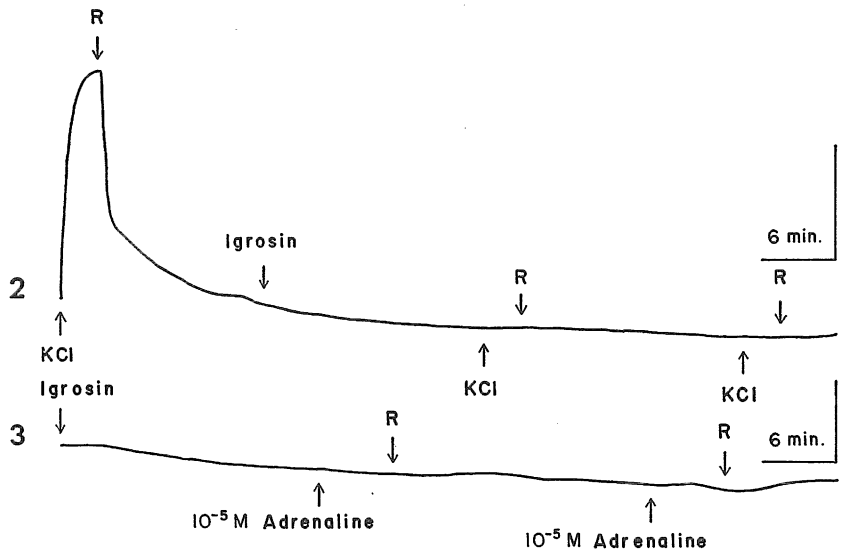
Fig. 1. A diagram of the experimental apparatus for recording melanophore responses *in vitro*.
CF, color filter; CL, condenser lens; CS, cover slip; EPR, electric paper recorder; FRD, diaphragm for field restriction; GT, glass trough; IP, inlet pipette for experimental solution; LS, light source; MS, microscopic stage; OL, objective lens; PCC, photoconductive cell; RIP, plane of real image; RM, reflecting mirror; SP, scale preparation.

g/ml Mersalyl, Sodium salicyl-(γ-hydroxymercuri-β-methoxypropyl) amino-O-acetate, (Takeda chemical Industrial LTD.), N-ethylmaleimide (Tokyo Kasei Co.). These agents were also dissolved in the saline. A 10^{-3} M or 10^{-2} M solution of cysteine hydrochloride (Kanto Chimical Co.) was made up in the saline before use. The pH of the solution was adjusted back to neutrality with a few drops of 0.1 N NaOH.

The experiments were carried out at room temperature between 19 and 24°C.

Results

Isotonic solution of KCl or 10^{-5} M adrenaline induces the full pigment aggregation in the innervated melanophores of *Oryzias latipes*. In these cases, *K* ions act on the pigment aggregating nerve endings and then the transmitter released from the endings brings about the melanosome aggregation in the cell. On the other hand, adrenaline, which is thought as a transmitter substance from the aggregating nerve endings, acts directly on the melanophores, thus causing centripetal migration of the



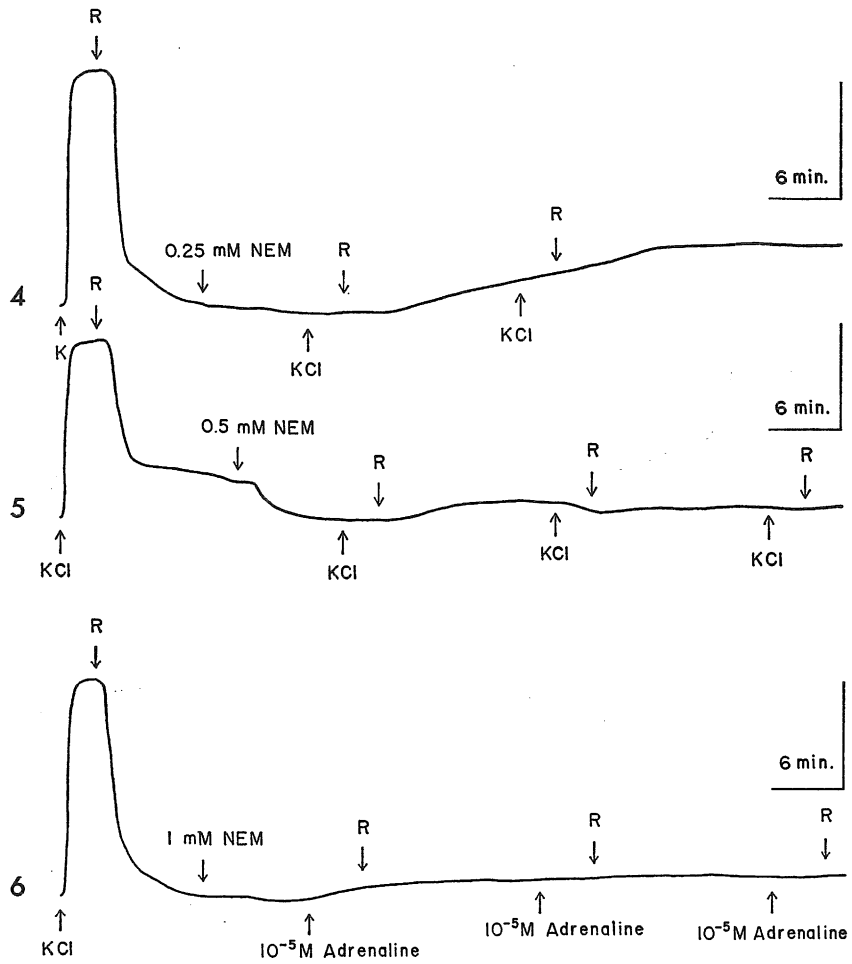
Figs. 2 and 3. The effect of Igrosin (10^{-3}M mersalyl) pretreatment on the response of melanophores to isotonic KCl or 10^{-5}M adrenaline.
R, Ringer solution (physiological solution). Calibration, Vertical lines indicate 50 percent of the maximal pigment aggregation. The same indications of the calibration are applied in all figures (Figs. 2–12).
Room temp., 20°C.

melanosomes.

Pretreatment of the solution of 10^{-3} M Igrosin (which contains mersalyl in a concentration of 10^{-3} M, the term of 10^{-3} M Igrosin will be used hereafter in stead of 10^{-3} M mersalyl for convenience) for 15 to 21 minutes blocked completely melanosome aggregation in the cell caused by application of KCl solution or also of adrenaline. The effect of Igrosin could not be removed by washing with the physiological saline. Typical examples of the effect on the pretreatment of 10^{-3} M Igrosin were shown in figures 2 and 3. Figure 2 indicated that 18 minutes' pretreatment with 10^{-3} M Igrosin blocked completely melanosome aggregation to isotonic solution of KCl and that the effect was not washed out by perfusion with the saline for another 18 minutes and even for 60 minutes, though it was not drawn in this figure. The same is true on the block to the action of adrenaline (Fig. 3).

Pretreatment of N-ethylmaleimide (NEM) caused also inhibition of melanosome aggregation in the cell to KCl or adrenaline. In the case of NEM, the action of KCl was effectively blocked by pretreatment of 0.25 mM NEM for 9 minutes. However, the treatment in this concentration induced slow, slight melanosome aggregation during the following perfusion with the saline (Fig. 4). This spontaneous aggregation of melanosomes did not observed with the pretreatment of NEM in higher concentra-

tion than 0.5 mM (Figs. 5 and 6). The pigment aggregating response of the melanophores to adrenaline was totally lost by pretreatment of 1 mM NEM for 9 to 15 minutes (Fig. 6). The effect of NEM could not be washed out by the saline, as shown in Fig. 6.

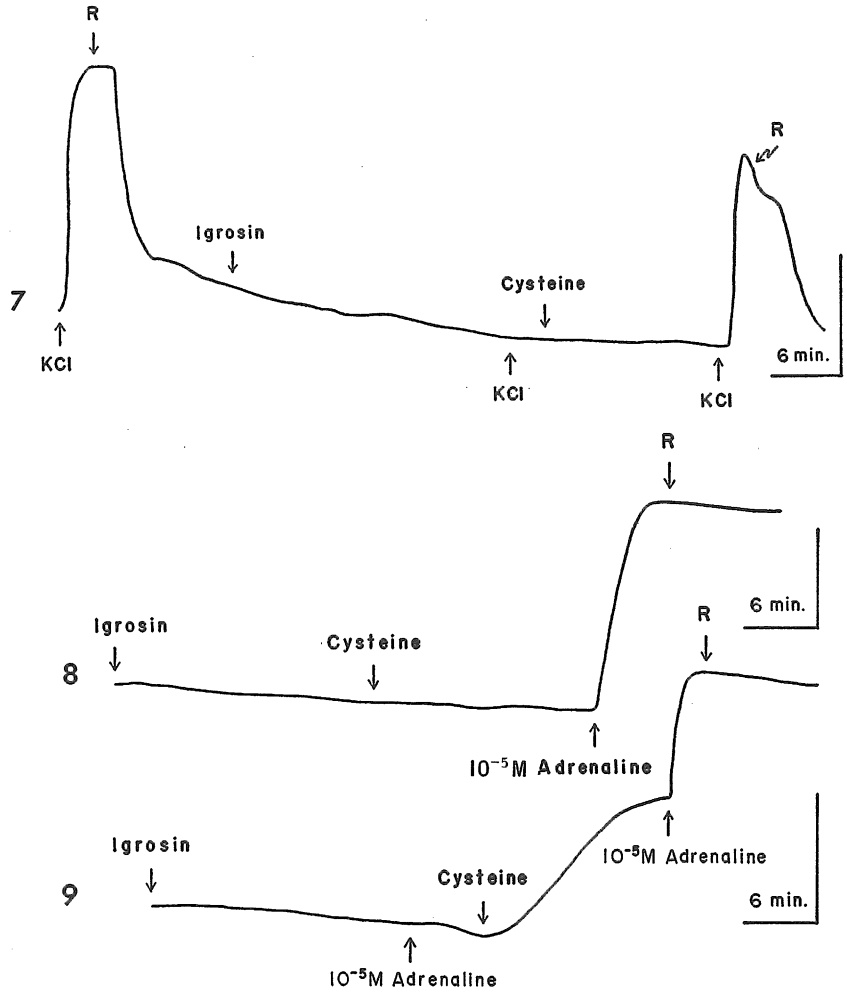


Figs. 4–6. The effects of N-ethylmaleimide (NEM) pretreatment on the response of the melanophores to KCl or 10^{-5} M adrenaline. R, Ringer solution. Room temp., 21°C.

Incubation in 10^{-2} M cysteine hydrochloride alone following the treatment with 10^{-3} M Igrosin had no effect on the melanophores. However, the effect of Igrosin on the response to KCl or adrenaline was removed by a 15 minutes' incubation in 10^{-2} M cysteine (Figs. 7, 8 and 9). That is, the melanophores which had been pretreated with Igrosin responded to KCl or adrenaline with pigment aggregation after

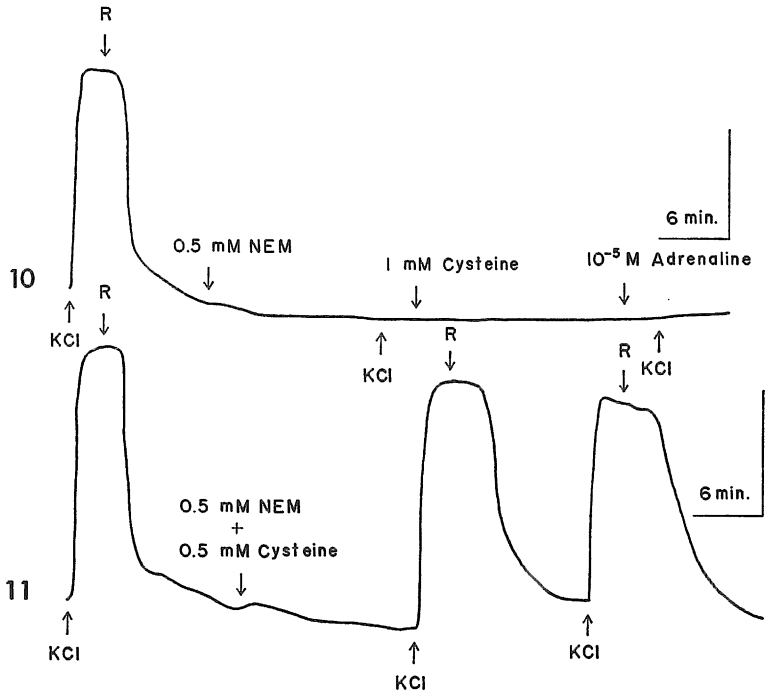
the adequate time of the incubation in cysteine. If adrenaline had been applied prior to cysteine incubation to determine the effect of Igrosin, then pigment aggregation in the cell began from 2 to 6 minutes after the application of cysteine (Fig. 9). As cysteine by itself had no effect on aggregation of melanosomes, thus such a sign of beginning of pigment aggregation may reflect the length of time required for the reversal by cysteine of the pigment aggregation mechanism in the cell which was impaired with Igrosin treatment.

The effect of NEM could not be removed by the incubation in cysteine (Fig. 10).



Figs. 7–9. The effects of Igrosin (10^{-3} M mersalyl) on the response of the melanophores to KCl or adrenaline and the reversal of its effects by cysteine (10^{-2}M). R, Ringer solution. Room temp., 21°C.

However, if NEM was applied in combination with cysteine, the effect of NEM was counteracted completely: the melanophores responded with pigment aggregation to KCl solution as the melanophores untreated with the mixture did (Fig. 11).



Figs. 10–11. The effects of NEM on the pigment aggregating response of the melanophores and the effect of cysteine. Fig. 10. The effect of postincubation in cysteine. Room temp., 23°C. Fig. 11. The effect of simultaneous application of NEM and cysteine. Room temp., 24°C.

Both Igrosin and NEM produced pigment dispersion in punctate adrenaline-treated melanophores. When the solution of 10^{-3} M Igrosin was applied to the punctate melanophores which were induced by pretreatment with 10^{-5} M adrenaline, melanosomes began to disperse within 2~3 minutes and attained to full dispersion within 10 minutes after the application of Igrosin (Fig. 12). The similar rapid dispersion of the melanosomes was also observed by 0.5 mM NEM, when the solution was applied on panctate adrenaline-treated melanophores. The speed of pigment dispersion was conspicuously greater than that produced by the physiological saline alone. After dispersion of the pigment the melanophores continued their expanded state in the saline. If the saline was exchanged with the solution of cysteine, pigment aggregation began after few minutes (Fig. 12). This aggregating response may depend on the remaining effect of adrenaline previously applied with the reversal of the effect of Igrosin

by cysteine. If Igrosin or NEM was applied on the melanophores which had kept the pigment dispersion in the saline in combination with adrenaline, temporary pigment aggregation was followed by rapid pigment dispersion. When the mixture in equimolar concentrations of NEM and cysteine was applied on punctate adrenaline treated melanophores, a pigment dispersion in the cells was also produced with the same rate as that produced by NEM alone. Cysteine by itself had no effect on dispersion of pigment: 0.5 mM cysteine had the same effect as the saline on punctate adrenaline treated melanophores.

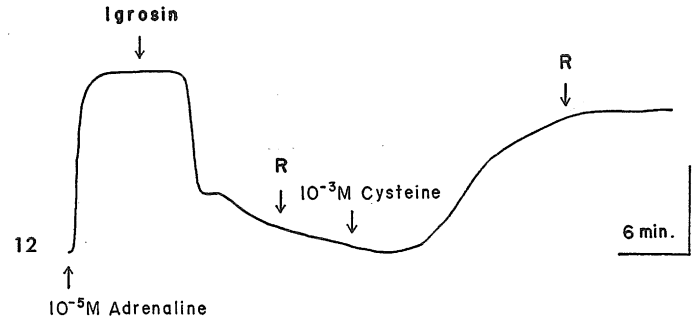


Fig. 12. Pigment dispersing effect of Igrosin (10^{-3}M mersalyl) on the punctate adrenaline-treated melanophores.
R, Ringer solution. Room temp., 20°C.

Discussion

On two sulfhydryl inhibitors used in the present experiments, there are differences in molecular structures and chemical properties. Mersalyl is one of organomercurials in its structure and blocks protein –SH groups through mercaptide formation. NEM departs from the organomercurial structure and acts to block –SH groups through an alkylating mechanism. Furthermore, NEM has greater selectivity for accessible –SH groups on protein molecules and passes easily through cell membrane (Jacob and Jandle, 1962; Kristen and Kuperman, 1970).

Pretreatment of each of the inhibitors blocked completely pigment aggregation in the melanophores of *Oryzias lapites* in response to KCl or adrenaline. It seems to be some differences in the inhibitory effects between two agents. The effect of the agents could not be removed by subsequent rinses in the saline alone. The effect of Igrosin was reversed with postincubation in cysteine. These results were similar to those of p-chloromerculibenzoate (PCMB), which is an organomercurial –SH inhibitor, observed by Yamada (1963) on the scale melanophores of *Oryzias latipes*, who found that pretreatment of PCMB suppressed pigment aggregation in the melanophores to KCl or adrenaline and the effect of PCMB was reversed by cysteine. Examining the action of colchicine, mersalyl or mercaptoethanol on the *Fundulus* melano-

phores, Wikswo and Novales (1968) found that preincubation of the melanophores in each of these agents resulted in a decrease in the rate of aggregation of melanosomes to adrenaline. From the inhibitory effects of sulfhydryl inhibitors, it is indicated that these agents act directly on the melanophores, blocking the pigment aggregation mechanism. The concentration in NEM by which melanosome aggregation in response to KCl was blocked was lower than that required for the block in response to adrenaline. Spontaneous, slow melanosome aggregation was observed during perfusion with the saline after treatment of 0.25 mM NEM. Assuming that such a pigment aggregation was induced by the transmitter leaked out from the nerve endings which had lost their normal physiological function by the effect of NEM, these phenomena will be understood. Thus, NEM may affect also the nerve endings to cause damage to their function. The blocking effect of NEM on pigment aggregating response could not be reversed even by cysteine, once the pigment aggregating mechanism was impaired with this agent. However, simultaneous application of NEM and cysteine had no effect on the pigment aggregating response to KCl. This indicates that the action of NEM involves a reaction with sulfhydryl groups. Thus, it is concluded that elements of protein –SH groups relate to the pigment aggregating mechanism in the melanophores.

Colchicine decreased the rate of pigment aggregation of *Fundulus* melanophores to adrenaline and its effect was reversed by cysteine (Wikswo and Novales, 1968, 1969). The similar effect of colchicine was observed in *Oryzias* melanophores (Iga, unpublished). Electron-microscopic studies revealed that microtubules in some teleost melanophores are disrupted with colchicine and the loss of microtubules in colchicine-treated cells correlates with changes in the rate of melanosome migration in response to adrenaline (Wikswo and Novales, 1972; Murphy and Tilney, 1974). As the inhibitory effects of sulfhydryl inhibitors on pigment aggregation were dramatic and in addition were completely reversed by cysteine, it is interesting to examine changes in the ultrastructural elements in the cells, which may be caused with sulfhydryl inhibitors, because it is still left to decide whether the elements of protein –SH groups which sulfhydryl inhibitors affected are something like microtubules or any one of another physiological processes.

Sulfhydryl inhibitors Igrosin and NEM produced pigment dispersion in punctate adrenaline-treated melanophores of *Oryzias latipes*. These inhibitors caused *in vitro* darkening of the skin of the lizard and the frog by dispersing melanosomes within dermal melanophores (Horowitz, 1957; Potter and Hadley, 1969). The dispersing effect of sulfhydryl inhibitors on *Oryzias* melanophores could not be inhibited by cysteine. Cysteine hydrochloride by itself had no effect on pigment dispersion. This may indicate that the action of sulfhydryl inhibitors on melanosome dispersion does not depend on a tying up of sulfhydryl groups.

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