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COLCHICINE AND CYTOCHALASIN B: A FURTHER CHARACTERIZATION OF THEIR ACTIONS ON CRUSTACEAN CHROMATOPHORES USING THE IONOPHORE A23187 AND THIOL REAGENTS

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Among the various examples of intracellular movement, one of the more easily studied is the migration of pigment granules within the cytoplasm of chromatophores. By controlling the distribution of the pigment within these cells, an animal possessing chromatophores is able to adjust its coloration to suit such purposes as camouflage (Sumner, 1935), temperature regulation (Wilkens and Fingerman, 1965), or protection from ultraviolet light (Coohill, Bartell, and Fingerman, 1970). When the pigment of a given chromatophore is dispersed throughout the cytoplasm, thereby covering a greater surface area, the tissue bearing the chromatophore takes on the tint of the latter. Alternatively, when the pigment is concentrated into the center of the cell, it contributes little to the coloration of the tissue.

As is the case for many other forms of intracellular movement, several studies have implicated microtubules and/or microfilaments as the agents responsible for translocation of pigment granules in chromatophores of vertebrates, crustaceans, and echinoderms. These studies have frequently employed colchicine, which has long been held to selectively disrupt microtubules (Borisy and Taylor, 1967a, b; Shelanski and Taylor, 1967) and cytochalasin B (CB), which has been promoted as a probe for microfilament involvement in cell functions by Wessells, Spooner, Ash, Bradley, Luduena, Taylor, Wrenn, and Yamada (1971).

Reports on CB action on chromatophores have been variable. The drug inhibits both pigment dispersion and concentration in crustacean and echinoderm chromatophores (Lambert and Crowe, 1973, 1976; Robison and Charlton, 1973; Fingerman, Fingerman, and Lambert, 1975; Dambach and Weber, 1975), enhances both dispersion and concentration in fish melanophores (Ohta, 1974), and inhibits pigment dispersion in frog melanophores while having variable effects on pigment concentration (Malawista, 1971b; McGuire and Moellmann, 1972; Novales and Novales, 1972; Magun, 1973; Fisher and Lyerla, 1974).

Colchicine has been found to inhibit pigment concentration in all chromatophores studied, in both vertebrates (Wright, 1955; Malawista, 1965, 1971a; Wikswo and Novales, 1969, 1972a; Schliwa and Bereiter-Hahn, 1973; Murphy and Tilney, 1974) and crustaceans (Lambert and Crowe, 1973, 1976; Fingerman *et al.*, 1975). The reported effects of colchicine on pigment dispersion are more variable. In frog melanophores the drug enhances the action of melanotropin (melanocyte-stimulating

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hormone, MSH) in bringing about pigment dispersion, and also causes dispersion itself (Malawista, 1965; Fisher and Lyerla, 1974). A similar situation has been reported for the teleost, *Fundulus heteroclitus* (Wikswo and Novales, 1969). In contrast, colchicine inhibits hormone-induced dispersion in erythrophores of at least one species of crustacean (Fingerman *et al.*, 1975) and does not itself cause pigment dispersion in melanophores of another (Lambert and Crowe, 1976). It is clear that, so far as pigment dispersion is concerned, colchicine's action on crustacean chromatophores differs markedly from its action on those of vertebrates. In contrast, pigment concentration by both types of chromatophore is inhibited by the drug. Wikswo and Novales (1972b) have suggested that colchicine exerts its inhibitory action by reacting with thiol groups, presumably of microtubules. To determine whether colchicine inhibits pigment concentration in crustaceans in the same manner in which it acts on vertebrates, the interactions of thiol reagents with the melanophores of *Uca pugilator* are examined in the first part of this paper.

That neither colchicine nor CB is as specific as once thought is now well established. For instance, both colchicine and CB have been found to inhibit a variety of plasma membrane transport systems (Mizel and Wilson, 1972a, b; Bos and Emmelot, 1974) and CB has even been found to bind with high affinity to the hexose transport proteins of the erythrocyte membrane (Lin and Spudich, 1974a, b). One of colchicine's non-microtubular actions appears to be interference with the mobility of intramembranous particles (Wunderlich, Müller, and Speth, 1973; Furcht and Scott, 1975). On the basis of experiments in which lumicolchicine, an isomer of colchicine which does not bind tubulin, was as effective as colchicine in inhibiting pigment concentration, Lambert and Fingerman (1976) suggested that: first, the inhibition of pigment concentration by colchicine was not due to microtubular disruption; and second, that colchicine might act at the level of the plasma membrane, perhaps interfering with the behavior of the hormone-receptor complex. Considering the diverse effects of CB in various systems, it was suggested that this drug might also act at the plasma membrane level (Fingerman *et al.*, 1975).

One way to distinguish whether or not these drugs act at the level of the hormone receptor, rather than on the pigment-moving machinery itself, is to examine their effects on pigment movement when the hormone-receptor system is bypassed. Such a bypass system has recently been devised, utilizing the calcium ionophore A23187 (Lambert and Fingerman, 1977). This ionophore has served as a useful tool for investigating the role of Ca^{++} in a variety of physiological processes, acting by shuttling Ca^{++} (or other divalent cations) across a biological membrane down the cation's concentration gradient (Pressman, 1976). When applied to erythrophores of the prawn, *Palaemonetes pugio,* the ionophore appears to mimic the action of the red pigment-concentrating hormone (RPCH), both agents presumably causing an influx of Ca^{++} which somehow activates the pigment-concentrating mechanism in the erythrophores (Lambert and Fingerman, 1977). The ionophore has also been recently applied to amphibian melanophores, where in contrast to the prawn erythrophores, pigment dispersion is induced (van de Veerdonk, Worm, Seldenrijk, and Heussen, 1977; Novales, 1977). In the second part of this paper, the effects of colchicine and CB on ionophore-induced pigment translocation are described.

Materials and Methods

Two crustacean species were used in this study; the fiddler crab, *Uca pugilator,* obtained commercially from the Gulf Specimen Company, Panacea, Florida, and the prawn, *Palaemonetes pugio,* collected near Irish Bayou, Louisiana. With the fiddler crab, the chromatophores studied were the melanophores on the merus of the third and fourth walking legs, using the perfusion technique of Fingerman (1956). This involved removing a pair of legs from each crab, inserting a 26 gauge needle into the distal end of each leg, and perfusing 0.1 ml of a given solution through the leg. One member of the pair would receive the test substance (*e.g.,* colchicine), the other a control solution (*e.g.,* physiological saline). Following perfusion, each leg was placed in a 0.1 ml drop of the same type of solution that had been perfused through it. This drop was placed in a petri dish whose air was saturated with water by the inclusion of pieces of filter paper soaked in distilled water. The saline used for the crab experiments was that of Pantin (1934). For experiments with the prawn, the large dark erythrophores of the ovaries were the chromatophores studied. The ovaries were removed and hemisected, one half being incubated in a control solution, the other half in the experimental solution. The physiological saline for these experiments was that of van Harreveld (1936). For both species, the responses of the chromatophores were recorded according to the scheme of Hogben and Slome (1931), in which stage 1 represents maximal pigment concentration, stage 5 maximal dispersion, and stages 2, 3, and 4 the intermediate conditions.

Of the substances tested, colchicine, cysteine hydrochloride and mersalyl acid were obtained from Sigma and glutathione was purchased from Calbiochem. CB (Aldrich) was dissolved in dimethyl sulfoxide (DMSO) and stored at 4° C as a stock solution of 1 mg/ml. The ionophore A23187, a gift from Dr. R. Hamill, Eli Lilly Co., was also dissolved in DMSO, to give a 5 mM stock solution which was stored at 4° C in the dark.

Preparation of melanophores of *Uca pugilator* for electron microscopy involved fixation in 6% glutaraldehyde in 0.1 M cacodylate buffer, osmolarity adjusted to 1100 with NaCl, the fixative being delievered to the cells by perfusing it through isolated legs on ice for 45 min. Post-fixation with OsO_4 was omitted to avoid its degrading action on actin filaments. Following fixation, the tissue was dehydrated through an acetone series, embedded in Spurr's (1969) epoxy resin, and sectioned with a Porter-Blum MT-2 microtome. Sections were collected on formvar-coated grids, then stained with 4% methanolic uranyl acetate at 65° C for 15 min, followed with lead citrate. The stained sections were examined with a Siemens Elmiskop 1A transmission electron microscope at an accelerating voltage of 80 *k*V.

Results

Sulfhydryls

The suggestion by Wikswo and Novales (1972b) that colchicine exerts its inhibitory action by reacting with thiol groups resulted from their studies with,

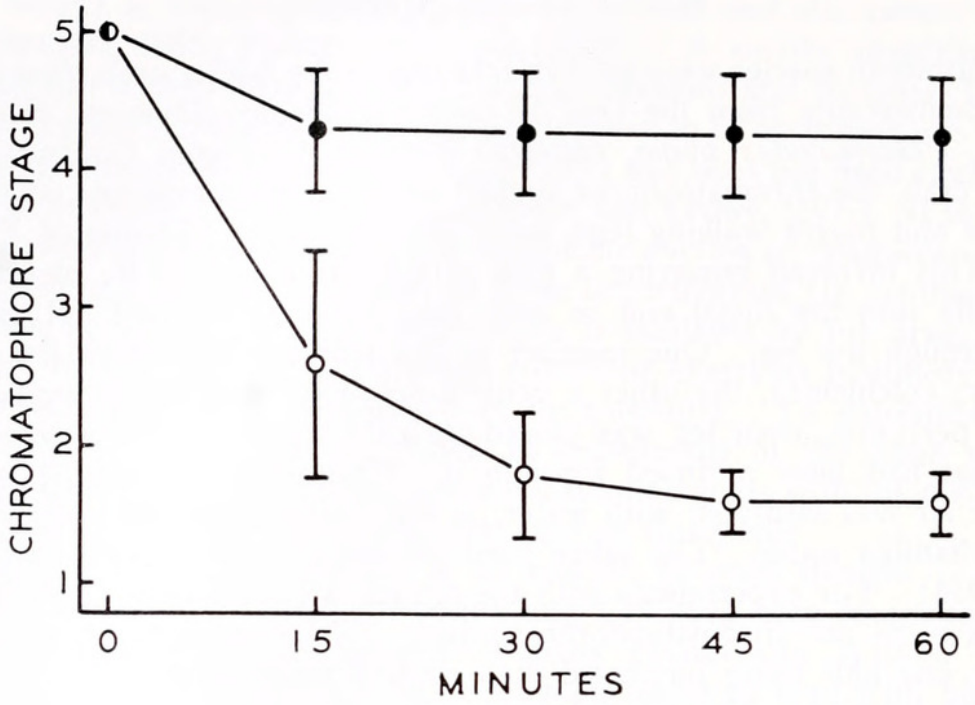

FIGURE 1. Effect of mersalyl acid on pigment concentration in leg melanophores of *Uca pugilator*. Opposite legs were removed; the experimentals (dots) were perfused wth saline containing 37 mM mersalyl acid, the controls (circles) with saline alone. Five pairs were used. Vertical lines representing one standard deviation on each side of the mean are included in this and the following graphs.

among other reagents, glutathione, cysteine hydrochloride, and mersalyl acid. To further characterize the site of action of colchicine in crustacean chromatophores, these reagents were applied to the melanophores of *Uca,* using the isolated leg-perfusion technique. As has been described previously, perfusing legs with saline brings about pigment concentration; when high concentrations of colchicine are included in the saline, this pigment movement is inhibited (Lambert and Crowe, 1973). To determine whether glutathione or cysteine hydrochloride could prevent colchicine's inhibition of pigment concentration, as was reported for the melanophores of *Fundulus* (Wikswo and Novales, 1972b), these reagents were mixed with colchicine and perfused through isolated legs. The control legs were perfused with colchicine alone. At the three concentrations used (50, 100, and 155 mM), glutathione was unable to prevent inhibition by 15 mM colchicine (data not shown). Similarly, 210 mM cysteine hydrochloride did not counteract inhibition by 21 mM colchicine (data not shown). In contrast to the differences between the melanophores of *Uca* and *Fundulus* described above, pigment concentration is inhibited in the melanophores of both upon treatment with mersalyl acid. As seen in Figure 1, perfusing mersalyl acid (37 mM) through isolated legs greatly inhibits pigment concentration in the melanophores of these legs, as compared to the controls perfused with saline alone. Lower concentrations of mersalyl acid (10 mM) were ineffective (data not shown). In contrast to colchicine's action, the inhibitory potential of mersalyl is negated by mixing it with 155 mM glutathione before perfusing it through the legs (Fig. 2).

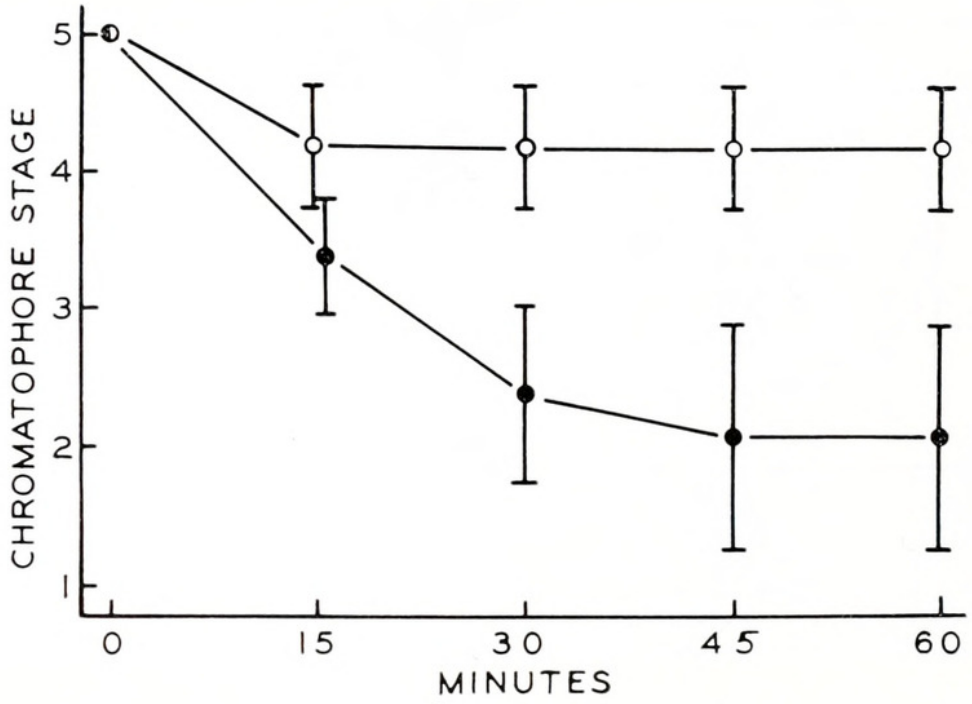

FIGURE 2. Effect of glutathione on mersalyl acid inhibition of pigment concentration in leg melanophores of *Uca pugilator*. Opposite legs were removed; the experimentals (dots) were perfused with saline containing 37 mM mersalyl acid plus 155 mM glutathione, the controls (circles) with saline containing 37 mM mersalyl acid alone. Five pairs were used.

A23187/colchicine effects

To determine whether colchicine acts at the hormone-receptor level or at some more distal site (*e.g.*, microtubules), its ability to prevent ionophore-induced pigment movements was examined, using the ovarian erythrophores of *Palaemonetes pugio*. As seen in Figure 3, I, erythrophores treated with 25 mM colchicine responded as well to the ionophore as untreated cells, even after a 30 min preincubation in the colchicine. The presumed influx of Ca^{++} from the medium into the cells caused rapid pigment concentration in both cases. In contrast, colchicine appears to inhibit the pigment dispersion that normally occurs when A23187-treated erythrophores are exposed to Ca^{++}-free/EGTA-containing saline (Fig. 3, II). The erythrophores on four of the five control ovary halves used (*i.e.*, those not treated with colchicine) dispersed their pigment, presumably due to the efflux of Ca^{++}, while all of the colchicine-treated erythrophores remained in a concentrated condition. The degree of re-dispersion was variable among the controls, probably due to damage to the erythrophores when the ovaries were bisected. Ionophore-treated cells appear to respond to Ca^{++}-free/EGTA saline best when treated very gently.

Cytochalasin B (CB) and A23187

To characterize further the inhibitory action of cytochalasin B on pigment concentration in crustacean chromatophores, its effect on A23187-induced pigment concentration was studied using ovarian erythrophores of *Palaemonetes pugio*.

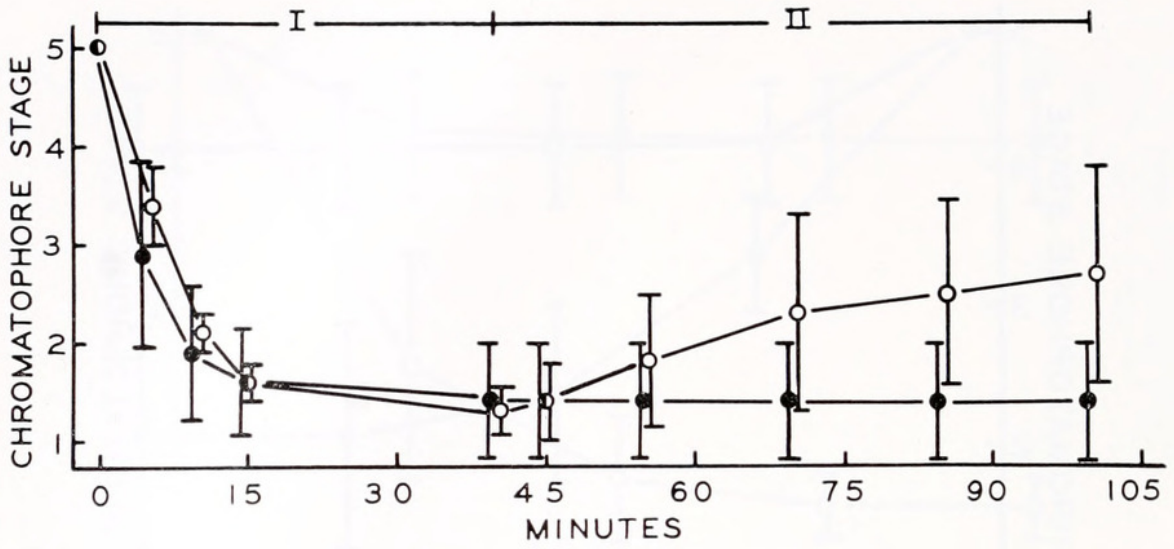

FIGURE 3. Effect of colchicine on ionophore A23187-mediated pigment movement in ovarian erythrophores of *Palaemonetes pugio*. Ovaries were removed and bisected, the experimental halves (dots) being pre-incubated in saline containing 25 mM colchicine and the controls (circles) in saline alone. After 30 min (at t = 0 min), the ionophore was added to all incubation media to a concentration of 25 μM, starting phase I. Forty minutes later, the test solutions were replaced with Ca^{++}-free/5 mM EGTA saline containing (dots) or lacking (circles) 25 mM colchicine, starting phase II. Five ovaries were used.

As seen in Figure 4, those erythrophores treated with CB respond less well than untreated cells to ionophore-induced pigment concentration. This inhibition is, however, partially reversed by the removal of CB and addition of fresh ionophore.

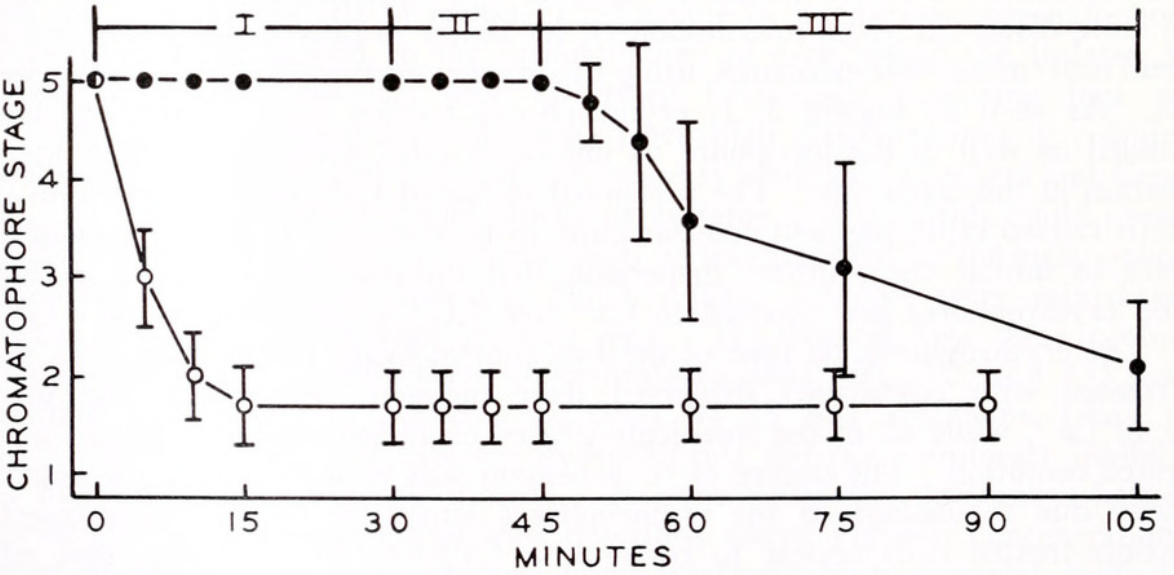

FIGURE 4. Effect of cytochalasin B on ionophore A23187-induced pigment concentration in ovarian erythrophores of *P. pugio*. Ovaries were removed and bisected, the experimental halves (dots) then being pre-incubated in 0.2 ml saline containing 1% DMSO and 10 μg/ml CB and the controls (circles) in saline containing 1% DMSO alone. After 20 min pre-incubation (at t = 0 min), the ionophore was added to all test solutions to a concentration of 25 μM, initiating phase I. At the start of phase II, all ovary halves were washed with fresh saline. At the start of phase III, this saline was replaced with fresh saline containing 25 μM A23187 and 0.5% DMSO. Ten ovaries were used.

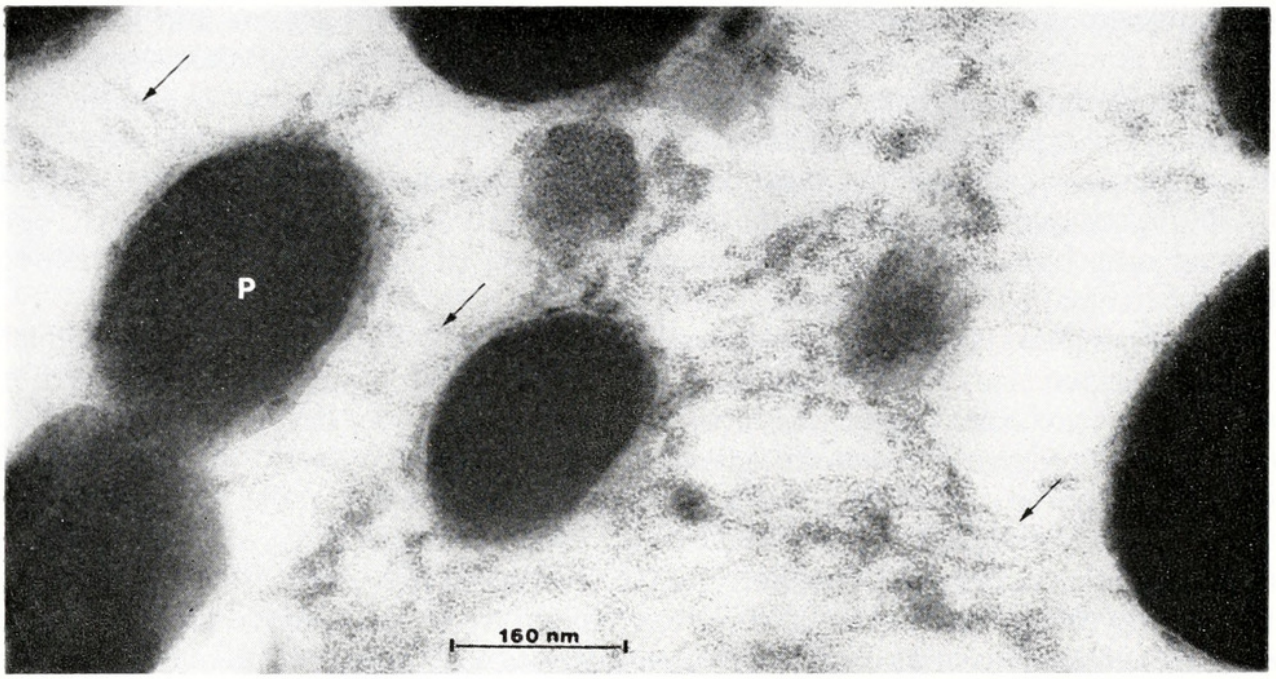

FIGURE 5. Electronmicrograph of a melanophore process from *Uca*. Several pigment granules (P) are seen in this portion of a process. They appear to be interconnected by the elements of the filamentous matrix (arrows). Bar represents 160 nm.

Electron microscopy

Earlier attempts at finding actin-like filaments in crab melanophores through the use of electron microscopy have been unsuccessful (Lambert, 1975). A possible explanation for this was recently provided by the observation of Maupin-Szamier and Pollard (1978) that OsO_4 degrades purified actin filaments. Since OsO_4 was included in the earlier fixation procedures for the crab melanophores, it was decided to try another fixation using only glutaraldehyde. Using the fixation procedure described under Materials and Methods, the cytoplasm of the melanophores of *Uca pugilator* was seen to consist of irregular filamentous elements which appear to contact, and interconnect, the pigment granules (Fig. 5). The dimensions of these elements are variable, ranging from 2.5 to 25 nm. In addition, they appear to coalesce frequently, both with each other and with more amorphous aspects of the matrix.

DISCUSSION

The attempt of this study to characterize further the modes of action of colchicine and CB on crustacean chromatophores has provided further evidence that microtubules are not necessary for pigment concentration, though a role in dispersion remains a possibility. The site of action of CB remains unclear.

A number of lines of evidence now indicate that colchicine's inhibitory action on pigment concentration in crustacean chromatophores is not mediated by microtubular disruption. One is the observation by Fingerman *et al.*, (1975) that very high doses of colchicine (25 mM) are required to inhibit pigment concentration in erythrophores of *Palaemonetes vulgaris,* while much lower doses (1 mM)

are sufficient for microtubular disruption in these cells (Robison and Charlton, 1973). Another is the observation that pigment concentration in melanophores of *Uca pugilator* is inhibited by high concentrations of colchicine (Lambert and Crowe, 1973), but similar concentrations of lumicolchicine, the UV-light derivative of colchicine which does not bind tubulin, have been shown to be equally effective in inhibiting pigment concentration (Lambert and Fingerman, 1976). If colchicine were acting by disrupting microtubules, then one would not expect lumicolchicine to mimic its action.

The ionophore studies reported here and elsewhere (Lambert and Fingerman, 1977) also tend to discount microtubular involvement in pigment concentration in crustacean chromatophores. It is apparent from these studies that an influx of Ca^{++} causes pigment concentration, yet Ca^{++} has been found by Weisenberg (1972) to inhibit microtubule polymerization and by Haga, Abe and Kurokawa (1974) and Gaskin, Cantor, and Shelanski (1975) to cause disassembly of existing microtubules. In fact, the ionophore A23187 has recently been employed by Schliwa (1976) to cause shortening of the axopodia of the heliozoan, *Actinosphaerium eichhorni,* this shortening being accompanied by a Ca^{++}-mediated depolymerization of microtubules. Since colchicine inhibits pigment concentration in crustacean chromatophores, whereas ionophore-induced Ca^{++} influx causes pigment concentration, and since both agents depolymerize microtubules, it is difficult to see how microtubules can be involved in pigment concentration in these chromatophores.

As explained below, employing the ionophore in conjunction with colchicine has helped to localize the site of action of the latter. When erythrophores of *Palaemonetes pugio* were treated with colchicine before exposure to A23187, it was found that the colchicine had no effect on ionophore-induced pigment concentration (Fig. 3). It appears then that the high concentrations of the drug necessary for its inhibitory action on hormone-induced pigment concentration (Fingerman *et al.,* 1975) may in fact be interfering with the initial events of hormone action—those preceding Ca^{++}-mediated actions and not involving microtubules. One target would be the plasma membrane, since colchicine has been found to interact with non-microtubular plasma membrane proteins of other cells (Mizel and Wilson, 1972a; Wunderlich, Müller, and Speth, 1973; Bos and Emmelot, 1974; Stadler and Franke, 1974; Furcht and Scott, 1975). Exactly how the drug would interact with the chromatophore membrane remains to be determined, however. The earliest action that it might have would be interfering somehow with the hormone's interaction with its receptor. Another possibility would be events immediately following the binding, such as those mediated by the movement of integral proteins within a fluid mosaic membrane (Singer, 1974). If, as we have suggested earlier (Lambert and Fingerman, 1977), RPCH acts in conjunction with its receptor as an ionophore, high concentrations of colchicine may prevent proper association of the integral proteins which, upon binding RPCH, normally form the ionophore complexes.

It should be noted that, whereas colchicine does not inhibit A23187-induced pigment concentration, it does inhibit somewhat the dispersion of Ca^{++}-concentrated pigment that normally results from the removal of Ca^{++} from erythrophores via an EGTA/ionophore saline (Fig. 3). Hence, the possibility remains that microtubules could be involved in pigment dispersion, although the inhibition observed

may have simply been due to non-specific poisoning by the long exposure to the high concentration of the drug.

While it seems clear that colchicine does not inhibit pigment concentration in crustacean chromatophores via disruption of microtubules, it is premature to extend this interpretation to the chromatophores of vertebrates: the results of the sulfhydryl experiments reported here, contrasted with those of Wikswo and Novales (1972b), indicate that colchicine may act on different targets in vertebrates and crustaceans. These investigators reported that both glutathione and cysteine hydrochloride prevent colchicine from inhibiting pigment concentration in melanophores of *Fundulus heteroclitus*. In contrast, these agents have no such effect when applied to the melanophores of *Uca,* even at concentrations much higher than those used by Wikswo and Novales. There is, however, some similarity between the melanophores of these two species: pigment concentration is inhibited by mersalyl acid in both cases, and this is prevented by glutathione (Figs. 1, 2).

CB has now been found to inhibit A23187-induced pigment concentration in prawn erythrophores (Fig. 4) in addition to its reported effects on RPCH action (Robison and Charlton, 1973; Fingerman *et al.,* 1975). It is of possible interest that the drug also inhibits ionophore action on amphibian melanophores, where the ionophore induces pigment dispersion rather than pigment concentration (Novales, 1977). In the present experiment, the effects of CB could not be reversed simply by exposing the erythrophores to fresh saline lacking the drug. A similar delay in reversibility was reported by Fingerman *et al.,* (1975) for CB inhibition of RPCH-induced pigment concentration. Only after a second medium change, to saline now containing RPCH (Fingerman *et al.,* 1975) or A23187 (Fig. 4, III), do the erythrophores recover their ability to concentrate their pigment. The delayed response may be due to the time or number of washes necessary for the cytochalasin to diffuse out of the cells to yield an intracellular concentration low enough to permit pigment movement. Alternatively, the additional stimulus by the second application of hormone or ionophore may be required to override the drug's inhibitory action. Since CB inhibits both hormone- and ionophore-induced pigment concentration, it seems probable that it is acting on the pigment concentrating machinery itself. A similar situation may apply to the pigment dispersing machinery: Lambert and Crowe (1973, 1976) have found that CB inhibits both hormone- and cAMP-induced pigment dispersion in melanophores of *Uca pugilator,* indicating that its action is exerted at some step beyond the postulated hormonal activation of adenylate cyclase, possibly at the level of the pigment-dispersing machinery. It is possible, therefore, that the same organelles may be involved in both pigment dispersion and concentration.

In contemplating the nature of these organelles, the rapid Ca^{++}-mediated contraction of muscle, as well as the possible control by Ca^{++} of actomyosin systems in many non-muscle cells (see Clarke and Spudich, 1977, for recent review) comes to mind. It is worth noting that, while CB has a variety of other actions, there is good recent evidence that the drug interferes with the actin networks of several non-muscle cells (Weihing, 1976; Hartwig and Stossel, 1976; Weber, Ranthke, Osborn and Franke, 1976; Osborn, Franke, Weber, 1977). To date, attempts to convincingly demonstrate the presence of actin filaments in crustacean chromatophores have been unsuccessful, although this may simply be due to

technical problems. It is worth noting, however, that there is a precedent for the occurrence of non-actin, non-microtubule systems in cellular movement: the contractile organelle of vorticellid ciliates, the spasmoneme, consists primarily of proteins, called spasmins, which appear not to be actin (Routledge, Amos, Gupta, Hall, and Weis-Fogh, 1975; Amos, Routledge, and Yew, 1975). Interestingly, these spasmins bind Ca^{++}, and this binding apparently results in a conformational change in the proteins, culminating in contraction of the entire organelle. It is tempting to speculate that the filamentous matrix seen interconnecting the pigment granules of crustacean chromatophores might behave in an analogous manner, Ca^{++} binding to the subunits of the network, causing conformational changes which lead to contraction of the network, and hence pigment concentration. In this regard, Byers and Porter (1977) have recently described a three-dimensional "trabecular network" interconnecting the pigment granules in erythrophores of *Holocentrus ascensionis*. This network appears to contract upon pigment concentration, pulling the granules with it. A similar network has been reported by Schliwa and Euteneuer (1978) to be present in the melanophores of *Pterophyllum scalare;* these latter investigators suggest that this network may be the principal agent involved in pigment granule concentration in fish chromatophores, the microtubules perhaps assisting in guiding the pigment movement. So far as pigment dispersion in crustacean chromatophores is concerned, one might speculate further that microtubules are involved, since colchicine inhibits pigment dispersion induced by red pigment-dispersing hormone (RPDH) (Fingerman *et al.,* 1975) and, possibly, by A23187/EGTA-mediated Ca^{++} efflux.

In keeping with the scenario proposed earlier (Lambert and Fingerman, 1977), that RPCH acts via Ca^{++} influx and RPDH via cAMP production, the following heuristic model of hormonal control of pigment granule translocation is proposed. Upon RPDH binding, adenylate cyclase is stimulated and the resultant cAMP increase stimulates microtubule polymerization. The elongating microtubules push the pigment granules out into the processes of the cell, either directly or via the filamentous matrix. Upon binding the opposing hormone, RPCH, Ca^{++} enters the cells and binds to the filamentous matrix (whatever its makeup), causing it to contract and pull the pigment granules toward the center of the cell. In addition, the Ca^{++} influx depolymerizes the microtubules, further enhancing pigment concentration. RPCH action would, therefore, easily override that of RPDH. Redispersion of pigment would occur when RPCH is removed, allowing the intracellular Ca^{++} level to return to its low "resting" value and RPDH-increased cAMP levels to re-stimulate microtubular polymerization. This model, while merely a working hypothesis, contains a number of testable ideas, and may prove useful in further examination of the physiology of crustacean chromatophores.

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Summary

1. Using the prawn, *Palaemonetes pugio,* and the fiddler crab, *Uca pugilator,* the modes of action of cytochalasin B and colchicine in inhibiting pigment granule movement were further characterized.

2. Colchicine was unable to inhibit, in ovarian erythrophores of *Palaemonetes pugio,* pigment concentration caused by the ionophore A23187. It did, however, inhibit somewhat the pigment dispersion that normally occurs upon placing A23187-treated erythrophores in Ca^{++}-free, EGTA-containing saline.

3. Inhibition by colchicine of pigment concentration in melanophores of *Uca pugilator* was not prevented by high concentrations of glutathione or cysteine hydrochloride. Pigment concentration was inhibited by mersalyl acid, and this was prevented by glutathione.

4. Cytochalasin B inhibited ionophore A23187-induced pigment concentration in ovarian erythrophores of *Palaemonetes pugio.*

5. Electron microscopy revealed a filamentous matrix interconnecting the pigment granules of *Uca pugilator.*

6. Speculation is presented as to the modes of action of cytochalasin B and colchicine, and a heuristic model for the control of pigment movement is put forward.

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