STUDIES ON THE MECHANISMS OF PIGMENT TRANSLOCATION IN FISH CHROMATOPHORES

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Contents

I. General introduction

Body coloration of lower vertebrates is formed by integumental pigment cells called chromatophores (1). The chromatophores are generally divided into three categories, melanophores, erythrophores or xanthophores, and leucophores or iridophores, which respectively attribute to black to brown, red to yellow, and white to reflective coloration. The coloration of chromatophores is formed by four different types of pigments specific to each category, melanin in the first category, pterin and/or carotenoid in the second and purine in the third. All these pigments are distributed within specific cytoplasmic granules called melanosomes, pterinosomes and/or carotenoid vesicles, and iridosome or reflecting platelets (1).

These chromatophores display synchronous centripetal (aggregation) or centrifugal (dispersion) translocation of pigment organelles in response to stimuli provided by neurotransmitters (epinephrine, norepinephrine), or hormones [melanocyte-stimulating hormone (MSH), melatonin or melanin concentrating hormone (MCH)] (1, 2). Rapid body color changes seen in lower vertebrates are caused by intracellular translocation of pigment granules occurring in their integumental chromatophores. This type of color change is called physiological color change and discriminated from slow morphological color changes which result from an increase or decrease of pigment cells and their pigments.

Pigment translocation in fish chromatophores has provided a unique model of cell motility in that centripetal and centrifugal migration of pigment granules alternate repeatedly within the cell, running in the certain direction under the influence of a definite neurotransmitter or hormone. For this reason, pigment translocation in chromatophores is considered to be an excellent, unique model for studies of non-muscle cell movement.

A number of hypotheses have so far been presented about the mechanisms of this movement as reviewed in a current article (3), emphasizing either an interaction of the electric charge of melanosomes and the membrane potentials (4) or the functional role of microtubules (5, 6, 7, 8) and microtrabeculae (9) or the active participation of microfilaments as a contractile machinery (10, 11, 12). Recent studies on pigment translocation of fish chromatophores have emphasized possible participation of dynein-microtrabecular systems (8, 9) but their results seem still immature for complete elucidation of the mechanisms of this movement (3, 13).

This study, I attempt to clarify the mechanisms which generate the motive force for pigment migration, with a particular reference to the role of actin. The emphasis on the role of actin in this movement is prompted by the evidence obtained from swordtail (Xiphophorus helleri) erythrophores. During an early phase of this study, pigment displacement in these cells was found to be inseparably

associated with streaming of cytosol (14). It is a well-known fact that cytoplasmic streaming is generated by actin filaments installed along cytoplasmic corteces in Nitella (15) and slime mold (16). Based on these findings, actin is taken as one of the most promising functional elements in this unique motility.

In part 1 of this report, the fundamentals of pigment translocation are studied using swordtail erythrophores as the material. These cells are considered to be an excellent material for the studies of this movement, inasmuch as they contain two different kinds of pigment organelles, pterinosomes and carotenoid vesicles in a single cell, both of which migrate toward the same direction simultaneously despite their different morphological properties and biochemical compositions (17). The presence of pigment granules different from melanosomes is expected to provide new information different from that on melanophores containing one kind of pigment granules. Simultaneous translocation of two kinds of pigment organelles with different characters will support the view that pigment granules move passively along a cytoplasmic flow which conveys all movable cytoplasmic ingredients without selection of particular particles such as melanosomes or pterinosomes. Along this line of thought, possible involvement of microfilaments and actin in the generation of such cytoplasmic flow is investigated. In this part, the following subjects are dealt with : 1) the kinetics of pigment movement as analyzed by means of autorecording microphotometry and

cinematography. 2) changes in cell shape and surface structures during the movement as observed by means of scanning electron microscopy (SEM). 3) changes in the intracellular localization of microfilaments or actin filaments during the movement as examined by means of transmission electron microscopy (TEM), immunofluorescence and immunocytochemical electron microscopy, 4) experimental modulation or blockade of pigment displacement by means of microinjection of the anti-actin antibody produced against carp skeletal muscle actin and 5) experimental demonstration of the occurrence of a cytoplasmic flow as studied by tracing intracellularly microinjected exogeneous paraffin droplets.

In part 2 of this study, pigment translocation variants of goldfish erythrophoroma cells (a neoplastic counterpart of erythrophores) are used to study the implication of actin isoforms in aquisition of the ability for pigment translocation. This approach becomes possible by the establishment of permanent cell lines of goldfish erythrophoroma cells and subsequent development of the method to induce melanogenic, translocation-variants from these cell lines (18). The biochemical studies on cell motility of normal pigment cells have long been disabled by the difficulty of obtaining them as a homogeneous material sufficient for such study. Recently, it has become possible, however, to separate enough amounts of various cell clones with different pigmentary characters and motile response from erythrophoroma cell lines (19). The most

useful variant clones induced from these cell lines are melanogenic. Some of these clones manifest responsiveness to neurotransmitters or hormones in a fashion similar to normal melanophores, and others show no responsiveness and motility. In addition, stem cell lines are also isolated from parent population of these cell lines, which have the potential to differentiate into melanogenic, translocation variants by chemical induction of differentiation. Using such variants including stem cell (melanoblastoma cell) clones, non-motile melanogenic (melanocytoma), and motile melanogenic (melanophoroma), the relationship between the composition of contractile proteins, particularly actin, and acquisition of the ability for pigment translocation is studied.

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II. Part I

A study on swordtail erythrophores

Section 1

Kinetics of pigment movement and its related morphological changes

1-1. Introduction

Pigment translocation in fish integumental melanophores has provided us with a unique model of cell motility. In the cell, melanosomes repeatedly migrate centripetally and centrifugally and their migratory direction is constant under the influence of a definite neurotransmitter or hormone (1, 2, 3, 4, 5, 6). Accumulated information discloses that a wide variety of non-melanogenic pigment cells of fish, erythrophores, xanthophores and iridophores, also translocate their own pigment organelles. These organelles are different in their morphology and pigment composition according to the cell type, i. e., melanosomes, pterinosomes, carotenoid vesicles or droplets and iridosomes or reflecting platelets (1, 6, 7, 8, 9, 10, 11). This pigment displacement is thought to be based on the cellular mechanisms which carry away every motile cytoplasmic component, without imposing any restriction on the nature of pigment particles.

The erythrophore in swordtail fish Xiphophorus helleri is an excellent material for studies of this motility. Because their pigmentation is composed of two kinds of pigment granules, pterinosomes and carotenoid vesicles, both pigment granules migrate synchronously in response to the same stimulus. SEM revealed that these cells change their shapes and surfacial structures markedly upon pigment displacement and that the behavior of the cell surface is apparently different between the two phases of this

movement (12). From these observations, pigment particles seem to be conveyed by a kind of cytoplasmic flow which is generated through activities coupled with cell membranes and cortices. It is also suggested that the procedures for generating such a flow are different between pigment aggregation and dispersion.

As to the mechanisms of pigment migration, a number of hypotheses have so far been presented. The main hypotheses are 1) a theory of electrophoresis in which it is believed that melanosomes are moved by an electric charge between melanosomes and membrane potentials (13), 2) a sliding theory which proposes that melanosomes migrate along microtubules, 3) a theory of microtrabeculae which proposes that structural changes of microtubule-based meshworks cause pigment movement (6, 13-26). Considering the cell surfacial changes in swordtail erythrophores undergoing pigment displacement (12), it is likely that cytoplasmic microfilament is a key functional element in this motility. This study will deal with: 1) the kinetics of pigment movement in swordtail erythrophores, 2) the changes in their cell shape and surfacial structures at this movement.

1-2. Materials and methods

Experimental treatment of erythrophores

Homogeneous red-colored swordtail fish (Xiphophorus helleri) were provided from an inbred stock maintained in our laboratory. Pigment aggregation in erythrophores (both cultured and in isolated scales) was induced by treating them with $5x10^{-4}$ M epinephrine (Merck, Darmstadt) dissolved in either isotonic physiological buffered saline (PBS)(123.2 mM NaCl, 2.4 mM KCl, 7.3 mM Na_2HPO_4, 1.3 mM KH_2PO_4 pH 7.3) or complete culture medium at 25°C (8)(cf. the method for cell culture). The response of erythrophores was monitored under a dissecting or culture microscope. Inasmuch as cell pigments were tightly aggregated in the cell center within a few minutes of such treatment, cells exposed to epinephrine for 10 minutes were taken to have completely aggregated pigments. For examination of intermediate phases of pigment aggregation, cells were exposed to this catecholamine for varying lengths of time up to 10 minutes and observed at optimum times as shown in the Results.

Cell culture of integumental erythrophores

Monolayer cultures of erythrophores were prepared first by digesting scales and skin pieces with 0.25% collagenase (Worshington, Type III, Freehold, N.J.) in CMF-PBS for 30 minutes with mild agitation at room temperature. Second digestion was performed with 1.0%

trypsin (Difco, 1:250, Detroit) under the same conditions. Dissociated erythrophores were collected by low-speed centrifugation and plated on sterile 10 mm^2 plastic (Falcon, Oxford) or 18mm^2 glass cover slips with or without coating of reconstituted collagen (8). Most cells were plated at a suitable density to avoid overlapping and maintained in a milieu composed of 7 parts L-15 medium (GIBCO, New York), 2 parts fetal calf serum (Microbiological Association, Maryland), 1 part redistilled water, 100 µg/ml streptomycin (Meiji, Tokyo) and 100 IU/ml penicillin (Meiji, Tokyo). Experiments were performed on the third to fourth day after plating.

Scanning electron microscopy (SEM)

The erythrophores firmly anchored on the glass cover slips were fixed without any treatment to observe the dispersed state. For the observations of the aggregated state, cells were fixed at an approximate interval of 2 minutes after the exposure to 5 x 10^{-4} M epinephrine, dissolved in either PBS or complete culture medium. The effects of colchicine (Sigma, St. Louis), cytochalasin B (Aldrich, Milwaukee) and ouabain were examined by exposing several specimens of the cultured cells to culture media containing these drugs at concentrations of 10^{-3} M, 2 x 10^{-5} M and 10^{-3} M, respectively, before the treatment with epinephrine. Prefixation was made in 2.5% glutaraldehyde (Polysciences, Warrington) in 0.1 M cacodylate buffer, pH 7.2 for 30 minutes at room temperature and then rinsed in the above buffer.

Photomicroscopy was used at this stage of processing with selected erythrophores to record the location of moving pigments. The cells were then subjected to postfixation, dehydration, critical-point drying and metal coating according to the method described by Matsumoto and Oikawa (27). SEM was performed on the erythrophores with the aid of address marks recorded in photomicrographs, using a JOEL JSM-S1 microscope.

Quantitative analysis of pigment movement as mass movement and movement of a single granule

The intracellular movement of pigment particles in swordtail erythrophores in isolated scales was recorded automatically by a specially designed microphotometer (28). The microphotometer had three main parts ; light source, photodiode and recorder. The spectrophotometric characteristics of pterinosomes and carotenoid vesicles in these cells were determined on living cells using an Olympus DMSP-II microphotometer equipped with a measuring spot size of 1.5 μm. As shown in Fig. 2, the transmittance properties of these particles were clearly different, and so the spectrum of the light source was adjusted to a wave length of 456 nm at the peak transmittance. Therefore, movement recorded can be regarded as that of pterinosomes. The photodiode used for light passed through pigment cells was a Sharp SBC-530 with 1 cm^2 area. The voltage generated in the photodiode by the passed light was recorded automatically as pigment mass movement. The

measurement was carried out on single cells using an isolated scale fixed by Scotch adhesive tape inside the Rose chamber, which was then filled with a medium flowing continuously at the rate of 0.47 ml/min. For the analysis of the aggregating process, $5x10^{-4}$ M epinephrine was administered through tubing attached to the Rose chamber at 25±2°C. For the analysis of pigment dispersion, 10^{-3} M theophylline was applied in the same manner following slight washing with PBS.

The movement of single pterinosomes was analysed using 16-mm movie film, taken at a magnification of x150 to x250, and the motion of an individual pterinosome was traced by means of a motion analyzer.

1-3. Results

Kinetics of pigment movement

The intracellular movement of pigment particles in swordtail erythrophores in isolated scales, as illustrated in Fig. 1, was studied with the use of a specially designed autorecording microphotometer. As the spectrophotometric characteristics of pterinosomes and carotenoid vesicles in these cells were different, as shown in Fig. 2, and those of the light system in the microphotometer were as shown in the same figure, the movement recorded was interpreted as that of the pterinosomes. A typical example of the recordings in which pigment displacement integrated as two-dimensional mass movement per cell is given in Fig. 3.

When the amount of pigment movement in these recordings was replotted on a log scale against the reaction time on an ordinary scale, it was revealed that the relation between these two factors was approximately linear for aggregation but not for dispersion (Fig. 4). This is taken to mean that the patterns of pigment movement are different between aggregation and dispersion. The average time needed for the completion of pigment aggregation was on the order of several minutes, migrating at the average velocity of 1 μm/sec., while that for dispersion was more than 30 minutes, at 0.1-0.03 μm/sec., in both cases at room temperature (25°C ± 2°).

The analysis of the movement of single pterinosomes

using a 16-mm movie film and the motion analyzer disclosed that each migrated linearly along the periphery-to-cell-center axis, in pigment aggregation. This pattern is essentially similar to the mass movement of aggregating pigments as recorded by the microphotometer. In dispersion, particles moved in a zig-zag fashion, changing migratory velocity irregularly as shown in Fig. 5. These observations indicate that the recorded pattern of pigment dispersion as mass movement per cell is an integration of such irregular movement of individual particles. The kinetics of pigment movement in swordtail erythrophores strongly indicate that pigment aggregation and dispersion are based on different principles, and are not in a direct relationship in which one is the reverse of the other.

Changes in cell shape and surface morphology during pigment movement

SEM of monolayer cultured swordtail erythrophores disclosed that with the onset of pigment aggregation there were marked changes both in shape and surfacial structure of these cells. The cells in dispersed states assumed flat, stellate shapes, without forming any particular surfacial configurations such as microvilli or blebs (Fig. 6). When pigment aggregation was initiated by exposure to epinephrine, an apparent squeezing or flattening first occurred, starting in the outer peripheries of their cytoplasmic processes or sleeves. Then the flattening propagated over their whole bodies. Paralleling these changes, a gradual protrusion occurred in the cell centers,

finally shaping them into hemispheres or spheroids. The cells at the end of pigment aggregation thus had an appearance similar to "sunny side up eggs", or a ball with thin frills and projections attached (Fig. 7). The surface morphology of these cells in pigment aggregation was characterized by an image of increased tension, an absence of special surface configurations and clear visualization of pits in the cell body.

When erythrophores in aggregated states initiated pigment dispersion after being transferred into a standard medium, free from any aggregating agents an abundance of fine convexities or short microvilli appeared over the surface of protruded cell bodies packed tightly with collapsed pigments. Administration of theophylline intensified the formation of such structures as well as pigment dispersion (Fig. 8). With the progress of pigment dispersion, a number of microprojections appeared along the edges of the cell peripheries and sleeves, gradually elongating them. Pigment dispersion proceeded accompanied by the swelling of flattened cell sleeves or dendrites, engulfing newly formed microprojections. The deformation of cell contours after one complete cycle of pigment aggregation and dispersion appeared to result from the procedures employed in this movement.

Pigment displacement in fish pigment cells is known to be impaired by colchicine, cytochalasins and ouabain (24, 29, 30). Therefore the effect of these drugs on the motility of pigment granules in swordtail erythrophores

was also examined in relation to the changes in cell shape and surface morphology. The exposure of these cells in a dispersed state to 10^{-3} M colchicine caused marked flattening of the cell bodies (Fig. 9). It is thus apparent that the changes in shape seen in these cells under the influence of this alkaloid are closely associated with the status of microtubules and their related cytoplasmic organelles. Such colchicine-treated cells successfully performed pigment aggregation, even in the continued presence of 10^{-3} M colchicine, upon exposure to $5x10^{-4}$ M epinephrine; the cell shape changes were as observed in untreated cells (Fig. 10). The treated cells were dispersed their pigments very slowly even in the presence of theophylline, and often failed to achieve full dispersion. It was observed that colchicine often impaired the dispersion of the pterinosomes more decisively than that of carotenoid vesicles.

The administration of cytochalasin B (2×10^{-5}M) to these cells with dispersed pigments resulted in a slight to moderate degree of pigment aggregation <u>per se</u> (Fig. 11), often leaving a small cluster of pigments at dendrite tips. Further administration of epinephrine to such cytochalasin B-treated cells enhanced the completion of pigment aggregation, though the effects of the neurotransmitter were often obscure due to preexisting semi-aggregation. The substitution of epinephrine in the medium with theophylline caused a slight loosening of tightly collapsed pigments.

Ouabain, added at a concentration of 10^{-3} M to PBS, had little effect on either pigment aggregation or dispersion, which were initiated by administration of epinephrine or theophylline respectively to the above medium. Such ouabain-treated cells often displayed clumping of pigments at the tips of the dendrites after pigment aggregation. These clumps spread out upon the initiation of pigment dispersion (Fig. 12).

1-4. Discussion

Studies on the kinetics of mass movement using swordtail erythrophores indicated that pigment aggregation was different in its modality from pigment dispersion. From the analysis of the behavior of single pigment granules, linear motion from cell periphery to cell center and irregular zig-zag migration were shown in the aggregation and dispersion processes, respectively. The behavior of single pigment granules in these two phases well reflected the changes of pigment granule localization in whole cells. The integration of the behavior of single pigments coincided with the changes of mass movement in whole cells.

On the kinetics of melanosomes in the melanophores, there is no information available. It is suggested that melanosomes in fish melanophores move under the different modalities in aggregation and dispersion as observed with pterinosomes in swordtail erythrophores, for the following reasons, 1) fish melanophores with aggregated pigments are subject to cell shape changes similar to those of swordtail erythrophores (31), 2) melanosomes are similar in size and buoyant density to pterinosomes (32, 33) and 3) the composition of cytoskeletons and their behavior during pigment displacement in melanophores are similar to those of erythrophores, as judged from available information (20).

SEM observations on swordtail erythrophores at

varying stages of pigment displacement indicated that pigment particles migrated centripetally as if all the motile cytolasmic component was squeezed away from the cell periphery. The drastic changes in cell shape under this movement cast into doubt the possibility that pigments moved within a rigid, stable cytoplasmic framework, though many such views had been set forth by other investigators (16, 21). The changes in cell surface during pigment dispersion were apparently different from those during pigment aggregation. It is a well known fact that microvilli contain actin microfilaments with a definite organization. Considering this fact, the appearance of these surface configurations over the cell body at the beginning of pigment dispersion would seem to imply that the reassembly of microfilaments occurred in these portions of the cell at this period of the movement.

Evidence obtained here suggests that pigment aggregation and dispersion are controlled by different mechanisms, and are not simple reversible processes as regards time and direction. Evidence also suggests that actin plays a key role in pigment translocation.

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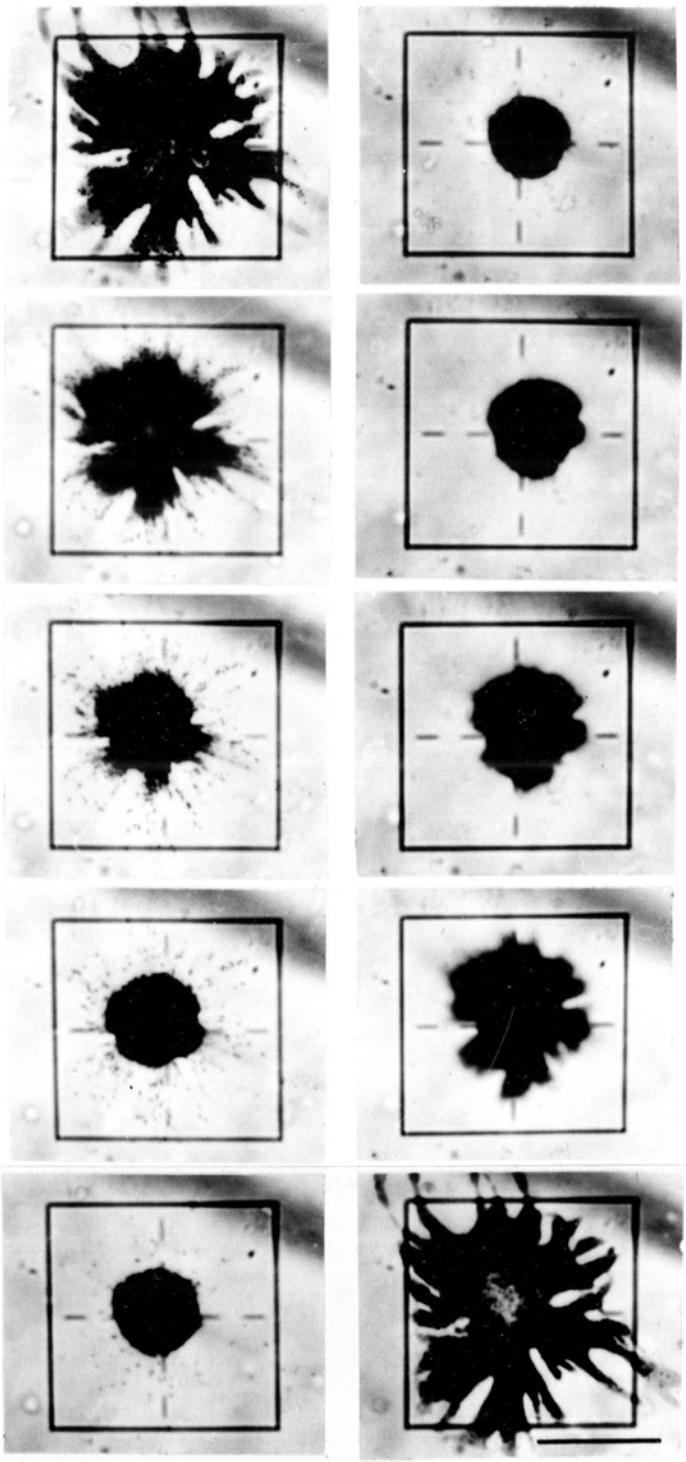
1-6. Figures and Figure legends



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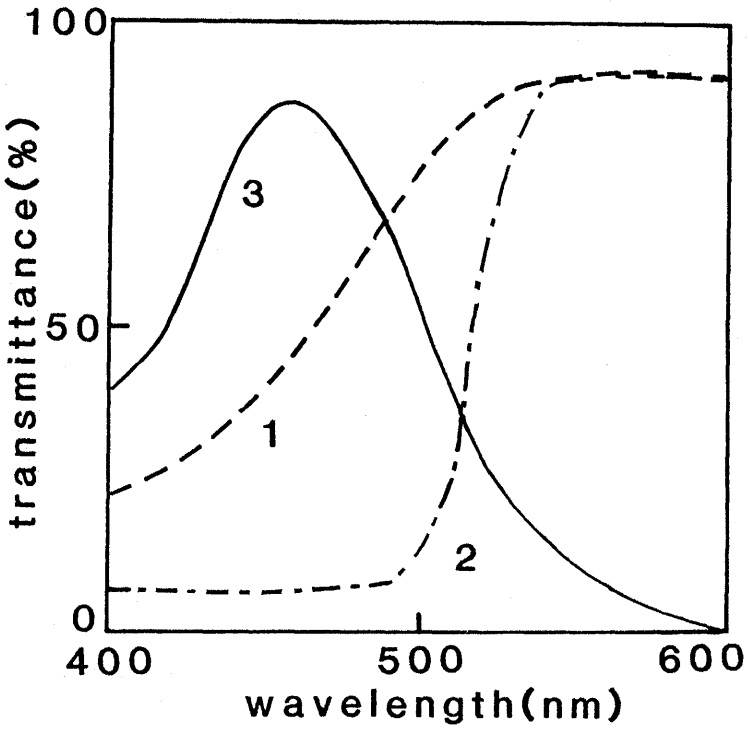

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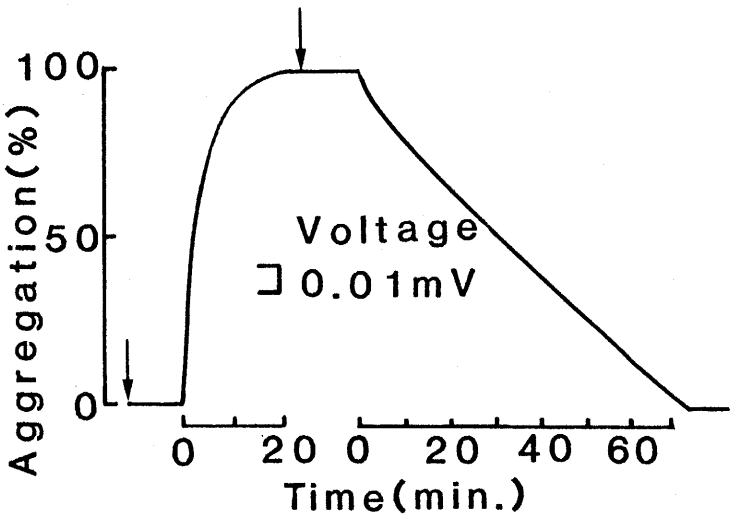

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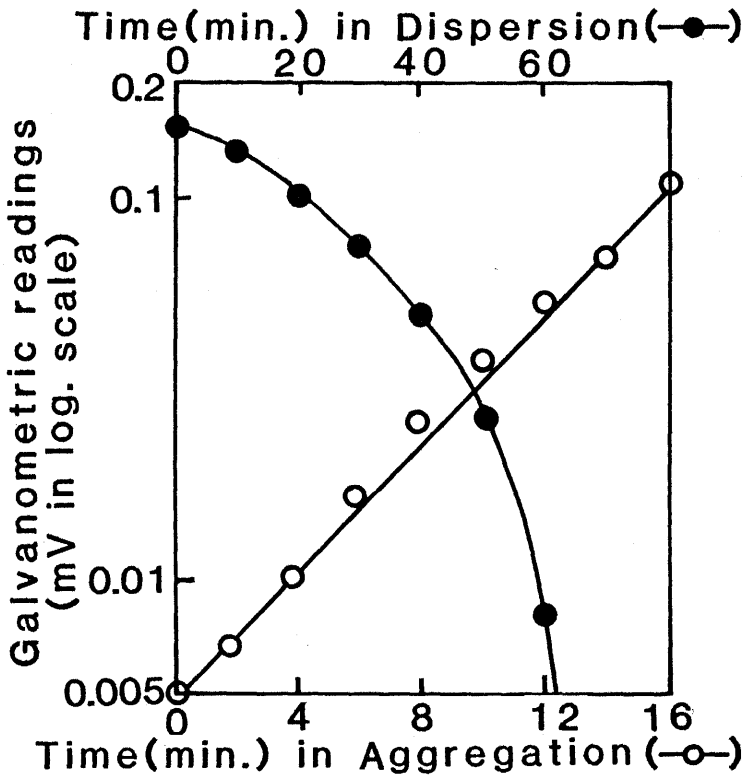
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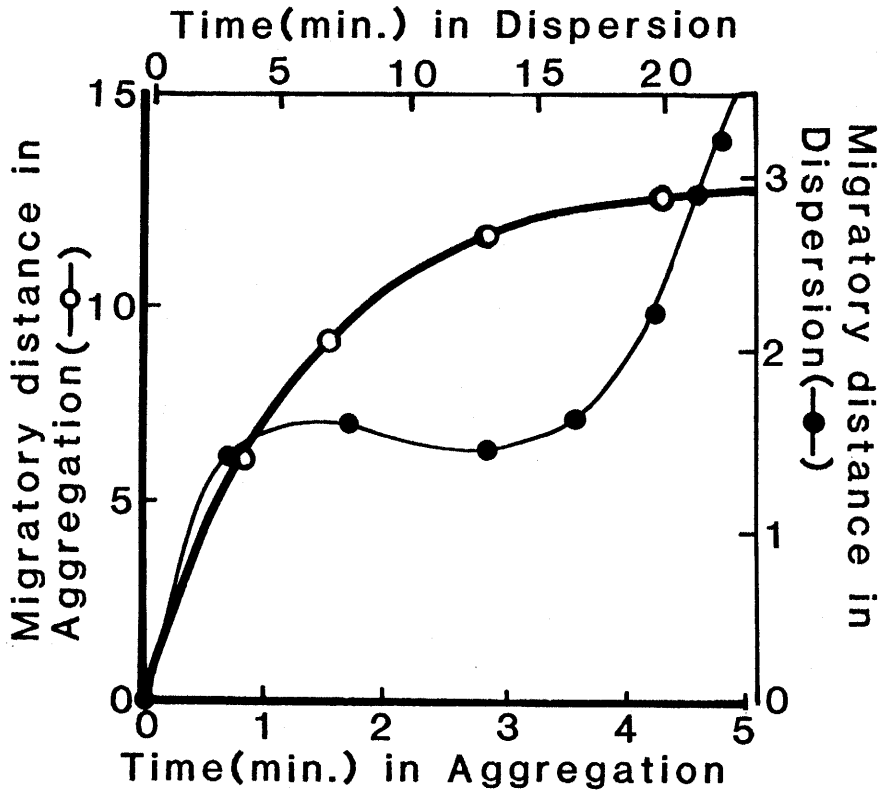
Time(min.) in Aggregation (—o—)

Fig. 4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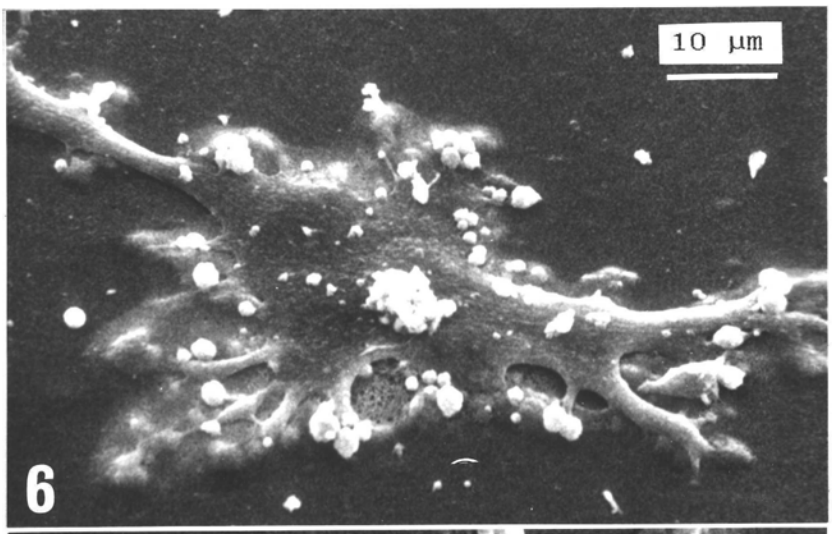
Time(min.) in Aggregation

Fig. 5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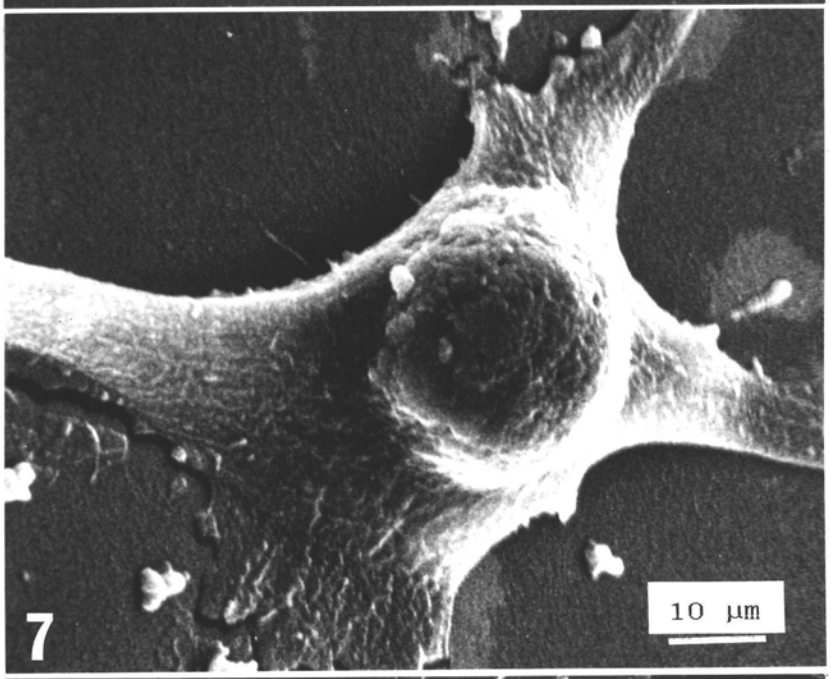
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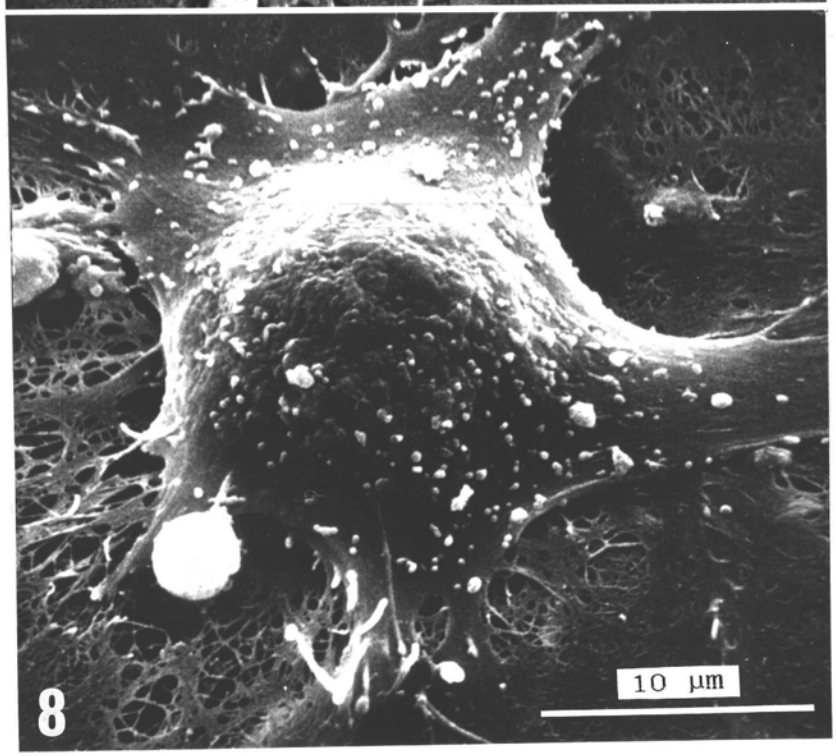
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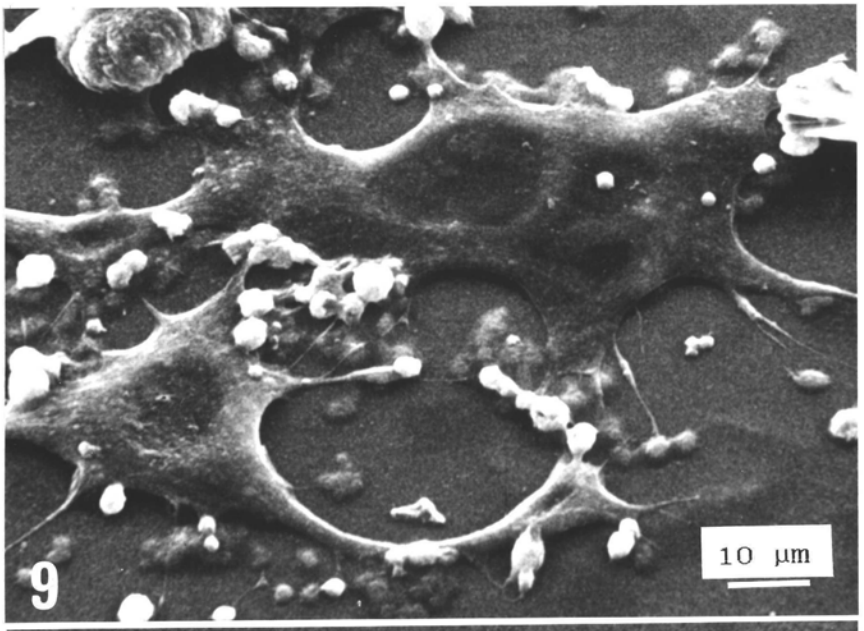
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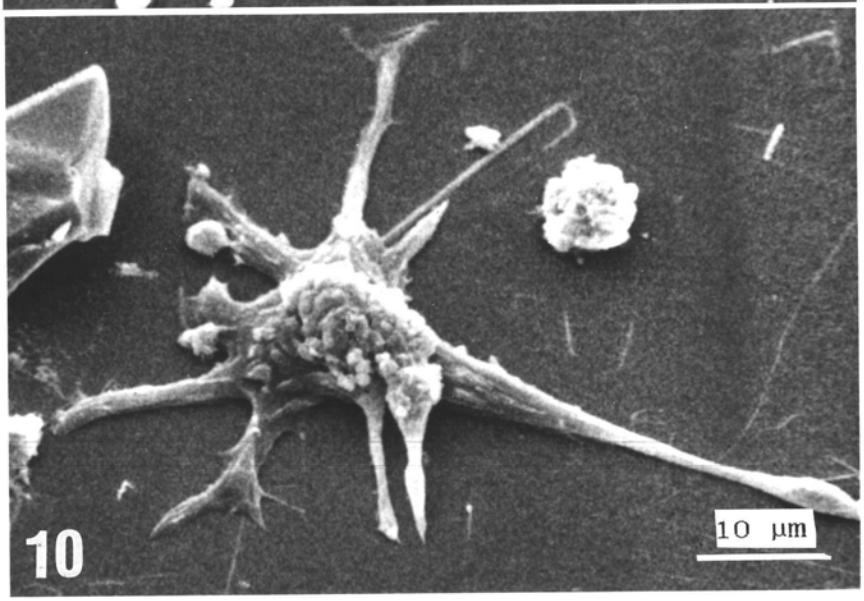
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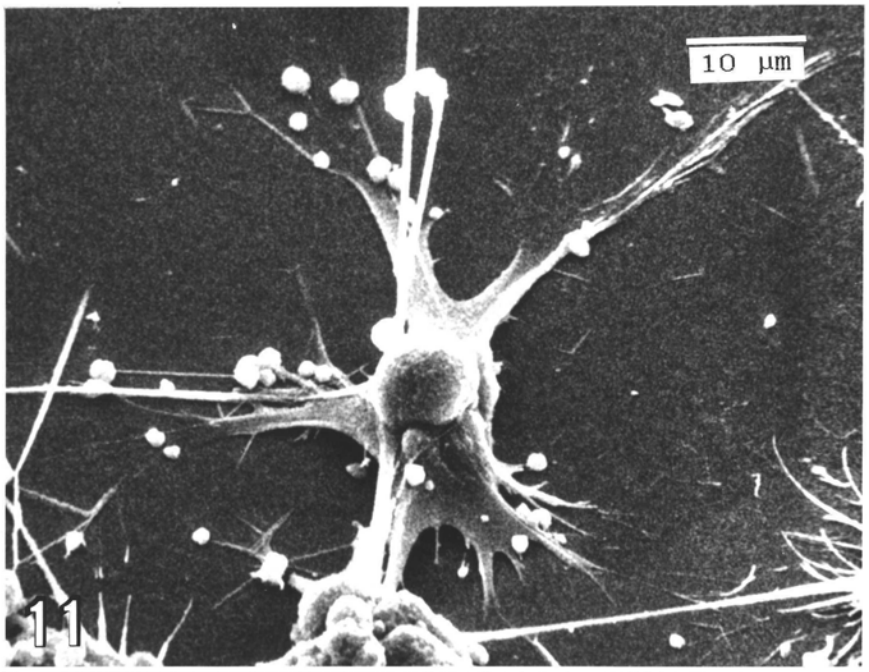
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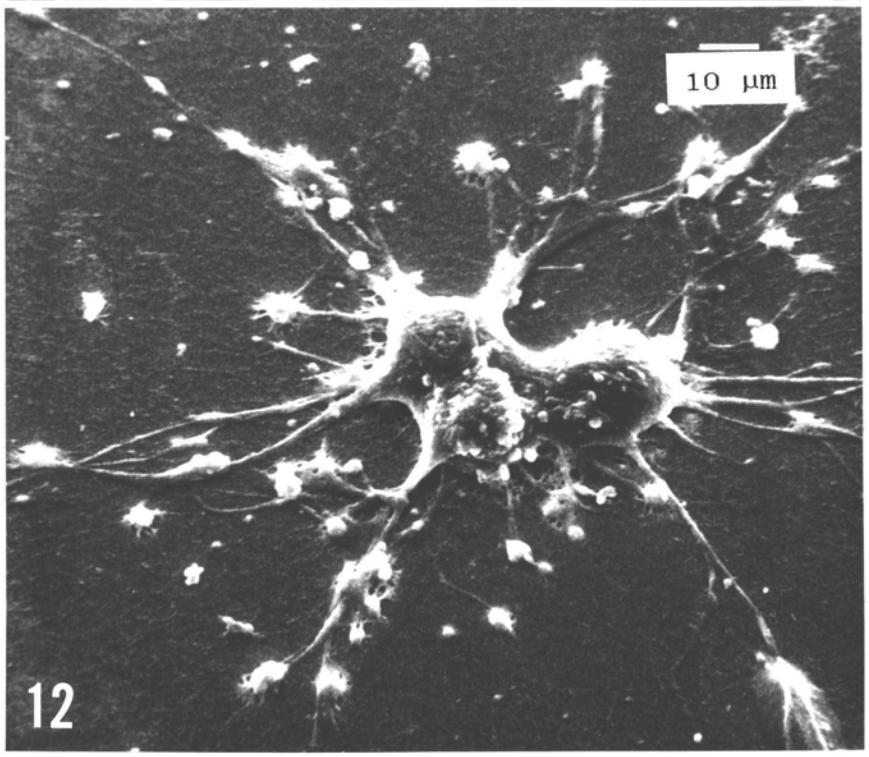
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12

Fig. 1 Photomicrographs of a swordtail erythrophore undergoing pigment displacement

The left row shows five stages of pigment aggregation from the dispersed state in physiological salt solution (top) to an aggregated state (bottom) upon administration of 5×10^{-4} M epinephrine. The right row shows the stages of pigment dispersion from an aggregated state induced by epinephrine (top) to the fully-dispersed state (bottom) upon administration of 10^{-3} M theophylline.

Fig. 2 Spectrophotometric properties of pigments in swordtail erythrophores

The transmission curves of pterinosomes (1) and carotenoid vesicles (2) were determined for living cells using an Olympus DMSP-II microspectrophotometer equipped with a measuring spot size of 1.5 μm. The spectrophotometric characteristics of the light system (light source ; tungsten lamp 6V 5A, glass filter; Toshiba B-47) of the autorecording microphotometer used are represented by curve (3).

Fig. 3 Photoelectric recording of pigment displacement in swordtail erythrophores

Measurement was made on single cells in isolated scales using a specially designed autorecording microphotometer at 25°C±2°C. Pigment aggregation or dispersion was elicited by the administration of epinephrine (arrow in left corner) or theophylline (arrow at top), respectively, though tubing attached to the Rose chamber. The time interval between

theophylline application and subsequent pigment reaction was in the magnitude of several to ten minutes. See reference 28 for further details on the instrumentation.

Fig. 4 Difference in the modality of pigment movement between aggregation and dispersion

Pigment movement per cell (log scale) against time (ordinary scale) was approximated to a linear line for aggregation but not for dispersion.

Fig. 5 Movement of a single pigment granule at aggregation and dispersion

The motion of an individual pterinosome was traced on a 16 mm movie film taken at a magnification of x150 to x200 by means of a motion analyzer.

Figs. 6-12 SEM of swordtail erythrophores in monolayer culture

Fig. 6 : In a dispersed state in a standard medium. Fig. 7 : In an aggregated state. Fixed after administration of epinephrine. Fig. 8 : Undergoing pigment dispersion. Fixed 5 minutes after administration of theophylline. Fig. 9 : Treated with 10^{-3} M colchicine for 30 minutes in a dispersed state. Fig. 10 : The same as Fig. 9 after administration of epinephrine. Fig. 11 : After treatment with 2×10^{-5}M cytochalasin B in a dispersed state, and then with epinephrine. Fig. 12 : At the onset of pigment dispersion after exposure to 10^{-3} M ouabain for 30 minutes

in an aggregated state and then to theophylline. Note the appearance of microextensions or spikes around the clumps present at the tips of the dendrites.

1-7. Summary

The changes in cell surface morphology at pigment displacement and their relation to the kinetics of this motility were studied using swordtail erythrophores. Kinetics studies on single pigment particles and their masses per cell indicated that pigment aggregation was different in its modality from pigment dispersion. SEM studies disclosed that the two phases of this movement were not in a reciprocal relationship with regard to the sequence of cell surfacial changes: Pigment aggregation was accompanied by squeezing of the cell peripheries and protrusion of the cell body whereas pigment dispersion was accompanied by the formation of microvilli over the cell body and microprojections along the cell peripheries. The effects of colchicine, cytochalasin B and ouabain were also different for the two phases of the movement: Colchicine failed to block aggregation and related cell shape changes although it slowed down dispersion markedly. Cytochalasin B caused weak to moderate pigment aggregation *per se*, accompanying protrusion of the cell body, whereas it impaired pigment dispersion. All these findings indicated that microfilaments play a key role in this motility, while the action of microtubules was supplemental. It is suggested that cell surface activities associated with actin microfilaments generate a cytoplasmic flow which conveys all motile cytoplasmic ingredients including pigments.

Section 2

Implication of actin filaments in pigment aggregation of swordtail erythrophores

2-1. Introduction

Swordtail erythrophores contain two kinds of pigment organelles: red pterinosomes and yellow carotenoid vesicles (1). These pigment organelles migrate simultaneously within the cells, either centripetally or centrifugally, in response to neurotransmitters, hormones or their related messengers (2). The simultaneous translocation of pigment organelles of different characters led to the conclusion that pigment movement is due to a cytoplasmic flow. The flow of cytoplasm is likely to convey all movable ingredients without selecting particular particles like pterinosomes. As shown in section 1, SEM studies provide evidence in support of this view : in pigment aggregation, the cell peripheries are progressively squeezed toward the cell center, almost simultaneously with the centripetal movement of pigment, whereas in dispersion, the markedly protruding cell body is flattened by the centrifugal migration of pigment (3, 4). Recently, it has been shown in several organisms manifesting dynamic cell motility that actin filaments play a key role in generating intracellular streaming (5). Considering these facts and taking into account the subtle susceptibility of swordtail erythrophores to cytochalasin B, led to the conclusion that actin is implicated in pigment translocation.

The aim of this study is to clarify the intracellular localization of actin filaments in erythrophores during the two stages of pigment aggregation and dispersion.

For this purpose, the localization of microfilaments was observed first by transmission electron microscopy (TEM) in both aggregation and dispersion states. Secondly, an antibody was prepared against carp skeletal muscle actin and then the antibody was used to examine the localization of actin filaments during pigment translocation through immunofluorescence and immunoelectron microscopy.

2-2. Materials and Methods

Transmission electron microscopy (TEM)

Monolayer cultured erythrophores or erythrophores in isolated fins or scales were washed in PBS three to five times, and treated with epinephrine according to the method described in section 1 to obtain the knowledge of cells in aggregated stage. Specimens were then fixed in 1% osmium tetraoxide in 0.1 M phosphate buffer, pH 7.5, for 1.5 hours at 4°C and then embedded via an alcohol series in Epon according to the ordinary procedure for tissue handling in electron microscopy. Sectioning was done with a Porter-Blum MT-2 and examination with a JEM100S electron microscope.

Purification of actin as antigen

Actin was isolated from carp skeletal muscle according to a slight modification of the method of Hirabayashi & Hayashi (6). Fresh skeletal muscles were dissected, immediately after total bleeding, from two commercially available carps, each weighing about 1.5 kg, minced into small pieces and ground down at 4°C. Ground tissue was then suspended in Guba-Straub solution (0.3 M KCl, 0.1 M KH_2PO_4 and 0.05 M K_2HPO_4, pH 6.5) for 20 minutes with continuous stirring in order to extract the myosin. The actin-containing fraction was collected as pellet from the suspension by centrifugation at 8,000g for 15 minutes at 4°C and then washed repeatedly with

ice-cold redistilled water, once with 4g/l sodium bicarbonate aqueous solution and twice with acetone successively. Acetone powder of muscle thus prepared was stored at -20°C until further purification started. Actin was extracted by rinsing one part of the acetone powder of muscle in 10 parts of cold 0.1 mM adenosine triphosphate (ATP) sodium salt aqueous solution for one hour with continuous shaking. The extract was centrifuged at 23,000 g for 30 minutes in order to remove undissolved residues and then adjusted so as to be in 5 mM Tris-HCl (pH 8.0) and 0.1 M KCl. F-actin was prepared in the solution by leaving it at 4°C for 15 hours with continuous stirring. Following centrifugal collection at 100,000g for 60 minutes, the pellet was resuspended in 0.1mM ATP solution and sonicated briefly to depolymerize F-actin. The suspension thus obtained was added again with concentrated Tris-HCl buffer and KCl so as to make the above-mentioned concentrations for the respective reagents. Polymerization of F-actin was accomplished by incubating the mixture for one hour with moderate agitation at room temperature. After repeating another cycle of dissociation and polymerization of F-actin, the precipitate was dialyzed against the solution containing 0.1 mM ATP and 5 mM Tris-HCl (pH 8.0) overnight in the cold, and termed tentatively as native actin.

Further purification of actin was performed by means of sodium dodecylsulfate (SDS) polyacrylamide gel electrophoresis (PAGE)(7). After incubating the sample with 8M

urea, 0.5% SDS, 5mM ethylenediaminetetraacetic acid disodium salt (EDTA), 0.1M ß-mercaptoethanol, 20mM Tris-HCl, pH 8.5 for one hour at 60°C, each 200 µl of actin solution, dissolved in 0.1 mM ATP and 5mM Tris-HCl mixture at a concentration approximately equivalent to 0.3 µg protein per µl, was loaded on the top of a 0.5 x 10.5cm disc containing 50mM Tris-glycin buffer and 7.5% gels and subjected to a constant current of 2 mA per gel for 2.5 hours. After electrophoresis, each disc gel was split into two parts along its electron flow direction, providing one for staining and another for collection of isolated actin. Each disc half was stained with 0.25% coomassie brilliant blue dissolved in 50% (v/v) methanol and 7.5% (v/v) acetic acid, mostly followed by decoloration in the mixture containing 20% (v/v) methanol and 7.5% (v/v) acetic acid. The location of actin in the gel was identified by comparison with that of authentic actin isolated from rabbit skeletal muscle and run simultaneously on a separate disc. With the aid of the stained half, actin band in the unstained half was carefully segmented with a razor blade and pooled for immunization experiments.

Preparation of antibody against carp actin

This was made based on the procedures described by Nishino and Watanabe (8): Gel segments obtained from seven discs (approx. 210-420 µg protein) were combined, homogenized *per se* and dialyzed against phosphate buffered saline (PBS) containing 145 mM NaCl, 7.5 mM $Na_2HPO_4 \cdot 12H_2O$

and 2.4 mM $NaH_2PO_4 \cdot 2H_2O$ at pH 7.2. Dialyzed gel homogenate was then mixed with an equal volume of Freund's complete adjuvant (Difco 063860, Detroit) and, after emulsification, injected at the same time subcutaneously into multiple sites of the dorsal portions of two male New Zealand rabbits. By repeating this procedure, immunizations were performed successively five to eight times at intervals of one week until the antibody for carp actin was formed. After the seventh immunization, one rabbit indicated an apparent antibody formation when examined by the Ouchterlony test (9). Blood was immediately collected from its neck artery. The serum was separated by clotting the collected blood, after standing for 0.5 hours at room temperature and then for 12 hours in the cold in the presence of 1% thymerosal (Maruishi, T710, Osaka Japan). γ-globulins in the serum were precipitated and purified by adding ammonium sulfate to it at a final concentration of 30-33% at 4°C and dialysed against the above-mentioned PBS after suspension with PBS.

Fractionation of swordtail erythrophores

The reactivity of the prepared anti-carp actin antibody to swordtail erythrophores was examined first by the Ouchterlony test using cell specimens obtained by enzymatic digestion of skin tissue and subsequent ultracentrifugal fractionating in a Ficoll-density gradient: Skin and scales detached from ten adult swordtail fishes were digested at 25°C first in 0.25 % collagenase solution

for 30 minutes and then in 1 % trypsin (Difco 1 : 250) for 15 minutes, both dissolved in Ca^{++}, Mg^{++}-free PBS (CMF-PBS). The procedures thereafter were essentially similar to those reported in the reference 2. Dissociated cells were then suspended in 3 ml of CMF-PBS and each 1ml of the suspension was layered on the top of a continuous Ficoll density gradient, the composition of which was modified from those by Ide and Hama (10) as shown in Fig. 1. Upon centrifugation at 98,000g for 90 minutes at 4°C using a Hitachi 55P ultracentrifuge and its RPS 40 swinging-bucket rotor, erythrophores were separated as deeply red-colored pellet at the bottom of a centrifuge tube. After cutting at the position shown in the Fig. 1 with use of a tube slicer, red pellets were pipetted out, subjected to freezing and thawing three times, sonicated briefly at 0°C and then provided for immunodiffusion examination or SDS-PAGE.

Indirect immunofluorescence assay

The localization of actin within swordtail erythrophores was visualized by a method of indirect immunofluorescence after Lazarides and Weber (11). The cells on a glass-slip were fixed first in 3.5% formaldehyde in PBS for 20 minutes at room temperature and, after brief washing in PBS, then in pre-chilled acetone at -20°C for 5 minutes. These specimens stood for a while in the moist chamber for removal of acetone and were then treated with rabbit anti-actin antibody (IgG fraction) which was diluted

15-fold (1.8 mg/ml) with phosphate buffered saline. Following a one-hour-incubation at room temperature, the cover slips with cells were carefully washed five times with PBS. Then the slips were treated with fluorescein isothiocyanate (FITC)-labelled goat anti-rabbit γ-globulin (Fuji zouki, Tokyo) diluted 20-fold in PBS for 45 minutes at room temperature, washed again five times in PBS, and mounted with use of glycerin on glass slides. For the control specimens, cells were treated with either antigen-absorbed anti-actin antibody or PBS in place of the antibody. Cells without any FITC-conjugated anti-rabbit IgG antiserum treatment were also examined. The specimens were examined under a Nikon fluorescence microscope (Model FT, Transmission type) epuipped with FITC interference filters (12) (Sakura Seiki). Photomicrographs were taken using SAKURA or KODAK ASA 400 color film.

Immunoelectronmicroscopy

Small pieces of fin web, about 3 mm square, were dissected from dorsal or tail fins about 3.5-cm long adult fish and slightly exposed to 0.2% collagenase in sterile CMF-PBS for removal of the epidermis. After removal, tissue pieces were further trimmed to 1 mm^2 and then subjected to labeling after or without exposure to epinephrine. After brief rinsing in CMF-PBS, they were fixed in Zamboni solution for one hour at 4°C (13), washed in cold CMF-PBS five times, and then assayed with the antibody at a concentration of 1.8 mg protein per ml for two hours

at room temperature. As the control, tissue pieces were assayed with antigen-absorbed antibody or simply with PBS. All specimens were mixed with peroxidase-conjugated anti-rabbit IgG antiserum (Miles Yeda Ltd. Israel) at ten-fold dilution in PBS. Following a two-hour incubation at room temperature, excess antibody was washed out by rinsing in PBS, and then a diaminobenzidine (DAB) reaction was conducted in Karnovsky solution for 10 minutes at room temperature (14). Specimens were washed in cold PBS five times, and then treated by the routine method for electron microscopy (cf. the method on TEM described above).

Estimation of distribution density of actin microfilaments

Counting of peroxidase labels along microfilaments was achieved on the erythrophores sectioned horizontally through the cell center to the tip of the dendrite. Topographical differences in the density of actin microfilaments were examined by dividing each dendrite into five septa along the proximo-distal axis (the length from the presumed cell center to the tip of the dendrite was divided into five equivalent fractions on a printed electronmicrograph) and then by comparing average counts per unit area of sectioned cell plane. The numbers of aggregates of thin filaments having a diameter of more than 0.5 μm were also counted in each fraction. The electronmicrographs used in such counts were printed at a magnification of x25,000 as exemplified by Fig. 14a.

Each count of labels and filamentous aggregates was expressed per square μm of actual sectioned plane of the cell.

2-3 Results

Ultrastructural foundation for pigment movement

TEM on swordtail erythrophores *in situ* and of *in vitro* culture disclosed that they contained an abundance of microfilaments, 6-7 nm in width, and microtubules, and that the distribution of these types of cytoskeleton were different between the phases with dispersed and aggregated pigments. In a cell with dispersed pigments, these cytoskeletons were intermingled in a parallel fashion along the axis of the dendrites (Fig. 2) and converged into the membranous complex in the cell body (Fig. 3). As in Fig. 2, within the dendrites, microfilaments were distributed densely near the cell surface whereas microtubules were situated along the central axis.

In cells with aggregated pigments, microfilaments existed densely in the form of a loose meshwork connecting small clumps of irregularly twisted filaments in the peripheries of the pigment aggregates present in the cell center. Whereas the amount of microtubules was reduced markedly in the dendrites and moderately in the cell body. Within the dendrites where all pigments were evacuated, almost all cytoskeleton disappeared, leaving only a few microfilaments (Fig. 4).

Specificity of the antibody against carp actin

Carp actin used as the antigen for antibody formation in this study was prepared by the method originally

designed for isolation of skeletal muscle actins of homeothermic vertebrate origin. In this method, the purity of actin was increased by repeating monomer-polymer conversion. In SDS—PAGE, a main component of actin fraction obtained from carp muscle extracts presented mobility similar to actin prepared from rabbit skeletal muscle by the same procedure (Fig. 5). This implied that rabbit and carp actins as monomers have similar molecular weights (15). The carp actin provided herein for antibody formation was further purified by the dissection of the band after SDS gel electrophoresis. Main bands of carp actin were cut out from split unstained halves of gels, and approximately 4.8 mg protein of carp actin was utilized in one series of immunization experiments. The protein was finally collected from 120 gels for 10 immunizations. All specimens thus obtained yielded only a single band upon separation by SDS electrophoresis, which exactly corresponded to the main component present in the starting extracts.

Upon successive immunization of rabbits with gel segments containing isolated carp actin, specific antiserum for this antigen was successfully prepared. As shown by the Ouchterlony immunodiffusion test, the antiserum thus obtained apparently reacted to carp actin, yielding one precipitation line against native carp actin and two against the SDS polyacrylamide gel-electrophoresed specimen used as the antigen, viz. denatured carp actin (Fig. 6a). A similar precipitation pattern was obtained with actin

extracted from swordail skeletal muscle by similar procedures (Figs. 6bc). Swordtail erythrophores were separated by means of enzymatic tissue dissociation and subsequent centrifugal fractionating in Ficoll-density gradients and then examined by the Ouchterlony immunodiffusion test after their mechanical homogenization. The reaction obtained was exactly the same as that for carp actin, yielding one precipitin line for the native extract (Fig. 6d). Even though erythrophore contained a small amount of non-pigment cell, this result can be taken as subsidiary evidence for the occurrence of actin in integumental non-muscle cells of this species. Similarly, little precipitation was observed between the antibody and other protein fractions of swordtail muscle appearing ahead of actin in SDS gel electrophoresis (Figs.6bc).

All these results indicated that the anti-carp actin antibody prepared in this study was specifically reactive to swordtail actin having molecular weight near 42,000 (Figs.6bc).

Localization of actin filaments in erythrophores with dispersed or aggregated pigments as revealed by immuno-fluorescence

The antiserum produced in this study was reactive with high specific activity to swordtail actin (Fig. 6). Immunofluorescence using this antibody revealed that erythrophores with dispersed pigments were labeled

diffusely throughout the cytoplasms, being interspersed randomly with tremendous amounts of densely labeled dots (Fig. 7a). The occurrence of labeling in linearly stretched filaments was rather rare and, if present, obscure. These observations indicated that most actin in cells in the dispersed state occurred as amorphous or diffused mesh-works. In erythrophores with aggregated pigments, labeling was recognized in two forms of filaments. One of these appeared as thin linear bundles running from the cell center to the pheripheries. The other appeared as fairly thick bundles distributed around pigment aggregates in the centrospheres (Figs.7b-e). The former assumed an astral arrangement as a whole, in which thin bundles around the main cell body diverged into numerous thinner filaments toward the cell peripheries. Some of them ran directly from the cell center to the margins of cell peripheries. Crossbridge-like structures were occasionally observed between these divergent thin filaments running parallel to the axes of the dendrites (Fig. 7e). The latter existed in a ring shape as a whole along the outskirt of collapsed pigment masses (Fig. 7b). These observations apparently indicated that with the onset of pigment agregation, the localization of actin filaments was subjected to marked change, and the filaments were transformed into two forms either as static cytoskeletons in an astral alignment or as motile elements drifting with pigment translocation.

The fates of actin filaments with motile characteristics were examined at varying stages of pigment

aggregation. It was shown that they emerged at first as relatively loosely organized, broad bundles of filaments along the cell margins (Fig. 7b), and thereater shifted their locations centripetally, encircling coalescing pigment masses. The appearance and subsequent behavior of such circular bands were more distinctly observed in discoid-shaped cells than in stellate-shaped ones, though similar events were more or less observed in every cell under aggregation.

Swordtail erythrophores contain in their cytoplasm a large amount of drosopterin-laden pterinosomes, which fluoresce orange by themselves under a fluorescence microscope. The recognition of FITC-labeled structures in assayed specimens, however, was greatly facilitated by the use of interference filters designed for fluorescein excitation. Labeling was clearly distinguished by the greenish-yellow fluorescence of FITC from the dim orangish fluorescence of endogeneous pigments (Fig. 7f). This difference was presumed to be mainly due to the absence of overlapping in the absorption peaks of this fluorescent dye and drosopterins (12, 16).

In the controlled experiments exposed to either nonspecific rabbit γ-globulin before FITC-labeling or FITC-labeled goat anti-rabbit γ-globulin antibody without pretreatment with the anti-carp actin antibody, little FITC-specific fluorescence was observed in any location (Fig. 7f).

Visualization by immunoelectronmicroscopy of actin filaments within aggregating erythrophores

Immunoelectronmicroscopy using peroxidase disclosed that swordtail erythrophores were distributed with tremendously large amounts of thin filaments 6-7 nm in width. These filaments were apparently decorated with reaction products. In the cells with dispersed pigments (Fig. 8), such labeled thin filaments occurred mostly in the form of twisted spherical masses having a diameter of about 0.5 μm. These masses were distributed at almost equal and moderate frequencies throughout the entire erythrophore cytoplasm, and were intermingled with varying types of cytoplasmic organelles such as pterinosomes, small smooth-surfaced vesicles (mostly carotenoid vesicles), mitochondria. Little decoration was observed on linearly-stretched thin filaments which were occasionally found in a dispersed state. In cells with completely aggregated pigments, large amounts of spherical-shaped masses of twisted thin filaments were distributed in regions circumscribing the collapsed pigment masses present in the centrosphere (Fig. 9a, 10, 11a; arrows). Decorations appeared clearly on these filaments at certain frequencies according to the length of the filaments (Fig. 9b and c). Most of these masses were situated in the vicinity of pterinosomes in the peripheries of pigment aggregates, and some were situated among compactly collapsed carotenoid vesicles in the middle of pigment aggregates. These structures were seldom recognized within pigment-evacuated

peripheral dendrites. Within these cells, labeling of reaction products was also observed on linear thin filaments stretching in parallel along their axis inside the dendrites (Fig. 12).

The cytoplasmic matrix of these peripheral dendrites of aggregated cells exhibited clearer images than did cells with dispersed pigments. This might be due to either the removal of movable cytoplasmic ingredients upon pigment aggregation, or a lack of labeled amorphous structures, or both. Clear visualization of decorated thin filaments in cells subjected to pigment aggregation might be also due to reassembly of preexisting fragmentary actin filaments into organized structures or polymerization of G-actin.

Cells under pigment aggregation were often distributed with coarse meshwork comprised of compact whorls of twisted thin filaments (Fig. 11) and connecting linear filaments or thin bundles (Figs. 11, 12). Some thin filaments stretching from such structures run deeply into the peripheral dendrites along their axes. Thin filaments of such meshwork were frequently in proximity to or connected to small endoplasmic vesicles nearby (Fig. 10). The locations of these twisted masses and related meshwork as revealed by immunoelectronmicroscopy were considered to correspond to those of the circular bands appearing in the distal peripheries of gathering pigments by immunofluorescence assay (Figs. 7bc). The distribution of densely labeled dots over diffusely labeled cytoplasm in FITC-labeled dispersed cells (Fig. 7a) was also

considered to coincide well with observations obtained by immunoelectronmicroscopy. The presence of numerous thin bundles of actin filaments in astral arrangements appearing after pigment aggregation, as revealed by immunofluorescence, was also confirmed by immunoelectronmicroscopy.

Changing distribution of actinfilaments during pigment aggregation

The number of peroxidase labels on microfilaments and of microfilament aggregates per unit area was counted along proximo-distal axis in cells at various intermediate stages. The analysis verified that actin filaments become distributed densely in two particular locations, the distal margins of an aggregated pigment mass and the tips of the dendrites. A typical example is depicted in Fig. 14b, in which pigment aggregation is in the middle of a complete reaction. In Fig. 14, the stage of pigment aggregation was visualized solely by the location of the pterinosomes. This was mainly due to the fact that carotenoid vesicles which migrated simultaneously with or slightly behind the pterinosomes were not an adequate positional marker of pigment displacement in electronmicrographs. Actin filament-rich zones in respective dendrites were always recognized in similar locations within a single cell (Fig. 14c).

Actin filaments in such densely accumulated zones existed in the form of irregularly twisted aggregates

or elongated, strayed single strands or thin bundles. All these microfilaments appeared to be assembled as a loosely organized meshwork. The average interval between filament aggregates in such meshwork measured 0.6μm. The comparison of cells at various stages immediately after epinephrine administration to slightly before the completion of pigment aggregation indicated that actin filaments-rich zones seen near the distal margins of aggregating mass migrated toward the cell center with progress of pigment aggregation (Fig. 4). The average of labeling counts per unit area over the entire cytoplasms at these intermediate stages of pigment aggregation was markedly (approximately 60%) increased as compared with the stages before and after aggregation. At these intermediate stages, the number of filament aggregates per unit area was also augumented. Inside actin filament-rich zone these filaments were often connected with carotenoid vasicles and smooth ER and located very close to the pterinosomes. It must be noted that microfilaments in the cells at such intermediate stages assume a more solid appearance with a clear fringe under the same conditions of treatment than those in cells at a dispersed state (Fig. 14a).

2-4. Discussion

Evidence reported herein indicats that swordtail erythrophores contain an abundance of actin filaments. These filaments are different in their intracellular distribution and modality of assembly in the dispersed and aggregated states.

From the TEM, the presence of abundant microfilaments and microtubules is understood. In the dendrite of the cell at a dispersed state, microfilaments were distributed in a parallel fashion to the long axes of the dendrites and the locations of microfilaments were closer to pterinosomes than those of microtubules. In cells with aggregating pigments, microfilaments densely located circumscribing aggregating pigments in the form of a loose meshwork. The changes of the localization of microfilaments associating the changes of pigment distribution, are thought to be more dynamic and flexible than those of microtubules. Furthermore, in order to detect actin unequivocally and to know the changes of the distribution of actin filaments, the author prepared the antibody against carp skeletal muscle actin and conducted an immunological examination. The antiserum produced was reactive with high specific reactivity to swordtail actin. Because the characteristic labeling with FITC or peroxidase products was hardly recognizable in controlled specimens, including those exposed to either antigen-absorbed anti-actin antibody or FITC- or peroxidase-conjugated goat

anti-rabbit γ-globulin antibody without anti-actin antibody pretreatment. Therefore, the labeled filaments were considered to be made of actin. The intracytoplasmic distribution of labeling was considered to coincide well with the immunofluorescence and immunoelectronmicroscopic observations. In the dispersed state, the densely fluorescing dots in immunofluorescence were thought to correspond to sparsely distributed masses of twisted actin filaments in immunoelectronmicroscopy. In an aggregated state, the fluorescing circular bundles around pigment masses were thought to correspond to more populated spherical masses of twisted actin filaments, and the labeled linear filaments in astral arrangements corresponded to the parallel-arrayed thin bundles of filaments within collapsed dendrites. These apparently confirm the occurrence of actin filaments in swordtail erythrophores and these results well accorded with the evidence of microfilaments obtained from TEM. This section leads to the conclusion that actin filaments in swordtail erythrophores occurrs in two forms which are either motile or static in nature. The former appear as either circular-shaped broad bundles in immunofluorescence or wide zone rich in twisted masses of thin filaments in immunoelectronmicroscopy, along the peripheral margins of aggregating pigment masses. Because of their almost simultaneous translocation with aggregating pigment masses, these filaments are probably associated in some ways with centripetal pigment movements. The latter forms, on the

other hand, appear as radially-arranged linear thin filaments inside pigment-evacuated cytoplasms. These are considered to be engaged in the maintenance of the stellate cell shape as a kind of cytoskeleton or the so-called "stess fibers" (17). At present, little is known about whether pigment aggregation accompanies conversion of G-actin to F-actin or not. Judging from the increased amounts of actin aggregating cells from semi-quantitative analysis based on immunoelectronmicroscopy, it seems probable that actin in the amorphous or monomer state is assembled into filaments in either twisted masses or cytoskeleton-like forms. If myosin or related elements possessing ATPase activities participate in these arrangements, local cytoplasmic gelation or contraction would occur within the cells, generating cytosol streaming. The progressive occurrence of such events along the periphero-central axes in all the dendrites would explain the occurrence of synchronous centripetal pigment migration.

SEM revealed simultaneous centripetal migration of squeezing waves and pigments during pigment aggregation (3). Such squeezing waves were presumed to appear in the positions where circular zones of actin filaments appear. Thin filaments stretching out from masses of twisted filaments were often connected directly with endoplasmic reticula or vesicles within such regions. This would imply possible participation of actin filaments or their meshwork in cell shape changes during pigment aggregation. Intimate topographical association between motile actin filaments

and squeezing waves leads me to believe that actin in swordtail erythrophores is present in a loosely organized, coarse meshwork throughout the entire cytoplasm in a dispersed state. Upon onset of pigment aggregation, actin filament becomes transformed progressively into a rather tightly organized meshwork. In this process, spherical masses of twisted actin filaments possibly serve as terminals responsible for mechanical support or as a reservoir of mesh-forming material. It would be of particular interest to know if similar events occur in actin localization in fish melanophores which show cell-shape changes during pigment displacement (18, 19) and in goldfish xanthophores which translocate carotenoid vesicles selectively (20).

All these observations emphasize the implications of actin filaments in pigment aggregation of swordtail erythrophores, whatever the intrinsic mechanism for the generation of cytoplasmic flow conveying the two kinds of pigment particles might be.

2-5. References

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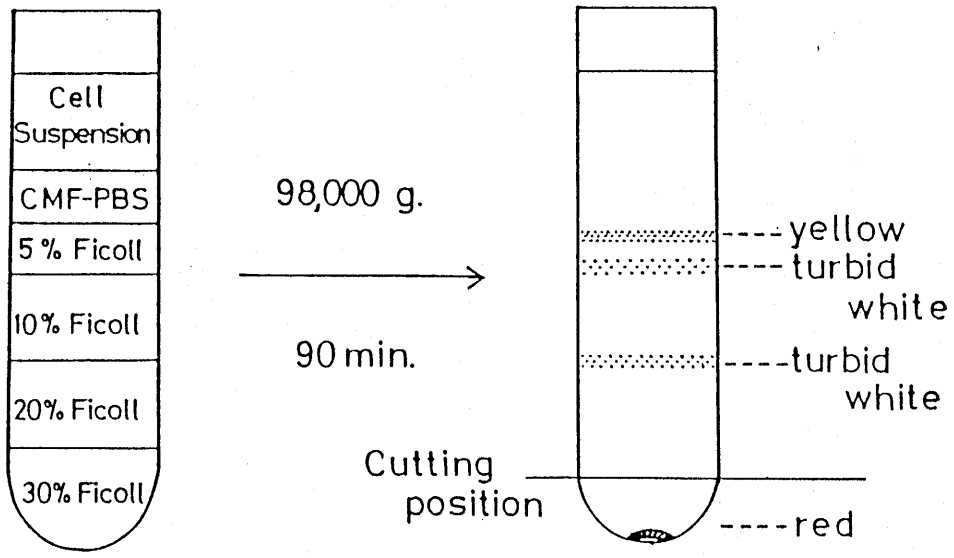
2-6. Figures and Figure legends



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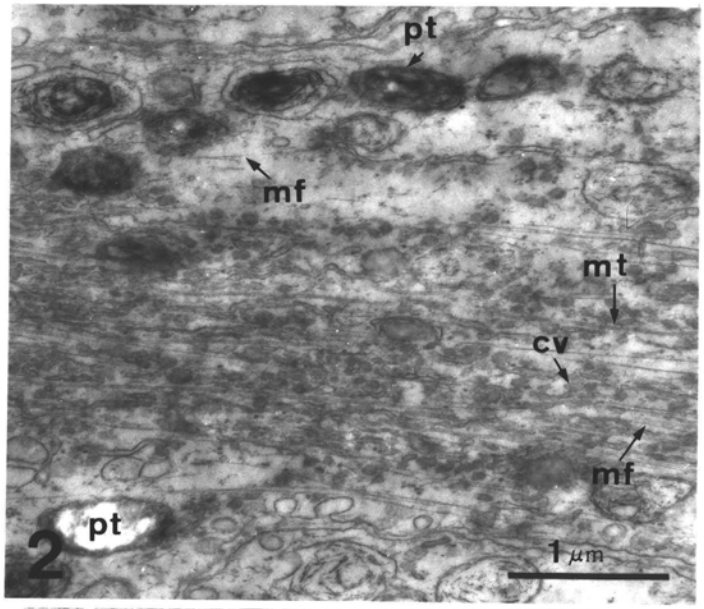
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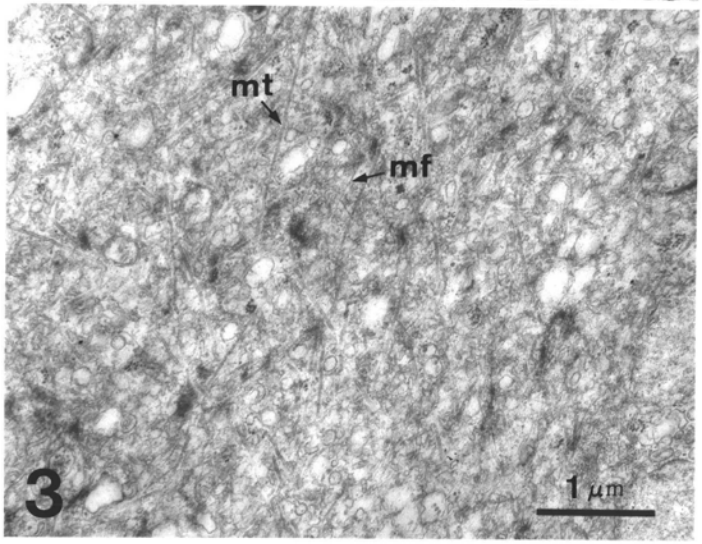
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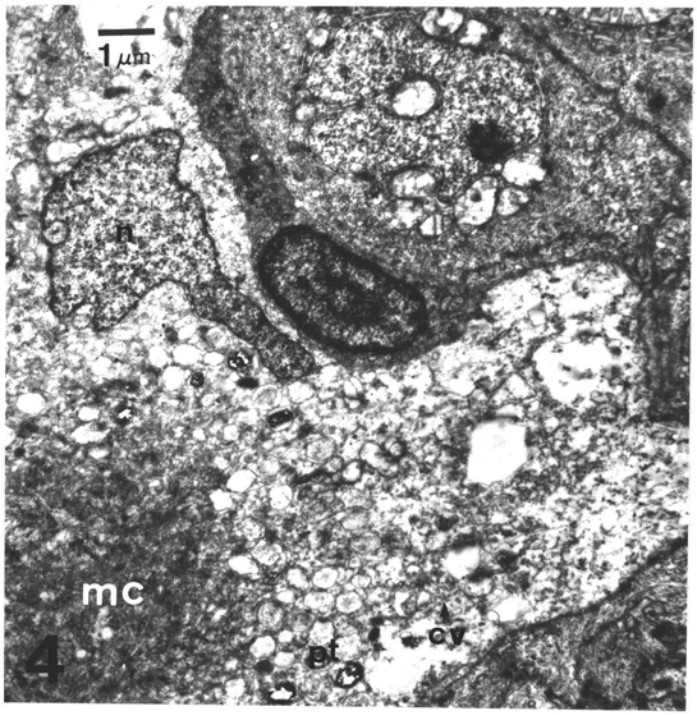
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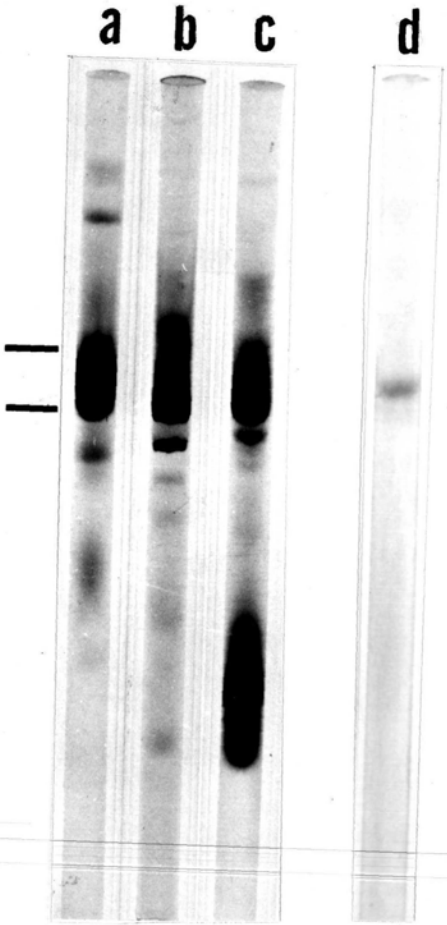

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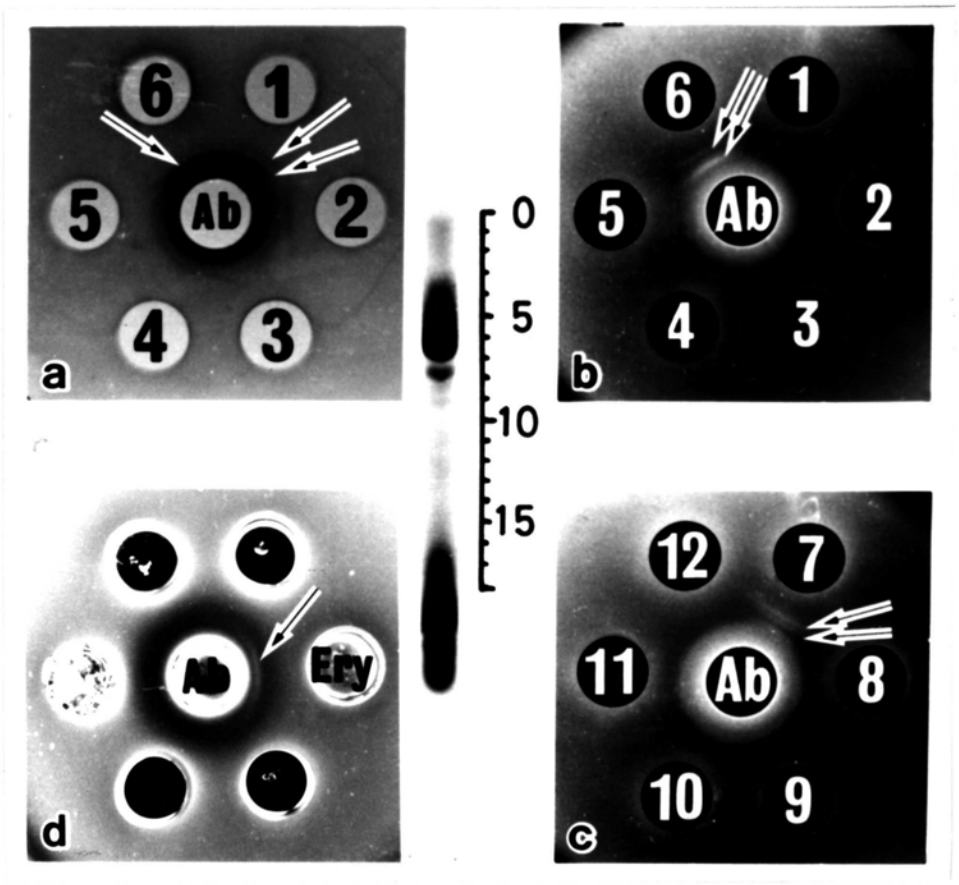

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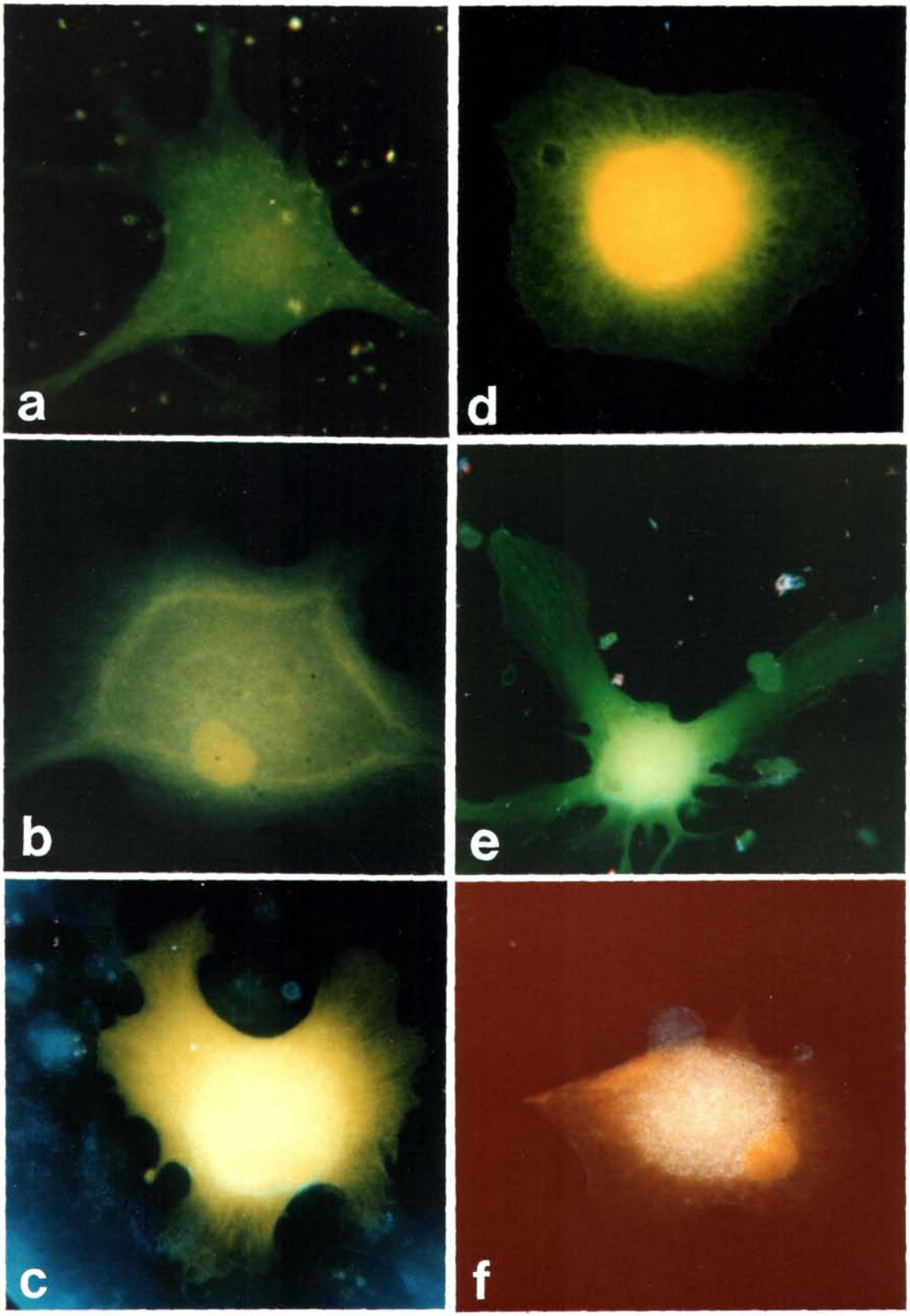

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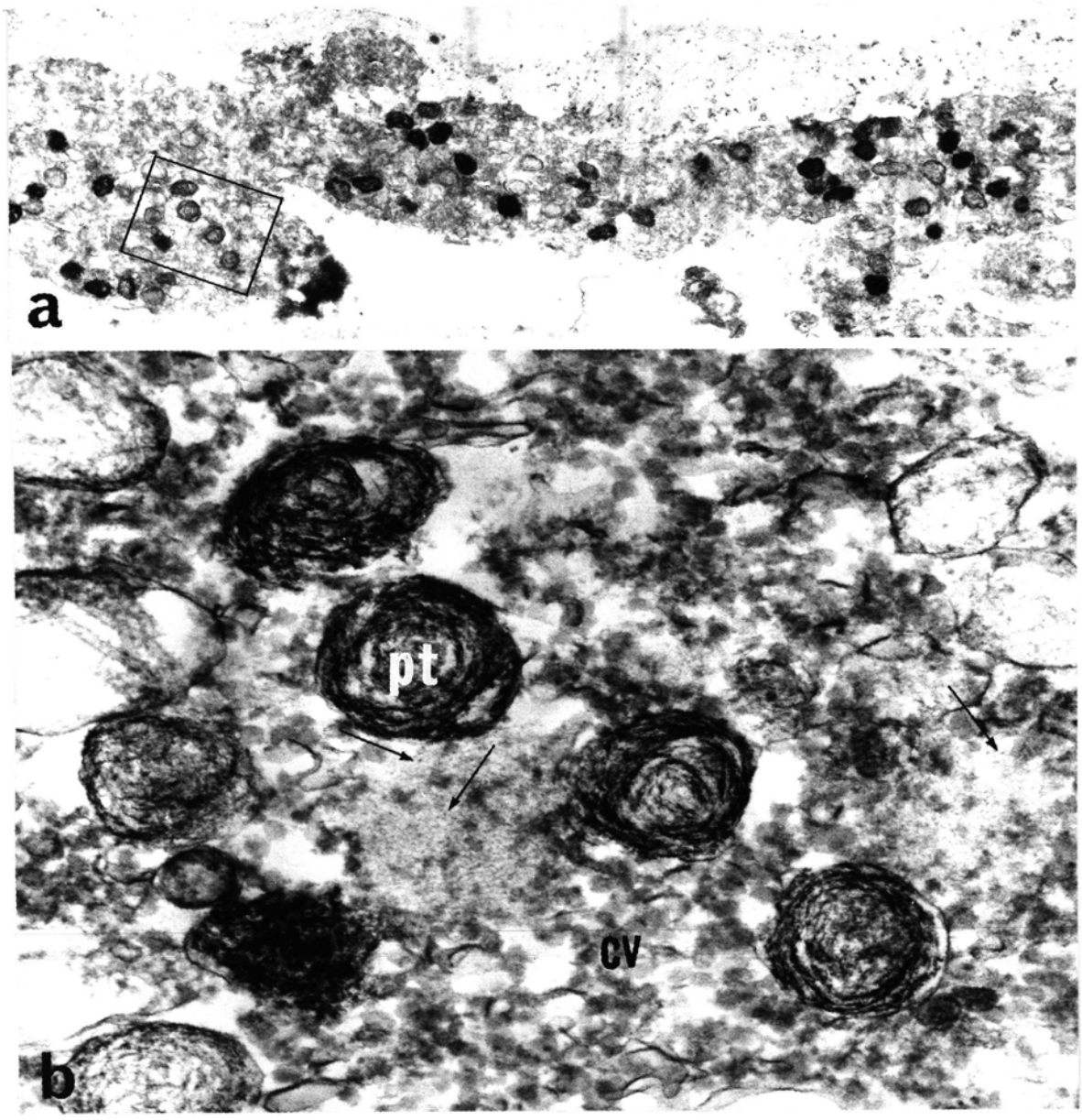

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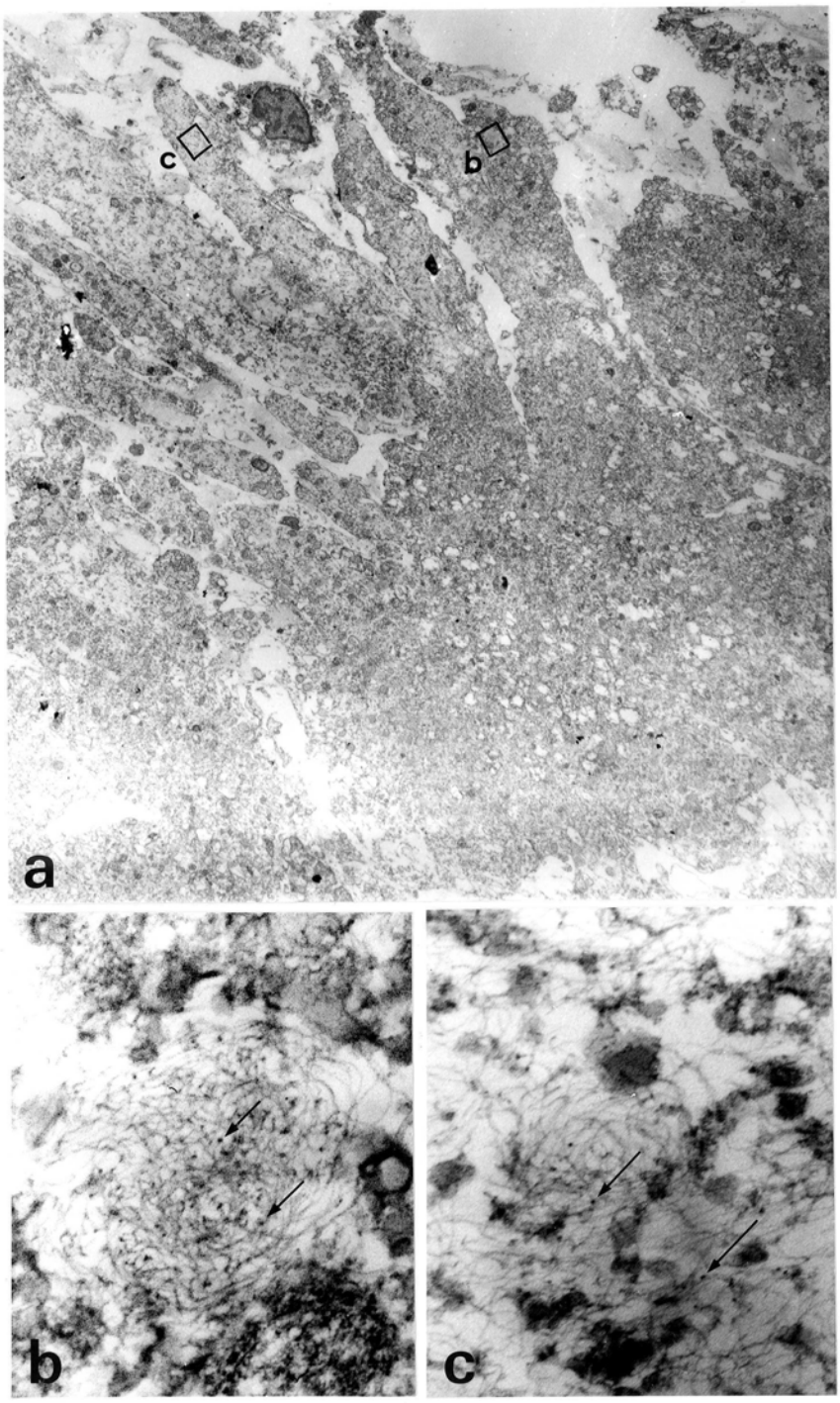

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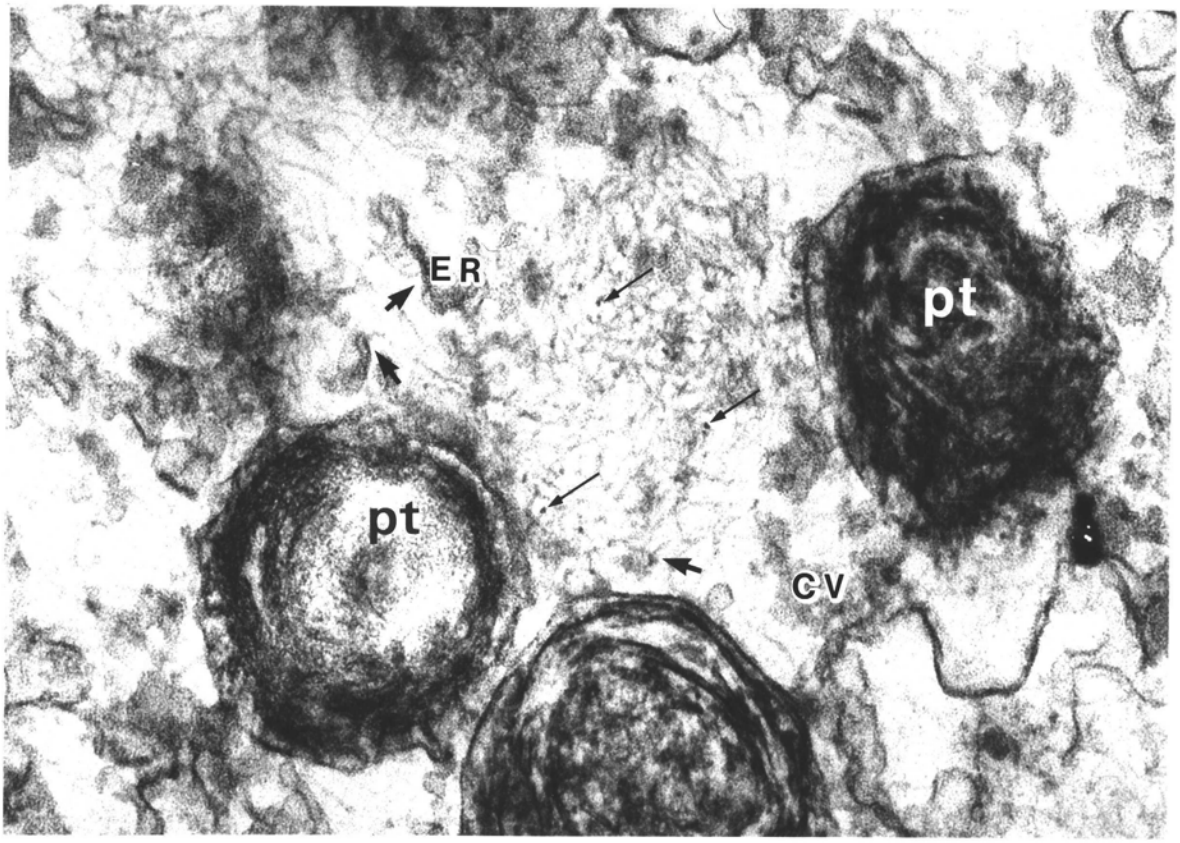

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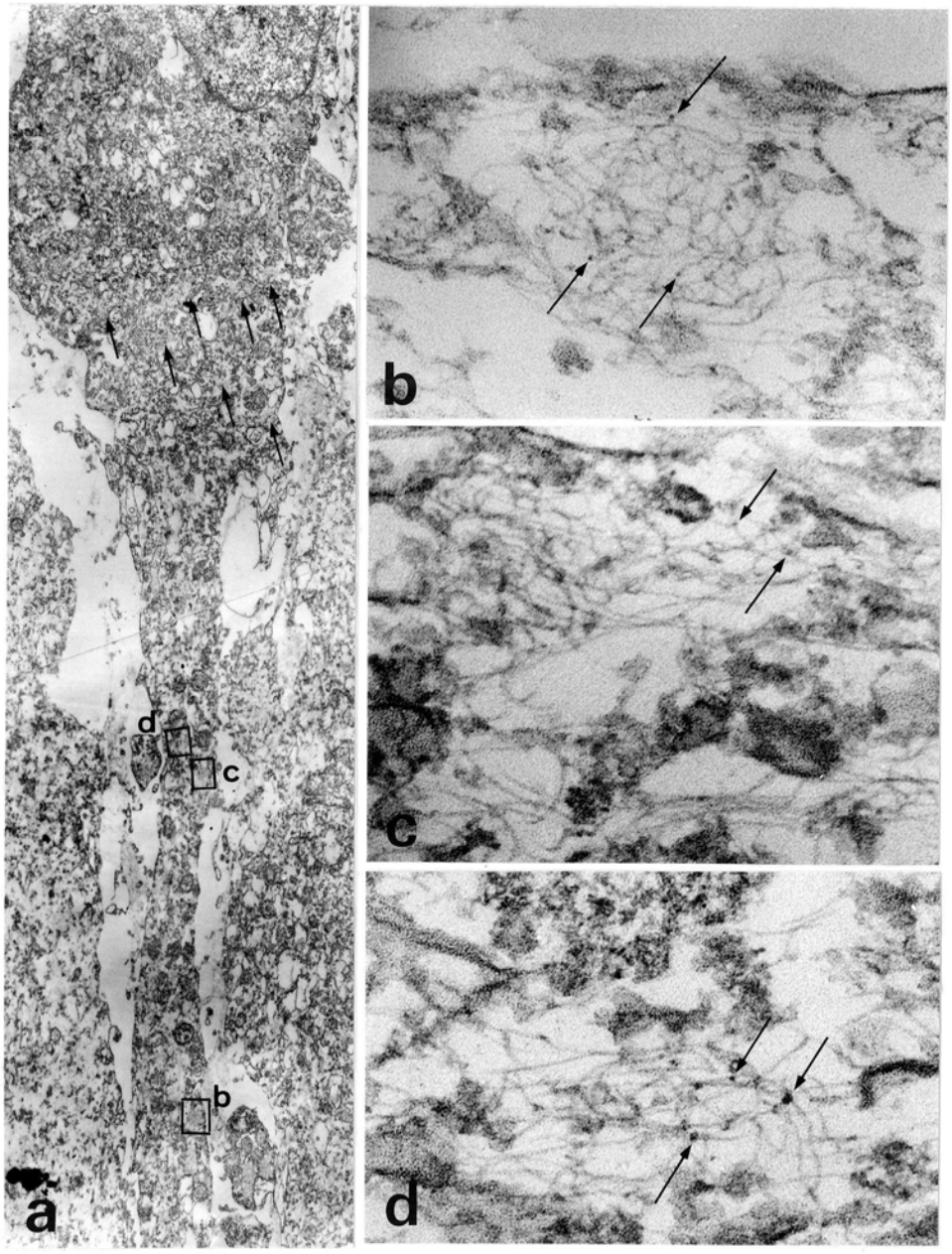
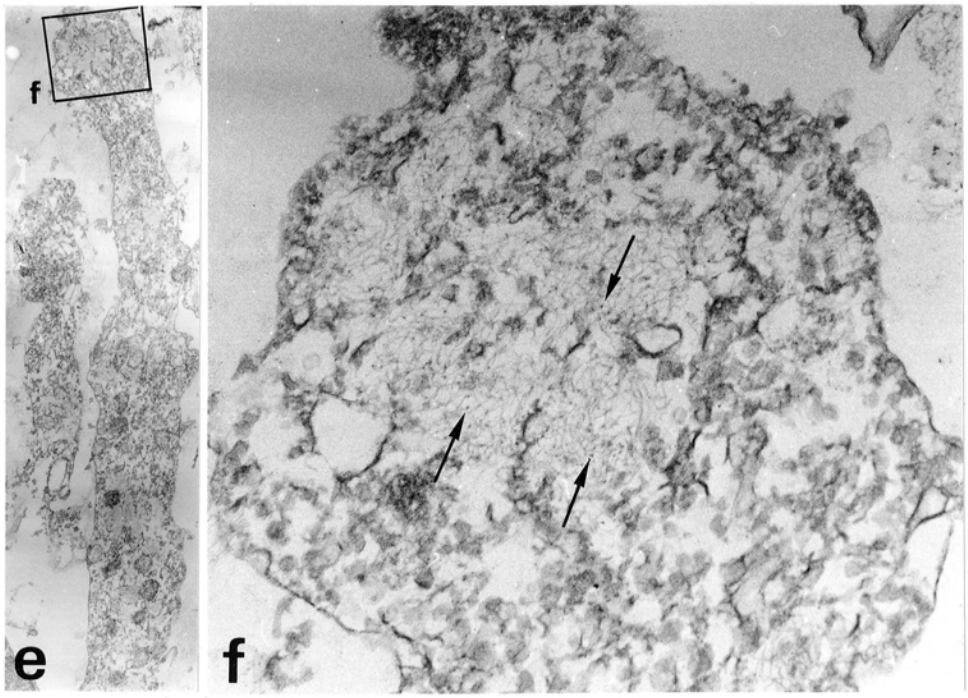


Fig. 11



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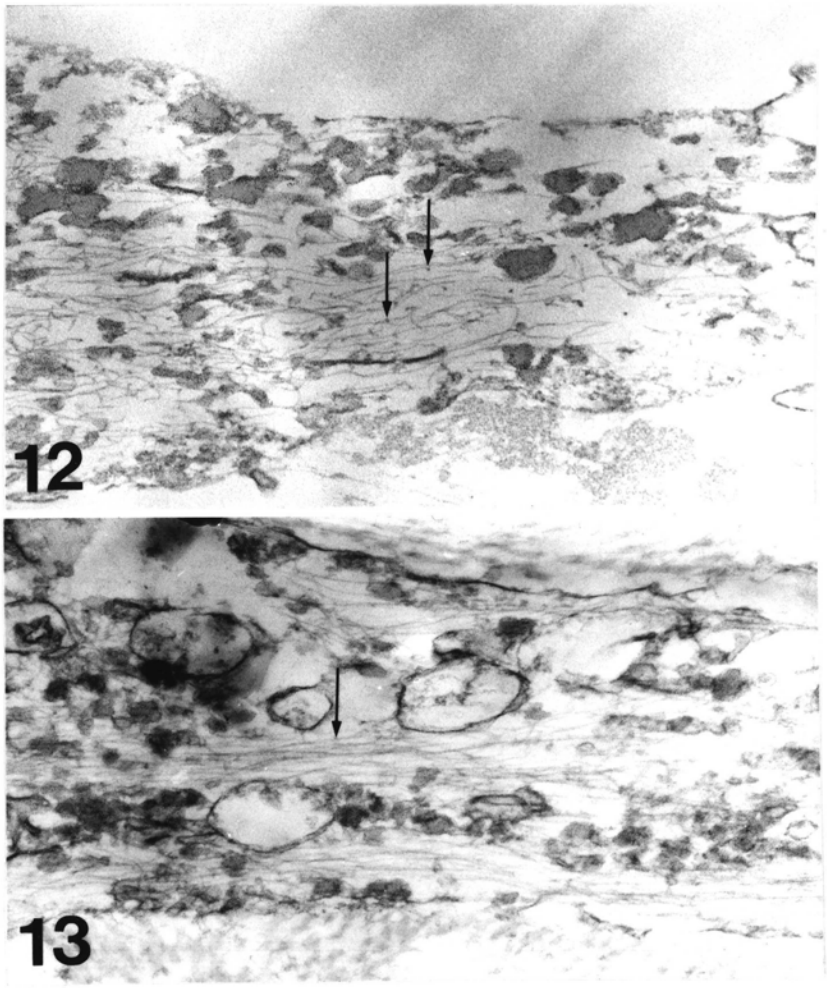

Fig. 12, Fig. 13

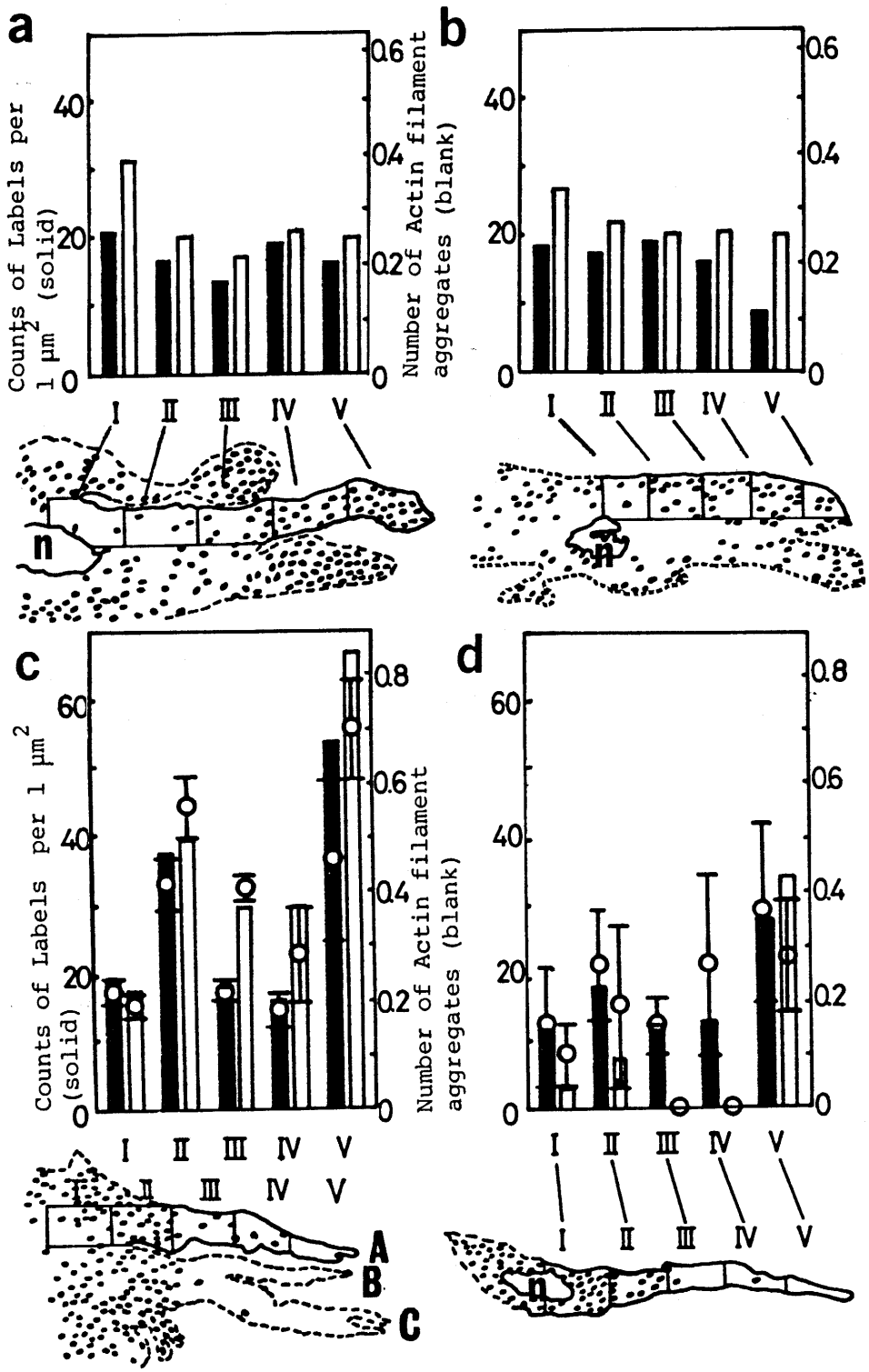
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Fig. 14

Fig. 1 Diagrammatic representation of a Ficoll density gradient used for centrifugal separation of swordtail erythrophores

Each 1 ml of 30%, 20% and 10% Ficoll solution and 0.5 ml of 5% Ficoll solution, all being dissolved in Ca^{++}, Mg^{++}-free phosphate buffered saline (CMF-PBS), was layered serially from the bottom of a centrifuge tube (Hitachi Model RPS-40) and allowed to stand for 20 hours to make a continuous gradient. Cells dissociated enzymatically from skin tissue were suspended in 1 ml of CMF-PBS and layered, with a spacer of 0.5 ml of CMF-PBS, on the top of density gradient. By centrifuging at 98,000g for 90 minutes at 4°C, erythrophores were precipitated as a red pellet in the bottom of a density gradient as shown in the figure on the right.

Figs. 2-4 TEM of swordtail erythrophores

Fig. 2 : A portion of the horizontally sectioned dendrites of the dispersed cell. Fig. 3 : A portion of the cell body of the same cell. Fig. 4 : A portion of the horizontally sectioned cell body and dendrites of the aggregated cell. Abbreviations indicate as follows; pt: pterinosome, cv: carotenoid vesicles, mt: microtubule, mf: microfilament, mc: membranous complex, n: nucleus.

Fig. 5 Electrophoretic separation of carp actin used as the antigen in a SDS polyacrylamide gel

(a) Actin fraction from carp skeletal muscles. Partially purified fraction (60 µg protein) was loaded on the top

of each gel column and electrophoresed. A main band of carp actin (arrow) was identified primarily by comparison with the mobility of rabbit actin run simultaneously in a separate column. (b) Actin fraction from rabbit skeletal muscles. Partially purified fraction (70 µg protein per a gel column) was subjected to similar electrophoresis. (c) Actin fraction from swordtail skeletal muscles. Partially purified fraction obtained by one cycle of G-F conversion (100 µg protein per a gel column) was electrophoresed for comparison. (d) Carp actin fraction reextracted from electrophoresed gels (8µg per a gel column). After electrophoresis of carp actin [lane (a)], each gel column was split into two parts along the electron flow direction, providing one for staining of protein bands and another for collection of actin. With the guidance of the stained half, the main band of carp actin in remaining half of a gel column was cut out in the positions shown by two lines in (a). The gel pieces were pooled as a purified antigen in -90°C deep freezer. The containing protein was also reextracted with PBS and analyzed again by electrophoresis (d).

Fig. 6 Standardization of anti-carp actin antibody by Ouchterlony immunodiffusion test

(a) Reactivity against the antibody : The antibody (Ab) produced in the present study using electrophoretically purified carp actin was placed in the center well and subjected to immune reaction against the antigen and

related fractions placed in side wells : 1 = gel septum containing a main band of carp actin and used as the antigen ; 2-5 = gel septa cut out serially at thickness of 3 mm from the fore tip of the main band toward the bottom of a gel ; 6= actin fraction before SDS treatment. As shown in Fig. 6a, a single precipitin line (arrow) appeared against native antigen (well #6), two precipitin lines (arrows) against denatured antigen placed in the well #1, one of which being common to native one. Faint line appeared for #2 but no precipitin line was observed for #3-#5. Gel plate was stained after diffusion test. (b and c) Reactivity against swordtail skeletal muscle actin : Actin fraction (100 μg protein per gel column) separated from swordtail skeletal muscles was subjected, as shown in the center of the figure, to further separation by means of SDS-PAGE. This gel column, as described in a legend for Fig. 5, was split into two parts and, with a guidance of stained half, trimmed at thickness of 3 mm (one increment in the scale), each septum being numbered serially from the top of column to the bottom. Slices of a gel column were placed in peripheral wells with PBS and subjected to immmunodiffusion test against anti-carp actin antibody (Ab) placed in the central well. Precipitin lines (arrows) appeared along wells, #6 and 7, which corresponded to the main band of actin in a gel column. No precipitin line was observed for slices after 12. (d) Reactivity against swordtail erythrophore actin : Erythrophore-rich non-muscle cell fraction

separated from swordtail skins by enzymatic tissue dissociation, and subsequent ultracentrifugation of Ficoll density gradient was subjected to freeze-and-thaw treatment or brief sonication and then placed in a side well (Ery). Note the presence of a single precipitin line between the antibody (Ab) and erythrophore fraction (Ery).

Fig. 7 Photomicrographs showing the distribution of actin filaments in swordtail erythrophores at varying stages of pigment displacement as visualized by indirect immunofluorescence

(a) A cell with fully dispersed pigments. x987 (b) A cell at the beginning of pigment aggregation (3 min after administration of epinephrine). x977 (c) A cell half-way through pigment aggregation (5 min after administration of epinephrine). x562 (d) A cell in the last phase of pigment aggrgation (8 min after administration of epinephrine). x848 (e) A cell at the completion of pigment aggregation, kept in epinephrine-added medium for more than 10 min. x562 (f) A cell with dispersed pigments, incubated without first and second antibodies. x987

Fig. 8 Electronmicrographs of erythrophores with dispersed pigments assayed with anti-actin antibody and peroxidase-conjugated second antibody

(a) Horizontal section. x5,000 (b) Enlarged image of rectangle portion in (a). x50,000 Arrows indicate labels.

pt; pterinosome, cv; carotenoid vesicles.

Fig. 9 Electronmicrographs of erythrophore with aggregated pigments assayed in the same way as in Fig. 8
(a) Horizontal section. x2,500 (b) Enlarged image of rectangle (b) portion in (a). x57,500 (c) Enlarged image of rectangle (c) portion in (a). x45,000 Arrows indicate labels.

Fig. 10 Immunoelectronmicrograph of a peripheral margin of pigment aggregate rich in the pterinosomes in erythrophores after pigment aggregation
Arrows indicate labels present on actin filaments in a form of spherical aggregates. Actin filaments are often connected with carotenoid vesicles (cv) or elements of smooth endoplasmic reticulum (ER) (short arrows) and situated very closely to pterinosomes (pt). x70,000

Fig. 11 Immunoelectronmicrographs of erythrophores under an intermediate phase of pigment aggregation
(a) Low magnification of a longitudinal section of a cell. Arrows indicate the locations of spherical aggregates of actin filaments. x5,400 (b) High magnification of portion (b) in (a), indicating the presence of meshworks of actin filaments. x75,000 (c) High magnification of portion (c) in (a), indicating stretching out of actin filaments in a parallel fashion. x75,000 (d) High magnification of portion (d) in (a), indicating frequent occurrence of actin filament aggregates at the tips of

the dendrites. x75,000 (e) Low magnification of a longitudinal section of the dendrites of another cell, indicating the abundant occurrence of filament aggregates in its tip. x11,500 (f) High magnification of portion (f) in (e). x35,000 Arrows in (b-f) indicate labels.

Fig. 12 Electronmicrograph of dendrite of aggregated erythrophores assayed in the same way as in Fig. 8. x45,000

Fig. 13 Electronmicrograph of a dendrite of aggregated erythrophores assayed with actin-absorbed anti-actin antibody. Note absence of label (arrow). x45,000

Fig. 14 Changes in distribution of actin filaments during pigment aggregation

(a) Before pigment aggregation. (b) At a onset of aggregation. (c) At a middle intermediate stage. (d) After completion of pigment aggregation. The distribution density of the filaments is indicated by the numbers of peroxidase labels on microfilaments (right ordinate and solid column) and of spherical aggregastes of filaments (left ordinate and blunk column) per unit area of a sectioned cell plane. The mean (open circle) and standard deviation (bar) are calculated from three estimates obtained on different cells at similar stages of pigment aggregation. Line drawings indicate an intracellular location of pterinosomes (black spots) which were traced from respective electronmicrographs. n; nucleus.

2-7. Summary

The localization of actin filaments in swordtail erythrophores in monolayer cultures or isolated scales or fins was examined in relation to their pigment (pterinosome and carotenoid vesicle) displacements by means of TEM, immunofluorescence and immunoelectronmicroscopy. The observations by TEM disclosed that abundant microfilaments with 6-7 nm width existed in these cells.

These filaments were apparently confirmed to consist of actin by immunohistochemical studies using anti-actin antibody. From the observations by immunofluorescence, these filaments appeared as either cytoskeletons in an astral array radiating from the cell center to the peripheries or as the bundles of motile character which circumscribed migrating pigment mass. These results clearly accorded with the evidence from immunoelectronmicroscopy.

The observations by immunoelectronmicroscopy were also presented as a semi-quantitative determination of the filaments, performed by counting the numbers of peroxidase products and aggregates of actin filaments per unit area in sectioned cell planes. Actin filaments in cells with dispersed pigments appeared mostly in a form of aggregates which were dispersed throughout the entire cytoplasm without forming definite meshworks. With the onset of pigment aggregation, their distribution density was markedly increased in two locations, the tips of the dendrites and the peripheries of aggregating pigment

masses, taking a loosely organized three-dimensional meshwork. A tendency was observed that actin filament-rich zones appear first in the distal portion of each dendrite and then split into these two locations, that is, the tips of the dendrites and the peripheries of aggregating pigment masses. The actin filament-rich zone which was in the peripheries of the aggregating pigment mass gradually moved centripetally with the progress of aggregation. Upon completion of pigment aggregation, the distribution pattern of actin filaments returned gradually to that of a cell at a resting state. A view was set forth that actin filaments gave rise to a tightening of cell peripheries, through which pigments translocated in the flow of cytoplasmic matrix.

Section 3

The blockade of pigment displacement by microinjection of anti-actin antibody

3-1. Introduction

Actin has been implicated as an important component in a wide range of non-muscle cell movements, including amoeboid movement and protoplasmic streaming of green algae (1, 2, 3, 4), but the functional roles of these molecules in the pigment displacement of cold-blooded vertebrate chromatophores remain to be clarified. Recent studies of the pigment displacement in fish erythrophores have focused on the participation of dynein-microtubular or microtrabecular systems (5, 6).

As described in previous sections, the pigments of the integumental erythrophores of swordtail fish can be displaced centripetally (aggregation) or centrifugally (dispersion), depending on the inducing signals (7). In section 2, combined immunofluorescence and peroxidase labeling immunoelectronmicroscopy were used to show that swordtail erythrophores contain an abundance of actin filaments and that the intracellular distribution of these filaments changes markedly upon pigment aggregation (8). Although I had revealed an intimate correlation between actin filament distribution and pigment displacement in erythrophores, I had not proved that actin is directly involved in this intracellular movement.

The present study of this section was meant to clarify the putative role of actin as the crucial functional component in erythrophore pigment movement. The problem was approached technically by a microinjection method

used to deliver, intracellularly, an antibody produced against carp skeletal muscle actin. Phalloidin, which promotes F-actin formation and stabilizes the molecule in this form, and DNase I, an inhibitor for polymerization of G-actin, were also microinjected into living erythrophores in order to examine whether G-F conversion plays any roles in pigment displacement (9, 10, 11, 12). The results indicate unequivocally that actin is the functional element in both centripetal and centrifugal pigment displacements in these cells.

3-2. Materials and Methods

Physiological treatment for microinjection study

Monolayer cultures of swordtail erythrophores were prepared on 9 x 17 mm plastic slips (Lux No. 5409) according to the methods shown in section 1 (7). Large erythrophores (macroerythrophores), with a diameter of 28-33 μm when rounded up, and containing many red pterinosomes and yellow carotenoid vesicles, were selected for microinjection. Under standard culture condition, pigments in the macroerythrophores were dispersed throughout the cytoplasm. Pigment aggregation was initiated by exposing these cells to (transferring a culture slip into) 5 x 10^{-4} M epinephrine (Sigma No. E-4375), and was completed within 1.5 minutes. Pigment redispersion was elicited as follows. Cells with aggregated pigments were washed briefly in phosphate-buffered saline (PBS) and then rinsed in 10^{-3} M theophylline. Pigment movement responses remained unaltered during the entire week of culturing, and 3- to 5-day-old cultures were routinely utilized. Epinephrine and theophylline were dissolved in either PBS or a standard culture medium.

The pigment movements of cultured macroerythrophores were examined under a light microscope (E. Leitz, Watzlar, LABORLUX II). Fields of view were selected at a magnification of x160 for observation, if they contained several responsive cells of similar or smaller size clustered together. Preliminary tests of responsiveness were made

by exposing the cells first to epinephrine and then, after recognition of complete aggregation, to theophylline for redispersion. Photographic records of each cell cluster were taken at three stages of such treatments: resting, aggregation, and redispersion. Recovery of the cell shape after two cycles of the treatment was fairly good; but severe modifications occasionally occurred during the second cycle. When noticeable cell shape change occurred in the injected cell upon examination of responsiveness after microinjection, mainly owing to detachment of its periphery, the assay was rejected from the valid data.

Microinjection Microinjection was achieved with the use of a Leitz micromanipulator (No. 520120) equipped with glass microneedles having tip diameters of less than 0.5 μm. Microneedles were made using an accessory puller fitted with an improved platinum heater. The micromanipulatory systems were filled with liquid paraffin which transferred, precisely, the pressure of the cylinder to the tip of the needles. The volumes of chemical solvents injected were calibrated from the diameters of liquid paraffin droplets ejected from an equivalent increment of the same microneedle and were measured as being about 2 pl; this volume corresponded to less than 10% (mostly 8.5%) of the cell volume. Preliminary experiments indicated that this dosage does little mechanical damage to most of the cells and does not perturb their pigment response. However, in less than 10% of the experiments,

pigment dispersion failed after cells in the aggregated state were injected. The successful introduction of antibody or related chemicals into the cells was assured by observing, intracellularly, either the instant diffusion of methylene blue mixed with the injected solvent (final concentration, 5%), or the presence of liquid paraffin droplets injected directly after the solvent. The former was achieved in every injection since all the solutions to be injected contained methylene blue, whereas the latter was achieved in about half of the total injections for respective assays. Microinjection into a protruded cell body of aggregated cells often resulted in mechanical cell breakage, possibly owing to their increased surfacial tension. Such technical difficulty provide the reason for relatively fewer cell numbers in the examination of aggregated cells than dispersed.

Chemicals for microinjection The anti-actin antibody used in this section was produced by a rabbit given repeated subcutaneous injections of purified carp skeletal muscle actin. The preparation of this antibody was described in detail in section 2. This antibody was microinjected after its concentration had been adjusted to 2.53 mg protein/ml. The Ouchterlony immunodiffusion test, using serially diluted antigen, indicated that the antibody at 2.53 mg protein/ml binds with 0.74 mg protein/ml of actin.

As a control, the same antibody, at a similar

concentration (2.32 mg protein/ml), but saturated with purified actin (absorbed antibody), was injected. Absorbed antibody was prepared by mixing the antibody with an excess of actin isolated from carp skeletal muscle. Protein concentration was determined according to the method of Lowry et al. (13); bovine serum albumin fraction V (Sigma) was the standard.

Phalloidin (Calbiochem, 516640) and DNase I (Sigma, E.C. 3. 1. 4. 5.) used for microinjection were dissolved in PBS at the final concentrations of 0.5-10 mM and 1-5 mg/ml, respectively. The intracellular concentrations of the drugs used in this study were technically limited and could not be increased. First, the concentrations of the injected solution must be sufficiently low that their viscosity does not impede pressure microinjection. Second, the total volume of solvent injected must be kept smaller than 10% of the cell volume to preclude mechanical damage. All solutions of the antibody, absorbed antibody, and the two drugs contained methylene blue at a final concentration of 5%. The microinjection technique used in these experiments was critical since all of the chemicals tested are impermeable to normal cell membranes.

3-3. Results

When anti-actin antibody was injected into 22 dispersed erythrophores, pigment aggregation inducible by epinephrine administration was inhibited totally in 77.3%, partially or incompletely in 18.2%, and negligibly in 4.5% (Table 1, Fig. 2). In all experiments, adjacent, noninjected erythrophores responded to this neurotransmitter with instantaneous pigment aggregation. Microinjection of the same anti-actin antibody into cells in an aggregated state, followed by the administration of theophylline, resulted in total inhibition of pigment dispersion in all of eight examined cells. Relatively few cells were used in the latter experiments because satisfactory injection of the antibody into aggregated cells, without cell destruction, was technically difficult. Nevertheless, the anti-actin antibody, once introduced successfully into a spherical cell body, did block pigment dispersion totally.

When similar concentrations of the saturated antibody were microinjected into erythrophores with dispersed pigments, nine of 11 examined cells exhibited complete pigment aggregation upon exposure to epinephrine (Table 1, Fig. 3). Microinjection of the same absorbed antibody into cells with aggregated pigments, and subsequent exposure to theophylline, resulted in complete pigment dispersion in nine of 13 examined cells.

The time required to complete the reaction, and the degree of pigment aggregation or dispersal at the end,

were virtually identical between cells injected with an absorbed antibody and cells not injected at all. Thus, the vast majority of cells microinjected with the absorbed antibody are capable of pigment displacement as the noninjected ones. Equivalent volumes of PBS or liquid paraffin, microinjected into dispersed cells, had little effect on pigment aggregation, but the same treatment on aggregated cells sometimes (10-15% of injected cells) blocked pigment dispersion (Table 1). Therefore, microinjection itself must cause virtually no mechanical damage since inert substances have no effect on pigment displacement.

The effects of microinjected phalloidin and DNase I on centripetal and centrifugal pigment displacements were different. Administration of these two drugs at two different concentrations into cells with dispersed pigments, followed by epinephrine exposure, totally inhibited pigment dispersion in about half of the examined cells; there was little effect in the remaining half. In contrast, injecting the same drugs into cells with aggregated pigments, followed by theophylline exposure, totally inhibited pigment dispersal in about 70% of the examined cells for phalloidin, and nearly half for DNase I. Both of these drugs seemed to be more distinctively inhibitory on pigment dispersal than on aggregation.

3-4. Discussion

Light and electron microscopic immunocytochemistry have disclosed that the intracellular distribution of actin microfilaments in swordtail erythrophores is different in the dispersed and aggregated states of the pigments (sectin 2) (8). To elucidate further the functional roles of actin in their pigment displacement, an anti-actin antibody and two motility inhibitors acting on actin were injected directly into living cells.

The present study decisively indicates that the anti-actin antibody produced against carp skeletal muscle actin, when microinjected into the erythrophores, blocks pigment movement totally, no matter the migratory direction. The almost complete absence of such blockade by the actin-absorbed antibody supports the idea that specific binding of the antibody to intracellular actin interferes with pigment displacement. The results all suggest that actin is a functional element in centripetal and centrifugal pigment displacement of erythrophores.

The study of this section has also confirmed that microinjected phalloidin and DNase I cannot completely inhibit either centripetal or centrifugal pigment displacement, and that pigment dispersion is more susceptible to these drugs than pigment aggregation. These findings suggest that the G-F conversion of actin molecules, or the assembly and disassembly of F-actin, is not essential process in pigment displacement. The different

susceptibilities to these drugs of aggregating and dispersing cells may be correlated with the previous findings that the surface changes accompanying pigment dispersion, including microvillus formation, are not merely a reversal of the changes seen during aggregation (refer to section 1) (14).

The incomplete inhibition of pigment movements by phalloidin and DNase I raises the possibility that their intracellular dosage was insufficient to produce an effect. In fact, the intracellular concentrations of phalloidin employed in this work are substantially higher than those used in the microinjection studies on Chara (15) and sea urchin eggs (16), because the volumes of the latter cells were larger than those of the swordtail erythrophores.

Since the actin content of swordtail erythrophores is unknown, the optimum dosage of DNase I to be injected is difficult to estimate. If the simplified assumptions are accepted that an erythrophore, when rounded up, has a diameter of 30 µm, a volume of 1.4×10^4 μm^3, a dry weight of 5×10^{-10} g, and 3.5×10^{-10} g of protein, including 1-5% of actin, then the actin content per cell would be about 10^{-11} -10^{-12} g. Since in vitro experiments indicate that DNase I binds with actins at a 1:1 molecule ratio (17), the dose of this drug that was injected (2 pl of 5 mg/ml solution) should have been approximately sufficient to mask endogeneous G-actin. These considerations support the notion that the incomplete

inhibitory effects of phalloidin and DNase I reflect the limited role of G-F conversion in pigment displacement of erythrophores. A similar observation concerning these two drugs was reported with angelfish melanophores by Schliwa et al. (18).

Recently, some inhibitors of dynein activity were shown to block intracellular transport of pigment in squirrelfish erythrophores (6). Evidently, swordtail erythrophores have two distinctive cytoskeletons : microtubules running along the central axis of their dendrites, and microfilaments distributed in the form of a meshwork (14). These cytoskeletons are known to be approximated topographically, and may even be linked structurally to each other. Thus, acting together, they may both contribute to complete and rapid pigment displacement. If this were the case, the discrepancy between observations on pigment transport would be explained reasonably.

3-5. References

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3-6. Figures and Figure legends

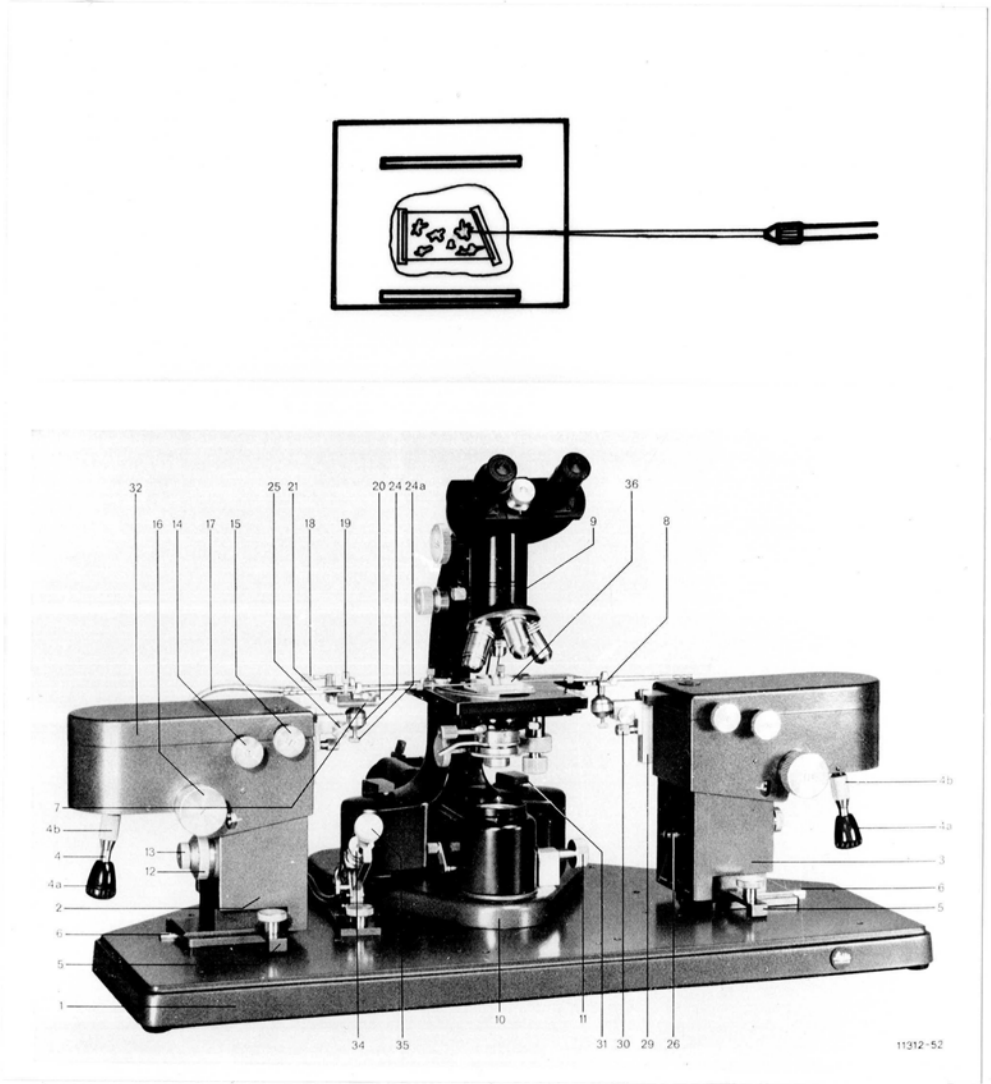

Fig. 1

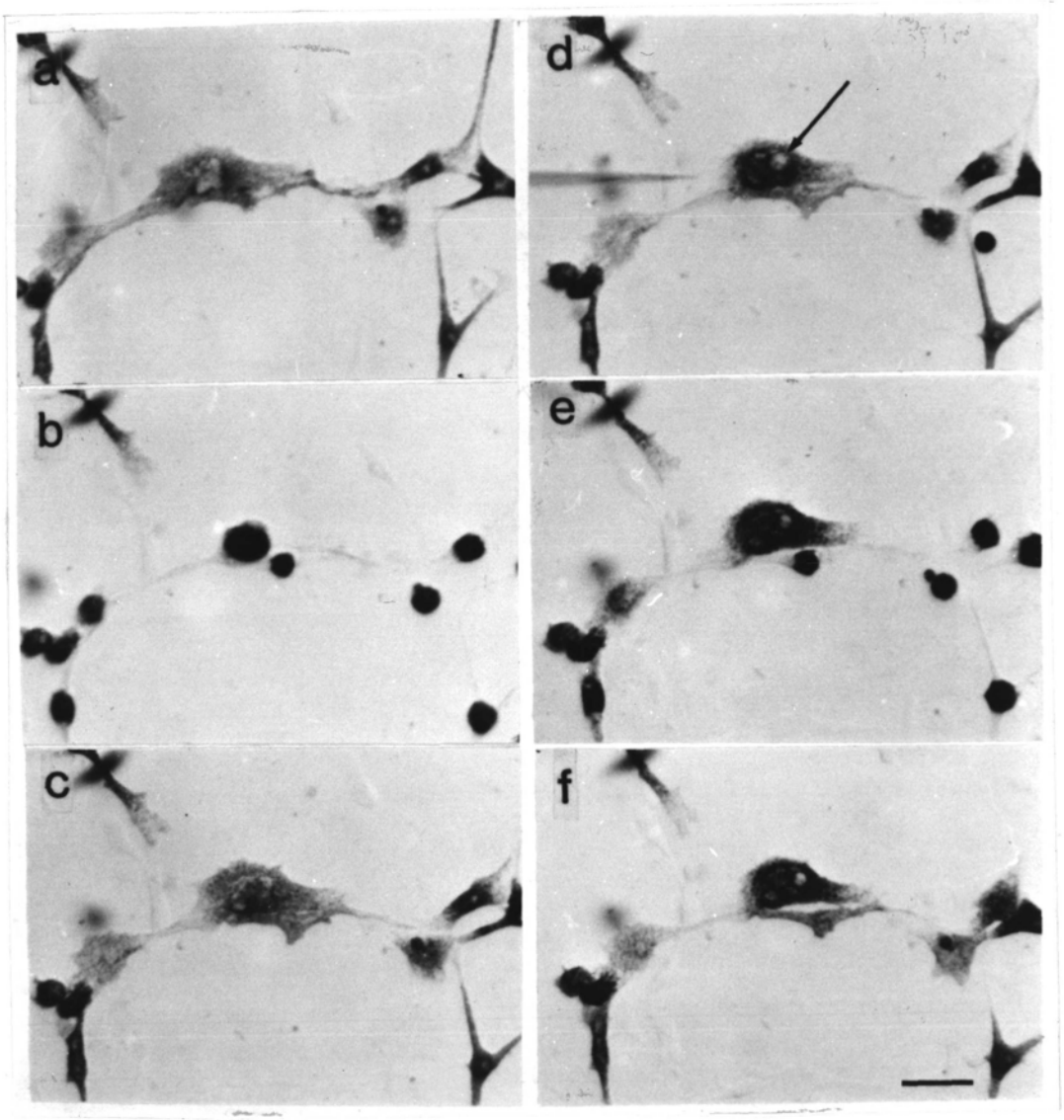

Fig. 2

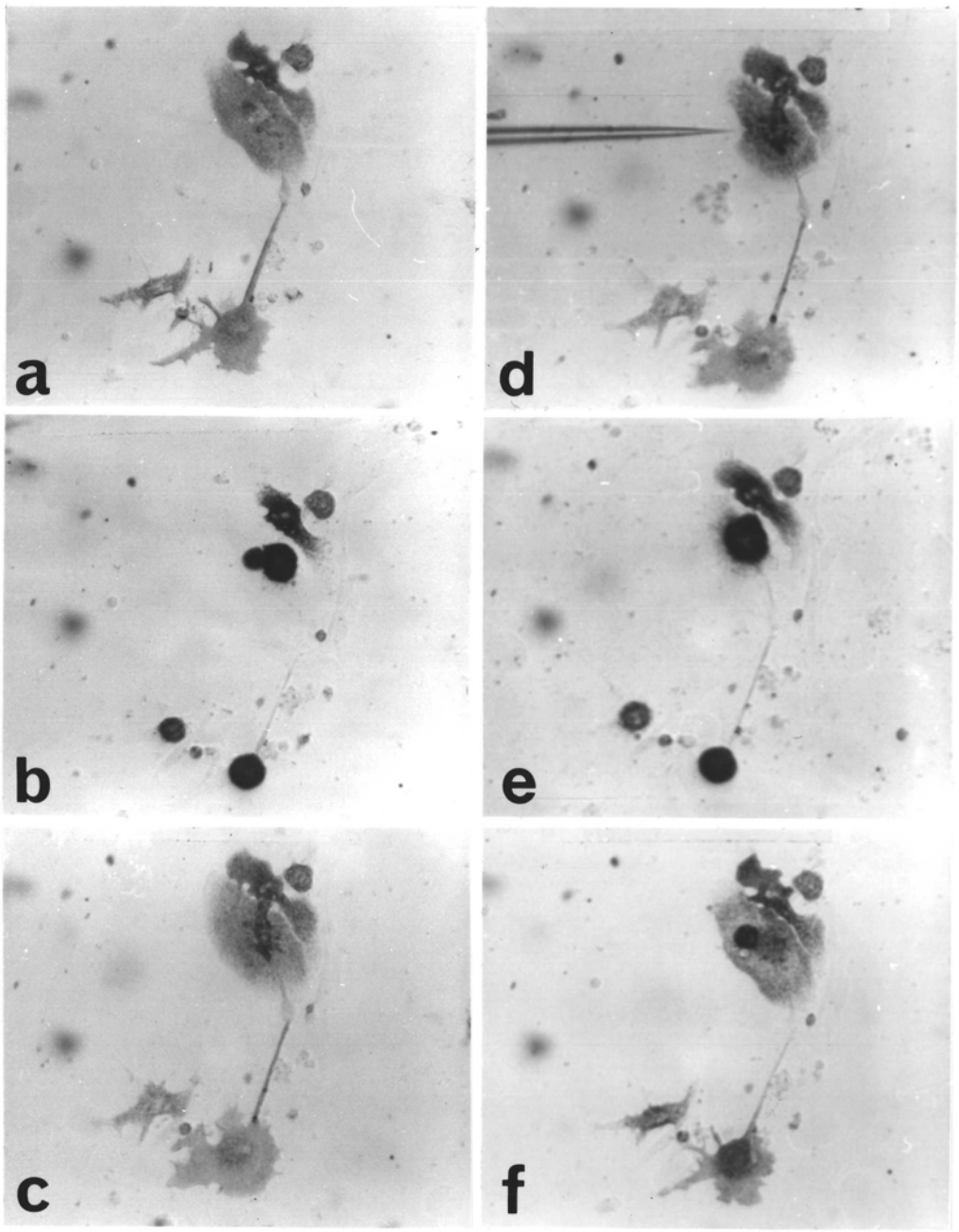

Fig. 3

Table 1 Effects of anti-actin antiserum, phalloidin and DNase I on pigment displacement of swordtail macroerythrophores

Chemicals & milieu injected	Type of pigment response	Numbers of cells			
		Examined	Totally inhibited (%)	Partially* inhibited (%)	No effect (%)
Anti-actin antibody	Aggregation	22	17 (77.3)	4 (18.2)	1 (4.5)
	Dispersion	8	8 (100.0)	0 (0)	0 (0)
Absorbed antibody	Aggregation	11	1 (9.1)	1 (9.1)	9 (81.8)
	Dispersion	13	1 (7.7)	3 (23.1)	9 (69.2)
Control (PBS or liquid paraffin)	Aggregation	18	0 (0)	0 (0)	18 (100.0)
	Dispersion	13	2 (15.4)	0 (0)	11 (84.6)
Phalloidin	Aggregation	(0.5-1mM) 10	4 (40.0)	1 (10.0)	5 (50.0)
		(10mM) 15	6 (40.0)	1 (6.7)	8 (53.3)
	Dispersion	(0.5-1mM) 8	6 (75.0)	1 (12.5)	1 (12.5)
		(10mM) 17	11 (64.7)	4 (23.5)	2 (11.8)
DNase I	Aggregation	(1mg/ml) 16	6 (37.5)	3 (18.8)	7 (43.8)
		(5mg/ml) 12	5 (41.7)	2 (16.7)	5 (41.7)
	Dispersion	(1mg/ml) 6	3 (50.0)	1 (16.7)	2 (33.3)
		(5mg/ml) 15	6 (40.0)	8 (53.3)	1 (6.7)

* Incomplete inhibition refers to responses in which induced pigment aggregation or dispersion occurs as judged by a slight to moderate retraction of dispersed pigments, or a loosening of peripheral of pigment aggregations, but the final stage is not reached.

Fig. 1 Illustrations of a Rose chamber (upper) and a micromanipulator (lower) used for microinjection

The micromanipulatory system was completely filled with liquid paraffin from the cylinder to the tip of a microneedle to transfer the pressure of the cylinder precisely. A trapezoidal-shaped plastic cover slip with monolayer cultured cells was fixed inside the Rose chamber with adhesive tape and rinsed in culture medium or epinephrine solution. The microneedle was injected into a cell at an angle 3-5 degree to the horizontal plane. The individual explanations for the parts of the micromanipulator which were indicated by numbers were omitted.

Fig. 2 Photomicrographs indicating blockage of pigment displacement by microinjection of the anti-actin antibody into swordtail erythrophores

(a) Before microinjection. Cells are in dispersed state in the standard culture medium. (b) The same as (a) after administration of 5×10^{-4} M epinephrine. All pigments are aggregated in the center. (c) The same as (b) after brief rinsing in PBS and subsequent administration of 10^{-3} M theophylline. Pigments are redispersed. (d) The same as (c) immediately after injection of the anti-actin antibody into a cell present in the center. Liquid paraffin droplet injected following the antibody solution was seen inside the cell (arrow). Note that the cell shapes of injected and noninjected cells were changed slightly after

one cycle of pigment responses. (e) The same as (d) which was exposed to epinephrine after injection. Note that the blockade of pigment aggregation was seen only in the injected cell whereas uninjected cells nearly present complete pigment aggregation. (f) The same as (e) which was then exposed to theophylline. No change was seen in the injected cell whereas the pigments in the others are redispersed. Scale bar 50 μm.

Fig.3 Photomicrographs showing an absence of blockage of pigment displacement in an erythrophore microinjected with absorbed antibody

Treatments in (a)-(f) are the same as in Fig. 2 except that the absorbed antibody was microinjected in (d). Note that pigment aggregation comparable to those in (b) did occur in a recipient cell (pointed at by a microneedle) with the absorbed antibody. Scale bar 50 μm.

3-7. Summary

When the anti-actin antibody produced against carp skeletal muscle actin was microinjected into monolayer cultured swordtail erythrophores, pigment displacement was almost completely blocked. Moreover, the injected antibody was equally effective at blocking the aggregation of dispersed pigment and the dispersion of aggregated pigment. If antibody saturated with carp actin was microinjected, no such blockade occurred.

Microinjection of phalloidin or DNase I into these cells brought about an incomplete inhibition of their pigment displacement. These two drugs tended to inhibit pigment dispersion more than aggregation, suggesting that actin is differently involved in the two phases of pigment movement. The results all indicate that actin is the functional element in the pigment displacement of swordtail erythrophores, and that G-F conversion of the molecule plays little or no role in pigment aggregation, but is implicated in some way in dispersion.

Section 4

The behavior of microinjected liquid paraffin droplets during motile response

4-1. Introduction

Swordtail erythrophores display two different pigment displacements; rapid aggregation of pigment granules in response to epinephrine and melatonin, and slow dispersion in response to melanocyte-stimulating hormone (MSH), theophylline and cyclic adenosine monophosphate (1). As shown in section 1, the migratory velocity of pigment granules is estimated to be on the order of 1 μm/sec for the former response and 0.03-0.1 μm/sec for the latter. A kinetic study of this cell motility disclosed that the mode of pigment movement is apparently different between aggregation and dispersion, suggesting the engagement of different procedures in the two phases of movement (see section 1) (2).

The distinctive feature of this intracellular movement is that two different kinds of cytoplasmic pigment granules present in these cells, red pterinosomes and yellow carotenoid vesicles, migrate simultaneously in the same direction in response to the same stimulus. The simultaneous translocation of these two types of pigment granules, which are entirely different in regard to morphology and chemical composition, would favor the view that this motility, at least in these particular pigment cells, is achieved by the mechanisms that carry all movable cytoplasmic materials.

In the section 1, scanning electronmicroscopic observations on the pigment cells fixed during pigmentary

responses disclosed that, upon pigment aggregation, a pronounced squeezing of the cell periphery and a protrusion of the perikaryon were brought about, shaping it into a hemisphere to which many thin dendrites or sleeves were attached, whereas, upon pigment dispersion, flattening of the cell body and thickening of the cell periphery occurred, restoring the ordinary shape with almost even thickness (3). These observations indicated that the vast majority of cytoplasmic components translocate back and forth between the perikaryon and the cell periphery at the pigmentary response. The objective of this section is to examine whether a particle of foreign material can migrate inside the dendrite along with endogenous pigment particles. For this purpose, liquid paraffin droplets of varying sizes were microinjected into monolayer-cultured swordtail macroerythrophores, and the behavior of such droplets inside the dendrite during pigment responses was observed under a light microscope. The results provided us with critical information as to whether pigment particles themselves have a motor for their translocation or whether cytoskeletal elements generate the motive force in this unique cell motility.

4-2. Materials and Methods

Analysis of the behavior of microinjected liquid paraffin droplets Integumental erythrophores were dissociated from scales of homogeneously red-colored swordtail fish maintained as an inbred strain in this laboratory by the method reported previously and then placed in sterile plastic slips (Lux #5409) for sparse monolayer culture (1). A varying amount (0.004-0.49 pl) of liquid paraffin (Wako Pure Chemical Industry, Tokyo) was microinjected into large erythrophores (macroerythrophores) (4) having a diameter of more than 70 μm. These cells, when stimulated, yielded a pigment aggregate having a diameter of 28-33 μm. The volume of liquid paraffin microinjected was calculated from the diameter of a droplet placed inside the cell. Details of the microinjection technique were described in section 3. The behavior of liquid paraffin droplets in pigment response was observed immediately after microinjection under a light microscope and recorded by photographs taken at intervals of 2 minutes. Pigment aggregation was elicited by exposing the cells to 5 x 10^{-4} M epinephrine dissolved in phosphate-buffered saline (PBS) and pigment dispersion by exposure to 10^{-3} M theophylline according to the method for the treatment of erythrophores, as described in section 1. The migratory span of microinjected liquid paraffin droplets was determined in relation to stable anchoring spots of the microinjected cell on printed color microphotographs at

a magnification of x330 (cf. Fig. 1a). Migration distances of the endogenous pigment granules near the liquid paraffin droplets were measured on printed photographs in a similar fashion and the average values calculated.

4-3. Results

Microinjection of liquid paraffin in varying amounts up to 0.49 pl was undertaken on 18 erythrophores with dispersed pigments and on 13 of those with aggregated pigments. In the former, all the cells (100%) aggregated their pigments upon administration of epinephrine (5×10^{-4} M), while in the latter, 11 out of 13 cells (84.6%) dispersed their pigments upon administration of theophylline (10^{-3} M). The response of liquid paraffin-microinjected cells to the amine or phosphodiesterase inhibitor was essentially similar in its rapidity and modality to that of the untreated cells. This apparently means that microinjected liquid paraffin at the dosage employed causes little mechanical damage and has little cytotoxicity in these cells.

When erythrophores in a dispersed state were microinjected with a droplet of liquid paraffin having a diameter of up to 2.5 μm (8.18 μm^3), mostly into a peripheral dendrite region, and then exposed to epinephrine, the droplet migrated toward the cell center together with endogenous pigment granules (Figs. 1a-d and 2, spots a-d). The migratory distance of these droplets was 7.34 μm on the average. When these aggregated cells were then exposed to theophylline (Figs. 1f-h and 2, spots f-h), the liquid paraffin droplets migrated toward the cell periphery and the average migratory distance was 10.57 μm (Table 1). These values were far smaller than

those of endogenous pigment granules (0.30 times for aggregation and 0.42 times for dispersion). In most cases, microinjected paraffin droplets, even though they could move short distances, were soon stacked at a part within the dendrite, while all the endogenous pigment granules present near the droplet migrated away into the cell center (Figs. 1d and 2d). Such droplets located in dendrites started to move toward the cell periphery upon the onset of pigment dispersion.

The migratory distance of a liquid paraffin droplet was shortened with increase in its size up to about 5 μm and, beyond this size, it failed to move. Upon pigment aggregation, such relatively large liquid paraffin droplets were subjected to deformation, where the regular sphere was rendered to a teardrop or pear-like shape, pointing its narrower tip toward the cell center (Figs. 1c, d and 3c, d). Another shape-deformation was observed at pigment dispersion, alternating its form from a sphere to a long spheroid (Figs. 1g and 2, spot g). During aggregation, liquid paraffin droplets which were too large to move were often torn into two or three pieces (Figs. 1e-g and 3, drops e-g) preceded by a severe shape-deformation. Such splitting of liquid paraffin droplets was rarely observed during pigment dispersion.

4-4. Discussion

The study of this section clearly indicates that: 1) liquid paraffin droplets do migrate at pigment response, though their migratory distances were far shorter than those of endogenous pigment particles, 2) the migratory direction of the droplets is in parallel to that of endogenous pigments, and 3) the droplets are deformed upon the onset of pigment movement, as if they were being pulled away along the migratory direction of endogeneous pigments, and their original regular spheres were restored with completion of pigment response. A liquid paraffin droplet is an unphysiological foreign substance. And further, pigment response was induced immediately after microinjection in order to prevent possible surfacial modification such as coverage by a protein. From these reasons, it seems reasonable that the motive force for its locomotion is provided by surrounding cytoplasmic components. It is clear that cytoplasmic components move synchronously in all the dendrites, along the cell center-to-periphery axes, upon initiation of pigment response. And it is also evident that endogenous pigment particles, as well as liquid paraffin droplets, migrate passively in association with such a movement or flow of cytoplasmic components. Occasional splitting of a droplet during migration would indicate that the driving force of the cytoplasmic movement is strong enough to overcome the surface tension of liquid paraffin.

A question naturally arises as to the nature of cyto-

plasmic components that generate motive force for translocation of their own, pigment particles and other movable ones, even foreign ingredients. The studies of section 2 by means of immunofluorescence and electronmicroscopic immunocytochemistry disclosed that swordtail erythrophores are equipped with an abundance of actin filaments, in addition to numerous microtubules. And the studies also indicated that, with the onset of pigment aggregation, actin filaments, which resided uniformly in the form of a loose meshwork, are subjected to marked changes in their distribution, forming a compact meshwork around aggregated pigments in the cell center (5). However, at the same time, microtubules in these cells are subjected to marked density reductions, suggesting strongly that actin rather than microtubules acts as a functional element in the operation of the pigment movement. In section 3, it has been indicated that when the antibody against carp skeletal muscle actin was microinjected into the cultured erythrophores, both pigment aggregation and dispersion were blocked almost completely (4). The same treatment with the use of the absorbed antibody caused little blockade. These results unequivocally indicate that actin is involved as functional machinery in both phases of pigment translocation. Recent advances in knowledge concerning cell motility indicate that contractility of the actin-involving system in certain non-muscle cells (6,7), as in protoplasmic streaming in the Chara species (8), was realized the the interaction with myosin as with

typical skeletal muscles. However, little is thus far known about the presence of myosin in swordtail erythrophores.

As to the organization of cytoskeletal components in fish pigment cells, Porter and his associates have reported the occurrence of an intracytoplasmic lattice named microtrabecula (9). This lattice was considered to be organized, in association with microtubules, into a cytoplast, a structural unit for pigment translocation (10). The microtrabecular lattice, although its exact molecular composition remains to be clarified, was reported to be free from actin. In squirrelfish erythrophores, pigment translocation was thought to take place in parallel to the drift of microtrabeculae along microtubules (11), while microinjected beads in angelfish melanophores failed to move along endogenous melanosomes (12). The results in this section were apparently different from these observations in that : 1) swordtail erythrophores were installed with an abundance of actin mostly in the form of meshworks and 2) exogeneous particles migrated together with endogenous pigments. Viewed from the cell shape change of swordtail erythrophores during pigmentary response as revealed by SEM (See section 1) (3) it was likely that contraction of intracytoplasmic meshworks, which were imparted mostly with actin filaments, yielded the motive force for centripetal translocation (aggregation) of motile intracytoplasmic ingredients, while reorganization of the meshworks caused a centrifugal shift (dispersion) of the ingredients. Relatively positive blockade of

microinjected DNase I on pigment dispersion of these cells would imply that actin was involved in the reorganization of the meshworks (4).

Studies on chromatophores obtained on species other than the swordtail favor the view that microtubules and dynein play a key role in pigment translocation. With swordtail erythrophores, it was shown that microtubules were necessary for well-ordered, rapid locomotion of pigment, inasmuch as the pretreatment of these cells with colchicine caused it to be irregular and extremely slow (2, 3). However, the motility of pigment particles in swordtail erythrophores appears to rely more on the actin-related system than on microtubules. Morphological visualization of the meshworks present in swordtail erythrophores, probably by high-voltage electronmicroscopy, is necessary. But even though the observation is an urgent task, the findings that artificial particles such as liquid paraffin droplets translocate inside the cells and change their shape at pigment response would lend good support to the above-mentioned model on pigment translocation.

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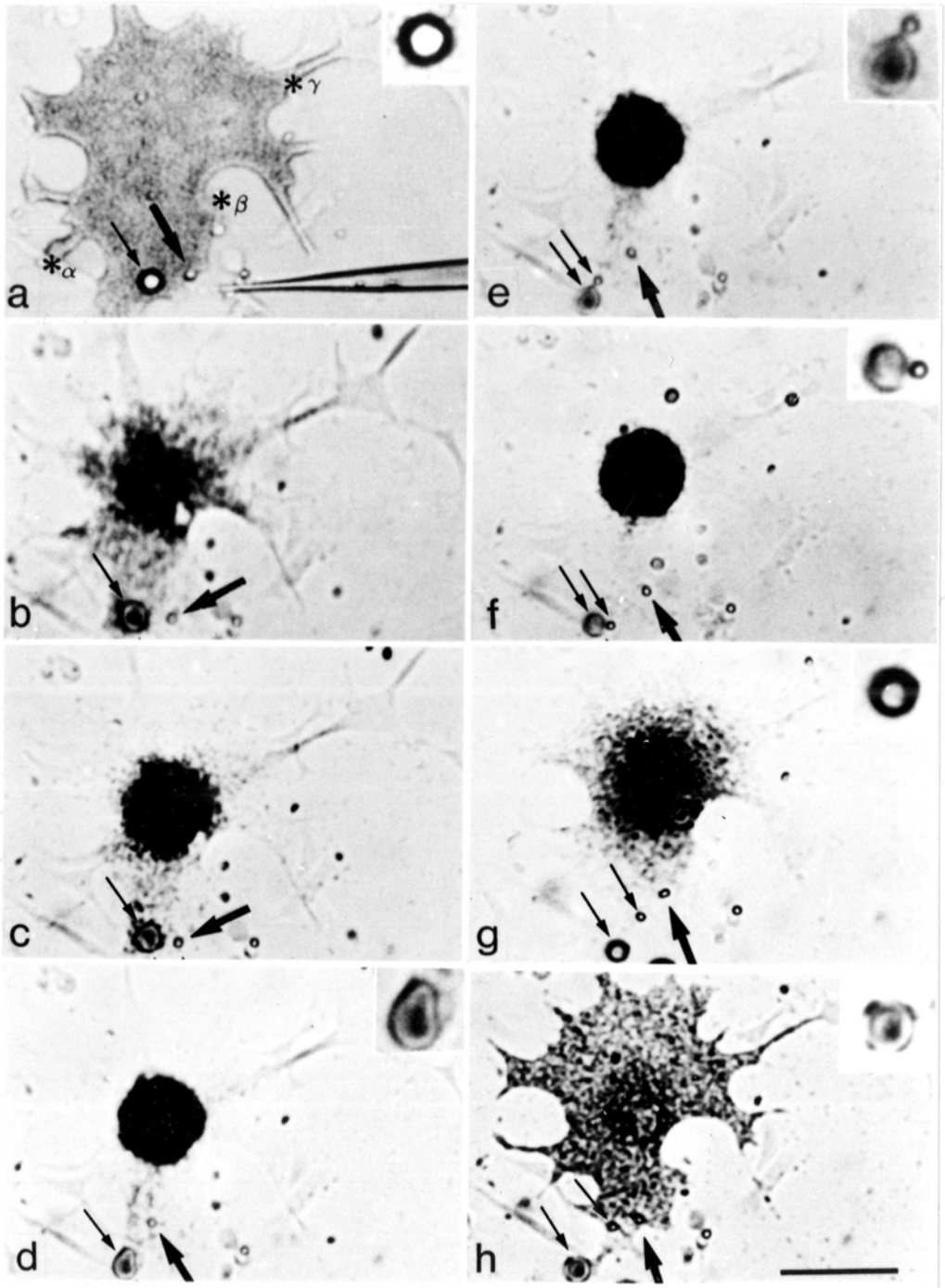
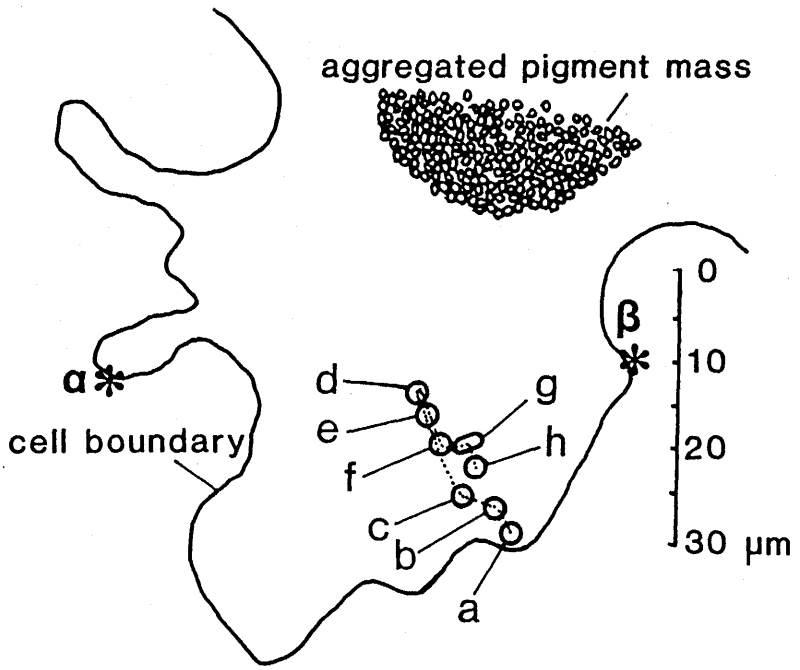


Fig. 1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cell boundary
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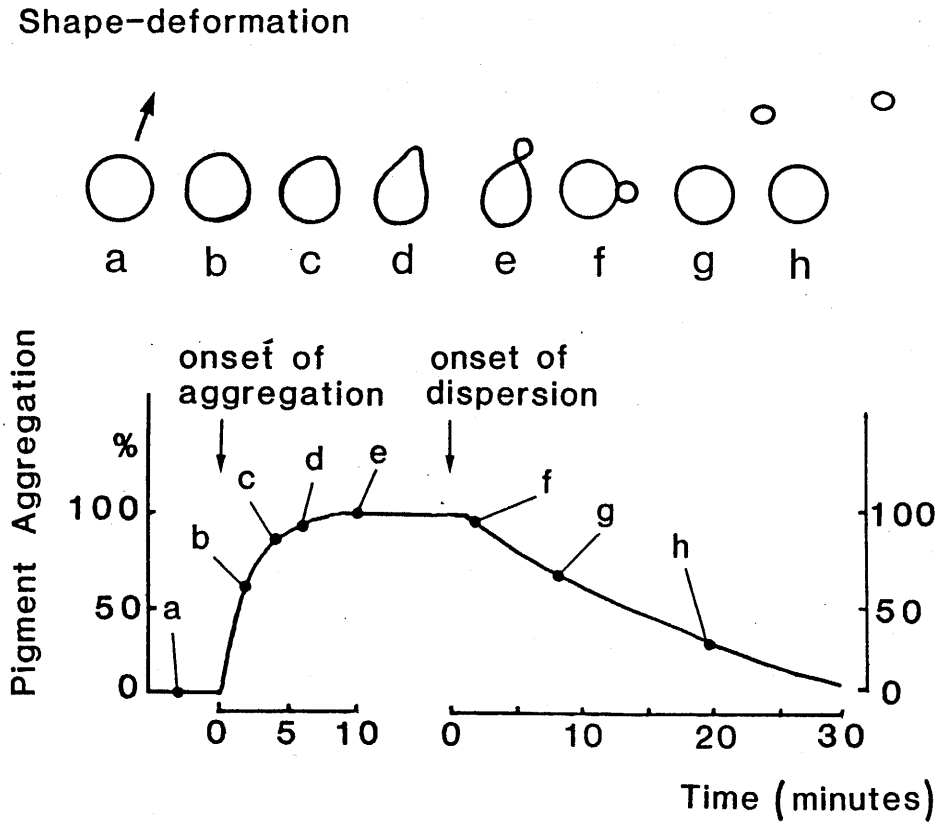
a
0
10
20
30 μm

Fig. 2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e
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g
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Pigment Aggregation
%
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50
0
onset of aggregation
onset of dispersion
a
b
c
d
e
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g
h
0
5
10
0
10
20
30
100
50
0
Time (minutes)

Fig. 3

TABLE 1. The span of migration of microinjected paraffin droplets in swordtail erythrophores during pigment-motor responses

Type of response	Exp. No.	Diameter of liquid paraffin droplet/μm	Span of migration/μm	
			Liquid paraffin droplet	Endogenous pigment*
Aggregation	1	4.4	2.5	21.0
	2	4.2	1.2	6.2
	3	4.3	10.0	17.3
	4	2.5	3.1	9.9
	5	1.9	14.5	16.0
	6	8.0	0.5	9.9
	7	1.9	12.0	15.0
	8	5.9	5.0	14.8
	9	2.4	7.4	9.8
	10	7.5	5.0	44.5
	11	8.8	2.0	38.0
	12	2.4	25.0	50.5
	13	8.0	0	23.0
	14	2.2	24.0	29.0
	15	9.8	0	45.0
	16	7.0	5.0	30.0
	17	6.0	10.0	30.0
	18	6.0	5.0	24.7
	Mean	5.2	$\bar{n}$= 7.34	$\bar{n}$=24.14
Dispersion	1	4.4	1.5	21.0
	2	4.2	25.0	27.2
	3	4.2	10.4	24.7
	4	2.5	12.0	16.0
	5	5.9	4.9	17.0
	6	8.8	4.9	29.0
	7	2.4	14.8	44.5
	8	4.4	13.0	20.0
	9	4.8	5.0	15.0
	10	6.0	10.0	29.7
	11	6.0	14.8	30.0
	Mean	4.9	$\bar{n}$=10.57	$\bar{n}$=24.92

* Estimated on pigments present near the microinjected paraffin droplet.

Fig. 1 Photomicrographs showing the migration and deformation of liquid paraffin droplets microinjected into a swordtail erythrophore during pigment-motor responses

(a) Liquid paraffin was microinjected into the cell with dispersed pigments in the standard culture medium. Two drops injected (arrows) were seen inside the cell. Asterisks indicate landmarks for tracing movement of droplets on microphotographs. (b) 2 minutes after administration of 5×10^{-4} M epinephrine. Pigments started to aggregate in the cell center. (c) 4 minutes later. A smaller droplet (thick arrow) moved toward a larger drop (thin arrow). Note that shape deformation occurred in the large one. (d) 7 minutes later. A smaller drop migrated toward the cell center (thick arrow), while a larger one was shaped like a pear (thin arrow). (e) 10 minutes later. Almost all pigments have aggregated in the cell center while a small droplet was stacked in the middle of the dendrite. A larger droplet was split into two drops (thin arrows) yielding a tiny droplet at the side facing the cell center. (f) The same cell was washed in PBS 3 times and then exposed to 10^{-3} M theophylline for 3 minutes. A newborn smaller droplet moved clockwise around the large droplet, indicating that the direction of the cytoplasmic flow was reversed. (g) 8 minutes later. Pigment dispersion was still under way, and one of the droplets was shaped like a long spheroid (thick arrow). (h) The locations of two smaller droplets were changed,

indicating turbulence of flows around periphery. Bar: 50 μm

Fig. 2 Superimposed tracing of the time-lapse photographs shown in Fig. 1, indicating the displacement of a liquid paraffin droplet microinjected into the cytoplasmic sleeve of a swordtail macroerythrophore. Detailed descriptions of the pigmentary response are given in the legend for that figure. Spots (α and ß) correspond respectively to those on Fig. 1a.

Fig. 3 Deformation of microinjected liquid paraffin droplets during pigment translocation

Upper: The arrow at the top of the droplet (a) indicates the migratory direction of endogenous pigment granules surrounding the droplet. The direction also applies to the other profiles of the droplet (b-h). The tiny droplet shown in (e)-(h) is that split off from the main droplet.

Lower: The stages of pigment displacemdent, at which the deformation of the droplet was recorded, are indicated in the photoelectric recording of the cellular response. As for the method of photoelectric recording of chromatophore responses, see section 1 (3).

4-7. Summary

The motility of artificial particles (liquid paraffin droplets) placed in a pigment-migration track of swordtail macroerythrophores was examined using a microinjection technique and an ordinary physiological procedure to elicit pigment-motor response. Microinjected liquid paraffin droplets less than 2.5 μm in diameter migrated centripetally upon administration of epinephrine and centrifugally upon that of theophylline, together with endogeneous pigment particles. The average span of the migration was 7.34 μm for aggregation (n=18), and 10.57 μm for dispersion (n=11). These figures were equivalent to 0.30 for aggregation and 0.42 for dispersion in comparison with an average migratory distance of neighboring endogenous pigments. Paraffin droplets which were immobile due to their larger sizes, were subjected to shape-deformation upon pigmentary response, changing form from regular spheres to tear-drops with their narrower tips toward the migratory direction of neighboring endogenous pigments. These observations suggested that the cytoplasmic matrix flows centripetally or centrifugally upon pigmentary response, and that all pigments, as well as artificial particles, move passively under cytoplasmic flow.

III. Part II

A study on translocation variants of goldfish erythrophoroma cells

Section 1

An association of actin isoforms with the expression of motile response in pigment translocation variants induced from goldfish erythrophoroma cells

Abbreviation:

CMF-PBS, Calcium, Magnesium-free phosphate-buffered saline; dbcAMP, dibutyryl cyclic adenosine monophosphate; EDTA, Ethylenediamine tetraacetate; FCS, fetal calf serum; FS, fish (carp) serum; MSH, melanocyte-stimulating hormone

1-1. Introduction

Intracellular translocation of pigment organelles in lower vertebrate pigment cells is a classical example of non-muscle motility. Investigations of a variety of such cells, particularly those of fishes, have revealed great complexity and variations, with the implication of the microtubule, the microtrabecular network, and of actin filament in organelle translocation (1, 2, 3, 4, 5, 6, 7, 8, 9). Recently, isolations of both normal and tumor chromatophores of goldfish have succeeded (10, 11, 12, 13). These include xanthophores, terminally differentiated melanophores, normal growing melanophores, a de-differentiated (no longer pigmented) erythrophoroma (xanthophoroma) cell line, and melanophoroma and melanocytoma (capable and incapable of undergoing epinephrine-induced melanosome aggregation respectively) from the erythrophoroma cell line. I wish to report here that, of three different clones derived from the same single cell, a stem cell-type clone (melanoblastoma) contains only ß-actin while both a melanophoroma clone and a melanocytoma clone contain both ß- and γ-actin. Further, both ß- and γ-actins are present in normal melanophores whereas a goldfish skin-derived fibroblast cell line (14) have only ß-actin.

1-2. Materials and Methods

Polypeptides of different iso-electric point (3.5-9.0) and molecular weight (12-280 kilodaltons) were purchased from BDH Chemicals Ltd., Pool, England. α-actin was prepared from carp skeletal muscle and ß- and γ-actins of chick origin was purchased from Sigma Co. St. Louis, USA, collagenase was from Worthington (Type III Freehold, USA). Soybean trypsin inhibitor, DNase I and MSH were from Sigma Co. Other reagents are as described previously (15) or the same as used by Hirabayashi (16).

The neoplastic cell lines (GEM 81) are derived from a goldfish erythrophoroma (10, 11, 12, 13) (See Results). A goldfish dermal fibroblast-like (RBCF) cell line was the generous gift of Dr. A. Shima, Tokyo University, Tokyo, Japan (14). Normal melanophores from Black Moor goldfish were isolated by a modification of the method of Lo et al. (12) with the following changes. After removal of the epidermis of scales by EDTA, the melanophores were released from the scales as follows. The scales were suspended first in a medium containing 100 mg collagenase, 5 mg DNase I, 10 mg soybean trypsin inhibitor, and 1.0 g bovine serum albumin per 100 ml L-15 at pH 7.4 for one hour and then, after brief washing in CMF-PBS, in 0.25% trypsin-0.1% EDTA for 10 minutes. Trypsin treatment was repeated three to four times until all melanophores were released. It was found that prolonged exposure of melanophores to trypsin was damaging. Therefore, the

melanophores were collected at 10 min intervals and immediately mixed with soybean trypsin inhibitor. The melanophores were separated from non-pigment cells by sedimenting through a 50% Percoll (20 min. at 1,000xg). Cell pellets were gently suspended in a small amounts of cold CMF-PBS and plated in a flask containing MSH (0.2 μg/ml)-supplemented, fibroblast-like cell-conditioned L-15 based culture medium (10). Reflecting platelets present in melanophore fraction were removed by renewing culture medium after cell anchorage. After examination of responsiveness to epinephrine, cultured pigment cells were harvested by trypsinization within one to five weeks after plating and stored in -90°C.

Two-dimensional polyacrylamide gel electrophoresis was by the method of O'Farrell as modified by Hirabayashi (16). Polypeptide standards were run on identical gels to determine pH and molecular weight.

1-3. Results

Isolation of melanophoroma, melanocytoma and stem cell type cell lines derived from the same cell : The isolation is based on the observation that some erythrophoroma clones (derived from single cells) can be induced by the combined treatment with dimethylsulfoxide and carp serum to give both melanophoroma and melanocytoma cells which can be cloned to give homogeneous cultures. For the present work, an uncloned culture of a goldfish erythrophoroma cell line (GEM-81) at 30th passage was cloned in soft agar containing carp serum. Individual colonies (unpigmented) were transferred to monolayer culture and later exposed for 4 days to carp serum medium containing 1.5% dimethylsulfoxide. From a culture which yielded both epinephrine-responsive and epinephrine-unresponsive melanized clones, subclones of melanophoroma and melanocytoma were obtained. These were re-cloned, dislodged from culture flasks by gentle trypsinization and purified by sedimenting from 50% Percoll to remove all non-melanized cells. The parent clone, although originally an erythrophoroma clone, is designated as a stem cell type (or melanoblastoma) in this paper for its ability to yield melanized cells. Cultures of these clones are shown in Fig. 1.

Morphology, pigmentation and motile response of stem cell type, melanocytoma and melanophoroma clones isolated from goldfish erythrophoroma cells : Light microscopic profiles

of cells of these three clones in monolayer culture are shown in Fig. 1. The stem cell-type clone consisted of bipolar- or tripolar-shaped, uniformly sized, non-pigmented cells (Fig. 1a). Prolonged maintenance of these cells beyond confluency often resulted in the appearance of faintly yellow pigments which exhibited no motile response to epinephrine. Melanocytoma clones consisted of amorphous shaped, heavily melanized cells in which melanosomes were densely distributed over the entire cytoplasm under an ordinary culture condition (Fig. 1b). The distribution of melanosomes in these cells was not effected by administration of $5x10^{-4}$ M epinephrine tartrate, indicating an absence of motile response to this neurotransmitter. Melanophoroma clones consisted of trigonal- or polygonal-shaped, moderately dendritic, heavily melanized cells whose melanosomes were either fully or partially dispersed under an ordinary culture condition (Fig. 1c). Exposure of these cells to $5x10^{-4}$ M epinephrine tartrate evoked rapid and synchronous melanosome aggregation (Fig. 1d). A melanophoroma clone examined showed similar rate of melanosome aggregation, reaching completion in 3-5 minutes. Repeated washing and subsequent maintenance of such cells in standard culture medium without epinephrine resulted in gradual dispersal of melanosomes, recovering to a fully dispersed state in 15-30 minutes.

Morphology, pigmentation and motile response of melanophores isolated from goldfish skin : Light microscopic profiles of normal melanophores in a primary monolayer culture are shown in Fig. 2.

Primary cultures of melanophores were maintained in a medium consisting of one part fresh FS, one part fibroblast-like cell-conditioned growth medium (10) and 0.2 μg/ml MSH. These cells assume variable cell shapes and are densely populated with melanosomes throughout cytoplasms (Fig. 2a). Administration of $5x10^{-4}$ M epinephrine tartrate to these cells evoked rapid, synchronous aggregation of melanosomes as observed in melanophoroma clones (Fig. 2b). Return of such cells with aggregated melanosomes to an ordinary culture medium after brief washing brought about pigment dispersal which completed within 30 minutes to one hour. The presence of MSH at a concentration of more than 0.2 μg/ml in a medium apparently enhanced pigment dispersion and such enhancing effect was observable with dbcAMP at $5x10^{-4}$ M.

Light microscopic examination of goldfish fibroblast-like (RBCF) cells disclosed that administration of 5×10^{-4} M epinephrine or 0.2 μg/ml MSH had no effect on their cell shape and distribution of cytoplasmic particles.

Two-dimensional (2-D) polypeptide profiles : The method used here differed from the original method of O'Farrell in that detergent was not used. This yielded a similar protein profile with the cytoskeletal components being the major proteins. The portions of the 2-D gels containing

these proteins obtained from the stem cell type, the melanophoroma and the melanocytoma lines are shown in Fig. 3. The actin isoforms are marked by letters α, ß and γ. There are two major differences in the proteins between the cell types : the amount of tubulin and the shape of the actin spots. The amounts of tubulin may however be due to differences in cell density as the uncloned GEM-81 cells, from which these cell lines are derived, show cell density-dependant variations in tubulin content (17, 18). In the case of actin, only one actin isoform is present in the stem cell type but two or three actin isoforms are present in melanized cells. The insets in panels (a) and (c) of Fig. 3 show the actin spots when carp skeletal muscle actin (α-actin) was added to the protein samples prior to electrophoresis in order to identify α-actin. In other gels, chicken gizzard ß- and γ-actins were added to identify these two actin isoforms (data not shown). The presence of multiple actin spots is not due to actin phosphorylation giving rise to iso-electric variants as all results with goldfish pigment cells showed that actin is not phosphorylated (17, 18). Fig. 4 shows actin isoforms of normal goldfish pigment cells and non-pigment cell, together with muscle actin as standard. It can be seen that normal melanophores have both ß- and γ-actins whereas the non-pigment fibroblast-like cells have ß-actin.

Although all the melanized cells have multiple actin isoforms, the relative amounts of these isoforms are variable. This is further illustrated in Fig. 5 where the actin spots of several melanophoroma clones are traced.

1-4. Discussion

Most studies on pigment organelle translocation have focused on the microtubules [for review, see Porter, (19), and Schliwa, (20)]. However, there is also concern on the possible role of actin [reviewed by Obika (21)]. Indirect evidences have been obtained that pigment organelle (carotenoid droplets) translocation in erythrophores/xanthophores may depend on actin. First, in the case of swordtail erythrophores, microinjection of antibody to carp skeletal muscle actin, an antibody which also recognizes swordtail erythrophore actin, inhibits both pigment dispersion and aggregation (7). Second, in goldfish xanthophores, carotenoid droplets have been seen to migrate into newly-formed filopodia which contain F-actin but no microtubules (22). These results logically led to the question whether actin is also involved in pigment translocation in melanophores.

Recently, following cell lines were obtained from the progenies of a single de-differentiated erythrophoroma (GEM-81) cell; (1) cell lines that contain melanosomes and respond to epinephrine by melanosome aggregation, (2) cell lines that contain melanosomes but do not respond to epinephrine, and (3) cell lines that remain unpigmented (11, 13, 23). These are designated here as melanophoroma, melanocytoma and stem cell type (melanoblastoma) respectively. The results presented have showed that the phenotypic differentiation into pigment cells, both normal and neoplastic, is accompanied by the appearance

of new actin isoforms, reminiscent of the development of myoblasts where phenotypic differentiation is accompanied by changes in actin isoforms (24). This suggests that the appearance of actin isoforms in the melanized cells may be to provide a motile apparatus for melanosome translocation. Such a hypothesis would be greatly strengthened if one were to find correlation of inability to translocate melanosome with the presence of only ß-actin. On the surface, the presence of multiple actin isoforms in the melanocytoma cell lines used here seems to be against this hypothesis. However, recent work (unpublished) has shown that the original designation of melanocytoma, based on unresponsiveness to epinephrine, is faulty in that these cells are capable of aggregating their melanosomes in response to melanin concentrating hormone (25). I am currently in the process of isolating true melanocytoma cell lines unresponsive to all of epinephrine, melatonin and melanin concentrating hormone. Among these clones, I hope to find the melanocytoma cell lines which are incapable of melanosome translocation due to the lack of multiple actin isoforms.

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1-6. Figures and Figure legends

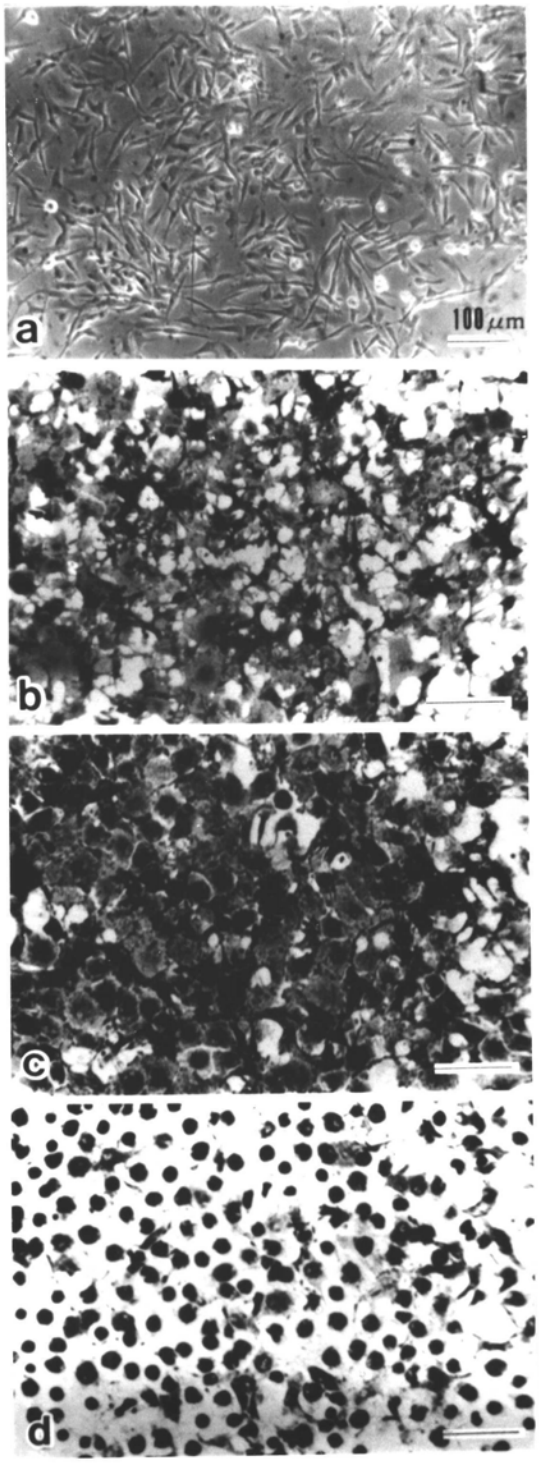

Fig. 1

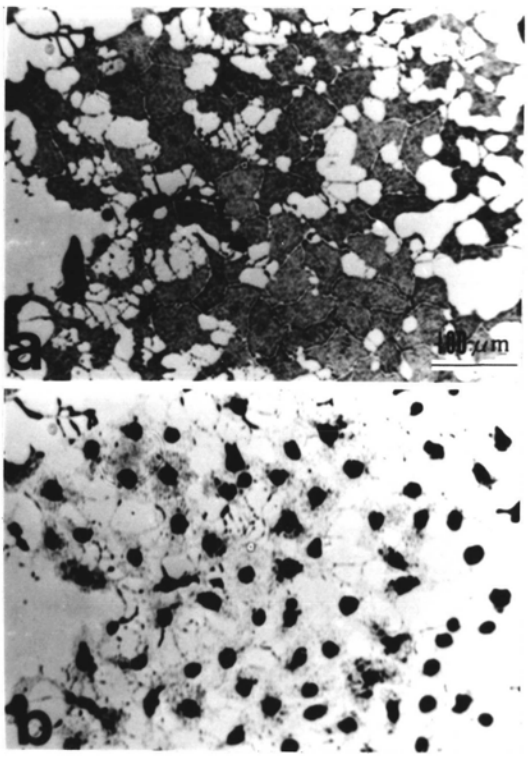

Fig. 2

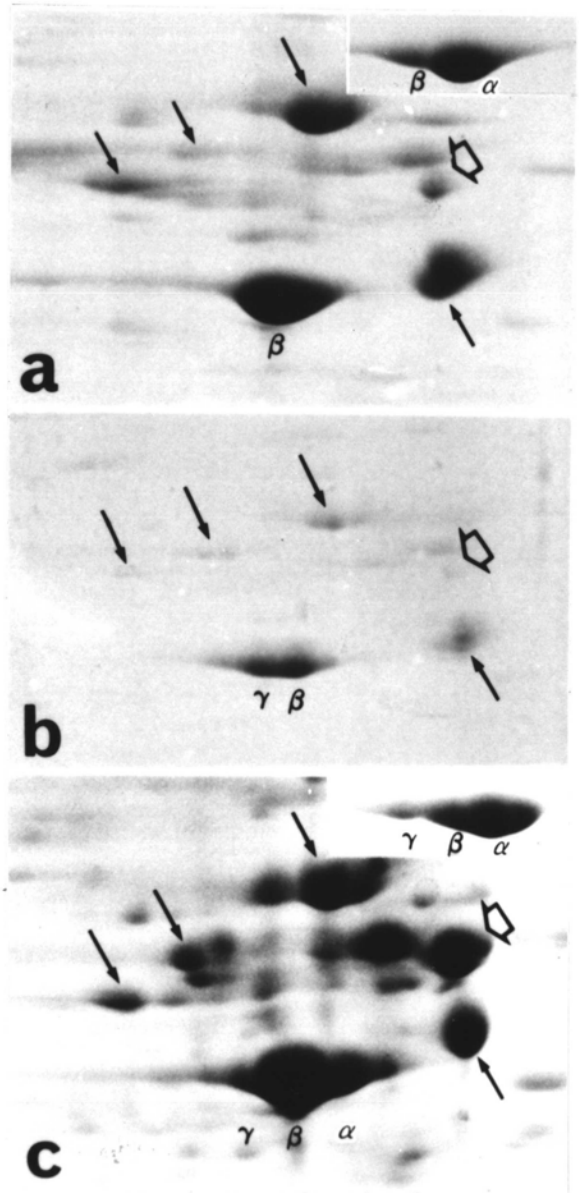

Fig. 3

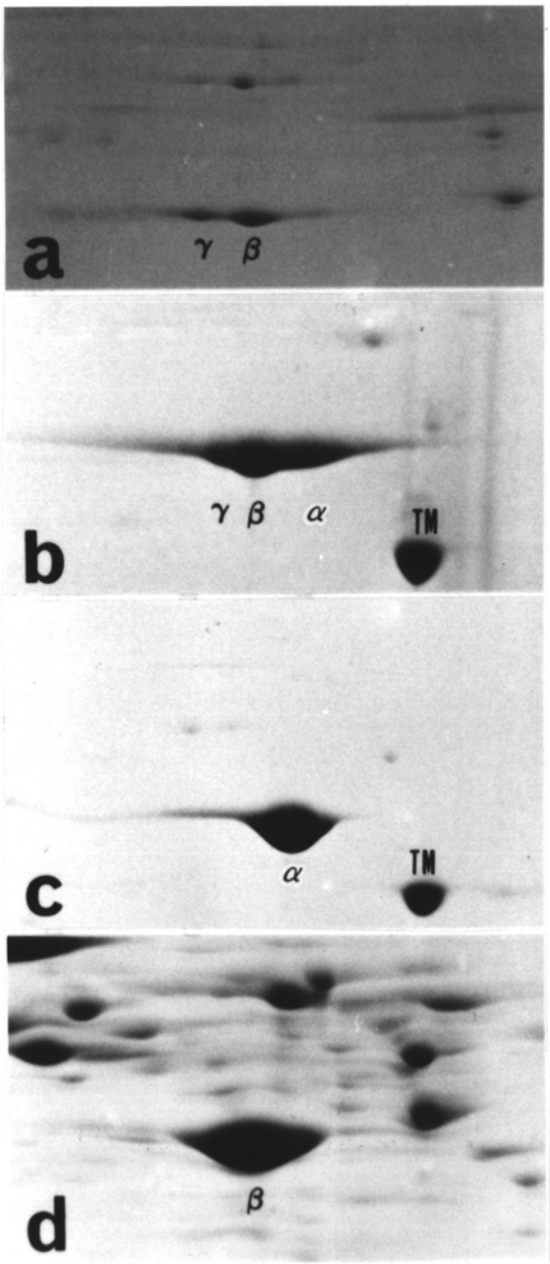

Fig. 4

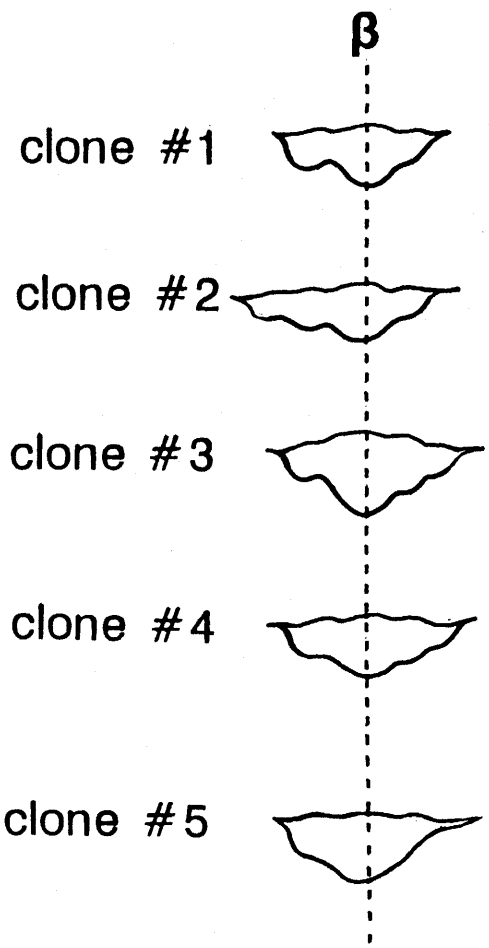

Fig. 5

Fig. 1 Photomicrographs of monolayer cultures of stem cell-type, melanocytoma and melanophoroma clones isolated from goldfish erythrophoroma GEM 81 cells
(a) Stem cell type. (b) Melanocytoma. (c) Melanophoroma and (d) Same as (c) after epinephrine treatment.

Fig. 2 Photomicrographs of goldfish xanthophores and melanophores in a primary monolayer culture
(a) Normal melanophores and (b) Same as (a) after administration of 5×10^{-4} M epinephrine.

Fig. 3 Portions of 2-D gels including the major cytoskeletal proteins
(a) Stem cell type. (b) Melanocytoma and (c) Melanophoroma. The letters α, ß and γ refers to the three actin isoforms. The identity of α-actin is shown in the insets to panels (a) and (c) where carp muscle actin was added to the sample prior to electrophoresis. Similar experiments using chicken gizzard ß- and γ-actins served to identify these two actin isoforms (data not shown). The proteins are tentatively identified as follows : solid arrows, intermediate filament proteins (26), open arrow, tubulin. For explanation, see text.

Fig. 4 Portions of 2-D gels showing the actin isoforms (a) Normal melanophores. (b) Normal melanophores together with carp skeletal muscle actin (TM: tropomyosin). (c) Carp skeletal muscle actin alone and (d) fibroblast-like RBCF cells. For explanation, see text.

Fig. 5 Tracing of actin isoforms from 2-D gels of five different melanophoroma clones : All these clones contain multiple actin isoforms but in different relative amounts.

1-7. Summary

Comparison of actin isoforms in unpigmented goldfish cells (a normal dermal fibroblast-like cell line, and an unpigmented erythrophoroma cell line capable of being induced to undergo melanization) and in normal and neoplastic melanized goldfish cells show that the melanized phenotype is accompanied by the presence of multiple actin isoforms. In contast, the unpigmented cells have only ß-actin. The possible significance of this to pigment organelle translocation is discussed.

IV. General conclusion

The objective of the present study was to clarify the mechanisms of pigment translocation in fish chromatophores, with particular reference on the role of actin and its associated cytoskeletons. The findings obtained in this report are summarized as follows:

1) Kinetic studies of pigment movement as a mass of pigment granules per cell and individual granules using swordtail erythrophores disclosed that the modality of pigment movement at aggregation was different from that at dispersion, suggesting that these two phases of the movement are operated by different mechanisms.

2) SEM studies of swordtail erythrophores undergoing pigment movement disclosed that (a) pigment translocation took place in a manner to convey all motile cytoplasmic inclusion toward a definite direction along the dendrites, (b) cell surfacial changes at aggregation did not proceed as a reversal of those at dispersion and (c) treatment with colchicine, a microtubule-disrupting agent, did not cause total inhibition of pigment translocation and its related cell shape changes, suggesting a limited role of microtubules in this motility.

3) TEM studies conducted parallel to SEM disclosed that the distribution of microfilaments abundant in these cells was subject to marked changes during transition from a dispersed state to an aggregate. A marked change was the formation of a loosely organized meshwork of these filaments with compactly packed pigment aggregates in

the cell center.

4) Immunofluorescence and immunocytochemical electron microscopic studies conducted on these cells using the anti-actin antibody, produced against carp skeletal muscle actin disclosed that (a) microfilaments observed under TEM were unequivocally decorated with this antibody, indicating their identity as actin filaments and (b) actin filaments were distributed dispersedly over the entire cytoplasm in cells with dispersed pigments. Upon elicitation of pigment aggregation, actin filaments were progressively migrating toward the cell center and finally accumulated densely in the periphery of aggregated pigment mass in the form of a ring.

5) Microinjection studies conducted on erythrophores using the anti-actin antibody and paraffin droplets disclosed that (a) the antibody brought about total blockade of pigment aggregation and dispersion, indicating an unequivocal involvement of actin in this motility as functional components, (b) liquid paraffin droplets placed inside cells translocated together with pigment granules, though their migration distances were shorter than those of neighboring pigments, thus indicating the occurrence of cytoplasmic flows during pigment response.

6) Studies on actin isoforms by means of two-dimensional electrophoresis using varying melanogenic translocation variants of goldfish erythrophoroma cells disclosed that the development of the ability for pigment translocation was closely associated with the expression of multiple actin isoforms.

Based on these findings, it can be concluded that actin or actin filaments play an important role as cytoskeletal components responsible for generating a motive force in pigment translocation of fish chromatophores.

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