

Reactivated Melanophore Motility: Differential Regulation and Nucleotide Requirements of Bidirectional Pigment Granule Transport

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Abstract. To study the molecular basis for organized pigment granule transport, procedures were developed to lyse melanophores of *Tilapia mossambica* under conditions in which pigment granule movements could be reactivated. Gentle lysis of the melanophores resulted in a permeabilized cell model, which, in the absence of exogenous ATP, could undergo multiple rounds of pigment granule aggregation and dispersion when sequentially challenged with epinephrine and cAMP. Both directions of transport required ATP, since aggregation or dispersion in melanophores depleted of nucleotides could be reactivated only upon addition of MgATP or MgATP plus cAMP, respectively. Differences between the nucleotide sensitivities

for aggregation and dispersion were demonstrated by observations that aggregation had a lower apparent K_m for ATP than did dispersion and could be initiated at a lower ATP concentration. Moreover, aggregation could be initiated by ADP, but only dispersion could be reactivated by the thiophosphate ATP analog, ATP γ S. The direction of pigment transport was determined solely by cAMP, since pigment granules undergoing dispersion reaggregated when cAMP was removed, and those undergoing aggregation dispersed when cAMP was added. These results provide evidence that pigment granule motility may be based on two distinct mechanisms that are differentially activated and regulated to produce bidirectional movements.

ORGANIZED intracellular particle transport in eukaryotic cells is a ubiquitous and functionally important phenomenon. The chromatophores of vertebrates provide an excellent model system for studying intracellular motility, which is characterized by the rapid, directed, and cyclic transport of thousands of pigment granules. Aggregation, the transport of granules towards the cell center, is of uniform velocity, while dispersion, or centrifugal pigment granule transport, is a saltatory process. These movements occur as temporally separate events and can be controlled both hormonally and chemically (Bagnara and Hadley, 1973; Novales, 1983). Thus, the cellular mechanisms involved in each direction of granule transport can be dissected and resolved.

Recent studies have revealed that bidirectional organelle transport can occur along a single microtubule (Hayden and Allen, 1984; Koonce and Schliwa, 1985; Schnapp et al., 1985) and in axoplasm may involve two distinct motors (Vale et al., 1985a, b). That the force generating motors might be associated with the translocating organelles is suggested by the observation that purified axoplasmic organelles exhibit bidirectional movements along dynein-free axonemal microtubules (Gilbert et al., 1985). A soluble protein, kinesin, purified from squid and bovine axoplasm (Vale et al., 1985a) and from sea urchin eggs (Scholey et al., 1985), induces the unidirectional movement, corresponding to anterograde ax-

onal transport, of latex beads and axoplasmic organelles along microtubules. Recent evidence has also indicated the existence of a presumptive retrograde translocator in axoplasm (Vale et al., 1985b). However, the mechanism by which the direction of movement is controlled during axoplasmic transport is unknown.

To examine the molecular mechanisms producing bidirectional movements, we have modified the procedures previously developed to study organized pigment translocation in lysed chromatophores. By permeabilizing melanophores from the killifish *Fundulus*, Clark and Rosenbaum (1982, 1984) obtained pigment granule aggregation in vitro and demonstrated an ATP requirement for this process in metabolically poisoned cells. In other studies, saltations of pigment in lysed erythrocytes of the squirrel fish *Holocentrus* continued in vitro, a characteristic movement of pigment in the dispersed state in intact cells. This system was used to study the sensitivity of saltatory movements to various ions, drugs, and antibodies (Stearns and Ochs, 1982). We report here the development of a lysis procedure for melanophores of the African cichlid, *Tilapia mossambica*, in which both aggregation and dispersion as well as saltatory movements of pigment granules, melanosomes, can be reactivated. Results of these studies indicate that each of these movements has a distinct requirement for ATP, that aggregation and dispersion are differentially supported by other nucleotides, and that the

direction of melanosome movement is controlled by cAMP. The results reported here and elsewhere (Rozdzial and Haimo, 1986) suggest that two distinct mechanisms may be involved in generating the opposing pigment granule movements.

A brief account of these results has appeared in abstract form (Rozdzial and Haimo, 1985).

Materials and Methods

Experimental Materials and Solutions

Live African cichlids, *Tilapia mossambica*, were purchased from Aquafarms International, Mecca, CA and maintained in freshwater aquaria at ~25°C. Adenosine triphosphate (ATPγS) was purchased from Boehringer Mannheim Biochemicals, Indianapolis, IN, and bretylium tosylate from Chemical Dynamics Corp., South Plainfield, NJ. All other reagents were purchased from Sigma Chemical Co., St. Louis, MO. Purified IgG fraction containing rat monoclonal antibodies against yeast tubulin has been previously characterized (Kilmartin et al., 1982) and was the generous gift of Dr. John Kilmartin, Medical Research Council, Cambridge, England.

Lysis buffer (pH 7.4) consisted of 30 mM Hepes, 10 mM EGTA, 0.5 mM EDTA, 5 mM MgSO₄, 33 mM potassium acetate, 2.5% polyethylene glycol (PEG; 20,000 mol wt), and 0.001% digitonin. Epinephrine, isobutylmethylxanthine (IBMX),¹ and reserpine were prepared as 100× stock solutions in dimethylsulfoxide and stored at 4°C. Calcium-free goldfish Ringer's (pH 7.4) was composed of 100 mM NaCl, 2.5 mM KCl, 10 mM sodium bicarbonate, and included 1 mM MgCl₂. Digitonin was freshly prepared as a 0.4% stock in 50% ethanol (Stearns and Ochs, 1982).

Preparation of Melanophores

Single scales were removed from fish with forceps and partially cut with dissecting scissors into several longitudinal sections. To facilitate the removal of the epidermis and expose the underlying melanophores, scales were then immersed and agitated in 5 mg/ml collagenase (Type II; Sigma Chemical Co.) in Ca⁺⁺-free Ringer's solution for 1.5 h at room temperature. The epidermal layer was then removed in Ca⁺⁺-free Ringer's. Subsequently, scales were transferred to fresh Ca⁺⁺-free Ringer's and cut transversely to free the sections with attached melanophores from the anterior portion of the scale. Depending on their size, most scales provided four to eight sections containing melanophores, allowing for numerous experimental and control procedures to be performed on tissue obtained from a single scale.

Cell Lysis Procedures

Permeabilized Cell Model. Melanophores were permeabilized with digitonin in a solution (see lysis buffer above) adapted from the axoneme reactivation solution developed by Witman et al. (1978) and modified as follows. Potassium acetate was used rather than KCl, since organic anions have been shown to improve the reactivated motility of sea urchin flagella (Gibbons et al., 1985). The lysis buffer also included 10 mM EGTA so that the pCa of the solution was <8.0 (Steinhardt et al., 1977), and the amount of PEG was increased to 2.5% (Cande and Wolniak, 1978). Although dithiothreitol was used for axoneme reactivation (Witman et al., 1978), motility was not enhanced by its presence, and, accordingly, it was not included in the lysis buffer. Before lysis, melanophores, still attached to portions of a scale, were transferred from petri dishes with a drop of Ca⁺⁺-free Ringer's and placed cell side up on a slide for light microscopy observations. A perfusion chamber was formed by the use of several pieces of cover glass to elevate an 18 × 18-mm coverslip over the scale. Melanophores were gently perfused with three to five changes of lysis buffer, which was drawn across the chamber with filter paper (No. 1; Whatman, Inc., Clifton, NJ). These permeabilized cell models were induced to aggregate pigment upon stimulation with lysis buffer containing 100 μM epinephrine, and to disperse pigment upon stimulation with lysis buffer containing 1 mM cAMP or 5 μM IBMX, without the addition of exogenous ATP.

Depleted Cell Model. Melanophores, with dispersed or aggregated pigment, were continuously perfused for ≥10 min with multiple (30–50)

changes of depletion buffer, which was lysis buffer containing 0.0015% digitonin. These nucleotide-depleted cell models only underwent pigment granule aggregation or dispersion upon reactivation with lysis buffer containing MgATP or MgATP plus cAMP (reactivation solutions), respectively. Unless otherwise noted, 3 mM ATP (or another nucleotide) and 1 mM cAMP were used in the reactivation solutions. To study pigment granule aggregation, populations of depleted melanophores with dispersed pigment were routinely obtained during lysis into depletion buffer. To obtain populations of melanophores with aggregated pigment for studies of melanosome dispersion, cells were first incubated in lysis buffer containing 100 μM epinephrine and then incubated in the depletion buffer.

Recording of Experiments

The responses of randomly chosen intact or lysed melanophores to drug stimulation or reactivation conditions were observed with differential interference contrast (DIC) microscopy and recorded with video, photographic, and photometric equipment. Melanophores were observed and scored for motility with a Plan 20× DIC objective on a Nikon Optiphot microscope. Video recordings were taken using a Plan 100× oil immersion objective, a Panasonic NV-8050, half-inch time-lapse recorder (Panasonic Co., Secaucus, NJ), and a camera equipped with a Newicon tube and circuitry for controlling gain, gamma, and black level parameters (67M Series; Dage MTI, Inc., Michigan City, IN). Rate measurements of pigment granule aggregation and dispersion were taken directly from recorded images displayed on a Panasonic WV-5360 monitor that had been calibrated with a stage micrometer. Real time output was provided by a time-date generator (VTG-33; FOR-A Co., West Newton, MA). Photographs were taken with a Nikon FX-35A camera on Plus-X Pan film developed in Rodinol (Agfa-Gevaert, Inc., Teterboro, NJ). Photometric measurements (see also Clark and Rosenbaum, 1984) of single cells were taken using the Plan 20× DIC objective and a Nikon PI photometer equipped with a 2-μm pinhole aperture and 530-nm filter (on generous loan from A. G. Heinze Co., Inc., Irvine, CA) mounted on the Nikon Optiphot microscope. Changes in light transmittance, recorded and displayed by the photometer digital output, were subsequently converted to changes in OD according to Beer's law. The progressions of pigment granule aggregation and dispersion were related to, respectively, the observed increases and decreases in OD. These changes in OD were normalized so that values for aggregation decreased from an initial value of 1.0 and dispersion increased from 0. In all figures, *n* refers to the number of cells recorded, and each point represents the average response of four or more cells.

Indirect Immunofluorescence

Aggregated melanophores, attached to sections of scales, were permeabilized with lysis buffer for various times and prepared for indirect immunofluorescence with minor modification of previously described procedures (Weber et al., 1975). Melanophores were fixed with 3.7% formaldehyde in lysis buffer for 6–8 min and then washed three times with 50 mM potassium phosphate and 150 mM NaCl (PBS), pH 7.4. The tissues were incubated for 1 h each at room temperature in 1:30 dilutions of rat monoclonal antibodies against yeast tubulin (Kilmartin et al., 1982) and fluorescein-conjugated rabbit anti-rat IgG (Miles-Yeda, Ltd., Rehovot, Israel) in PBS containing 1 mg/ml BSA (PBS-BSA). Each antibody incubation was followed by three washes in PBS-BSA for 30 min. Scale portions were then mounted on glass slides in 90% glycerol, 10% PBS, and 1 mg/ml *p*-phenylenediamine to prevent photobleaching (Johnson and Nogueira Araujo, 1981) and viewed by epifluorescence with a 40× fluorite or Plan 20× DIC objective. Long exposure fluorescence images were directly recorded on Kodak Tri-X Pan film and developed with Kodak D-76 developer.

Results

Conditions and Evidence for Cell Lysis

To study bidirectional intracellular organelle transport in melanophores, we sought to lyse cells under conditions in which both aggregation and dispersion as well as saltatory movements could be reactivated. Accordingly, melanophores were incubated in lysis buffer, a solution adapted from that used for axoneme reactivation (Witman et al., 1978), to optimize conditions for microtubule-associated mo-

1. Abbreviations used in this paper: DIC, differential interference contrast; IBMX, isobutylmethylxanthine.

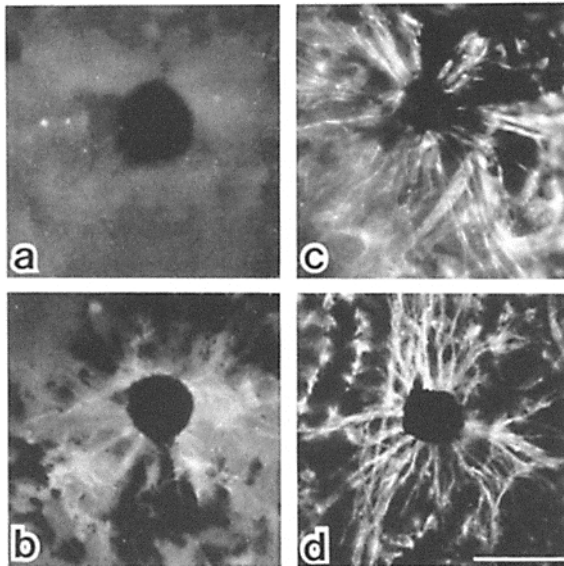


Figure 1. Indirect immunofluorescence staining of melanophores with tubulin antibody. Melanophores with aggregated pigment were incubated in lysis buffer in the absence (a) or presence of digitonin for 1.5 min (b), 2.5 min (c), or 30 min (d). Radially arrayed microtubules are visible only in the digitonin-treated preparations (b–d). Bars: (a, b and d) 50 μm ; (c) 75 μm .

tivity. Indirect immunofluorescence images (Fig. 1) demonstrate that when intact (Fig. 1 a) and lysis buffer-treated (Fig. 1, b–d) melanophores were incubated with antibodies against tubulin, the antibodies had access to the radially arrayed microtubules only in the lysed preparations. Time-course experiments revealed that within 15 min after initiation of cell lysis, tubulin antibodies stained the melanophores (Fig. 1 b) and that the microtubules were stable for at least 30 min in lysis buffer (Fig. 1 d).

Permeabilized Cell Model

As detailed in Table I, intact melanophores (Fig. 2 a) could be induced to aggregate or disperse pigment with 100 μM epinephrine or 5 μM IBMX, respectively. Melanophores, gently perfused with three to five changes of lysis buffer, initially responded by aggregating pigment. Within a few minutes, however, these melanophores spontaneously hyperdispersed their pigment granules (Fig. 2 b) and remained in this state unless challenged with 100 μM epinephrine, which induced aggregation (Fig. 2 c). Subsequent treatment of these cells with 1 mM cAMP, which had no effect on live cells (Table I; Abramowitz and Chavin, 1974), resulted in pigment granule dispersion (Fig. 2 d). These permeabilized cell models were able to undergo repeated cycles of pigment

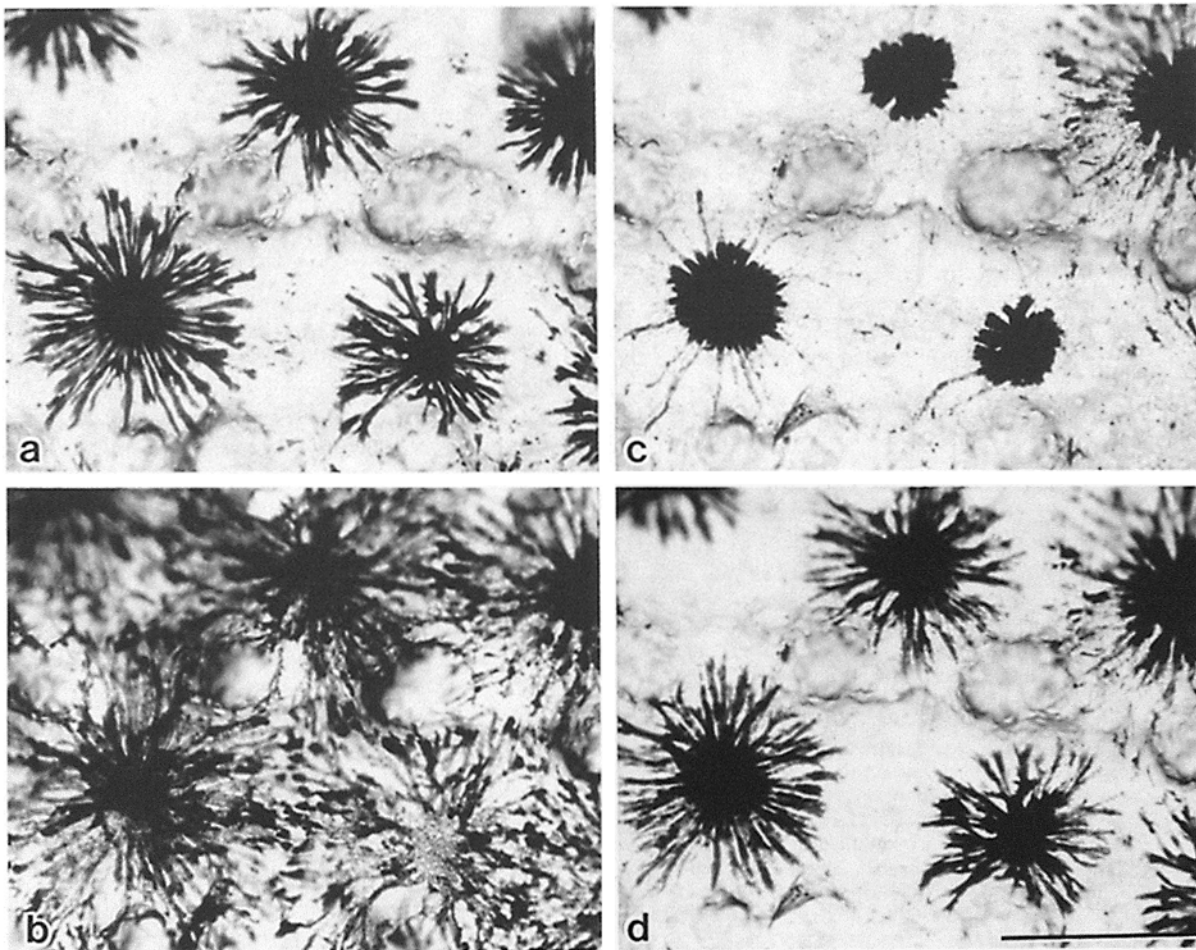


Figure 2. Response of permeabilized cell models to stimulation with epinephrine or cAMP. Sequence of light micrographs of melanophores before (a) and after permeabilization (b–d). Intact cells (a) hyperdispersed pigment after 5-min incubation in lysis buffer (b). These permeabilized melanophores underwent pigment aggregation upon treatment with lysis buffer containing 100 μM epinephrine (c) and subsequent dispersion upon treatment with lysis buffer containing 1 mM cAMP (d). Bar, 100 μm .

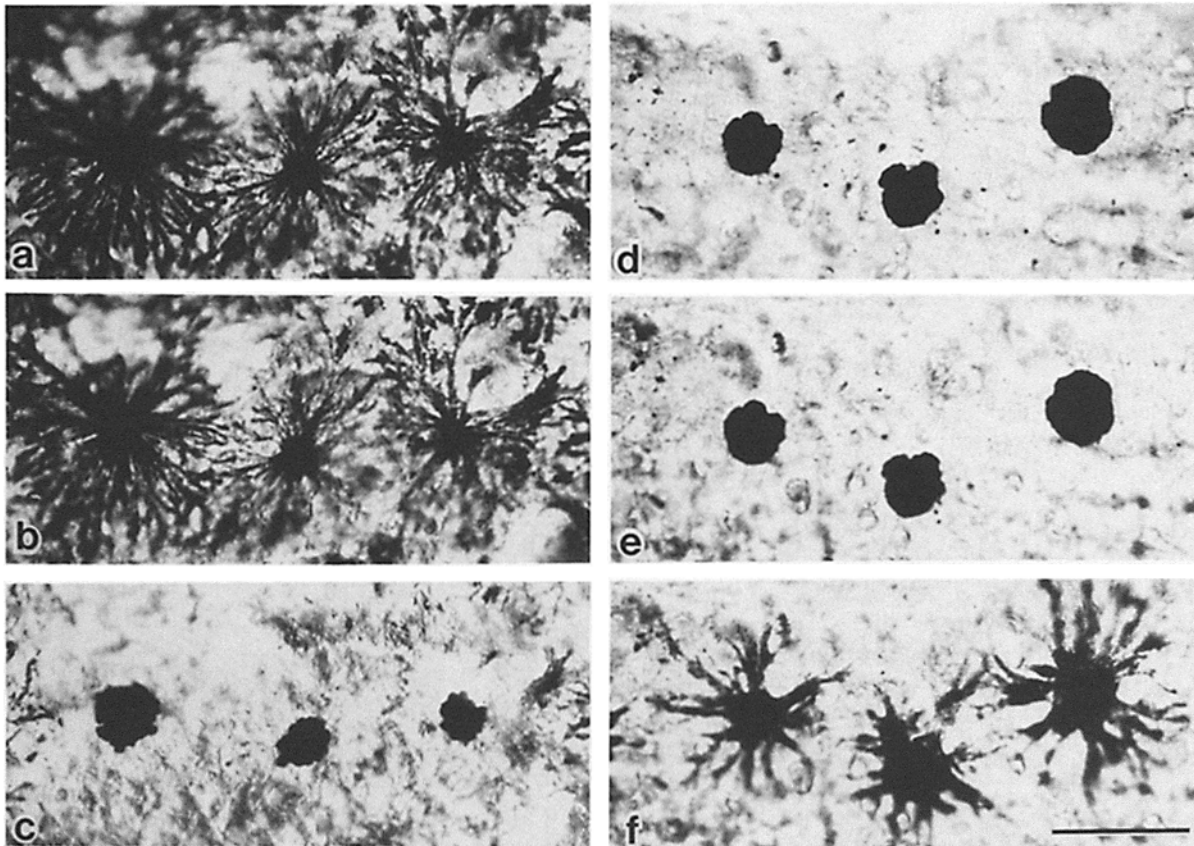


Figure 3. Reactivation of pigment granule aggregation (*a-c*) and dispersion (*d-f*) in depleted cell models. Each sequence of light micrographs begins with melanophores 10 min after extraction in depletion buffer. Depleted melanophores with hyperdispersed pigment (*a*) do not respond to epinephrine (*b*). Reactivation of pigment aggregation is induced only when these melanophores are treated with MgATP (*c*). Similarly, depleted melanophores with aggregated pigment (*d*) do not respond to cAMP alone (*e*). Dispersion is reactivated only upon addition of both cAMP and MgATP (*f*). Bars: (*a-c*) 50 μ m; (*d-f*) 72 μ m.

granule aggregation and dispersion for at least 1 h after cell lysis upon stimulation with 100 μ M epinephrine and 1 mM cAMP, respectively, without the addition of exogenous ATP.

Depleted Cell Model

To examine the nucleotide requirements of pigment granule movements, melanophores were perfused with 30–50 changes of depletion buffer over a 10-min period. These continuous perfusions, compared with only three to five changes of lysis buffer used to generate the permeabilized cell model, effectively and reproducibly depleted endogenous nucleotide levels, as cells treated in this manner (Fig. 3 *a*) did not aggregate hyperdispersed pigment upon stimulation with epinephrine (Fig. 3 *b* and Table I) and those with aggregated pigment (Fig. 3 *d*) did not disperse it upon addition of cAMP alone (Fig. 3 *e* and Table I). Indeed, in the depleted cell models,

aggregation could be induced only upon addition of a reactivation solution containing MgATP (Fig. 3 *c* and Table I) and dispersion only upon addition of a solution containing MgATP and cAMP (Fig. 3 *f* and Table I). Thus, each direction of transport requires ATP. The differences in the ability of intact, permeabilized, or depleted cells to transport pigment under various conditions is summarized in Table I.

Metabolically Inhibited Permeabilized Cells

A requirement of bidirectional pigment granule transport for ATP was examined also in permeabilized cell models by subjecting them to metabolic poisoning. As detailed in Table II, only ATP at a threshold concentration of \sim 2 mM was effective in restoring pigment transport in DNP-treated permeabilized cells. Neither 5 μ M reserpine, a drug that depletes catecholamines from nerve terminals (Miyashita and Fujii,

Table I. Inducers of Pigment Granule Transport in Intact and Lysed Melanophores

Cell	Induction of aggregation		Induction of dispersion		
	Epinephrine	MgATP	IBMX	cAMP	MgATP + cAMP
Intact	+	–	+	–	–
Permeabilized cell model	+	–	+	+	+
Depleted cell model	–	+	–	–	+

+ indicates induction of granule movements; – indicates no induction.

Table II. Rescue of Pigment Granule Movements in DNP-poisoned, Permeabilized Melanophores

Treatment	Resumption of aggregation/dispersion	
Epinephrine/cAMP and:		
—	—	—
3 mM ATP	+	+
3 mM ATP, 5 μ M reserpine	+	+
3 mM ATP, 100 μ M bretylium	+	+
3 mM NTP, or AMPPNP	—	—
3 mM NTP or AMPPNP, followed by 3 mM ATP	+	+

Melanophores were pretreated with 0.4 mM DNP for 60–120 min in Ca^{++} -free Ringer's, permeabilized with lysis buffer containing 0.2 mM DNP and subsequently challenged with either 100 μ M epinephrine or 1 mM cAMP in the presence or absence of 3 mM ATP or 3 mM NTP (GTP, CTP, ITP, UTP) or 3 mM AMPPNP.

+ indicates the rescue of granule movement; — granules remain stationary.

1975), nor 100 μ M bretylium, which selectively inhibits adrenergic nerve endings (Boura and Green, 1959), prevented this ATP reactivation (Table II). Accordingly, neurons were not the target of ATP rescue of melanophore pigment motility. Together, these studies on depleted and on metabolically inhibited cell models demonstrate that both pigment granule aggregation and dispersion require ATP.

Nucleotide Requirements

To determine if aggregation and dispersion require the same amount of ATP, depleted cell models were reactivated with various concentrations of MgATP (Fig. 4). Transport of pigment granules was measured quantitatively by use of photometry. Little or no pigment dispersion occurred below 40 μ M MgATP (Fig. 4 b and Table III) whereas some pigment granule aggregation could be reactivated in 1 μ M MgATP (Fig. 4 a and Table III). Thus, pigment granule aggregation can be initiated at an ATP concentration \sim 40 times lower than that required for dispersion. Most interesting was the observation that in the depleted cell models pigment dispersed without its characteristic saltations when the ATP concentration in the reactivation solution was \leq 3 mM. Only at higher concentrations did saltatory movements resume. These observations indicate that the ATP requirement for saltations is significantly greater than that of either aggregation or dispersion and suggest that dispersion itself can be dissected into two separate components in which saltations may be superimposed upon the transport process that disperses the melanosomes.

Rate measurements from video recordings of reactivated bidirectional pigment transport revealed that the increase in the velocity of pigment granule aggregation and dispersion with increasing MgATP concentration was saturable and followed Michaelis-Menton kinetics (Fig. 5 a). In intact melanophores the rate of pigment granule dispersion was \sim 60% that of aggregation, 1.59 ± 0.19 versus 2.54 ± 0.44 μ m/s, respectively. A similar rate difference was also observed with reactivated pigment dispersion (0.46 ± 0.15 μ m/s) and aggregation (1.11 ± 0.22 μ m/s). Double reciprocal plots of this data (Fig. 5 b) permitted the calculation of the apparent K_m for ATP for each of the two transport processes in this in vitro system. Each line was determined by linear regression and K_m s were calculated from the x -in-

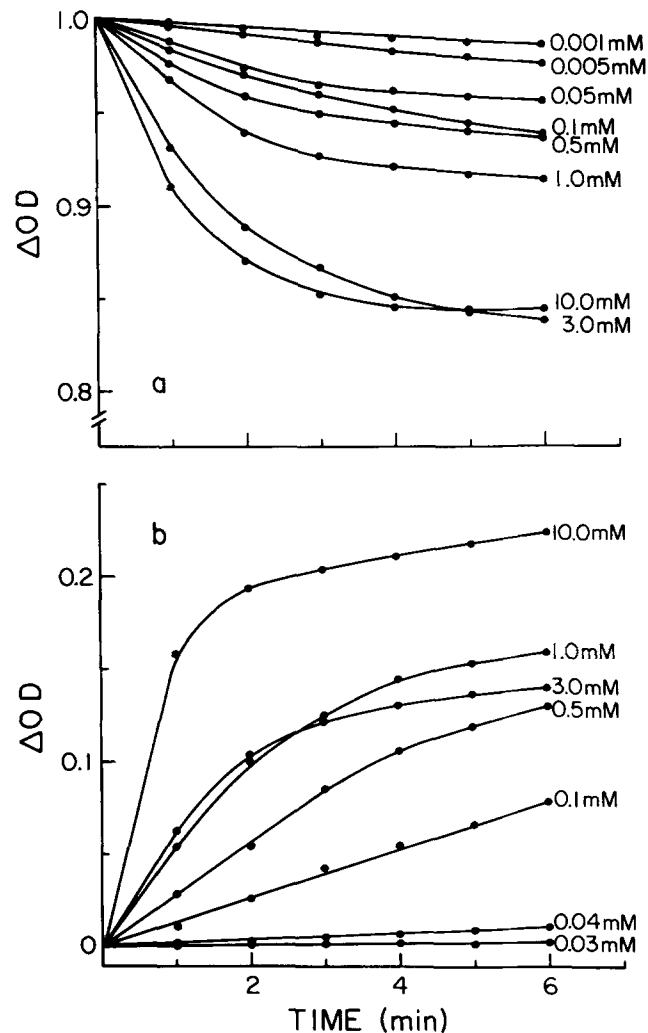


Figure 4. ATP concentration dependence of reactivated pigment aggregation (a) and dispersion (b) in depleted melanophore models. Photometric measurements are plotted as changes in OD of the cells with time. Each point represents the mean response of a minimum of four cells. Threshold concentrations of ATP for reactivated aggregation and dispersion are 1.0 μ M and 40 μ M, respectively. With 3 mM MgATP reactivated aggregation and dispersion are essentially completed within 4–6 min. In comparison, intact cells aggregate or disperse pigment completely within 1 min while permeabilized cell models require 2–3 min.

tercept. Similar K_m values were obtained when the data were plotted by the Eadie-Hofstee instead of the Lineweaver-Burke method. The process of pigment granule aggregation had an apparent K_m value (0.29 mM) almost half that measured for the process of pigment granule dispersion (0.51 mM).

Nucleotide Specificity

Differences in the nucleotide specificity for aggregation and dispersion were indicated in studies in which depleted cell models were challenged with reactivation buffer containing other nucleotides. Both the relative rates (Fig. 6) and extents (Fig. 7) of pigment granule aggregation and dispersion in GTP, CTP, UTP, ITP and the nonhydrolyzable ATP analog, AMPPNP were $<10\%$ of those measurements obtained for ATP-reativated motility. However, ADP or the thiophos-

Table III. Characteristics of Pigment Reactivation in Depleted Melanophore Models

	Aggregation	Dispersion
ATP threshold for reactivation	1 μ M	40 μ M
Stimulus for reactivation	None	≥ 1 μ M cAMP
K_m ATP	0.29 mM	0.51 mM
ADP threshold for reactivation	25 μ M	—
ATP γ S threshold for reactivation	>100 μ M	20 μ M

Threshold concentrations for reactivation were determined by photometric measurements on four or more cells.

phate ATP analog, ATP γ S, supported pigment aggregation or dispersion, respectively. Aggregation could be reactivated with 3 mM ADP but was about four times slower (Fig. 6) and never approached the extent (Fig. 7 a) observed with 3 mM ATP even after long incubations. A threshold concentration of 25 μ M ADP (Table III) was required to initiate pigment movement toward the cell center. No pigment dispersion occurred with ADP (Fig. 6 and 7 b) which, instead,

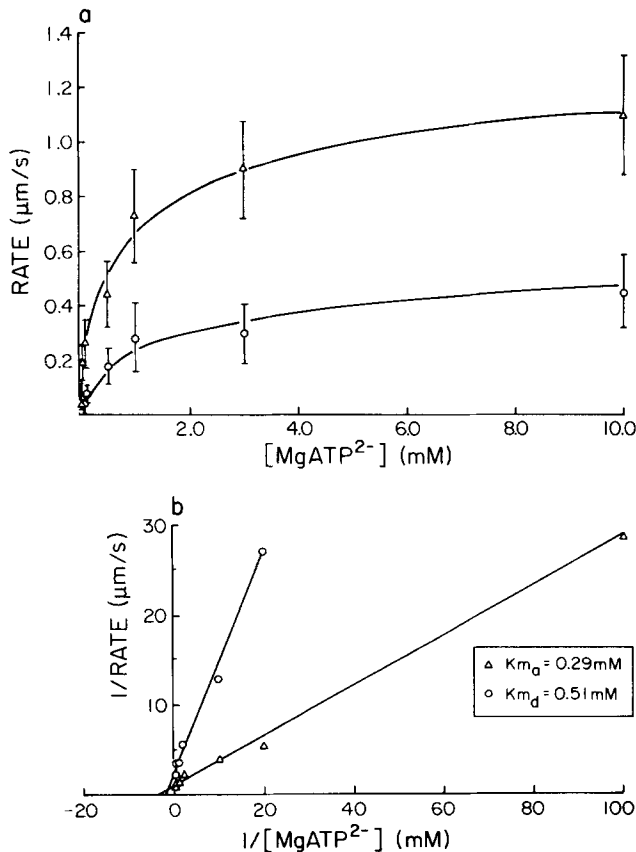


Figure 5. Rate of pigment granule movement as a function of MgATP concentration during reactivated aggregation (open triangle) and dispersion (open circle) in depleted melanophore models. (a) Kinetics of reactivated pigment transport. Bars, mean \pm standard deviation for at least 20 measurements taken from four melanophores at each MgATP concentration. (b) Double reciprocal plots of data presented in a. Each line is obtained by least squares method ($r = 0.998$, aggregation; $r = 0.996$, dispersion). x -intercepts represent K_m s for aggregation (K_{m_a}) and dispersion (K_{m_d}).

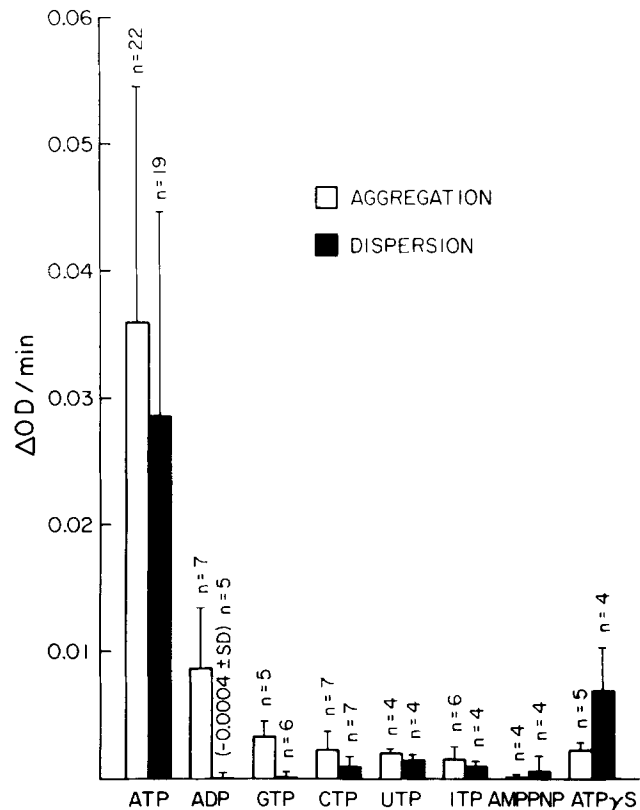


Figure 6. Nucleotide specificity of reactivated pigment aggregation and dispersion in depleted melanophore models. Relative rates of changes in OD per minute were obtained from photometric measurements taken at 6 min after onset of treatment with reactivation solutions containing either 3 mM ATP, ADP, GTP, CTP, UTP, ITP, AMPPNP, or ATP γ S. Bar shows standard deviation (SD); n, number of cells recorded.

condensed the pigment in the cell center so that the OD of the cells actually decreased (Fig. 7 b). ATP γ S, substituted in the reactivation solution, initiated very minimal pigment aggregation (Figs. 6 and 7 a), possibly as the result of ADP contamination in the ATP γ S (Cassidy et al., 1979; Brooks and Brooks, 1985), and the threshold concentration was at least 100 times that of ATP (Table III). Surprisingly, unlike aggregation, extensive pigment granule dispersion could be reactivated with ATP γ S (Figs. 6 and 7 b). The threshold concentration to initiate dispersion of 20 μ M was half that required using ATP (Table III). With 3 mM ATP γ S, dispersion occurred at about one-fourth the rate (Fig. 6) but, with long incubations (~ 25 – 30 min), to the same final extent of that observed with 3 mM ATP.

The extent and rate of recovery in ATP after treatment of cells with the other nucleotides also revealed differences between aggregation and dispersion. Complete rescue of pigment granule aggregation by ATP occurred routinely after incubation of the depleted cell models in nucleotides that did not initiate movement (Fig. 7 a), although a lag of up to 3 min was observed in the AMPPNP preparation. However, while dispersion in cells incubated in the nucleoside triphosphates could be completely rescued by ATP, dispersion in cells incubated in ADP or in AMPPNP exhibited only partial recovery (Fig. 7 b). A slight lag (≤ 1 min) was observed in the latter preparation. Potentially, the transport

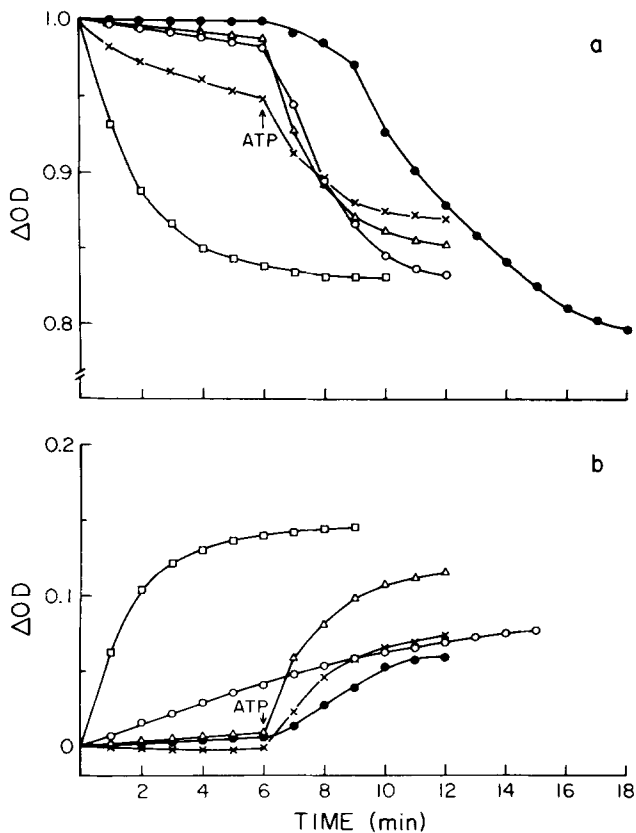


Figure 7. Nucleotide specificity of reactivated pigment aggregation (a) and dispersion (b) in depleted melanophore models as measured by changes in OD with time. Melanophores were treated with 3 mM ATP (open square), ADP (x), UTP (open triangle), AMPPNP (solid circle), or ATP γ S (open circle). At 6 min, cells that displayed little or no movement were challenged with ATP (arrows) to determine if cells could still transport pigment. Similar plots to that obtained for UTP (open triangle) were also observed with the other nucleotide triphosphates, GTP, CTP, and ITP, in which cells exhibited movement only upon subsequent ATP addition. Note that the ATP rescue of aggregation subsequent to treatment with AMPPNP is characterized by a significant lag while its rescue of dispersion after treatment with AMPPNP or ADP is incomplete. ATP rescue in melanophores dispersing in the presence of ATP γ S could be induced but is not shown, since movement occurred with this analog (b, open circles). Each point represents the average of at least four determinations.

processes are differentially affected by the various nucleotides, some of which may interfere with the interaction of ATP with the motile machinery for one or the other direction.

Direction of Pigment Granule Transport

Studies with depleted cell models revealed that both directions of transport required ATP while dispersion also required stimulation with $\geq 1 \mu\text{M}$ cAMP (Table III). Experiments were undertaken to determine if dispersion required the continuous presence of cAMP or, instead, if dispersion initiated in the presence of cAMP continued to completion in its absence. Accordingly, depleted cell models with aggregated pigment (Fig. 8 a) were induced to disperse with cAMP and ATP. During pigment dispersion (Fig. 8 b) the reactivation solution was exchanged for one lacking cAMP.

The pigment immediately reversed direction and reaggregated (Fig. 8 c), indicating that the cells must be continuously exposed to cAMP for dispersion to proceed. Similar studies were conducted to determine if cAMP added to cells aggregating pigment caused a reversal of melanosome direction. Depleted cell models with dispersed pigment (Fig. 8 d) were induced to aggregate with ATP (Fig. 8 e). During pigment aggregation cAMP was added and, again, the pigment changed direction and redispersed (Fig. 8 f). Thus, these studies demonstrate that cAMP alone is sufficient to induce a switch between the mechanisms generating the two opposing movements. While requiring ATP, dispersion occurs only if cAMP is continuously present, and aggregation occurs only if it is absent.

Discussion

Results of these studies demonstrate that bidirectional pigment granule transport can be reactivated in lysed melanophores. While both aggregation and dispersion required ATP, the K_m for ATP and the minimum concentration necessary for reactivation were greater for dispersion than

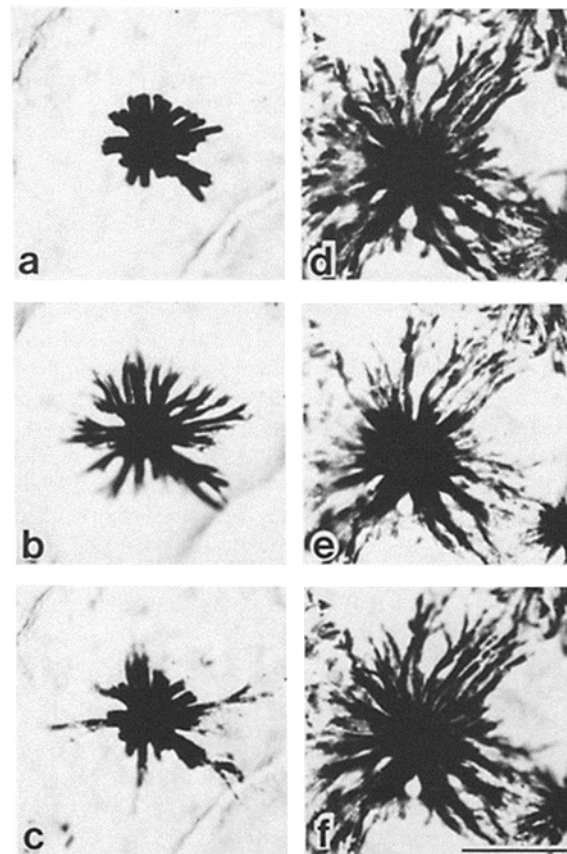


Figure 8. The direction of pigment transport is controlled by the presence or absence of cAMP. Depleted cell models with aggregated pigment (a) initiated pigment granule dispersion in the presence of reactivation buffer containing cAMP and ATP (b). Exchange of this reactivation buffer with one lacking cAMP induced the pigment granules to reverse direction and reaggregate (c). Depleted cell models with dispersed pigment (d) initiated pigment aggregation in ATP (e). Addition of cAMP to these cells also induced pigment to reverse direction and redisperse (f). Bar, 40 μm .

for aggregation. Moreover, aggregation and dispersion were differentially supported by other nucleotides, and dispersion required the continuous presence of cAMP. Previous studies have been conducted in which lysed chromatophore cell models were developed, but in each case these models were capable of undergoing only limited movements. Melanophores lysed with Brij 58 into a Pipes-buffered solution could undergo aggregation but not dispersion *in vitro* (Clark and Rosenbaum, 1982; 1984). Erythrophores lysed with digitonin also into a Pipes-buffered solution (Stearns and Ochs, 1982) were not amenable to an analysis of the process of aggregation or dispersion, but the spontaneous saltatory movements of granules in the dispersed state could be examined. In the present study both aggregation and dispersion, as well as saltatory motions, of pigment could be routinely obtained or reactivated with ATP in lysed cells. Because these movements can now be studied *in vitro* and the direction controlled by cAMP, the underlying mechanisms producing force in the two opposing directions can be analyzed.

The permeability of the melanophore plasma membrane is altered with digitonin, allowing free exchange of buffer solutions and molecules at least the size of immunoglobulins (see Fig. 1). That cells treated with lysis or depletion buffer are, in fact, lysed is indicated by several lines of evidence, including immunofluorescence staining with tubulin antibodies, hyperdispersion of pigment granules, and pigment transport upon addition of cAMP and/or ATP. Intact cells exhibit none of these responses. Furthermore, melanophores disperse pigment in the presence of cAMP \sim 1-1.5 min after onset of treatment with lysis buffer, the same time period in which the antibodies have access to the cell interior (Fig. 1 *b*).

In the permeabilized cell model, cycles of pigment granule aggregation and dispersion can be induced with sequential incubations in epinephrine and cAMP without the addition of ATP. These observations imply that sufficient levels of endogenous ATP are maintained to support continued pigment transport after lysis, thereby suggesting that mitochondria remain functional. Furthermore, the intact hormonal response of these permeabilized cell models to epinephrine indicates that sufficient numbers of membrane receptors are retained for the induction of pigment transport. Epinephrine, acting as an α -adrenergic agonist on these cells (Fujii and Miyashita, 1975), induces melanosome aggregation by reducing intracellular levels of cAMP (Abe et al., 1969; Andersson et al., 1984). Indeed, aggregation can proceed only in the absence of cAMP (see Fig. 8).

Depleted cell models, devoid of endogenous nucleotides, are prepared by extensive extraction of the melanophores. These cells can undergo pigment granule aggregation only when exposed to MgATP and do not require stimulation with epinephrine, suggesting that intracellular cAMP levels have also been depleted. In bypassing the receptor-mediated response, motility can be examined uncomplicated by possible effects of hormones on cell behavior unrelated to pigment transport. Dispersion in these depleted cell models requires the addition of both MgATP and cAMP. Surprisingly, at ATP concentrations \leq 3 mM saltatory melanosome movements are absent from cells undergoing reactivated dispersion, which, instead, occurs as a uniform and linear process. Thus, saltatory dispersion must be composed of two distinct

components in which saltatory motions are superimposed upon the continuous outward progression of pigment granules. Results of these studies demonstrate, therefore, that all pigment granule movements require ATP. Similarly, studies using permeabilized cells poisoned with DNP also reveal that aggregation (Clark and Rosenbaum, 1984) and dispersion can be reactivated only upon ATP addition.

Previous studies on live erythrophores have suggested that pigment granule aggregation is an energy-independent process of elastic recoil that is powered by the potential energy stored during the process of dispersion. Thus, it has been proposed that aggregation does not require ATP (Byers and Porter, 1977; Luby and Porter, 1980). In contrast, our results with lysed melanophores indicate that ATP is essential for both pigment aggregation and dispersion, although these two movements appear to be physiologically distinct processes. Reactivated dispersion requires cAMP, has a threshold concentration at least an order of magnitude greater, and has almost twice the apparent K_m for ATP that aggregation does. These differences may explain the observation that intact erythrophores aggregate pigment when cellular ATP is depleted by inhibitors of oxidative phosphorylation (Byers and Porter, 1977; Luby and Porter, 1980). As the ATP level falls below a critical value in metabolically poisoned cells, cAMP production and dispersion could no longer occur. Accordingly, requiring lower energy, aggregation becomes the favored transport.

A possibility exists that the observed ATP dependence of the bidirectional movements in melanophores may be due to the activation of adenosine receptors on the cell membrane (Miyashita et al., 1984). This response is unlikely, as ATP treatment of intact cells is ineffective in promoting pigment transport (Table I; Clark and Rosenbaum, 1984). Neither does ATP produce pigment granule transport by rescuing neuronal response, since reactivation occurs in the presence of nerve blockers (Table II). Although the precise role of cytoskeletal structures in pigment granule transport has not been determined (Bikle et al., 1966; Murphy and Tilney, 1974; Schliwa and Euteneuer, 1978, 1983; Porter and McNiven, 1982; Beckerle and Porter, 1983; McNiven and Porter, 1984), ATP might be required, not for the motile machinery of pigment transport, but, rather, for microtubule stability. However, GTP and UTP, which induce *in situ* tubulin polymerization in detergent-permeabilized 3T3 cells (Deery and Brinkley, 1983), cannot substitute for ATP to generate pigment movements in the depleted melanophore models. Thus, these studies indicate that ATP is utilized directly by the motile machinery producing bidirectional pigment transport.

Differences between the processes of aggregation and dispersion are indicated not only by their different ATP requirements but also by the studies showing that only dispersion can utilize the ATP analog ATP γ S as a substrate, while only aggregation can utilize ADP. When ATP γ S was substituted for ATP, a twofold lower threshold of reactivated dispersion was obtained. This threshold discrepancy probably reveals that other cellular processes are competing for ATP with the motile machinery, producing aggregation and dispersion for ATP, and thus may explain the relatively high apparent K_m values obtained for both movements. As ATP γ S cannot be used as a substrate for most enzyme systems that use ATP (Gratecos and Fischer, 1974), the dispersion threshold con-

centration of 20 μM ATP γS may be a closer approximation of the ATP threshold concentration actually required to initiate pigment movement toward the cell periphery. The ability of ADP to support limited aggregation, but not dispersion, may be the result of adenylate kinase activity (Brokaw and Gibbons, 1973) unable to convert sufficient ADP to the higher ATP threshold necessary for pigment dispersion. ADP may also directly inhibit dispersion, as this nucleotide induces protein kinases to dephosphorylate their substrates (Flockhart, 1983). In other studies we have shown that pigment dispersion requires protein phosphorylation (Rozdzial and Haimo, 1986). Further evidence of the disparate regulation of aggregation and dispersion is shown in the differential ability of these opposing movements to resume in the presence of ATP after treatment with the nonhydrolyzable ATP analog, AMPPNP, which induces a rigor-like attachment of vesicles to microtubules in axoplasm (Lasek and Brady, 1985). The rate of recovery was two to three times faster for dispersion than for aggregation (Fig. 7), and this disparity may reflect the dissimilar K_m s for ATP, and accordingly for AMPPNP, by the two motile processes. Moreover, the incomplete recovery of dispersion, but not aggregation, in cells treated with ATP after incubation in ADP or AMPPNP suggests that these nucleotides interfere with the motile machinery producing dispersion.

Results of these studies also demonstrate that cAMP is the switch that controls the direction of pigment transport. The addition or removal of cAMP induces melanosomes undergoing transport to reverse direction. If cAMP is absent and ATP present, aggregation occurs and requires no additional stimulus. If both cAMP and ATP are present, dispersion occurs. In other studies we have shown that cAMP induces dispersion by activating cAMP-dependent protein kinase that phosphorylates a 57-kD polypeptide, which is subsequently dephosphorylated during aggregation (Rozdzial and Haimo, 1986). Similar observations were recently reported in xanthophores (Lynch et al., 1986). The results reported here showing that cAMP must be continuously present suggest that a protein, potentially the 57-kD polypeptide, must be continually rephosphorylated during dispersion.

The data presented here and elsewhere (Rozdzial and Haimo, 1986) indicate that aggregation and dispersion may be mediated by two distinct mechanisms. Aggregation and dispersion have different nucleotide sensitivities, and only dispersion requires stimulation by cAMP. Although protein phosphorylation and dephosphorylation are required for melanosome dispersion and aggregation, respectively (Rozdzial and Haimo, 1986), they have not been implicated in axonal transport (Vale et al., 1985a, b). Thus, different mechanisms may be responsible for producing organelle transport in these different cell types. The system described in this paper in which pigment aggregation and dispersion can be reactivated under defined conditions should permit an elucidation of the motile mechanisms responsible for force production in the two opposing directions.

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