1965

Studies on the Granule-Moving System of Melanocytes of Fundulus Heteroclitus

Lorna Green

STUDIES ON THE GRANULE-MOVING SYSTEM OF MELANOCYTES OF FUNDULUS HETEROCLITUS

A thesis submitted to the Faculty of The Rockefeller Institute

in partial fulfillment of the requirements

for the degree of Doctor of Philosophy

by

Lorna Green, B. Sc.

Approved for Publication

Professor at Rockefeller University

May 1965

The Rockefeller Institute

New York, New York

PLEASE NOTE:

Several pages with glassine overlays are blurred and indistinct. Filmed as received.

UNIVERSITY MICROFILMS.

Preface

I would like to express my deep gratitude to:

Dr. Detlev W. Bronk, president of The Rockefeller Institute, for making possible to myself and others this unique apprenticeship.

Dr. Paul A. Weiss, my thesis advisor, for guidance and for the great independence of study he permitted.

Many have helped me with this work. I would like to thank Dr. T. Shedlovsky and Dr. L. G. Longsworth for many valuable discussions on mechanisms of granule movement; Dr. Keith Porter of Harvard University for discussion of electron micrographs; Miss Sonia Wohl, and Miss Mary Wissler of the American Museum of Natural History for their invaluable help in locating old manuscripts; Mr. McCall, instructor in Greek at Harvard University, for translation of the Greek fragments presented in Chapter I; Dr. Friedrich Kueppers for assistance in translating German manuscripts; Dr. Peter Woodford for his suggestions on the writing of the thesis; members of the illustrations department--Mr. R. F. Carter, Ruth Mandelbaum, Josephine Wolk, Muriel Fabrizio and Otie Kloesman for their careful preparation of the diagrams and photographs; Maggie and Philip Rhinelander and Walter Chapin for their hospitality during much of the writing of the thesis.

I owe much to friends who have greatly enriched my understanding of the sciences: Merrill and Bertil Hille, Barbara Alexander, Alan Steinbach for discussion and criticism; Peter Wolk who first set me to wonder what the inside of a cell was like; Dick Campbell who raised a question of general theory in biology.

I am beholden more than can easily be expressed in words to Dr. Murray Rosenberg and Dr. John Green for their extensive discussion of many facets of the problem, and to Donald Knight whose constant interest and encouragement saw the work to completion.

Finally, I would like to acknowledge a great debt to my mother, Winifred Maud Pascoe, and my father, John Joseph Green, who first fostered my interest in the natural world. To them this work is inscribed, in token of respect and friendship.

Abstract

Pigment granules in melanocytes of Fundulus heteroclitus exhibit both local movements and long distance intracellular migrations in response to hormones. These movements have been studied and a theory of the granule-moving system has been proposed: The granules are in a structured continuum which expands and contracts to move them; the state of the continuum is the resultant of a dynamic equilibrium between expansile and contractile forces manifested in the local movements of granules. The fine structure of the cell has been explored by electron microscopy; the prominent features of the cell are microtubules. Several alternatives for the specific granule-moving apparatus have been discussed.

In the light of these studies, previous theories of the mechanism of granule movement have been ruled unlikely.

TABLE OF CONTENTS

I. INTRODUCTION

A. ANIMAL COLOR CHANGES

1. Discovery of Color Changes.

Many common animals exhibit striking changes of color in response to variations in the background illumination. Fish and amphibians blanch and darken on white and black backgrounds respectively; octopi display vivid changes of hue--passing from grey into various shades of orange and red. These color changes have been known to men for hundreds of years. The king in Homer's Thebaid advises:

> Oh hero, Amphilochus, my child, having the mind of a polypous, adapt yourself to those among whose people you arrive.*

And a fragment of Sophocles reads:

> Consider, in relation to the true thought of a man, how the color of a polypous on a rock, changes.* (Iphigenia)

Shakespeare was later to write:

> Do you change colour?--Give him leave, madam; he is a kind of chameleon.
> (Two Gentlemen of Verona, ii, 4)

Only recently have these color changes been understood. The story of the scientific investigations of these phenomena is a long and fascinating one and begins in ancient times.

Aristotle first set down a description of color changes in scientific records. In Book II of his Historia Animalium he noted that the chameleon could change its color from black to green, a color change which occurred over the whole animal, including even the eyelids. And in Book IV of the same work, he observed that the adult octopus, when pursuing prey, blended its color to match its background. The color changes exhibited by this animal were even more readily observed in the young, which turned from white to red in alarm if they were prematurely broken free of their egg cases. Roman naturalists substantiated Aristotle's observations, and in addition, recorded color changes in fish. Pliny remarked in Book IX of his Natural History that the mullet, a rather ordinary looking fish from the Mediterranean Sea, assumed a variety of different hues as it died,

---

* 'Polypous' is the Greek word for 'octopus'.

and Seneca reports of this fish that:

> A mullet even if just caught is thought little of unless it is allowed to die in the hands of your guest. They are carried about in enclosed globes of glass and their color is watched as they die which is changed by the struggles of death into various shades and hues.
> (Physical Investigations, III, 100)

In 1715, Vallisnieri, an Italian naturalist, recorded for the first time that the common European frog, Rana esculenta, could change its tint, and in 1758, Roesel von Rosenthal made a similar observation on the tree toad. The Scottish naturalist, Stark, reported in 1830 that several species of European fish darkened on a dark background and blanched on a light one. Shortly thereafter, in 1842, Kröyer discovered color changes in the shrimp, Hippolyte. The observations of Kröyer completed the discovery of the five major groups of animals in which color changes occur--the cephalopod molluscs and the crustaceans among the invertebrates, the fish, the amphibians, and the reptiles among the vertebrates.

2. Basis of Color Changes.

The mechanism of color changes first excited serious attention in the early 19th century. Because changes of color in chameleons were often accompanied by inflation of the skin, some investigators believed that the skin contained tiny, colored grains of sand which were exposed when the skin was inflated with air and concealed when the skin was deflated (see Milne-Edwards, 1834). The famous French anatomist, Cuvier (1817), suggested that color changes of the octopus were under the control of fluids. His anatomical studies revealed that whereas the blood of this animal was deep purple, the walls of the blood vessels were yellow. It was natural to suppose that the color of the octopus depended on the amount of fluid in the vessels. Cuvier's hypothesis was overthrown two years later when Sangiovanni (1819), an Italian naturalist, demonstrated that the color changes of the octopus were the result of contraction and expansion of small, colored bodies in the surface of the animal. He names these bodies chromofori (in English, chromatophore) and introduced the terms 'systole' and 'diastole' to designate the movements of these bodies.

In 1834, Milne-Edwards reported similar colored bodies in the

skin of chameleons and grasped clearly the principles of color change in all animals. Through careful observation, he discovered that the skin of chameleons contained two layers of these colored bodies--a deep layer bearing black pigment, and a superficial layer bearing green pigment. Whereas the black pigment remained stationary, the green pigment could be spread out to cover the black pigment altogether, or else, withdrawn into clusters, exposing the underlying black pigment to view. Shortly after Milne-Edwards discovered pigment-containing bodies in chameleons, similar bodies were found in amphibia (Acherson, 1840), fish (Buccholz, 1863; von Siebold, 1863), and crustaceans (Sars, 1867).

The subsequent work of many investigators has established that there are two types of chromatophore in the animal kingdom (see Fingerman, 1965). The first, the more complex of the two types, is found only among the squids and octopi. It consists of a central elastic-walled sac, filled with colored pigments and attached by many radiating muscle fibres to the surrounding tissue (Bozler, 1928). The contraction of these fibres causes the central sac of pigment to spread out into a flattened disc which may have a diameter twenty times as great as that of the original sphere. Upon relaxation of the muscle fibres, the sac returns to its initial size, presumably due to elastic restoring forces within its walls. Each muscle fibre is innervated, and the behavior of the cephalopod chromatophores is entirely controlled by the central nervous system.

The second type of chromatophore occurs throughout the other groups of animals. It is a single cell containing granules of colored pigments. The prominent color changes of animals result from the movements of the pigments within these single cells; the pigment granules may be spread throughout the cytoplasm of these stellate cells or clumped together in a small sphere. Most animals possess chromatophores of two or three different colors,* and their overall coloration is a complex function of the distribution of granules within each kind, together with effects produced in combination with other permanent colors in the skin.

---

* On the basis of the color of the contained pigment, five different kinds of chromatophores are distinguished. There are melanophores (black pigment), erythrophores (red pigment), xanthophores (yellow pigment), guanophores (white pigment), and iridiocytes (silvery, guanine crystals) (Fingerman, 1965).

3. Control of the Chromatophores.

Prior to the experiments of Stark (1830), there were diverse theories of the function of color change. Some observed that it occurred when animals were alarmed and suggested that it accompanied emotional states; others suggested these changes were a symptom of illness (see Milne-Edwards, 1834). Daily rhythmic changes were also reported (Keeble and Gamble, 1904). But Stark's experiments showed clearly that fish changed color to match their backgrounds, and by the 19th century most investigators recognized that color change was a protective device which allowed animals to match their surroundings.

It was widely believed that adaptation to the background was mediated by the eye, and thence, by the central nervous system. However, proof of nervous control was lacking until 1852 when Brücke published a monograph which set a model for all later physiological investigations of these problems. In a series of experiments on the African chameleon, he showed that the destruction of nerves to a particular region resulted in a permanent expansion of the chromatophores of the region. Pouchet (1872) later obtained the same results in fish, i.e., denervated regions became permanently darkened due to the expansion of melanophores. These studies were confirmed and extended by von Frisch (1910) in the first part of this century.

Whereas nervous control of the chromatophores was readily demonstrated in fish and reptiles, it was not so readily demonstrated in other animals, and even at the time of von Frisch's decisive experiments, another current of thought was evident. The systematic sectioning of crustacean nerves by Pouchet (1872) failed to have any effect upon the state of the chromatophores. However, if their blood supply were cut off, the chromatophores expanded. They also became permanently expanded in response to removal of the eyestalks, an organ which contains many neurosecretory cells. These experiments suggested hormonal control of crustacean chromatophores (Keeble and Gamble, 1903). Decisive experiments were made by Koller (1925) who injected the blood of a dark shrimp into a light one and produced darkening of the latter. The reverse experiment--injection of the blood of a light shrimp into a dark--failed to produce blanching, but Perkins (1928) later accomplished this in shrimp which had darkened in response to removal of the eyestalk. Perkins subsequently demonstrated that darkening was con-

trolled by a hormone of the rostrum, lightening by a hormone of the eyestalk.

The chromatophores of amphibia were also shown to be under hormonal control. In 1898, Corona and Moroni discovered that when adrenalin was injected into the circulation of the frog, the melanocytes contracted. This observation was confirmed by Lieben (1906) and later by Redfield (1916) for lizards. Shortly thereafter, several investigators drew attention to the fact that amphibians lightened if their pituitary glands were excised (see Parker, 1930). Hogben (1924) and his school subsequently demonstrated that a hormone of the pituitary gland, the melanocyte-stimulating hormone (MSH), caused expansion of the chromatophores in these animals. Although other hormones have since been shown to affect the state of the chromatophore (Fingerman, 1965), MSH appears to be the single, most important controlling agent.

In summary, the work of many investigators has culminated in the following picture of chromatophore control (Fingerman, 1965): Cephalopod chromatophores are controlled by the nervous system, hormonal factors being of secondary or no importance. Both amphibian and crustacean chromatophores are controlled by hormones. Teleost chromatophores are controlled by both nerves and hormones, the relative importance of these two systems varying from one species to another--in Anguilla, both nerves and hormones are important; in Fundulus, the control of the chromatophores is primarily neural.*

By the 20th century, color changes in animals had been shown to be the result of changes in the distribution of pigment granules in single cells. These changes were triggered by neural and hormonal stimuli in response to changes in the background illumination.

---

* Modern investigations of the control of the chromatophores consist mainly in extending and elaborating the principles of regulation discovered by 19th century investigators. The picture of the control of the chromatophore in situ becomes more complex as an increasing number of physiological agents is shown to affect its state. In addition there is extensive investigation into the retinal control of color patterns (which must be exceedingly complex in many fish which can blend with a checkerboard background). Relationships of color changes to various other physiological functions--such as heat and water balance, and diurnal variations in color--are also being investigated. Reference to this contemporary work will be found in Fingerman (1965) and Waring (1963).

Investigators now turned their attention to the unit of color change, the single chromatophore.

B. THE SINGLE CHROMATOPHORE

Single chromatophores of fish may be easily seen by examining a fish scale with the light microscope. A scale of Fundulus heteroclitus, a marine teleost, is shown at the right. The black dots are melanocytes. These melanocytes are representative chromatophores and will be described in this section.

Fig. 1

1. Description of Teleost Melanocytes.

Melanocytes in situ. Mature melanocytes are large, stellate cells of the order of 180 microns in diameter (Figs. 2a, 2b). Most are multinucleate. They occur in the dermis of the skin--a thin layer of collagen overlying the scale--and are interspersed among xanthophores and often immediately underlain by large iridiocytes. There may be as many as two hundred melanocytes on a small scale 2 mm. in diameter (Fig. 1). The population of pigment cells is permeated by capillaries and liberally innervated. The nerves cannot be seen in the living preparations shown below (Fig. 2a, 2b), but they have been demonstrated in fixed preparations, and are often so numerous that each cell appears to be enclosed in a veritable basket of nerve strands (Ballowitz, 1893). A thin sheet of epidermis overlies the mat of collagen and pigment cells.

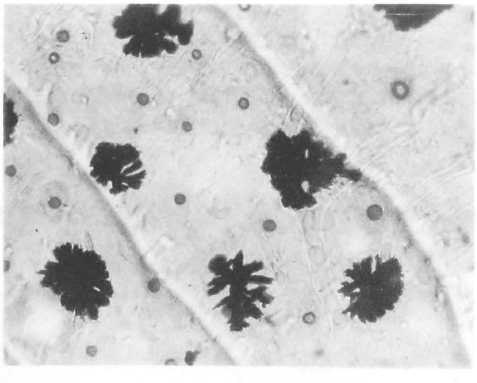


Fig. 2a. 200 x. Large, black cells are melanocytes. Round structures between are yellow pigment cells. Capillaries containing red blood cells can be seen among them.

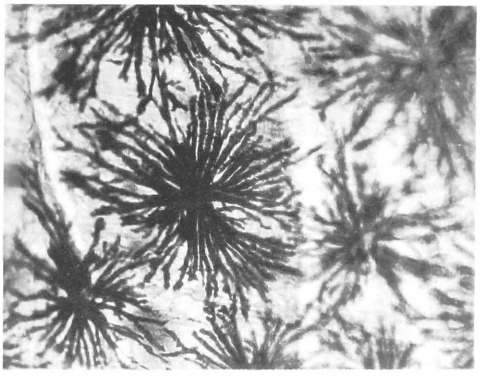


Fig. 2b. 200 x. Melanocytes with black pigment dispersed throughout the cytoplasm.

Origin of Melanocytes. Early embryologists recognized that the melanocytes originated from non-pigmented precursor cells which migrated to the scales from sites of origin deeper in the body (see Gilson, 1926). It was first thought that the cells were mesenchymal in origin and were a type of modified smooth muscle cell (Spaeth, 1916), but it has subsequently been established that they originate from the neural crest (DuShane, 1944; Rawles, 1948). The precursor cells are both non-pigmented and amoeboid. They migrate through the tissues to the scale and differentiate in situ, gradually losing their amoeboid properties as they acquire their characteristic load of black pigment. Once the melanocyte begins making melanin, it loses its properties of cell division, and mature melanocytes do not divide. New melanocytes continually arise in the adult fish by de novo differentiation on the scale (Gilson, 1926).

Pigment of Melanocytes. The black pigment is contained in small granules which may be seen in the mealnocyte shown in Fig. 3. The pigments are melanins--oxidation products of tyrosine. Considerable work has been done on the chemistry of these pigments (Fox, 1953; Lorinz, 1954), the biosynthetic pathways of their formation (Raper, 1928; Lerner et al., 1950), and the intracellular sites of pigment synthesis (Seiji et al., 1963).

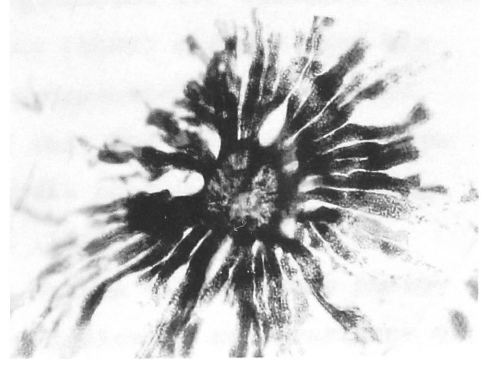


Fig. 3. 400 x. Large melanocyte granules can be seen. Two nuclei are visible in the central region of the cell.

2. Investigations into the Behavior of Single Chromatophores.

Fundulus heteroclitus blanches if placed upon a light background and darkens if placed upon a black one. These changes in color are due primarily to changes in the distribution of the pigment granules in the melanocytes. Depending on neural and hormonal influences, the granules may be clustered in a small sphere or spread in a stellate form. The fish

lightens and darkens accordingly.

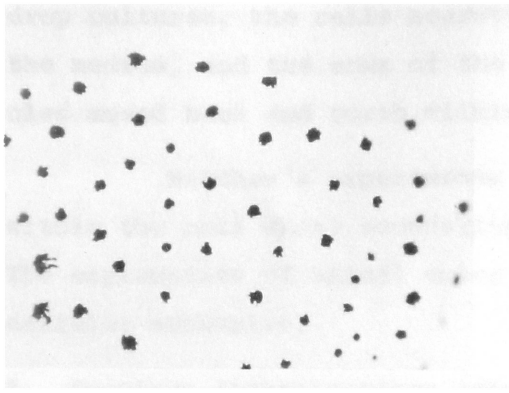


Fig. 4a. 75 x. Granules clustered.

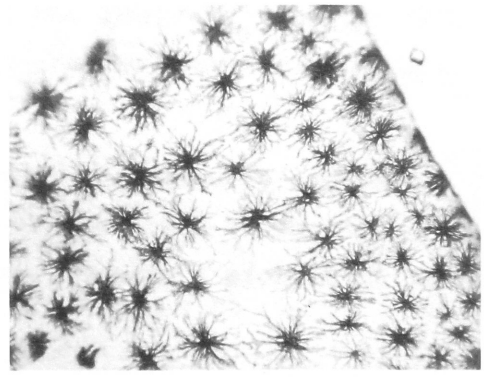


Fig. 4b. 75 x. Granules dispersed.

These events in melanocytes have attracted considerable attention in the past. The first question was whether the pigment movements were the result of a change of shape of the entire cell (amoeboid theory) or whether they represented an intracellular migration of granules (fixed-cell theory). Since no cell boundaries could be seen in living preparations, von Wittich (1854), Frohlich (1910), Hooker (1914), and others (see Parker, 1930) inferred that the cell was amoeboid, i.e., the granules were fixed in the cytoplasm, and movements of granules were due to extension or withdrawal of pseudopods. This theory was supported by the known occurrence of amoeboid activity in young melanocytes. Other workers contested the amoeboid theory. Brücke (1852), Zimmermann (1893), and Biedermann (1892) claimed that the cell boundaries could be seen in histological preparations and remained extended during granule migration. This meant that the movements of granules were intracellular. The opinion that granule movement was an intracellular migration was also given by Ballowitz (1914) on the basis of extensive observations of living cells, and by Spaeth (1913) whose photographs of successive expansions of living cells indicated an invariance of form with respect to the smallest branches.

This early controversy is discussed by Fuchs (1914), van Rynberk (1906), and Gilson (1926). Although the majority of workers favored the fixed-cell theory, Parker concluded in a review of the subject in 1930 that the existing evidence did not warrant a choice between either hypothesis.

The fixed-cell theory was put on a firm basis by Matthews in 1931. He discovered that the boundaries of melanocytes, which generally could not be seen, became visible if scales were kept for several hours in artificial medium. Furthermore, when small fragments of scales were placed in hanging

drop cultures, the cells nearest the edges of the fragments migrated into the medium, and the arms of the cell could be clearly observed. The granules moved back and forth within them.*

Matthew's experiments established that the granules migrated within the cell whose boundaries remained virtually fixed in position. The explanation of animal color change therefore became a problem of intracellular mechanics.

3. Previous Investigations into the Mechanism of Granule Movement.

Many previous investigators have endeavored to explain how the granules move. To this end melanocytes have been explored with a variety of techniques and several hypotheses proposed. Early German workers studied cell structure in fixed preparations and postulated that the granules were in a 'net' which contracted to move them (Zimmermann, 1893; Biedermann, 1892). Franz later postulated that the granules were sandwiched among rod-like structures which moved them by 'melting' and 'reforming'. Ballowitz, observing granule movements in living cells, suggested that the granules were in channels which moved them by peristalsis. Investigations of the physical properties of melanocytes has culminated in a sol-gel theory of movement--a theory that the cytoplasm of the cell consists of colloidal particles which aggregate or disperse (Spaeth, 1913; Marsland, 1944). Several Japanese workers studied the electrical properties of melanocytes and postulated that the granules were moved by electrical forces (Kamada and Kinosita, 1944). One study of the cell by electron microscopy has resulted in the theory that the granules are confined within an intracellular sac, and moved by the contraction of a surrounding ring of fibres (Falk and Rhodin, 1957).

Previously, there has been no way to discriminate among these the-

---

* Since Matthew's experiments, only one alternative to the fixed-cell theory has been offered. Using polarized light, Shanes and Nigrelli, in 1941, discovered that the melanocytes were connected to the scale by birefringent strands; they suggested that contraction of these strands might cause pigment movement. However, if their photographs are carefully compared with the living preparation, it is apparent that what they mistook for birefringent strands were actually the arms of iridiocytes lying just beneath the melanocytes (personal observation). These iridiocytes are almost invisible in the living preparation, but their crystals of guanine reflect polarized light, rendering the cell a silver color in the dark field.

ories of granule movement. Each is based upon a specific kind of information, and the very phenomenon which all have been proposed to explain--the movement of granules--has never been analysed in detail. Such an analysis would establish the boundary conditions to be met by any of these theories. In recognition of the need for an analysis of the movement of granules, this study has been undertaken in the present thesis, and in the light of such the theories outlined above are discussed in Chapter 5.

C. SUBJECT OF THE THESIS

1. The Phenomenon.

The phenomenon which is to be explained is the intracellular migration of granules. If adrenalin is given, the granules move through the cytoplasm of the cell to become concentrated in the central region; when the preparation is rinsed with fresh medium, the granules become re-distributed throughout the cell cytoplasm.* The visible phenomena are a concentration and a dispersion of granules, and these terms will be employed to designate these processes. The extreme states of granule distribution will be referred to as the concentrated and the dispersed states.**

2. The Problem.

The explanation of granule movement entails specification of the forces upon the granule, the cell structures in which these forces arise, and the molecular events, triggered by hormones, which give rise to these forces. At present, investigation into this problem in melanocytes is at the stage of specifying the forces on the granules and the cell parts in which these forces arise. The work to be presented in this thesis is a contribution to this investigation. As a result of the work to be presented, what a full explanation of the movement of granules will entail, in concrete terms, will be made clear at the end of this thesis.

---

* Many agents will trigger these processes. See Fingerman, 1965.

** Earlier investigators have employed such terms as systole and diastole, contraction and expansion, aggregation and dispersion. These terms most often refer to a particular theory of the mechanism of movement. The accurate designation of the processes is concentration and dispersion.

3. The Analysis.

The research to be presented consists of an extensive analysis of the movements of granules, together with preliminary observations of cell fine structure by electron microscopy.

An analysis of the movements of the granules could give considerable information about the mechanism of their movements. It would be possible to infer whether they moved actively--swimming or crawling along the substratum as mitochondria do--or passively. If they were passively moved, then the kinds of forces which could move them might be ascertained. Would electrical forces be sufficient to move these large granules over distances up to 60 microns? Are they carried in a fluid medium set in motion by hydrostatic pressure, or do they perhaps mark some structured matrix whose movements they parallel? Many questions concerning the granule-moving system might be answered by an analysis of the movements of granules. Moreover, the interpretation of granule movements would give rise to a framework of ideas within which the significance of information yielded by various techniques may be assessed, and systematic future investigation be undertaken.

The granule movements of Fundulus heteroclitus are more complex than implied in the preceding pages. If a living cell is examined, the first impression is of intense activity in the cell cytoplasm. The granules are in continual motion. Close inspection of resting cells reveals that the granules are moving to and fro in non-directed fashion in localized regions. When hormones are administered to the cell, the localized motion gives way to the directed, long distance migrations.

Melanocytes of Fundulus heteroclitus are excellent preparations in which to study both types of granule movement. The melanocyte is easily viewed on the scale of the fish. These scales can be kept alive in vitro in artificial medium for several days, while the behavior of melanocytes is observed by light microscopy. The same agents which cause concentration and dispersion of granules in vivo--adrenalin and acetylcholine--will elicit these migrations in vitro, and the granule migrations can be triggered at the will of the investigator. Moreover, melanocytes can be dissociated from the scale altogether and put into cell cultures (a technique developed during the course of these studies). In these cell cultures, single cells

(Fig. 5), now freed of all attachments to other tissue, exhibit their characteristic behavior in response to hormones. The movement of granules in both cell preparations may be observed by direct visual observation and filmed by time-lapse cinematography.

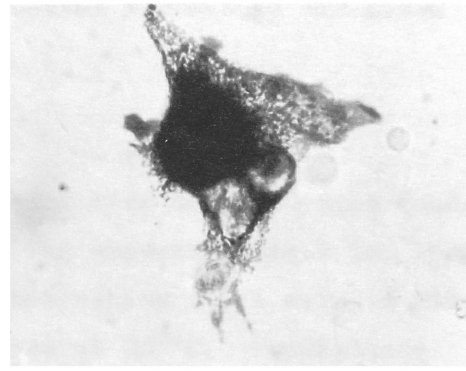


Fig. 5. Cultured melanocyte. The two ovoid bodies at the lower right-hand side of the granule mass are nuclei.

The thesis is present according to the following plan: Methods for culturing cells and studying living preparations, and in addition, the preparative methods for electron microscopy, are presented in Chapter II. The static, structural features of the melanocyte, determined using both light and electron microscopy, are described in Chapter III. The granule movements in living cells are described in Chapter IV. In Chapter V, the movements of granules are interpreted, and a theory of granule migration is presented and briefly elaborated in terms of cell fine structure.

II. METHODS

The techniques used in the study of melanocytes are described in this chapter. Two in vitro preparations of living cells are described in sections A and B; in section C are described the techniques of analysis by time-lapse cinematography. Methods for electron microscopy are given in section D.

A. MAINTENANCE OF FISH

Live specimens of Fundulus heteroclitus were obtained from Woods Hole Marine Biological Laboratory. Each fish was approximately 6 in. long. Groups of 6-8 fish were put into glass tanks containing a 1:1 aerated mixture of fresh water and sea water and maintained at 20° C. Populations were kept permanently light-and dark-adapted by keeping the tanks on white and black backgrounds respectively.*

B. PREPARATIONS OF LIVING CELLS

Living melanocytes were studied in two in vitro preparations. They were examined on the scale, a preparation which will be designated 'in situ'; they were dissociated from the scales and put into cell cultures, a preparation which will be referred to as the cell culture preparation. Both preparations are described separately below.

1. In Situ Preparation.

Preparation. The most convenient preparation is the in situ. A single scale, taken from the dorsal fin region of the fish, was placed tissue-side up in a drop of Eagle's medium (Paul, 1961a) on a glass slide. A cover glass supported by plasticine under the four corners was applied, and the preparation was observed with the light microscope.

Behavior of Cells in Situ. All melanocytes immediately responded

---

* Fish moved to a light background respond by an immediate concentration of melanocyte granules. During the next few days, the granules revert to a semi-dispersed state. If the fish are maintained for some time on a light background, the number of cells quantitatively decreases (Waring, 1963). In addition, cells examined after two weeks contain fewer granules than do cells on dark-adapted fish. Fish placed on dark backgrounds respond by immediate dispersion of melanocyte granules. In the next few days the granules revert to a semi-dispersed state and over succeeding days there is a quantitative increase in the number of melanocytes present and in the number of granules within any one melanocyte.

to in vitro conditions by a dispersion of the granules followed by granule concentration. The duration of the preliminary dispersion depended upon the state of the fish from which the cells were taken. The cells from light-adapted fish behave as if they were released from concentrating stimuli, whereas cells from dark-adapted fish behave as if released from dispersing stimuli: In cells from dark-adapted fish dispersion lasted only a few minutes, and then the granules re-concentrated. On the other hand, in cells from light-adapted fish, the granules dispersed and might remain in the dispersed state for as long as thirty minutes. Eventually the granules of all cells moved into the central region. Either they remained in a semi-dispersed state, or more often, successively concentrated and dispersed several times over the next few hours.

Light, heat, and alkaline medium are all weak concentrating agents. Since the Eagle's medium became alkaline due to loss of CO_2, fresh medium was constantly supplied by pipette under the edges of the coverslip. Small fluctuations in pH did not markedly affect cells, and any slight tendency to concentrate or disperse was taken into consideration in the characterization of granule movements (see Chapter IV).

Usefulness of Preparations. The in situ preparation was suitable for study by direct, visual observation and by short-term time-lapse cinematography. The major drawback to the study of granule movements in this preparation was that the scale and tissues beneath the cell obscured the details of the movements, and the environment of the melanocyte was not constant due to small fluctuations in pH. For this reason, the cells were dissociated from the scales and put into cell cultures.*

2. Cell Culture Preparation.

Preparation. Cell culture preparations were prepared in the following way:

A fish was killed by a blow on the head, and the scales were

---

* The in situ preparation was not suitable for studies of hormone action. Due to continual fluctuations in the pH of the medium, the environment of the melanocyte was not constant. Moreover, nerve endings were present. Since all previous investigations on the effects of added agents on melanocytes have been made in the in situ preparation, claims for the direct effects of drugs on melanocytes must be considered doubtful, due to the possible mediation of their effects by release of neural transmitters.

scraped off and washed in Eagle's medium (Paul, 1961a) containing penicillin (Lilly, 100 u.s.p./100 ml. of culture medium), streptomycin (Lilly, 0.01%), mycostatin (Squibb, 2,000 u.s.p./100 ml. of culture medium), and beef embryo extract (Microbiological Associates, Bethesda, 10 ml./100 ml. medium). Medium of the same composition was later used to culture the dissociated pigment cells.

The scales were washed in this medium, changed several times during a 30 minute period, and then rinsed with several changes of Ca-Mg free Earle's solution (Paul, 1961b) for 5 minutes. The scales were divided into two lots and placed in 10 ml. centrifuge tubes containing 6 ml. of Ca-Mg free Earle's solution. To each of these tubes was added a small portion (3-4 grains) of collagenase (Worthington Biochemical Corp., N. J.). (It was not found necessary to standardize this procedure rigorously; the amount used could be gauged by the eye and was that determined by trial and error to be just sufficient to dissociate most of the cells after 1-2 hours of incubation at 37° C.) The medium was rendered slightly alkaline (light pink by the phenol red indicator) by exposure to an air jet. The tubes were then sealed with silicon stoppers and incubated at 37° C. in tube rollers.

Dissociation could be followed with the eye. When most of the cells were dissociated, the tubes were removed from the incubator and centrifuged at 170 g. for 5 minutes at room temperature. The incubation medium was discarded, and the cells were re-suspended in Eagle's medium containing beef embryo extract and antibiotics and centrifuged for another 5 minutes. The medium was then discarded and about 1½ ml. of fresh medium were added to make a concentrated cell suspension.

The cells could be cultured in any regular culture chamber, but for the purpose of this study it was found convenient to culture them on coverslips 7/8 of an inch in diameter, so chosen to fit standard perfusion chambers. The coverslips were placed on filter paper in sterile Petri dishes in lots of 15 coverslips per dish.

A few drops of cell suspension together with a few drops of Eagle's medium were added to each coverslip. A high density of melanocytes in a small portion of medium was essential. These Petri dishes were stored in a vacuum jar containing water in the base through which was bubbled gas from an attached cylinder of CO_2-balanced air mixture. In this way the Eagle's medium was maintained at neutral pH. The vacuum jar was covered with a black cloth and maintained at 23° C. Unavoidable pH changes occurring during these operations did not appear to impair cultures.

The medium on the coverslips was removed by pipette every 2-4 days and replaced with fresh medium. It was changed more often if contamination increased, and such changes were found to be effective in keeping contamination at a minimum.

Coverslips could be examined with a dissecting scope without being removed from the Petri dishes. Those chosen for detailed observations were examined on glass slides or fitted into perfusion chambers. Coverslips could be sealed into chanbers with vacuum grease or even with

hot wax, provided the cultures were kept cool during this process by repeated changes of the medium.

Characteristics of Cultures. These cultures were by no means pure cultures of melanocytes but rather contained every type of cell which occurs on the scale. All cultures differed in longevity, in ratios of cell types present, and in the forms of melanocytes obtained, in a manner difficult to predict or control and regardless of whether cells were taken from light-or dark-adapted fish, or dissociated in the morning, afternoon, or evening.

The average survival time was 8-10 days. One set of cultures which survived for four weeks was characterized by a sheet-like growth of epidermis which almost entirely covered the coverslip surface.

Many different forms of melanocytes were obtained in cultures. Some cultures contained small, dendritic forms and others contained large, flattened cells. All the conditions determining these forms are not known. There seemed to be some correlation of the appearance of discoid forms with a slight acidity of settling conditions which were sometimes obtained due to slight variations in the pH of the different cylinders of CO_2-balanced air mixtures. Cultures containing many discoid cells tended to live longest--up to two weeks in some cases.

Other cells in the cultures eventually overgrew the melanocytes, and therefore films were made of granule movements one or two days after settling of these cells.

Use of Cultures. These cultures had many advantages over the *in situ* preparations. Melanocytes could be maintained in relatively constant conditions in their perfusion chambers for hours and even days. Without the obscuring scales and collagen, many more details of the entire cell could be seen, and the melanocytes could be observed by phase microscopy. The flattened forms of most cultured cells also permitted many more details of the movements of the granules to be seen.*

3. Comparison of Cells in the Two Preparations.

Cultured cells differ in appearance from cells on the scale. The

---

* The nerve endings were absent from these cultures, and drugs could be tested for direct effects on the melanocytes.

two cell types are illustrated diagrammatically in the following figure (Fig. 6). The cell in the upper right-hand corner is a cell on the scale. The four lower cells are cultured forms. Cultured cells are generally smaller than cells on the scale. They also have fewer but wider arms. Many cells flatten into discoid shapes like the one shown on the left. In these cells the granules are spread in a single layer throughout the cytoplasm.

Since an interpretation of the granule-moving system will be based upon observation of the movements of granules in both cell types, a question arises concerning the comparability of the two cultures. In answer to this question, it may be said that the characteristic movements of the granules is the same in both types, and all forms of cells exhibit granule concentration and dispersion in response to appropriate agents.

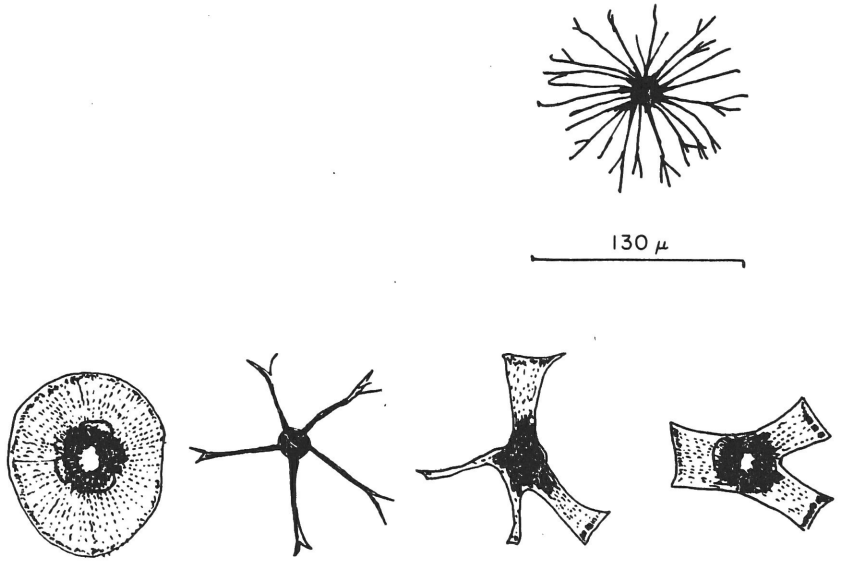


Fig. 6

C. ANALYSIS OF LIVING CELLS

The two cell preparations were studied by light microscopy. A Leitz binocular scope fitted with a low power and an oil immersion objective was used for the observation of *in situ* preparations. A Zeiss binocular fitted with phase optics was used for examination of cell culture preparations. A reticule calibrated in microns and mounted in the

eyepiece of the microscope permitted the measurement of cell dimensions with an overall precision of measurement of approximately 0.3 microns, and allowed the estimation of granule velocities.

Granule movements were analysed from films made by time-lapse cinematography using Kodak Plus X Reversal film. For the analysis of the shuttling motions, the time lapses were from 1/16 of a second to 40 seconds. For the analysis of the mass motions, the time lapses were of the order of 1/16 of a second to several minutes. Granule displacements were traced from the films and are presented as position-time charts; thus, frame by frame analysis of the films permitted both the displacements of single granules and the co-ordinated displacements of many granules to be analyzed in a quantitative manner.

D. METHODS FOR ELECTRON MICROSCOPY

Cells in both the concentrated and dispersed states were fixed and sectioned and observed by electron microscopy.

Scales removed from the dorsal fin region of Fundulus were rinsed in Eagle's medium at room temperature for 20 minutes. The granules dispersed; cells to be fixed in the dispersed state were transported immediately into the fixative. Cells which were to be fixed in the concentrated state were placed in Eagle's medium containing adrenalin for about 5 minutes or until noticeable concentration occurred. All cells were fixed for one hour in 6.5% glutaraldehyde (Sabatini, et al., 1963) made up in phosphate buffer, 0.05 M at pH 7.4. The entire scale was put into the fixative and cut into small pieces either during fixation or after dehydration. The difference in these procedures was found to have no affect on the fixation obtained. Tissues were then stained in 1% OsO_4 solution at room temperature, dehydrated in alcohol and embedded in epon (Luft, 1961).

Orientation and sectioning of the tissue proved difficult because of the presence of the scale; thus the tissues were cut out of the hardened blocks and re-embedded in epoxy glue mounted on the top of old blocks. The tissue was cut away from the scale, cut up into small squares containing single cells, mounted and oriented in the soft glue, and hardened overnight at room temperature.

Sections were cut with diamond knives on the LKB ultratome. The sections were carried on grids with a support of carbon film and stained with lead citrate (Reynolds, 1963), or with uranyl acetate (Watson, 1958). The sections were examined with an RCA (EMU-3F) microscope up to magnifications of 56,000X.

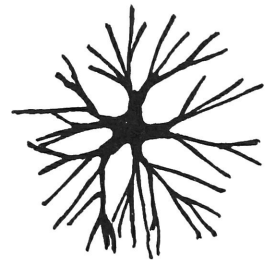
III. CELL STRUCTURE

The static features of melanocytes studied by both light and electron microscopy are described in this chapter.

A. MELANOCYTE FORM AND STRUCTURE

1. Forms of Melanocytes.

Melanocytes are distinguished by their black granules and by the intracellular migration of these granules in response to hormones. All such cells are highly branched in form; the four or five main arms which radiate from the small central part of the cell branch almost immediately into smaller arms, and as many as forty arms may be distinguished in the distal regions of the cell. Whereas the arms are very thin--3-4 microns in diameter--they may be up to 60 microns in length.



Melanocytes *in situ* exhibit variations in this form (see Fig. 6). In any population occur small, dendritic cells containing only a few granules and perhaps one or two nuclei,* together with many large, cartwheel forms stuffed with granules and exhibiting four to eight nuclei. Since melanocytes differentiate on the scale, these differences

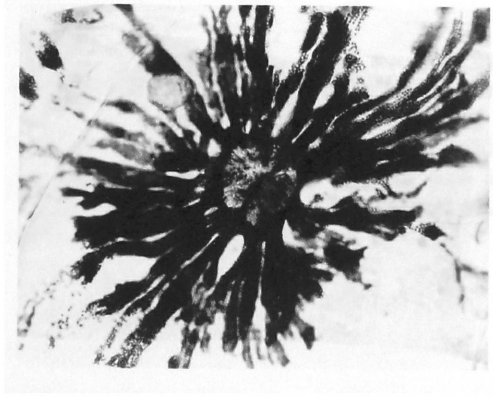


Fig. 7a. 400 x. Large, cartwheel form containing many granules.

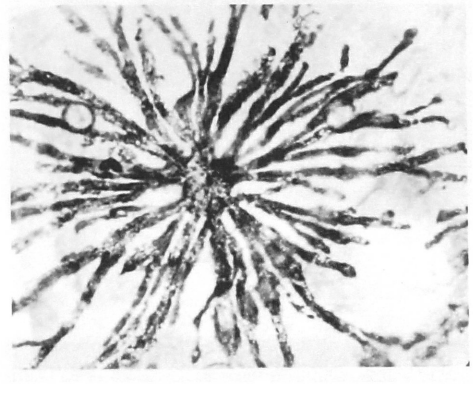


Fig. 7b. 400 x. Spider-shaped cell; thin arms and fewer granules.

* These cells are the best cells for the observations of movements of granules and occur in large numbers in a fish adapted to a dark background.

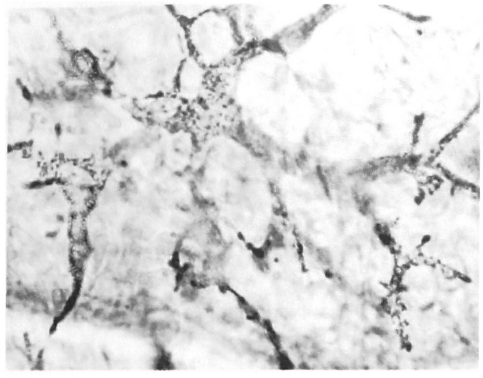


Fig. 7c. 600 x. Dendritic cell; few granules.

may indicate cells of different ages occurring together. Representative forms are shown in Figs. 7a-c.

Melanocytes in culture, settled on different substrata, exhibit an entirely different form. They are disc-shaped cells exhibiting few arms and are more flattened than cells in situ. Since the granules are often spread in a single layer through the cytoplasm, these forms are excellent for observing granule movements. Two such forms are represented in Figs. 8a,b.

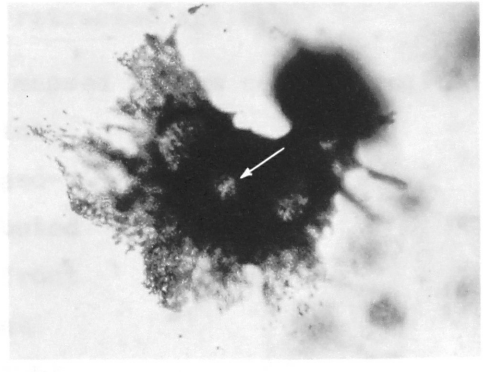


Fig. 8a. 500 x. Cultured cell with bulk of granules in central part; among them is seen the clear, gelled area. The nuclei are embedded in the granules.

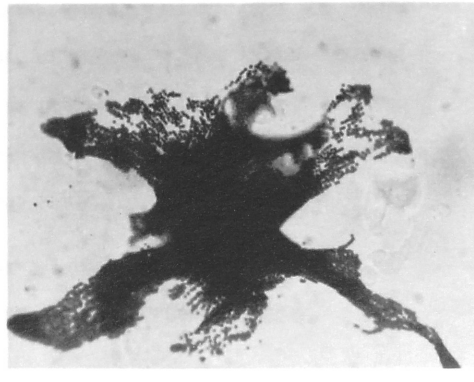


Fig. 8b. 500 x. Cultured cell with granules spread to the periphery. Cell form approximately same as in Fig. 8a.

2. The Parts of Melanocytes.

The terminology which will be used throughout this discussion is presented in Fig. 9. Additional terms which are used are: lateral--direction across the width of the arm; axial--direction along the length of the arm.

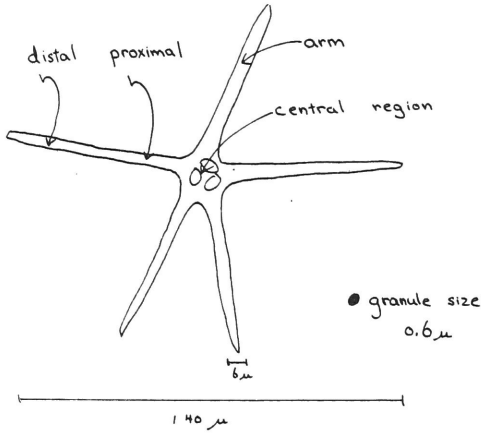
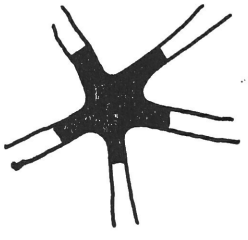


Fig. 9.

Melanocytes are bounded by a cell envelope whose surface area is very large in comparison with the volume of the cell due to the many branching arms. In cultured cells this boundary is the site of continuous activity. Ruffles and waves pass along the surface and microspikes are extended, waved about in the medium, and are retracted again.

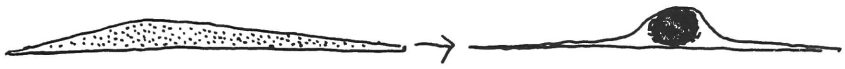
In cells in situ, the granules are massed in the central region of the cell and extend for a short distance into the cell arms. The mass of granules has a 'geometric boundary', being symmetrically distributed around the central region of the cell. The front region--the granules at the distal edge of the granule mass--is generally linear (see Fig. 30b).



In cultured cells, the bulk of the granules is massed in the central part of the cell around a clear, gelled area which can be seen in Fig. 8a (arrow). A thin layer of granules extends from this dense mass to the distal edges of the cell. In this area of the cell there often occurs a densely clustered row of granules, all motionless and apparently moored in the peripheral cytoplasm.

The entire cell is flattened in the plane of the scale, and in side view, is disc-shaped, being deepest in the central part. The

vertical dimension of the central part increases during concentration (Franz, 1935-36; Kamada et al., 1944).



The cell nuclei can be seen in the central regions, embedded in the granule mass or, occasionally, trapped within the cell arms. Worm-like mitochondria can be seen in cultured cells, particularly towards the distal regions of the cell.

3. Structures of the Cytoplasm.

It is one of the characteristics of melanocytes that no fine structure is visible at the level of resolution of the light microscope. The only visible features of the cell cytoplasm are the black granules. However, the arrangement and behavior of these granules may provide clues as to the structure of the cytoplasm. The granules are aligned in columns, a fact which suggests the presence of cytoplasmic partitions; the axial excursions of granules within these rows are limited, a fact which suggests the presence of structures in addition to the partitions which limit the movements of granules.

a. Evidence of Alignment.

The most striking feature of the cell is the peculiar arrangement of the granules. They are not randomly scattered about in the cytoplasm, but are neatly ordered into columns which radiate from the central region of the cell towards the distal edges. These columns of granules may be separated from one another by distances of up to 2 microns (Fig. 10). If, on the contrary, the columns are packed tightly together, func-

Fig. 10. 900 x. Cell from dark-adapted fish (5) (see Chapter IV for meaning of this designation). The granules at the distal end of the arm are moving actively. Those in the proximal and distal parts are highly aligned. Lateral separations up to 2 microns in width can be seen between granule columns.

tional independence is indicated by the movement of neighboring columns in opposite directions. In the photograph shown above, columns of gran-

ules alternate with cleared spaces. In the cultured cell traced below, these cleared spaces are less numerous and are separated from one another by masses of granules 6-8 granules deep. Cleared spaces radiate from the central region like the spokes of a wheel.

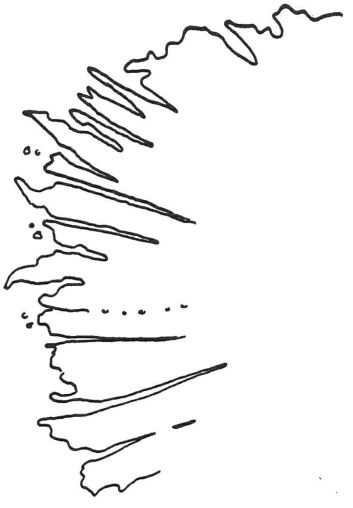


Fig. 11.

The granules are clearly ordered in columns in the proximal and distal parts of the cell. This arrangement may extend into the central region, but more often, the granules in this region are not ordered and move in random directions. Occasionally they exhibit sudden traverses along radial paths.

The columns of granules are separated from one another in the distal regions of the cell and merge together in the proximal regions. In cells undergoing granule concentration, the granules pile on top of one another and no alignment is evident.

In many flattened cells, the columns of granules are often curved, as if the granules in the distal region were moored in the cytoplasm, and the central part of the cell was rotating in relation to them. In one film made of the central region, five nuclei embedded in the granule mass and fixed at constant distance from one another were observed to rotate as a group through 180 degrees in a period of 4 hours.

The positions of granules within the columns are not rigidly defined. The rigidity of the alignment of granules is somewhat variable from one cell to the next. In cells from dark-adapted fish (see Fig. 13), the granules are rigidly aligned; they would neither change position within a row nor cross over into other rows. The maximum lateral deviations are of the order of $\frac{1}{4}$ of a granule length (approximately 0.2 microns). The granules are loosely aligned in cells from light-adapted fish (see Fig. 12) and can be seen to interchange positions within a row, or to cross over into other channels. Deviations from a longitudinal axis may

be of the order of 1-2 granule lengths (1.5 microns). The greatest lateral deviations occur in the distal regions of dispersed cells.

The ordering of the granules in columns suggests the presence of cytoplasmic partitions which channel the granules. Generally speaking, these structures cannot be seen directly. However, they may be occasionally seen in concentrated cells viewed by phase microscopy. Long structures, radiating from the central region, are sometimes barely visible in the distal granule-free cytoplasm.

b. Evidence of Structure Enmeshing the Granules.

Even within the channels, the granules are not free to move but are confined to a small region. Their paths are limited in the axial direction. This confinement of granules indicates the presence of structures in the cytoplasm; within the fine structures of the cell one might expect to find such structures which could limit the path of the granules to a few microns.

4. Fine Structure of Melanocytes.

The fine structure of melanocytes seen in the electron micrographs of both concentrated and dispersed cells is presented in this section. All sections of cells shown here were made parallel to the plane of the fish scale.

The melanin granules are black, ovoid bodies approximately 0.4-0.7 microns in dimension (Plates 1, 5). No substructure can be distinguished within them. Each is bounded by a membrane which in some instances appears closely applied to the granule surface; in other cases, it appears separated from the surface, up to distances of 400 A.

Dispersed Cells. The electron micrographs of dispersed cells show the granules to be in the columns which could be seen in living cells (Plate 1). Between the columns are the cytoplasmic partitions inferred from the arrangement of the granules, seen here as long, rod-like structures, parallel to the longitudinal axis of the arms. Between any two columns may occur large numbers of these structures approximately 400 A. or more apart. In Plate 2, these structures can be seen concentrated in the central region of the cell, and they radiate from this region out among the granules. The very central part of the cell is occupied by the cell centrioles, and the rods seem to originate from its vicinity. These rod-like

structures, the most prominent feature of melanocyte cytoplasm, have recently been found in other cells--of both plant and animal origin--when fixed in glutaraldehyde (Ledbetter and Porter, 1963), and are considered to be tubules on the basis of their electron-light core and electron-dense periphery. The tubules are approximately 220 A. in diameter although considerable variations (±30 A.) occur. Their surfaces are corrugated, and irregular variations in electron density along the length of the tubules give the appearance of striation. No connections are observed between granules and tubules. The straight appearance of the tubules suggests stiffness and a possible function as skeletal agents within the cytoplasm for channelling the granules.

The two small structures visible in living cells--the nuclei and the mitochondria--may be seen in the fixed cells. The nuclei are most often ovoid in concentrated cells and lobate, with many nuclear pores, in dispersed cells.

The fine structure of the granule-containing portion of the cytoplasm may be ascertained from these photographs. The most prominent structure is the smooth endoplasmic reticulum. Strands of this structure may be seen sandwiched among the granules and in some places almost completely encircle them (Plate 3). This endoplasmic reticulum appears the most likely structure to limit the paths of the granules, and it must permeate the mass of granules.

Another prominent feature of this part of the cell is the large, membrane-bounded compartments. These have proven to be variable in number from one cell to another (Plate 1).

In addition to these two major structures of the granule-containing cytoplasm--the tubules and the endoplasmic reticulum--other components are ribosomes (particularly in the vicinity of the nuclei) and particles of glycogen.

All structures mentioned above are in an electron-light matrix which is not resolvable into structure at this level of resolution.

The melanocyte is bounded by a thin, wavy membrane. Just beneath this membrane, in dispersed cells, are congregated masses of small vesicles (Plate 3). These are not pinocytotic vesicles but rather appear to be con-

fined to the surface and therefore resemble the pitting of the surface observed for smooth muscle (Porter and Bonneville, 1964, Plate 24). The collagen surrounding the cell can be seen in some of these plates (6,9,10).

Concentrated Cells. Concentrated cells are shown in Plates 4-10. The granules of the concentrated cell are packed tightly together in the central region of the cell and exhibit no particular pattern of aggregation (Plate 4). Distal to the granules, part of the granule-free portions of the arms can be seen. The large membrane-bounded components appear to be extruded from the granule mass (Plate 4).

There are two sets of microtubules in concentrated cells. One set appears to move with the granules; the other remains surface associated. Plates 6 and 8 show the front region of a concentrating cell. The granules have withdrawn from the distal regions of the arms, leaving behind them a cytoplasm free of granules and also relatively free of microtubules (Plates 8 and 9). Microtubules occur in high concentrations among the granules. These structures are shown at high magnification in Plate 8. They do not exhibit the rigid alignment of tubules in dispersed cells, but appear more randomly distributed. A second set of microtubules, just beneath the cell surface, remain surface-associated during the migrations. They can be seen cut tangentially in Plate 9 and are shown in long section in Plate 10. The structures of the cytoplasm in the distal regions of the arm cannot be discerned. There appear to be many membranous elements within that region.

Comparison of Concentrated and Dispersed States

CONCENTRATED STATE	DISPERSED STATE
1. Granules packed into the region of the centrioles.	1. Granules excluded from the centriolar region.
2. Few tubules visible in the central region.	2. Central region often packed with tubules.
3. Tubules among granules seem less aligned, less straight.	3. Tubules among the granules are aligned in parallel rows and are straight.
4. Granules in no particular pattern.	4. Granules aligned among the tubules.
5. Few vesicles observed.	5. Many vesicles observed.

6. High density of granule packing often observed, but not necessarily so.	6. Low density of granule packing.
7. Nuclei usually ovoid.	7. In cells dispersed for some time, nuclei are often lobate, and there appear many ribosomes in the cytoplasm.
8. Large, membrane-bounded compartments extruded from granule mass.	8. Large, membrane-bounded compartments found among the granules.

Summary of Fine Structure Studies. In summary, melanocytes appear to contain cell structures exhibited by most cells, i.e., ribosomes, glycogen, smooth endoplasmic reticulum. One feature recently appearing in many cells fixed with glutaraldehyde (Ledbetter and Porter, 1963) is particularly prominent--the microtubules. In addition, dispersed cells exhibit small vesicles congregated under the cell membrane.

5. Contributions of Previous Workers to Cell Structure.

Two previous studies of cell structure have been made. Franz (1939-40) studied both fixed and living cells by light microscopy and determined that the cytoplasm consisted mainly of rod-like structures. Recently, studies of cell fine structure by electron microscopy have been published (Falk and Rhodin, 1957). In melanocytes fixed with osmium, they found no cell structure among the granules; the granules appeared to be confined within an intracellular sac, itself surrounded with fibrils in the distal cytoplasm. On the basis of these studies, these workers have postulated that the granules move by contraction of the intracellular fibrils. Since this theory has been reported in a recent review of color change (Fingerman, 1965), it is important to note that the photographs presented in their work are not in good focus. The material which is suggested to be intracellular fibrils is, in fact, extracellular collagen; the sac around the granules is the cell membrane. That very little detail of cell structure is preserved is no doubt due to the fact that their cells were fixed in osmium, a fixation that destroys microtubules.

PLATE 1

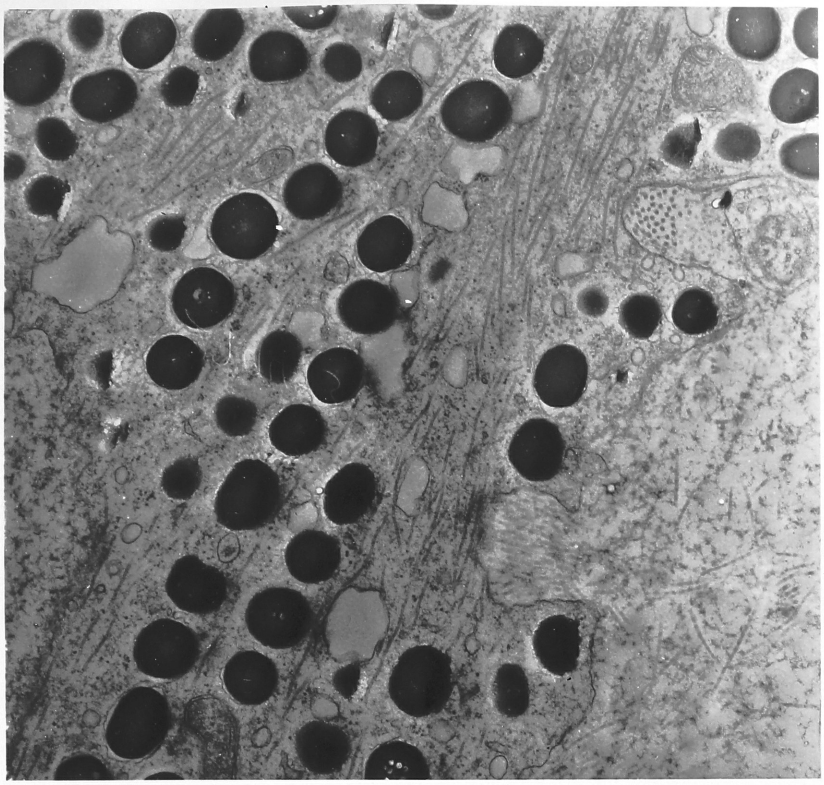
Dispersed Cell. Proximal region of arm. 10,000 x.

Section through the proximal region of the arm of a semi-dispersed cell (dark-adapted, 3--see Chapter IV for meaning of this designation). The granules are aligned in long columns between groups of microtubules, spaced about 400 A. apart. This micrograph was part of a series of serial sections which showed tubules at all levels of the arm. In addition to the prominent granules and tubules, there are many large, membrane-bounded compartments.

PLATE 2

Dispersed Cell. Central region. 25,000 x.

Section through the central region of a dark-adapted, 3 cell (see Chapter IV for meaning of this designation). The central part is crowded with tubules, and the granules are excluded from the area. In the light microscope, the granules in this central region are motionless.



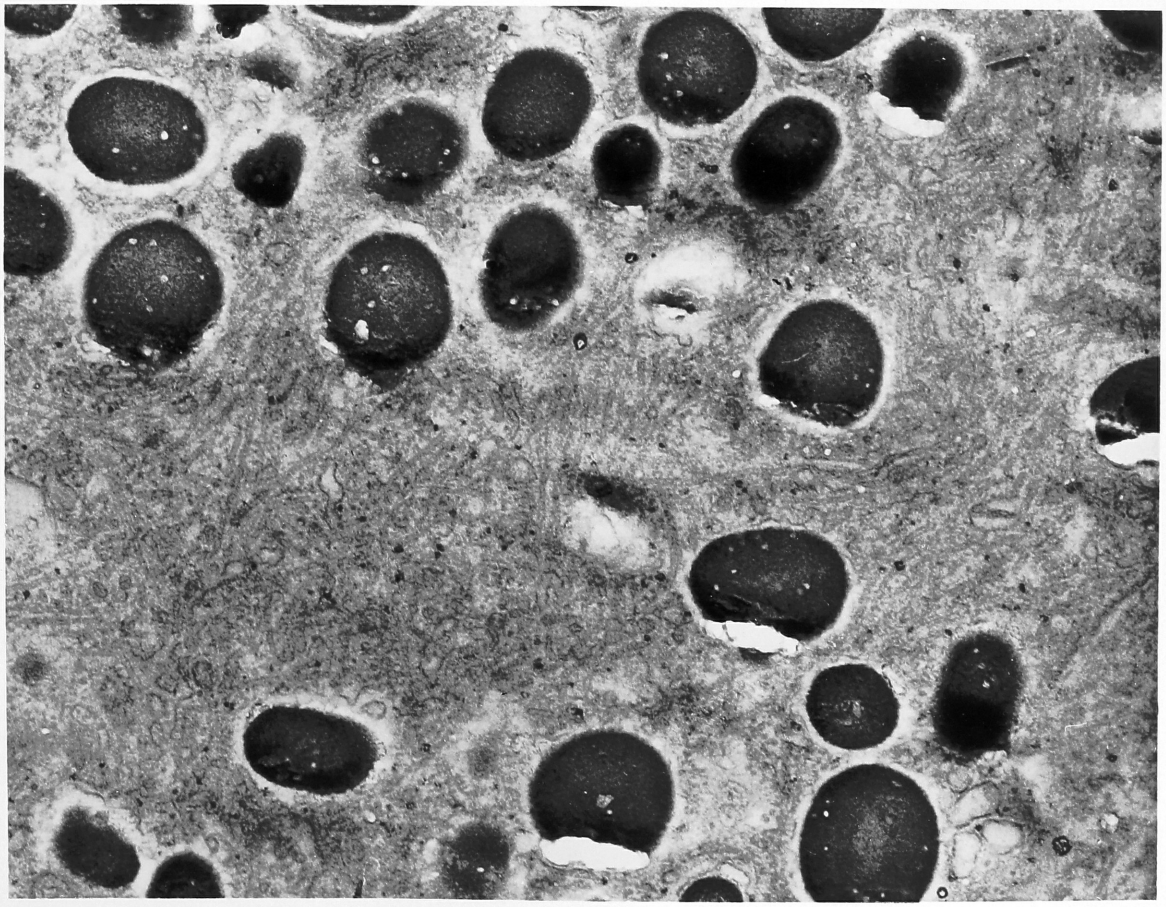


PLATE 3

Dispersed Cell. Distal part of the arm. 22,000 x.

Section towards the end of the arm. Tubules and membranes of the smooth endoplasmic reticulum are enmeshed among the granules. A prominent feature of dispersed cells is the population of small vesicles congregated just under the surface of the cell, between the cell membrane and the border of the granule mass. These vesicles ressemble the pitting of the surface found in cells of smooth muscle.

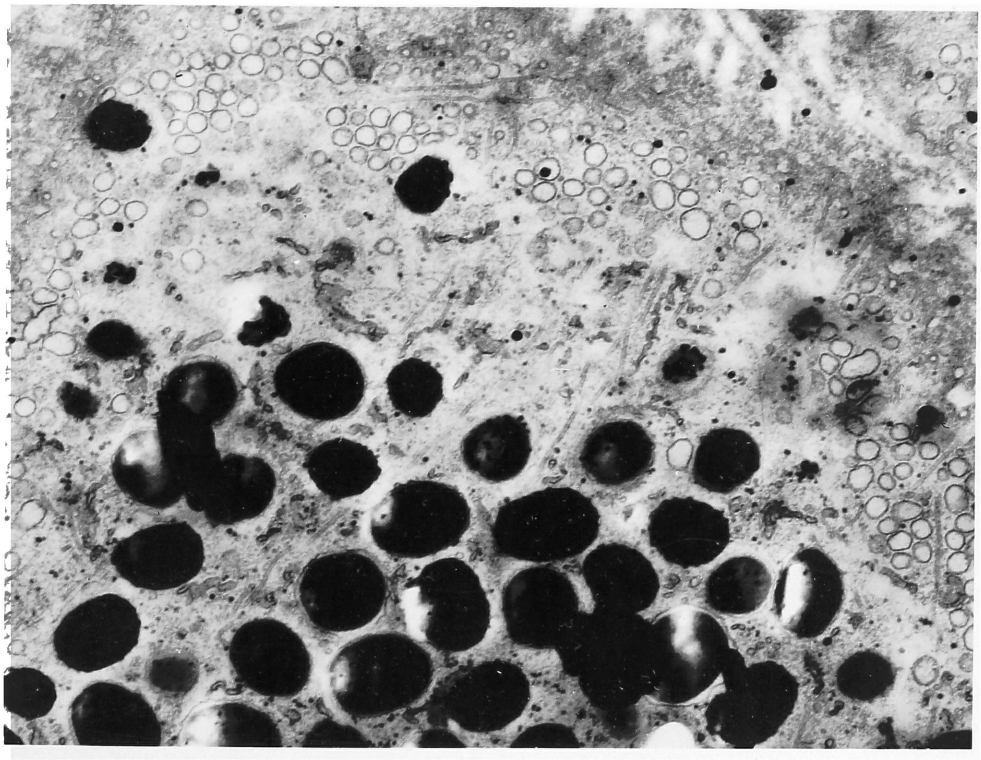


PLATE 4

Concentrated Cell. 5,000 x.

The granules are crowded into the central region of the cell. The granule-free arms are seen extended distally. Large, membrane-bounded components may have been extruded from the concentrated granule mass.

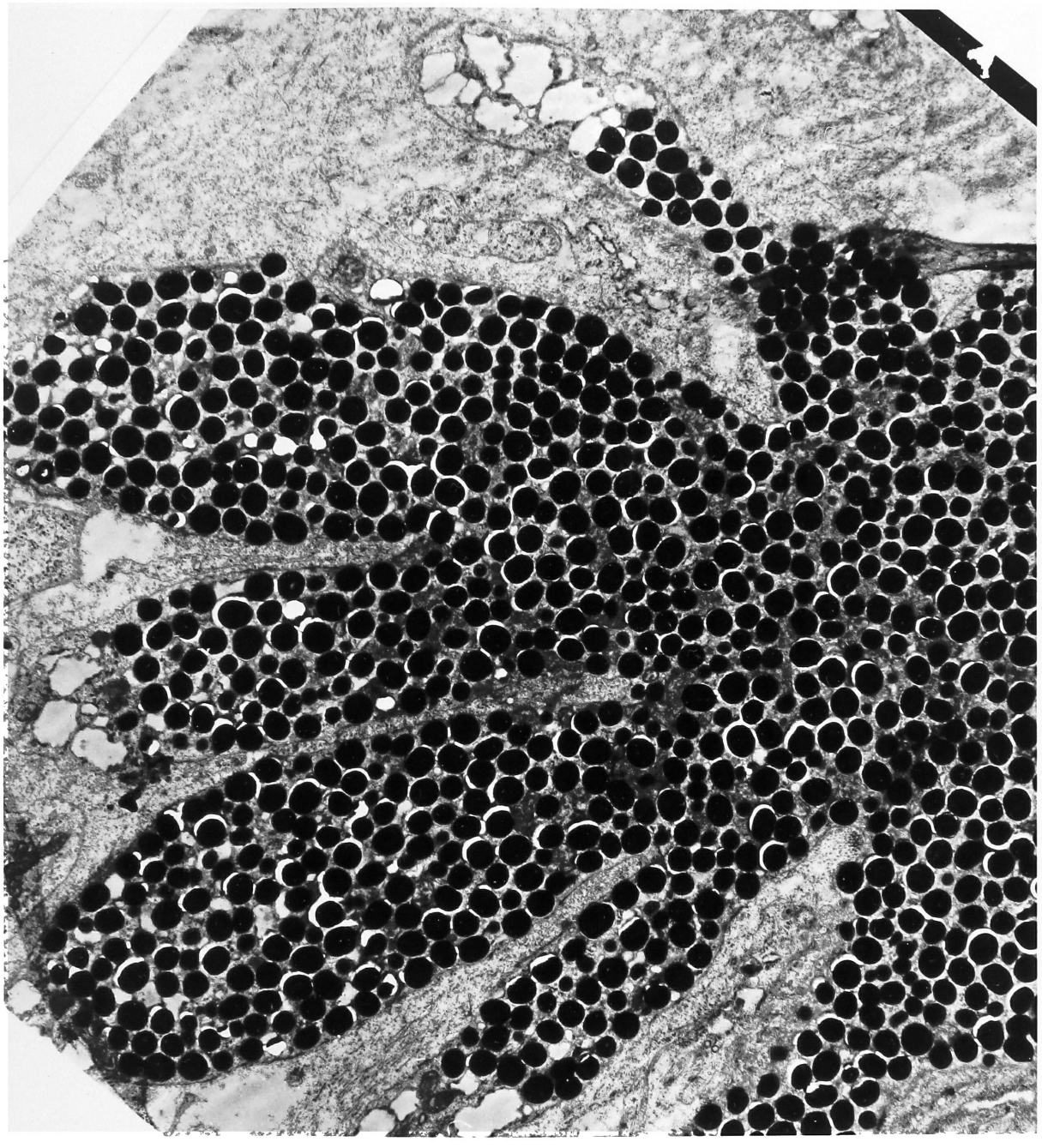


PLATE 5

Concentrated Cell. Central region. 45,000 x.

Cell centrioles occur in the central region of the melanocytes. In cells in situ a cleared area often visible in the centre of the granule mass marks their location. This clear, gelled area can be readily seen in cultured cells (Fig. 8a).

This photograph was taken by Dr. Sam Dales, The Rockefeller Institute.

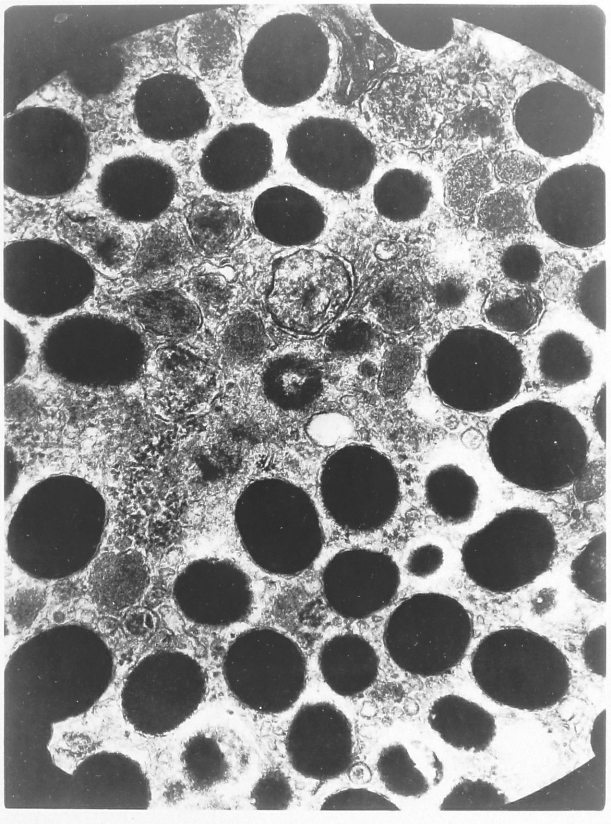


PLATE 6

Concentrated Cell. Proximal part of arm. 14,000 x.

Section through the proximal part of the arm during concentration. The granule front region is approximately linear. Membranous elements can be seen at the distal side of the granule mass. Even at this magnitude, microtubules are visible among the granules. The collagen in which the melanocyte is embedded can be seen around the periphery of the cell.

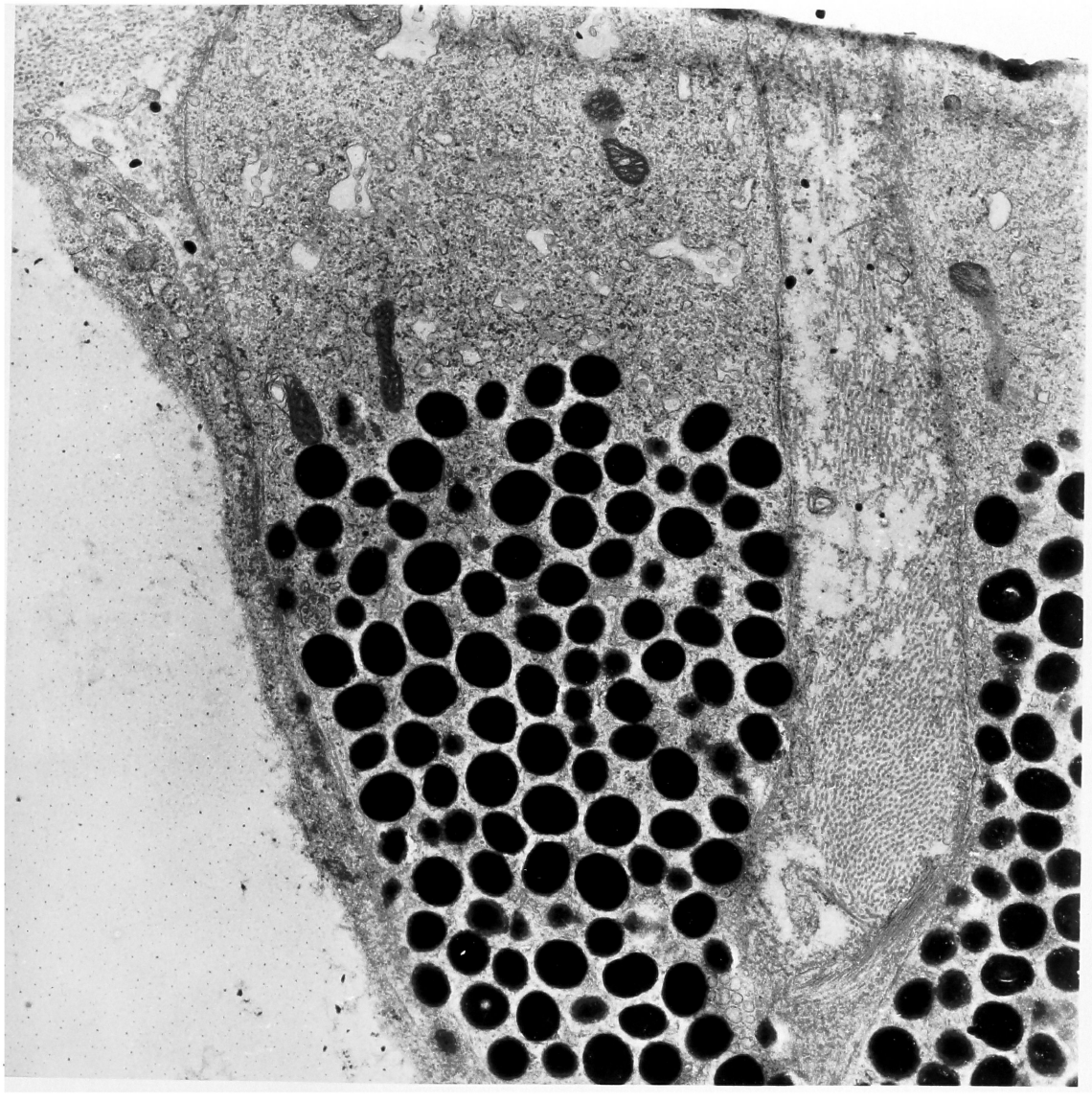


PLATE 7

Concentrated Cell. Central region. 25,000 x.

Among the granules occur large numbers of microtubules.

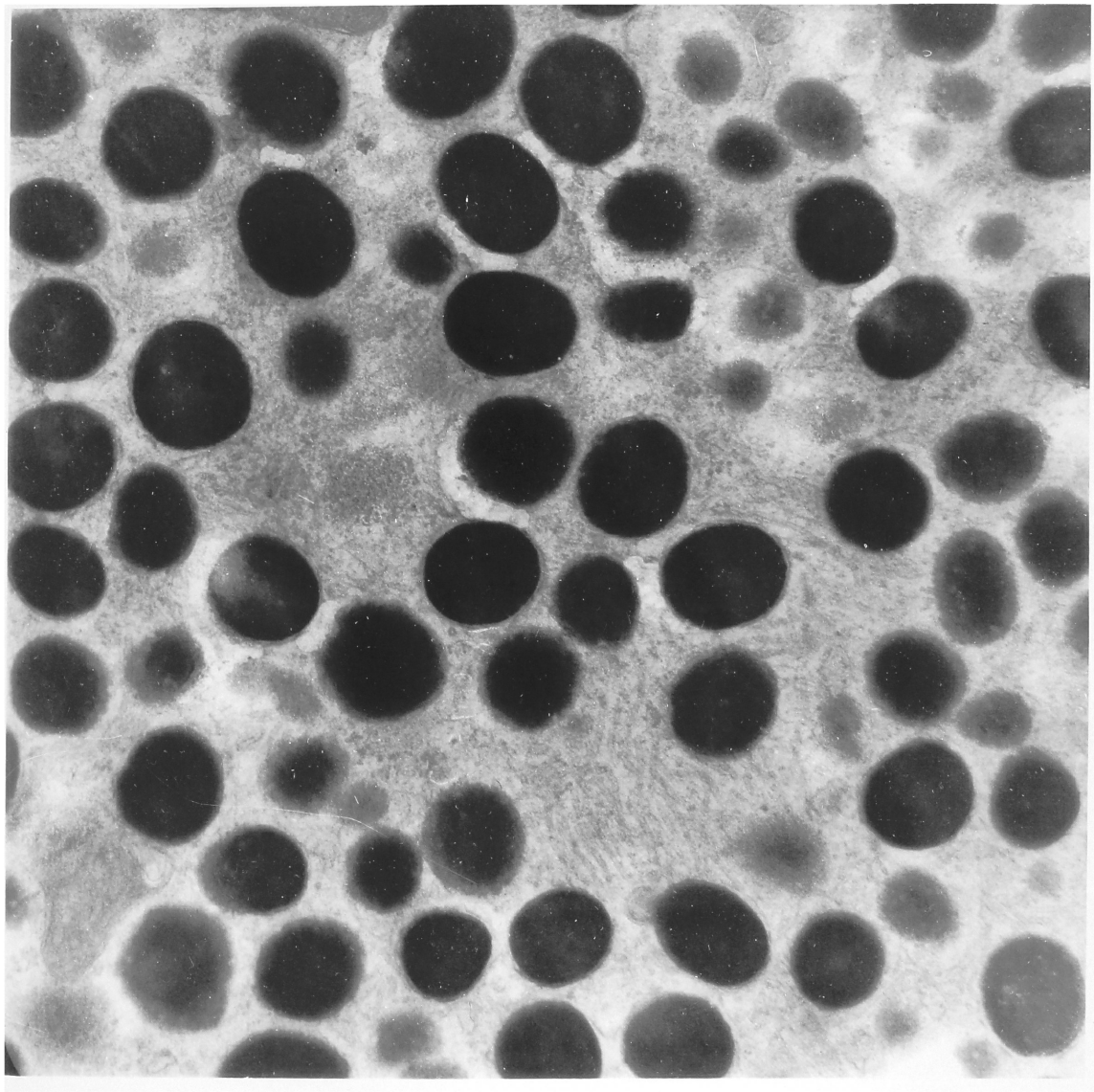


PLATE 8

Concentrated Cell. Basal part of the arm. 22,000 x.

Region between the granules and the granule-free portions of the arms. A large number of microtubules can be seen in the center of this micrograph.

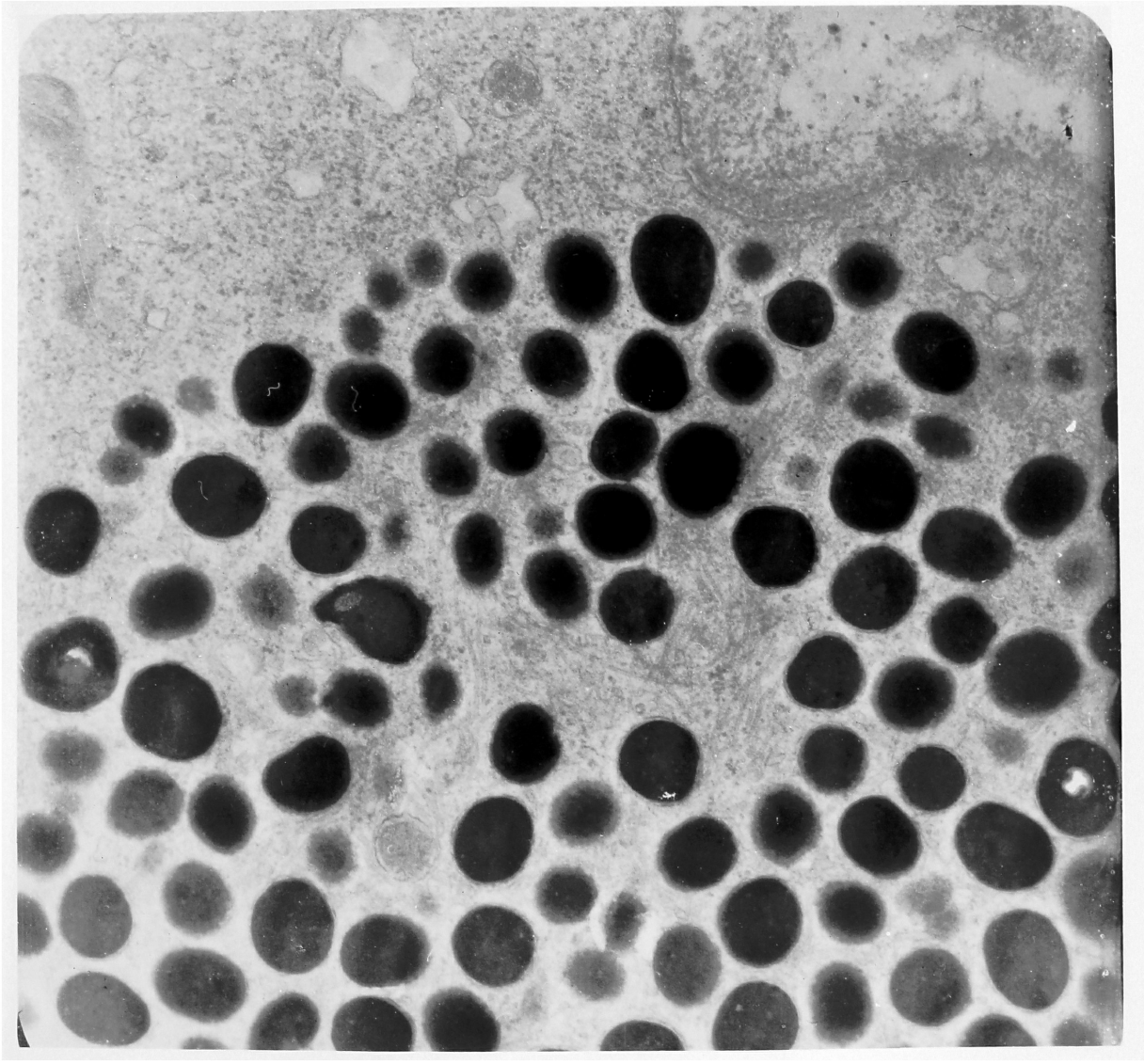


PLATE 9

Concentrated Cell. Distal region of arm. 45,000 x.

One set of tubules remains surface-associated during the movements of the granules and can be seen in this micrograph; the cell was cut tangentially just beneath the cell membrane. These tubules form a herring-bone pattern with the collagen outside the cell. Elongated mitochondria which have been left behind the concentrating granule mass are also visible.

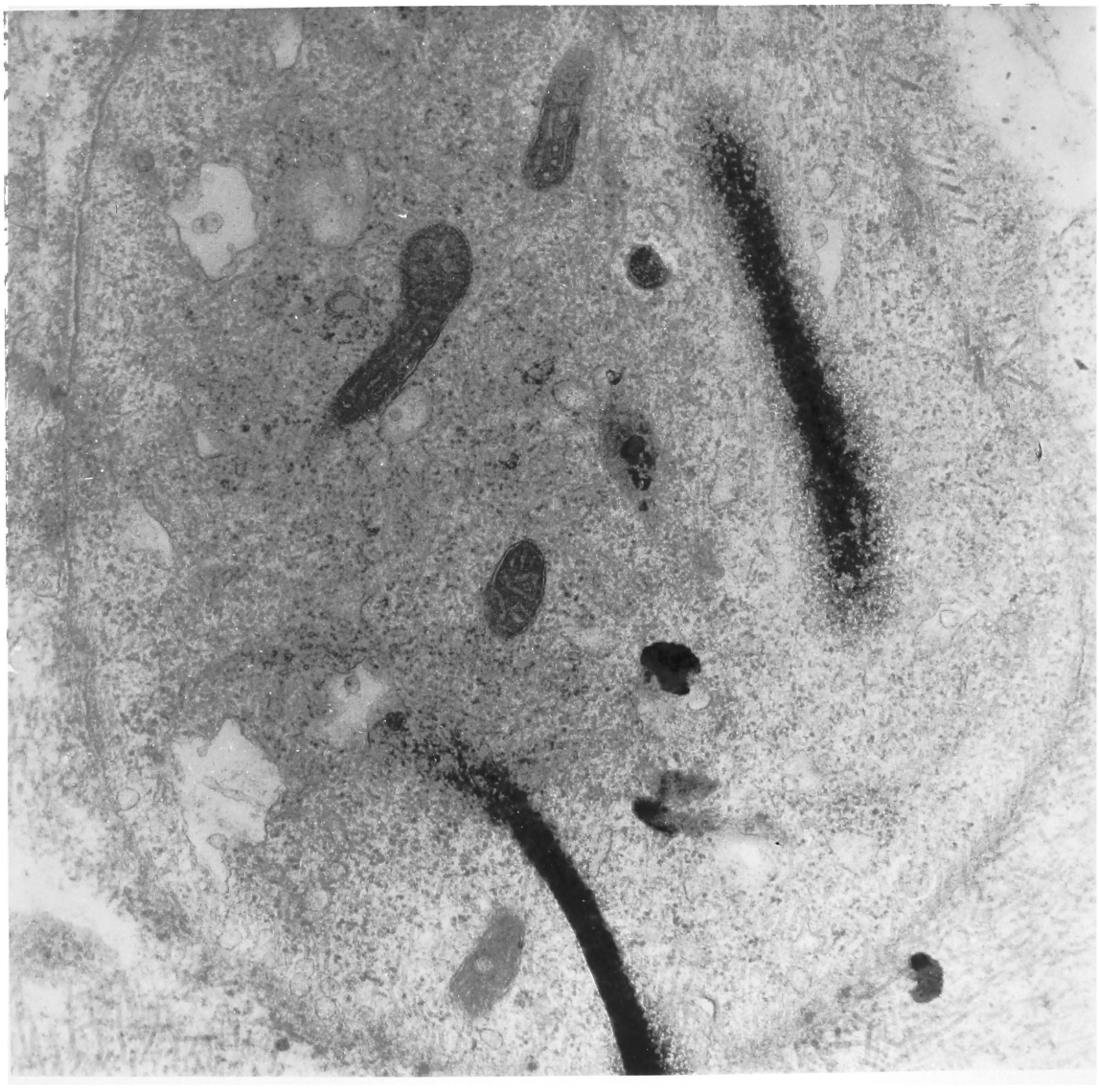


PLATE 10

Concentrated Cell. Distal part of the arm. 25,000 x.

Section through the distal region of the cell just beneath the cell membrane. Large numbers of tubules are aligned in parallel. Tubules appear to form a sheath beneath the cell envelope.

This photograph was taken by Dr. Sam Dales, The Rockefeller Institute.

IV. GRANULE MOTIONS

The motions of the granules are described in this chapter. Because of the complexity of the motions, it has been found convenient to distinguish between two types of motion. The first is a localized to and fro motion of a single granule within a fixed, small region. This will be referred to as the 'shuttling motion' of granules, a term proposed for it by Dr. Paul Weiss. The shuttling motions will be described in section A of this chapter. The second type of motion is the mass motion of granules during concentration and dispersion. This type of motion will be described in section B of this chapter.

Terminology. The following terminology will be employed throughout these descriptions:

The resting state is defined as one in which no net concentration or dispersion of granules is taking place.

The extreme states of granule distribution are: the concentrated state, in which granules are in the central region of the cell; the dispersed state, in which granules are distributed through both the central region and the arms of the cell.

The intermediate state of granule distribution will be referred to as the semi-dispersed state. Granules are distributed through the central part of the cell and through the proximal parts of the arms.

A. SHUTTLING MOTIONS

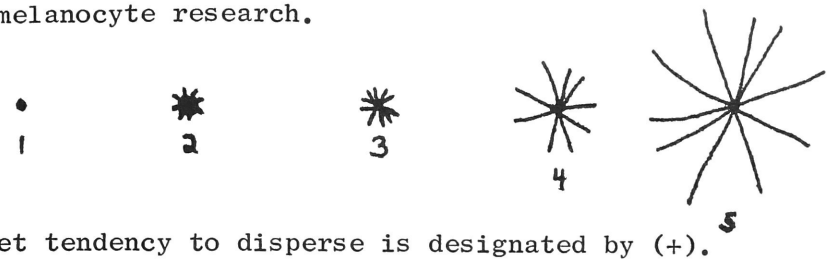
The shuttling motions of the granules in resting cells are described in this section. The motions have been described in living cells with the aid of two methods of classification.

1. Methods of Classification.

a. A classification of melanocytes into 'cell states' was drawn up to serve as a frame of reference for the description of granule motions. This classification was made on the basis that no cell is ever really in a resting state, but always exhibits some tendency to concentrate or disperse, tendencies which greatly affect the characteristics of the shuttling motions. This method of classification is given below:

1. The condition of the fish from which scale was removed is referred to as light-adapted or dark-adapted.

2. The distribution of granules is designated by a number (Healey,1951). This classification is in general use in melanocyte research.



3. A net tendency to disperse is designated by (+).
   A net tendency to concentrate is designated by (-).

Classification into cell states was useful for several reasons. It permitted the effect of net concentrating and dispersing tendencies on the local movements of granules to be taken into account. It also permitted attention to be focused upon certain features of the movements visible in some cells but not in others; e.g., rigid alignment in dark-adapted cells, or granule interaction in dispersed cells. This classification of living cells may also serve as a useful frame of reference for the study of fixed cells with electron microscopy, although full advantage of this was not taken in the present work.

Photographs of cells in different cell states are shown below:*

1. Maximally-dispersed, 5. Granules spread throughout cytoplasm.

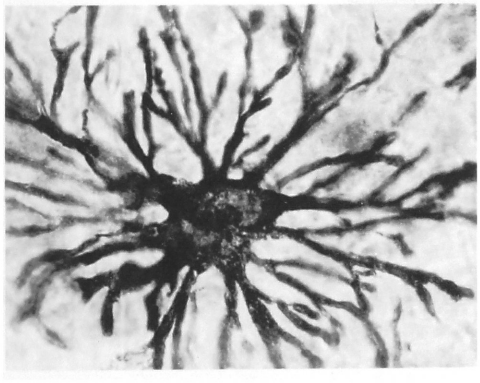


Fig. 12. Light-adapted. Thin, cylindrical arms. Granules in motion throughout cell. Few partitions.

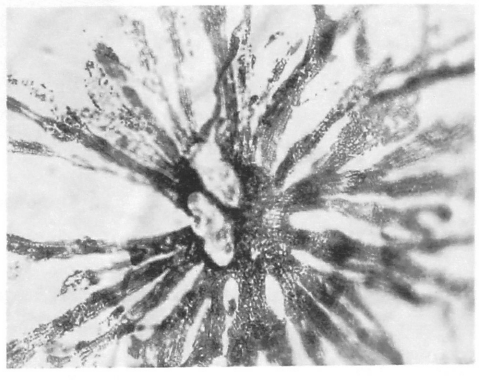


Fig. 13. Dark-adapted. Flattened arms. Many granules. Many partitions evident.

* Most of these states could be obtained as granules successively dispersed and reconcentrated in in vitro conditions. Maximally-concentrated states were obtained by administering adrenalin.

2. Semi-concentrated, 3 and 3-. Granules in central and proximal regions are stationary or concentrating.

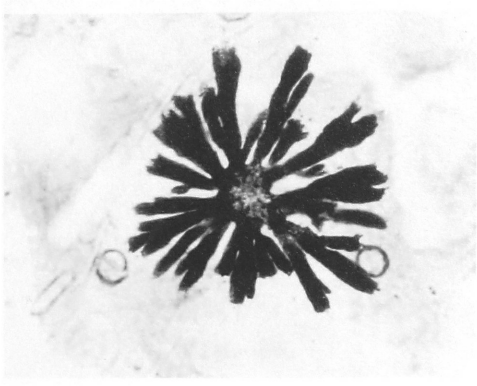


Fig. 14. Light-adapted. Granules in motion.

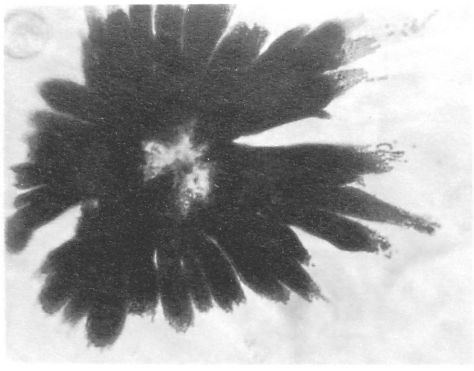


Fig. 15. Dark-adapted. Granules motionless in front region.

3. Concentrated, 1. Granules tightly packed into central region.

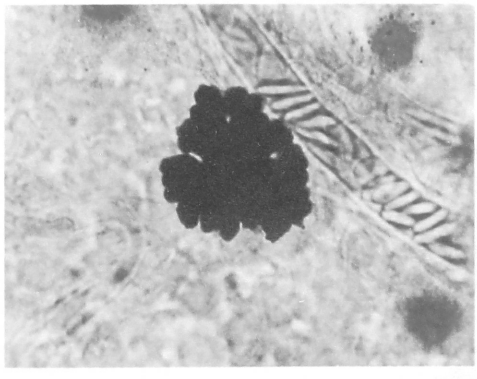


Fig. 16. Light-adapted.

4. Re-dispersing, 1+. Granules loosely constrained, dispersion commencing.

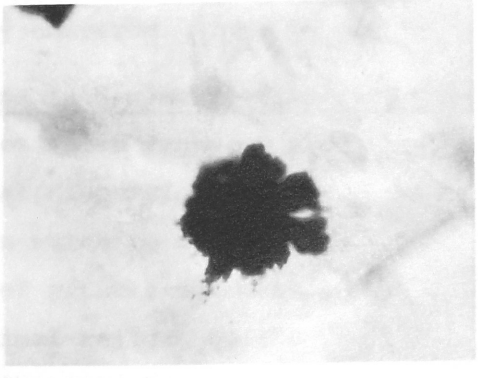


Fig. 17. Light-adapted.

5. Semi-dispersed, 3+. Granules in proximal region of the arms and dispersing.

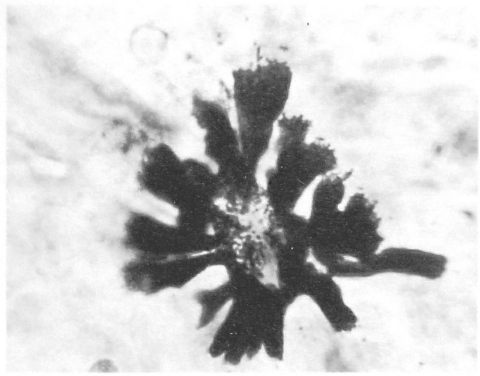
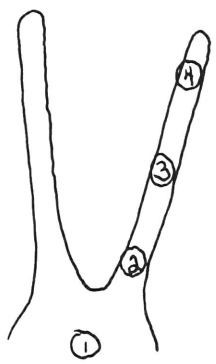


Fig. 18.

b. A second scheme of classification denotes the variables by which the movements of single granules were characterized and compared between one cell and another.



Cell state
Granule location -position 1,2,3,4.
alignment -rigid: lateral deviation 0.2 microns or less.
-loose: lateral deviation 1.5 microns or more.
path -length.
-time for proximal traverse.
-time for distal traverse.
-period.
-characteristics of the traverse.
Other characteristics
-Brownian motion.
Correlations between characteristic motion of granules in one part of the cell and in others.

The movements of granules will be described in the maximally-dispersed cell. The characteristics of the movements in other cell states will then be briefly compared.

2. Shuttling Motions in Maximally-dispersed Cells.

Melanocytes whose granules are in a state of maximal dispersion are the best preparations in which to ascertain the major features of granule movement. Whereas in the proximal regions of the arms of such cells the granules are densely packed in long columns, in the

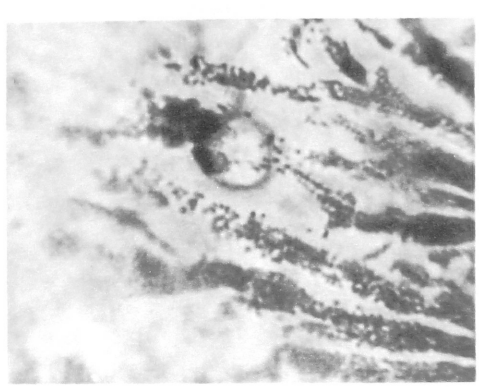


Fig. 19.

distal regions the granules have separated from one another and the movements of single granules may be clearly seen.

These single granules are by no means free to wander through the cytoplasm. In the first place, each is highly constrained in the lateral direction and appears to be in a channel within which it moves back and forth. These channels extend the full length of the arms; the granules in the distal region of the arms are in common channels, and often collide as they move in the axial direction. In the second place, in addition to being confined in the lateral direction, the granules are constrained in the axial direction as well. Each moves to and fro within its channel in a local region which, during any short time of observation, remains fixed in the cytoplasm.

These characteristics of the motions seem to be a function of cytoplasmic fine structure. Microtubules form channels in the arms, confining the granules in the lateral direction. In the axial direction, granules must be confined by some material between the tubules--perhaps endoplasmic reticulum or some amorphous matrix.

Due to the constraints by these structures, the granule moves in a small region. This region may be represented diagrammatically as an ellipsoid. The path and direction of movement of the granule within this region is represented by the arrow.

a. Characteristics of the Regions.

The length of regions varies between 0.8 microns and 4 microns in cells in situ. The longest regions are to be found in the central channels of the arms towards the distal region of the cell. In cultured cells the arms are broader and more flattened; the regions in such cells are often up to 10 microns in length. The lengths of regions shows a gradation, being longest in the central part and very short at the flattened edges of the cells (Fig. 21). The longest regions coincide with the deepest part of the cell arm. The cell flattens

at the edges and the lateral granules are motionless, trapped in the flattened cytoplasm.

The lateral dimensions of regions varies between $\frac{1}{2}$ and 2 microns. The granules in dark-adapted cells are rigidly aligned among the evident partitions in the arms (Fig. 13), and the lateral dimensions of the regions would be approximately $\frac{1}{2}$ to 1 micron. In light-adapted cells, on the other hand, the granules have greater freedom of motion. The lateral dimensions of the regions may be as great as 2 microns (Fig. 12).

There is no clear relationship between the axial and lateral deviations in the few observations made during this work. The excursions of granules in a cultured cell were traced during a short interval, and the regions are presented in Fig. 22. In the deep, central regions of the arm, where the granules seem to move most freely, some relationship is evident in the dimensions of regions marked 1 and 3. The region behaves like an elastic ring which has been distorted. But for the most part, no clear relationships are evident. The lateral constraint appears to be primarily due to the confining partitions, and the axial constraint, to other factors.

Although the regions are ellipsoid in cells <u>in situ</u>, they are not always this shape in cultured cells. In such cells which contain only a few granules, the granules move in circular orbits. Perhaps this circular movement is due to the fact that there are fewer partitions in these cells with few granules, or else, that the partitions would, in any event, be expected to radiate from the central part at wide angles to one another and therefore, have little affect in channelling the granules.

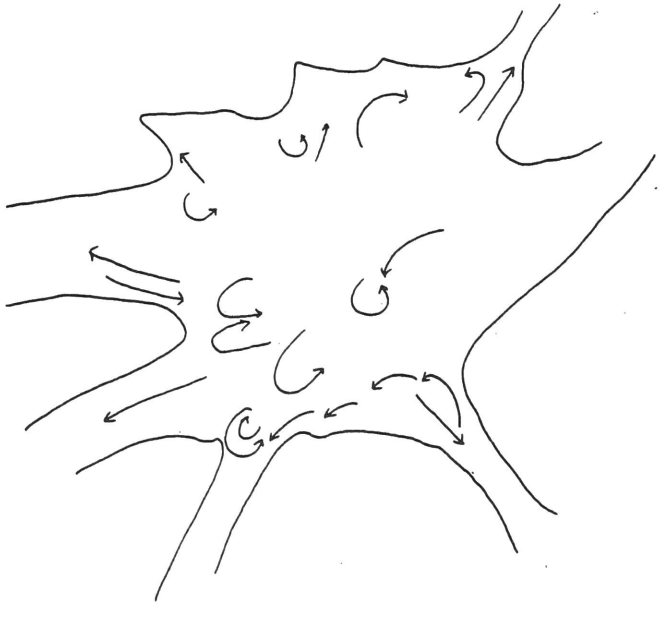


Fig. 20. Tracing of cultured cell containing few granules. Time lapse of 2 seconds was too great to permit continuous observation of single granules, but paths could be momentarily observed and are indicated by arrows.

Fig. 21. Granules in a wedge-shaped, flattened arm. The excursions of several granules were followed during $1\frac{1}{2}$ minutes of filming. These granules are numbered for convenience, and their axio-lateral excursions are plotted in Fig. 22. The cell was deepest in the mid-line.

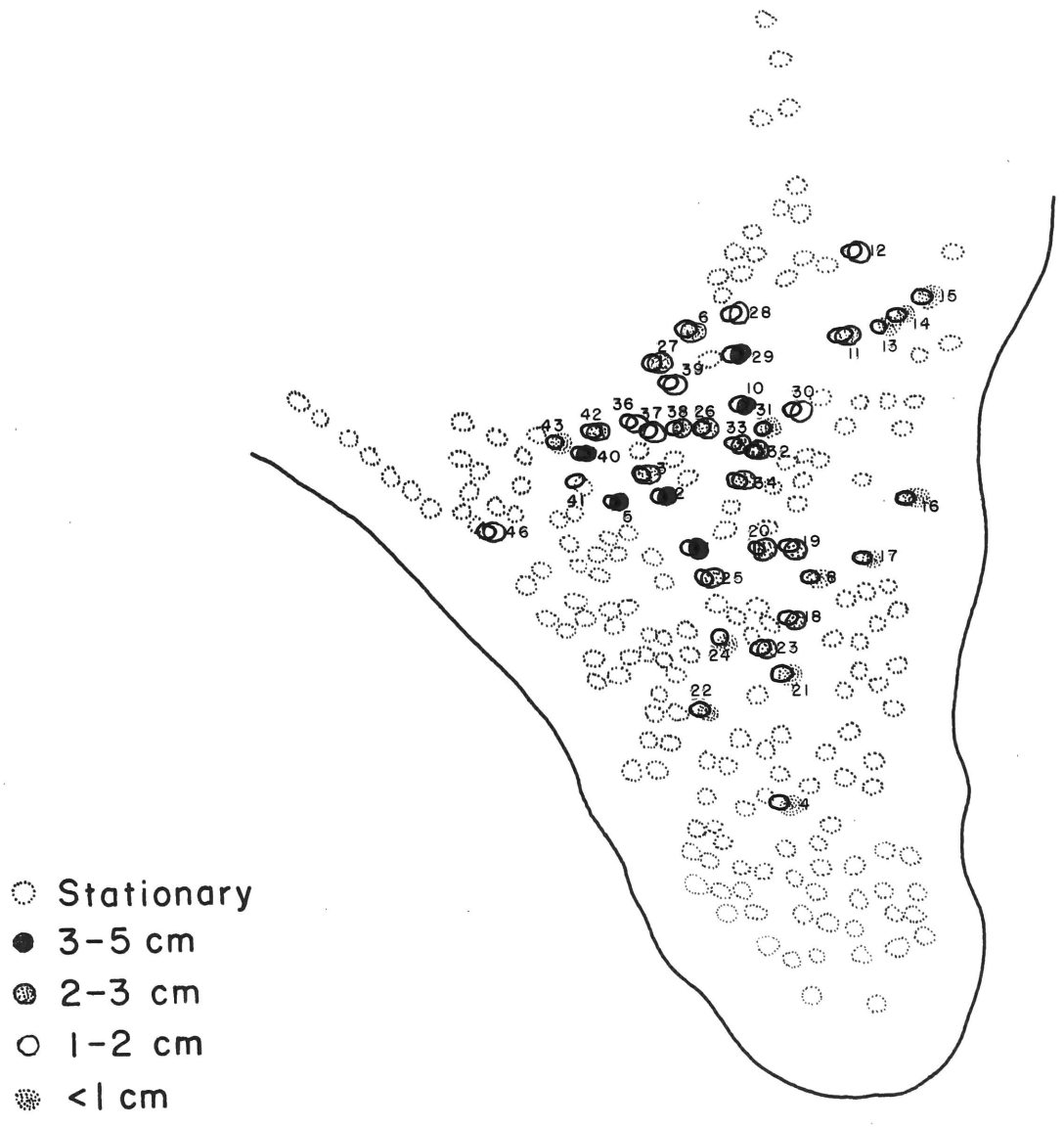


Fig. 21.

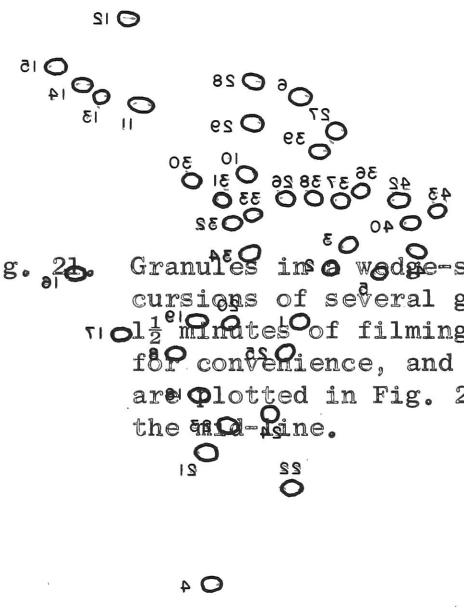


Fig. 21. Granules in a wedge-shaped, flattened arm. The excursions of several granules were followed during 1½ minutes of filming. These granules are numbered for convenience, and their axio-lateral excursions are plotted in Fig. 22. The cell was deepest in the mid-line.

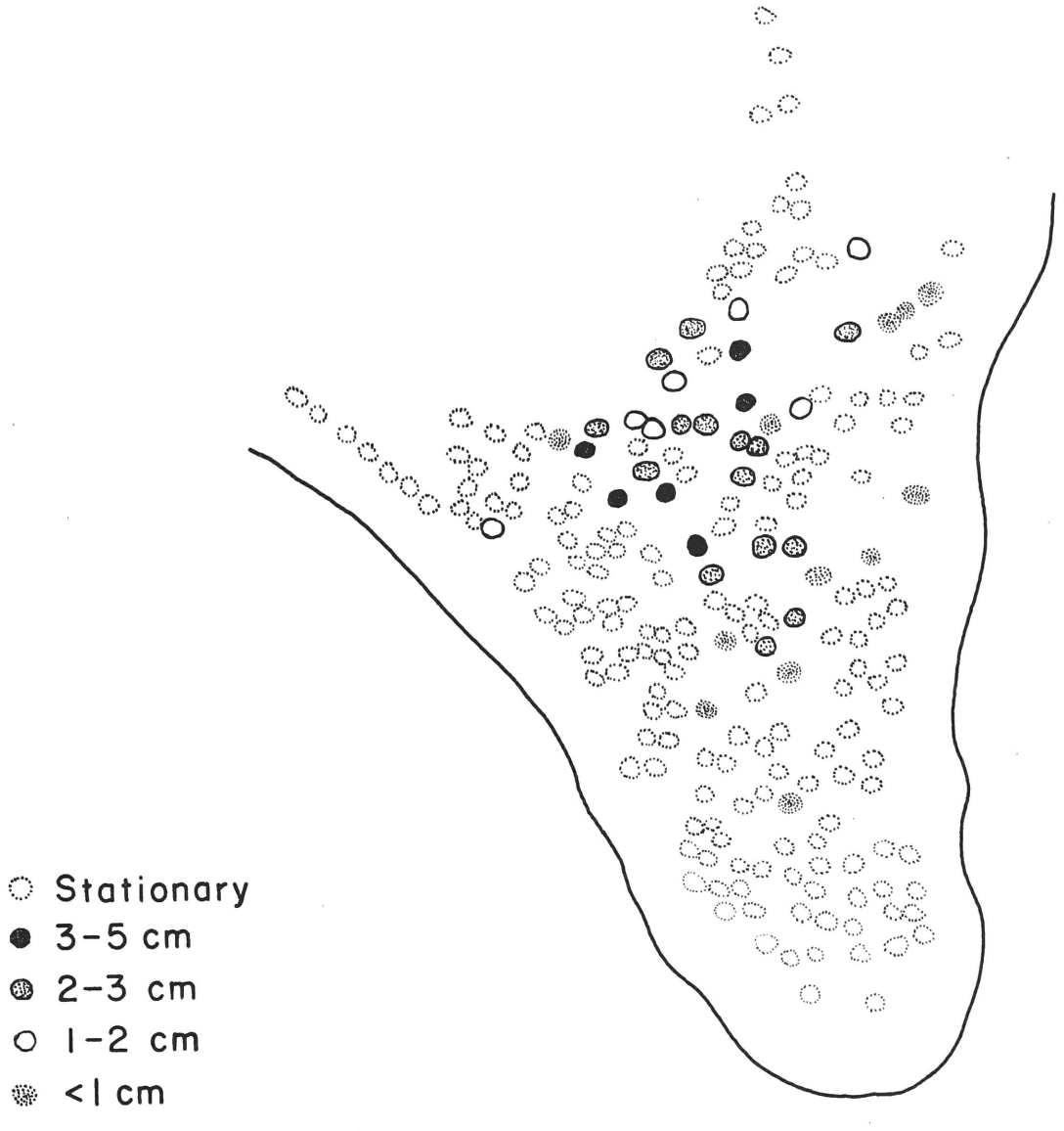
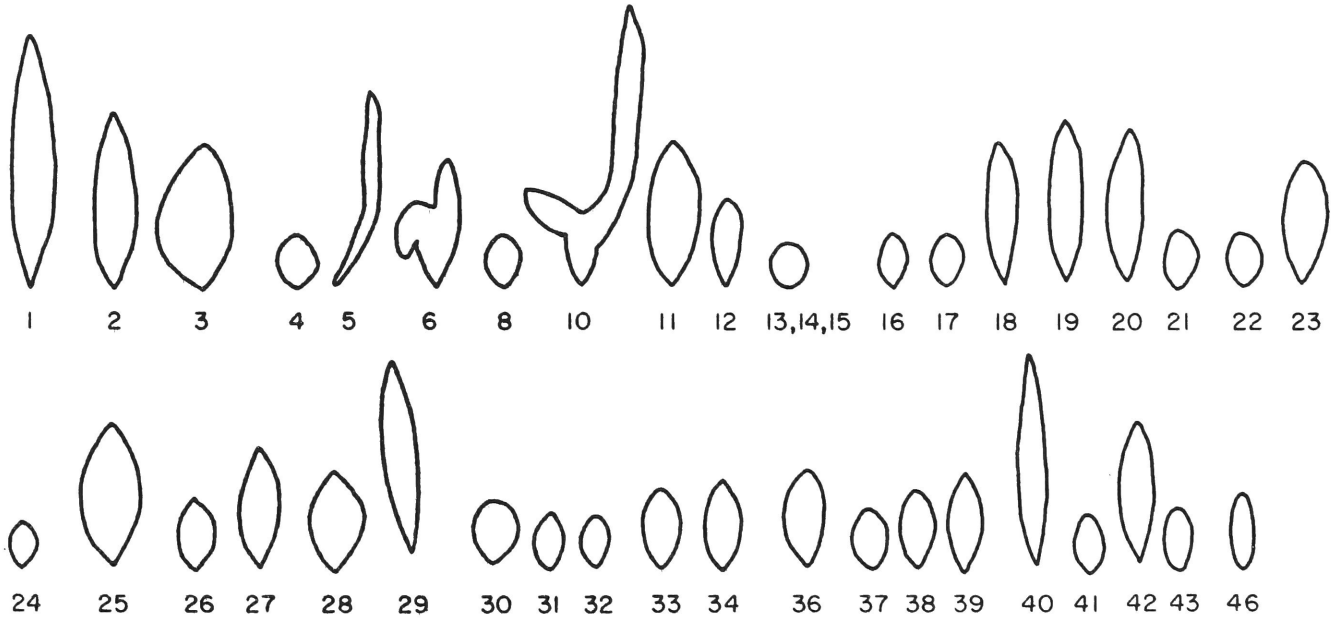


Fig. 21.

Fig. 22. Axio-lateral excursions of several granules in the arm of the cultured cell in Fig. 21. Traced for $1\frac{1}{2}$ minutes of filming.

Plot of Axio-lateral Excursions during Approximately One Minute of Filming



b. Characteristics of Motion.

Within its localized region, each granule moves to and fro in the proximo-distal direction. The granule generally does not oscillate to and fro continuously, but rather, may remain at one end of its path for some time, then traverse the region by a series of short starts and stops. It is convenient, therefore, to describe separately the characteristics of the distally-directed and the proximally-directed traverse.

Most often, the distally-directed path is traversed by a discontinuous motion--a series of short starts and stops. A granule may take up to 60 seconds to traverse the distal path over a distance of 2 microns. The proximally-directed path, on the other hand, is generally traversed continuously, and more rapidly. The granule commences the proximally-directed motion with a sudden, rapid acceleration from a resting position, moves rapidly (up to 3 microns/sec.) and traverses long distances in a single, continuous movement.

The characteristics of these motions are greatly affected by the presence of net concentrating or dispersing tendencies within the cell. Net concentrating tendencies have the effect of exaggerating the characteristics given above. The proximally-directed path may be very long--up to 6 microns--in cells <u>in situ</u>. The granule traverses its path rapidly and may accelerate all the way along the proximal traverse. Velocities of up to 4 microns/sec. have been recorded. The granule generally remains at the proximal end of the path for some time. The characteristics of granule paths in the proximal regions of a cell exhibiting a net concentrating tendency are shown in Fig. 23.

Net dispersing tendencies alter the characteristics of the distally-directed path. The distally-directed path is generally traversed continuously, and quite rapidly. The granule stops at the distal end of the path with a short rebound and remains bobbing about at the distal end of the path for some time. The move-

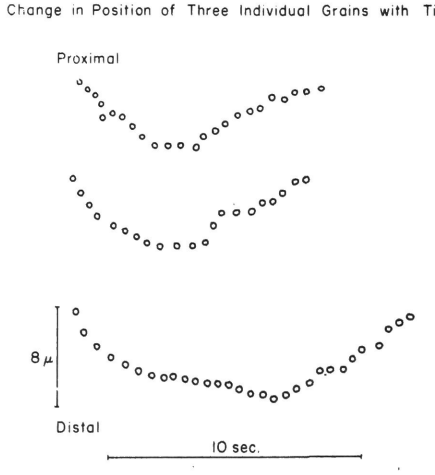


Fig. 23.

ments of granules in the distal part of a cell in which a net dispersing tendency was superimposed upon the shuttling motions are illustrated on the following page in Fig. 25. Granules remain longest at the distal ends of the path. The bobbing motion is difficult to see in these tracings, but is evident in some.

The characteristics of the shuttling motions when affected by overall net dispersing or concentrating tendencies are diagrammatically illustrated in Fig. 24. The distally-directed granule remains some time at the distal end of its path. The time-position plot of granules upon which a net concentrating tendency is superimposed gives a saw-tooth curve.

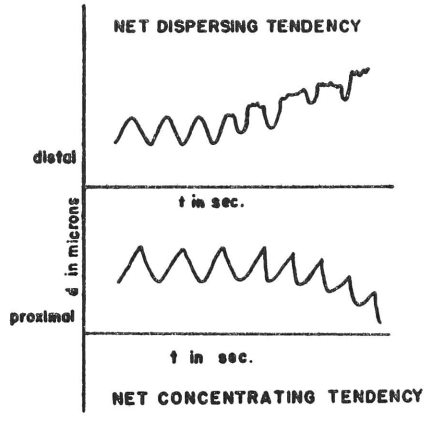


Fig. 24.

The total period is variable since the granule executes so many stops and starts. It may be of the order of a few seconds up to several minutes. It is reduced in duration when net concentrating tendencies are superimposed, since the time a granule spends at the distal end of its path is reduced, and in addition, the granule traverses the proximally-directed path more rapidly. It may be lengthened if weak net dispersing tendencies are superimposed, since the granule remains for some time at the distal end of the path. Strong net dispersing tendencies generally decrease the period. The period is very short, with the granules exhibiting a perpetual oscillation, when strong tendencies to disperse (evidenced by rapid translocation of shuttling regions) are countered with stimuli to concentrate. This is the case when cells from light-adapted fish, whose granules are dispersing in culture, are irradiated with strong light (by opening the diaphram of the microscope) or exposed to mildly alkaline medium. As net dispersing tendencies are replaced by net concentrating tendencies, the granules undergo rapid oscillation.

c. Relations Among Granules.

Certain characteristics of the shuttling regions, and of the

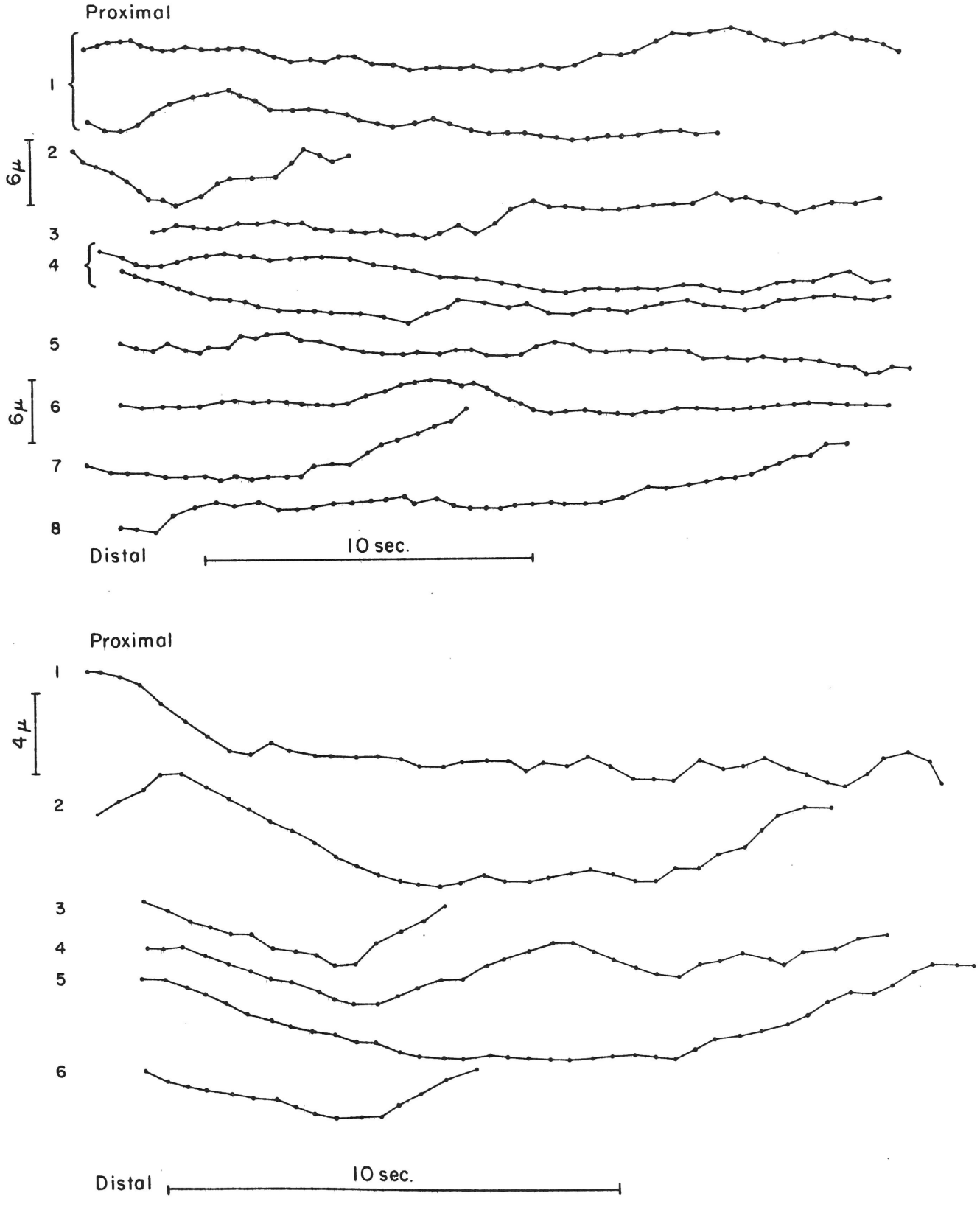

Proximal
1
2
3
4
5
6
7
8
6μ
6μ
Distal
10 sec.
Proximal
1
2
3
4
5
6
4 μ
Distal
10 sec.

Fig. 25

movements of a single granule within the region, have now been described. This section deals with the relations between granules--immediate interactions of granules with one another, the relationships between the shuttling regions, and the relationships between the motions of different granules.

Granule Interactions. The interactions of granules with one another may be most readily observed in the broad, flattened arms of cultured cells. The true impression of this movement is very difficult to convey. Ballowitz previously described it as kornschantz oder kugelspiel (a dance or play of spheres). Granules moving to and fro in common channels collide and move as a pair for a minute or two, and then part again; or they may collide, without rebound, the result being that both granules stop moving; or they collide, but glide by one another in the same channel or else turn around one another once or twice. Light reflectance from the granules suggests that they may be turning over, although this is very difficult to see. However, 180 degree flips of pairs of granules within the same channel are very commonly observed. Granules in the most distal regions of cultured cells are often in little clusters in the cytoplasm. The individual granules mill about one another, or the entire cluster turns slowly.

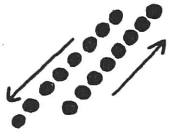
While certain features of this interaction--sticking together, gliding by one another--may be due to properties of the granules, the major features seem to be due to the underlying cytoplasm. The granules show no evidence of long-range attractions or repulsions. Collisions appear to be accidental, the result of movements of the underlying cytoplasm.

Relations Between Regions. The shuttling regions bear a relationship to one another. During any short period of observation, the position of regions remains constant within the cell, and constant with respect to the positions of neighboring regions. Their relative positions cannot be rigidly determined since granules are observed to interchange positions within a channel or to cross over into other channels. Co-ordinated displacement of regions is often seen in dispersion; the granules continually exhibit shuttling motions as the regions slowly change position within the cell.

Relationships Between the Granule Motions. The granules exhibit both independent and co-ordinated motions.

In maximally-dispersed cells, the motions of granules that are separated from one another by some distance are independent. Within a long column of granules, many granules may be motionless and others may be moving to and fro. Granules, one behind the other, within the same channel may move in opposite directions, and the shuttling motions of granules in neighboring channels seems unrelated. The independence of motions may be represented in the following manner:

Co-ordinated motion is observed when granules are close together. Single columns of tightly packed granules move as a unit in a direction opposite to that of neighboring columns. When granules are tightly packed in the proximal regions of the arms, co-ordinated movement appears as an arhythmic pulsation. Many columns of granules move in a co-ordinated manner back and forth over distances of 2-3 microns. The total period for the to and fro motion varies between 7 and 30 seconds. The proximally-directed path is traversed more rapidly than the distally-directed path. Typical times, appropriate for short periods, are 2-3 seconds and 5-6 seconds, respectively. Pulsation may involve the entire granule mass. On the other hand, single arms may pulsate, adjacent arms may exhibit independent pulsations, and even within a single arm, the granules in the proximal and distal regions may pulsate out of phase and with different periods.



Co-ordinated motion between granules separated by considerable distances is also observed. This may be seen in cells in situ when net concentrating or dispersing tendencies are superimposed upon the local shuttling motions and, in these instances, seem to indicate the simultaneous input of forces. They are also observed in cultured cells where, in the instances to be presented below, they indicate the presence of a

connecting substratum.

Tracings of the movements of granules in the distal regions of cultured cells are shown in Figs. 26, 27, 28. In both cells used in these figures, the distal-most granule was moored in the peripheral cytoplasm, and the movements of other granules in relation to this fixed marker could be observed. The first cell presented in Figs. 26, 27, is a resting cell; in the second cell (Fig. 28), a net concentrating tendency was superimposed upon the motion by rendering the medium slightly alkaline.

A tracing of the highly aligned granules in the distal region of the resting cell is shown in Fig. 26. The column of granules indicated by the arrow was traced by frame by frame analysis, and the change of position with time is presented in Fig. 27. The distal-most granule, #1, remains fixed in the cell cytoplasm. Granules 2-6 are also relatively motionless. The longest excursion is made by granule #7, which moves a distance of approximately 3 microns. Several types of relations among granules are observed. Shuttling motions, independent of one another both in time and direction, occur; shuttling motions independent in direction, but initiated at the same time (b), may be seen; co-ordinated movements of granules separated by considerable distances can also be seen (a, c). Since the latter occur when granules have undergone particularly long excursions, they seem to indicate the presence of a connecting substratum.

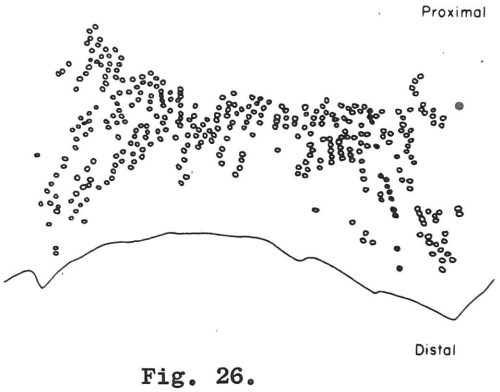


Fig. 26.

The presence of a connecting substratum is also evident in co-ordinated motions of the second cell (Fig. 28). The tracing of granule positions in the cell when it is in the resting state, and therefore has little shuttling motion, can be seen at (a) in Fig. 28. Shortly after the beginning of this tracing, a net concentrating tendency was superimposed on the shuttling motions (b). When this tendency appeared, the proximal granules moved rapidly towards the central region (c). The distal granule, however, remains moored in the peripheral cytoplasm, and the granules be-

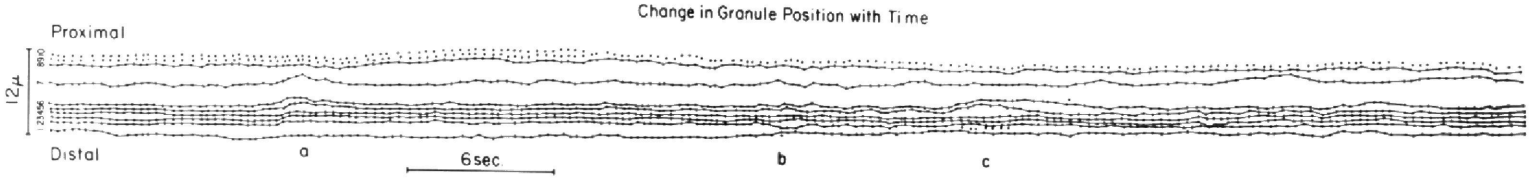

Change in Granule Position with Time
Proximal
12μ
8910
7
12456
Distal
a
6sec.
b
c

Fig. 27

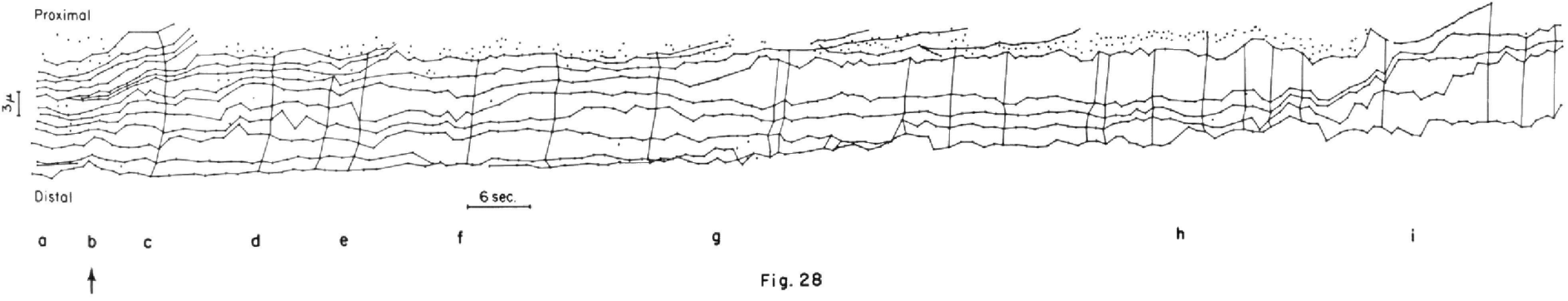
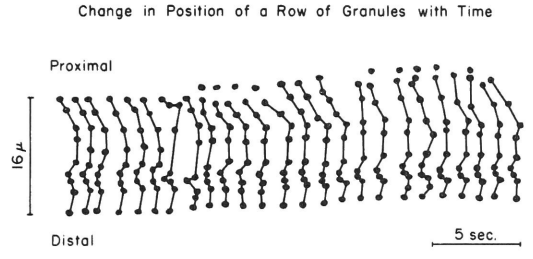

Proximal
3μ
Distal
6 sec.
a
b
c
d
e
f
g
h
i

Fig. 28

tween the two extremes oscillate back and forth (d), (e), and (f). At (g) one granule in particular is seen to be making its way towards the central region, and by (h), the distal-most granule seems to be losing its mooring. By (i) all but the distal-most granule are heading toward the central region of the cell.

The granules in a third cultured cell have been traced in Fig. 29. They indicate another type of co-ordinated movement--co-ordinated changes of axial and lateral positions--of such a nature as to indicate a connection between granules and possibly, a stiffening reaction in the substratum.

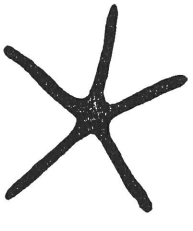
Fig. 29.



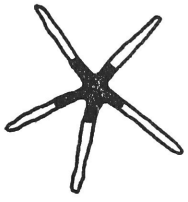
3. Comparisons of Shuttling Motions in Other Cell States.

The major features of shuttling motions have been described in maximally-dispersed cells. The characteristics described are modified only slightly in other cell states, with the exception of the concentrated state in which the shuttling motion is replaced by another type of motion or ceases altogether. The movements of granules in other cell states will now be described.

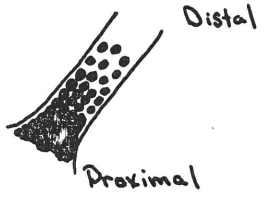
Maximally-dispersed cells, 5. The major difference between cells from light-and dark-adapted fish is the number of granules and the number of evident partitions between the granules (see Chapter II, p. 13, footnote). However, it may be due to this difference that the motions of the granules differ slightly in character. In light-adapted maximally-dispersed cells, the granules are few enough in number to be separated from one another and exhibit characteristic shuttling motions. On the other hand, granules in the dark-adapted cell are densely packed together and almost



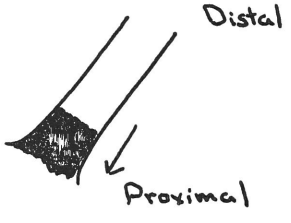
motionless.



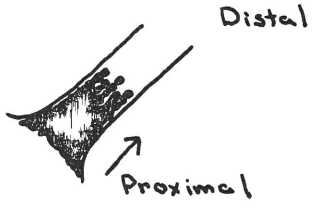
Semi-dispersed and semi-concentrated cells, 3. Several differ-cell states have in common the fact that their granules are densely crowded into the central and proximal regions of the cell. They are so densely packed that no details of the movements, or interactions, may be seen. Only the behavior of granules in the front regions may be observed. If the front is stationary (3) and there seem to be no net concentrating or dispersing tendencies, then the granules in the front region are very loosely packed together. They are aligned and move to and fro in a steady, shuttling motion. The path traverse may have the characteristics described in section 1, or the movements of granules may resemble a slow oscillation.



If a slight concentrating tendency is present (3-), the granules move towards the central part of the cell. The granules become tightly packed and apparently, highly constrained in the front region. Neither shuttling motions nor alignment can be seen, and the front region is almost linear.



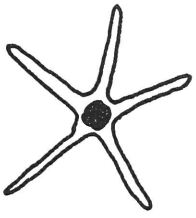
If a slight dispersing tendency is present (3+), then the front region is jagged in appearance. Granules move outwards with the characteristics described for motions which have superimposed upon them a dispersing tendency. Groups of granules move out, remaining in contact with one another, aligning at the distally-directed phase of the movement, losing alignment at the proximally-directed phase. Pulsation is frequent.



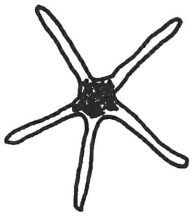
Very rapid movements of granules in the front regions are observed when cells which exhibit a strong dispersing tendency are placed in in vitro conditions and there encounter concentrating stimuli (light or alkaline medium). The granule movement is very rapid and appears as a continuous oscil-

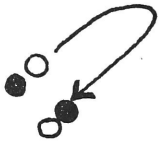
lation. Pulsation is frequently observed in these cells.

Concentrated cells, 1. The shuttling motions cease in concentrated cells and are replaced by slow, uni-directional movements which vary in acceleration. Granules are highly constrained, and if a granule does break away from the group, it moves spasmodically. Movement may cease altogether.



Re-dispersing cells, 1. The granules in re-dispersing cells are still in the central regions, but the constraints upon them seem to loosen. Granules and small groups of granules migrate out together for short distances. Commonly, pairs of granules will move out together, turn 180 degrees, and move back into the centre again with the same granule leading all the way.





4. Summary.

The major features of the shuttling motions have been described in the preceding sections. They can be summarized as follows: Each granule is confined within the cell to a fixed, small region. The granules may be motionless or else they may move to and fro in the proximo-distal direction with a variable period. The proximally-and distally-directed traverses are not mirror images of one another in that the proximally-directed path is the more rapidly traversed of the two.

In addition, the relations between granules have been described and in summary are: The regions bear a relationship to one another; the granules exhibit both independent movements (as in maximally-dispersed cells) and co-ordinated movements.

Five cell states have been designated for melanocytes on the basis of the distribution of granules and the predominant net direction of their motion.

B. MASS MOTIONS

The mass motions of granules are the long-distance, intracellular migration of granules which have been designated 'concentration' and 'dis-

persion'. Each of these processes will be described separately in this section.

It has proven extremely useful during the analysis of mass motions to consider that the cell behaves as a flip-flop circuit. The two extreme states of granule distribution, i.e., the maximally-dispersed and the maximally-concentrated states, are regarded as end states or states of rest. The granules may exist in one or the other and be made to pass between them at the will of the investigator by the administration of appropiate hormones. The process of concentration--the passage of granules from a state of maximal dispersion to a state of maximal concentration--is described in section 1. The process of dispersion is described in section 2.

1. Concentration.

The distribution of granules during concentration in a cell *in situ* is illustrated in Figs. 30a, b, c. In the maximally-dispersed state (Fig. 30a), the granules are scattered throughout the cytoplasm and are executing their continual shuttling motions. When adrenalin is administered,* the shuttling motions give way to continuous directed motion, and the granules migrate through the long arms (Fig. 30b) to become concentrated in the central region of the cell (Fig. 30c). The process is complete in 30-60 seconds.

The details of the process of concentration will be described in three parts, a, b, c, which follow. In the first will be described the characteristics of the movements of granules; in the second will be described both the relationships of the mass of granules with the cell boundaries, and the various changes in the position of these boundaries; in the third part will be described both the behavior of other cell structures during granule movement and ancillary observations on the process of concentration.

a. Movements of the Granules.

i. Initial events. The response of the granules to adrenalin appears to be initiated at the distal tips of the arms. The distal-most granules are the first granules to begin moving centrewards. The granules

---

* Although many agents will trigger concentration, adrenalin (1×10^{-5} M) was used throughout this study.

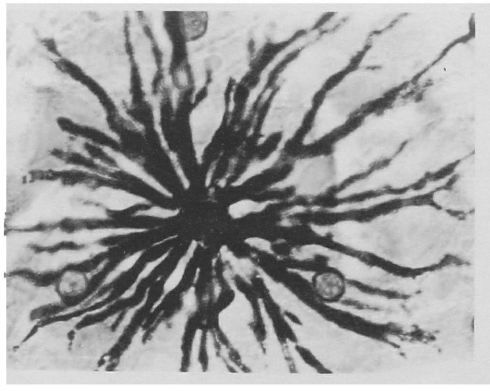


Fig. 30a. 400 x. A light-adapted, dispersed cell.

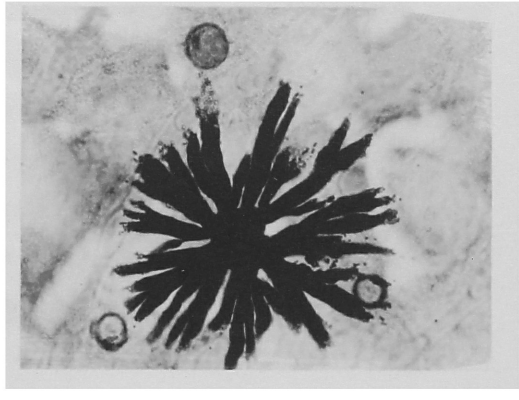


Fig. 30b. 400 x. Cell in Fig. 30a a few seconds after administration of adrenalin. Granules are migrating centreward and maintain a linear front. The arms of the cell are not visible although they remain on the scale during this process.

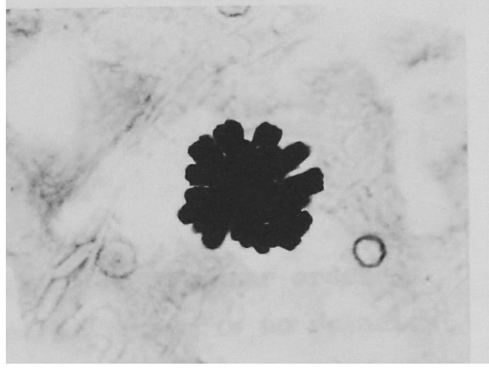


Fig. 30c. 400 x. Concentrated cell with granules crowded into the central region.

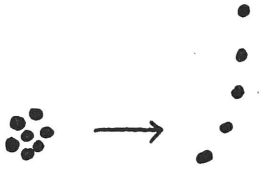
throughout the arm begin moving a few seconds later; they are set in motion, not by collision of the more distal granules with them, but as a result of some change in the cytoplasm in which they are situated. The initiation of movement proceeds as a travelling wave passing from the distal tips of the arms towards the central region. This wave travels with a velocity of the order of 10 microns/sec. in both light-and dark-adapted cells. This wave may not be visible when the granules have a great freedom of motion.

The shuttling motion of granules (present before adrenalin is

given) may give way immediately to continuous, directed motion when adrenalin is added. But more often the transition to continuous motion is marked by a few rapid shuttling motions characterized by long, proximally-directed paths. Within a few seconds after the first reaction to adrenalin, the granules throughout the cell are moving centrewards with a terminal velocity of 3 microns/sec.

ii. Later events.

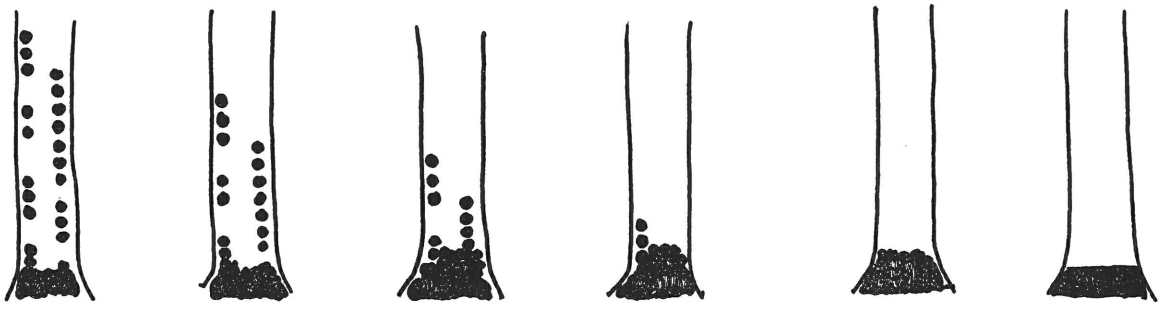
Peripheral region. As the granules begin moving centreward, changes in their relative positions may often be observed in cultured cells. Granules sometimes draw closer together in the lateral direction, and granules clustered together in the peripheral regions of cultured cells often become widely separated from one another and form columns which align in the proximo-distal direction. This latter process often gives rise in cells *in situ* to a characteristic movement which can only be compared to the uncoiling of a rope.



Distal region. The granules maintain their columnar order as they move through the distal regions of the cell. There is no velocity profile across the arms, and the granules in all columns move at a uniform rate which does not change even at the places of bifurcation of the arms. The spacing between granules remains relatively constant, and the granules appear to be carried along in a moving stream. However, a close inspection reveals several phenomena which indicate that this is not the case. E.g., among the proximally-moving granules may be seen many which are motionless, others which are moving in the distal direction. Also, there are some variations in velocity which can be correlated with the width of the arms--the granules accelerate as they move through wide, uncongested regions, and they slow as they move through narrow, congested regions. Such variations are the opposite of that expected in a fluid in

motion.

Proximal region. If there are few granules in the cell, they may move directly into the central regions and often accelerate to a velocity of 5-6 microns/sec. as they near the centre. More often the granules become highly congested in the proximal regions of the arms. This region appears to be a bottleneck since the granules become congestes in it even when the central regions of the cell are devoid of granules up to the very end of the process. In the proximal regions of the arms, the granules lose their columnar order and pile on top of one another. Granules which might have been formerly spread in a thin layer throughout the cytoplasm become crowded together in a 3-dimensional mass. The proximal region of the arms bulges in consequence. The movements of granules are most highly constrained in the front regions of the granule mass and only occasionally do granules escape and move into the distal region of the arms. However, such granules seldom remain separated long and return almost immediately, and often rapidly, to the granule mass.



The front region--the distal edge of the granule mass--is linear in concentrating, in situ cells, a fact which would seem to indicate a very great constraint upon granule movements. In cultured cells, on the other hand, the front region is generally crescent-shaped during concentration. The changing shape of the granule front in a cultured cell which is undergoing concentration is shown in Fig. 31. These differences in the shape of the front regions appear most likely to be the result of the dif-

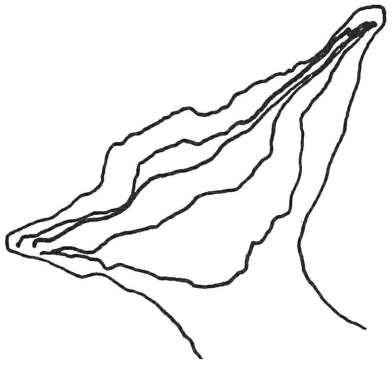


Fig. 31. Successive positions of the front during period of 15 seconds. Traced from time-lapse films.

ferences in the two cell types.

Central region. The granules crowd slowly into the central region which often remains cleared of granules until the very end of concentration. In cultured cells, the central region can be seen to 'hump up' as the granules enter. The movements of the granules become highly constrained and may cease altogether; shuttling motions are absent.

When the body of granules is concentrated in the central region of the cell, many granules are often still stranded at the distal edges of the cell. They either remain here for some time or migrate into the central region a few minutes after concentration is complete. They move into the central region slowly, with many starts and stops. In cultured cells, granules are frequently stranded at the distal edges which appear to have collapsed slightly as the mass of granules withdrew. Such stranded granules behave as if under constant tension from the central regions of the cell and finally move in over time. A tracing of three such granules is shown in Fig. 32.

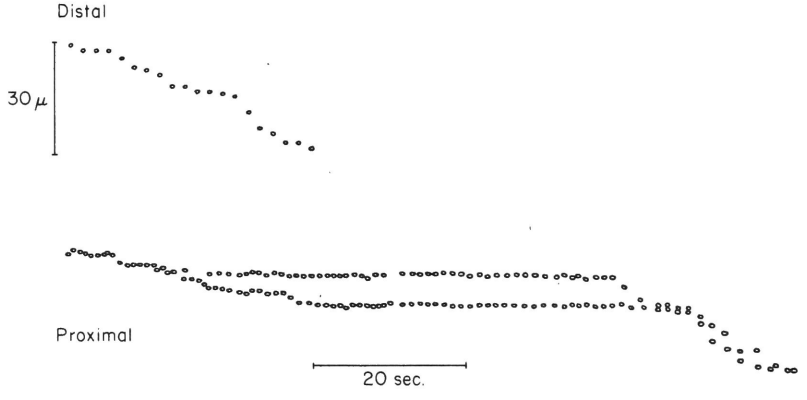


Fig. 32. A granule (top) and a pair of granules (lower) are shown moving into the central region after the main granule mass has completed concentration.

b. Cell Membranes.

i. Relationship of granule mass and cell membranes. The arms of in situ cells remain attached to the scale during granule migrations. Normally they cannot be seen but may be rendered visible if the cell is swollen with water (Fig. 33).

In cultured cells, the cell envelope can be clearly seen. It

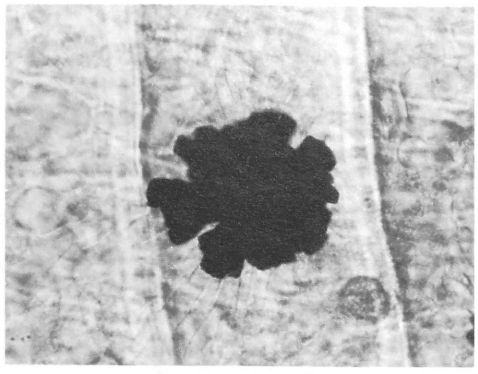


Fig. 33.

remains attached to the coverslip during granule migration, and the mass of granules withdraws from the cell boundaries like the hand from a glove. In the cultured cell shown in Fig. 34, the mass of granules, whose border was initially coincident with the cell boundary (solid line), is being pulled inwards and separating from the cell boundary in the region of bifurcation of the arms (dashed line, drawn in on left side). The concentrating mass (dotted line) preserves, to some extent, the shape of the whole cell, while the boundaries remain fixed to the coverslip. (Also, the angle between the arms decreases during concentration.)

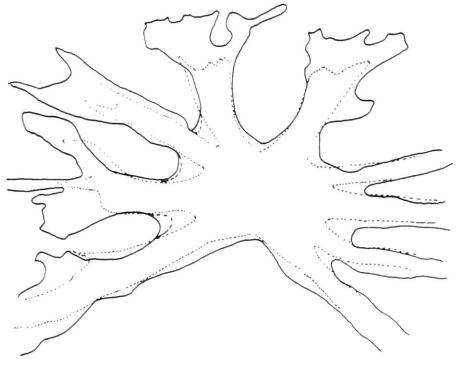


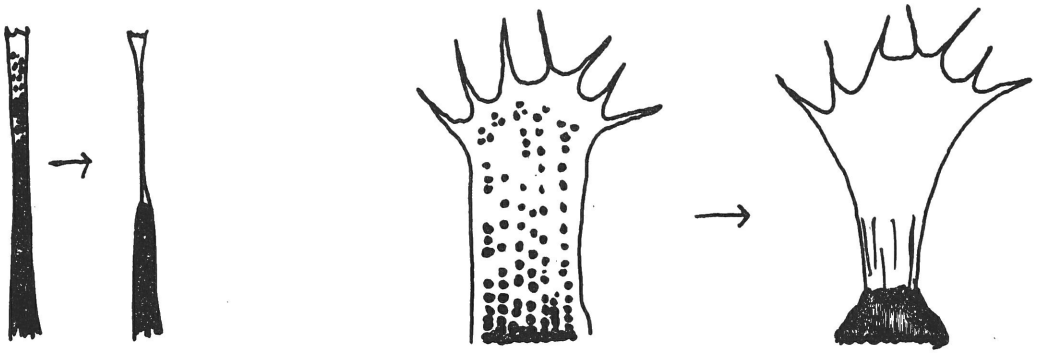
Fig. 34.

The mass of granules changes its shape within an envelope which remains extended in a stellate form. A considerable amount of the cell cytoplasm moves with the granules, and particularly in cultured cells, the arms of the cell appear to collapse as granules and cytoplasm withdraw into the central region.

ii. Changes in position of cell boundaries. Although the arms of the cell remain attached to the substratum, there are changes in the position of the boundaries and certain changes of shape of the cell envelope. Three types of changes are designated and described below:

Immediate Shape Changes. The arms appear to collapse as the

granules withdraw from them. Thin arms collapse into a delicate thread of protoplasm.* These shape changes indicate that the granules do not merely separate from one portion of the cytoplasm, but rather, a great deal of cytoplasm moves into the central regions with them. (The shape changes are represented diagrammatically below.)



Whether all the cytoplasm does move into the center is not known. Electron micrographs indicate that some granule-free cytoplasm is present in the distal region of the arms (Plates 9, 10). When observing cells with phase microscopy, material often appears to be extruded from concentrating granules.

No peristalsis has ever been observed. The bizarre shapes of cultured cells make its effectiveness in moving granules unlikely.

Secondary Shape Changes. Many changes in shape occur during the few minutes after concentration is complete. Flattened arms may narrow towards the mid-line. The angle of bifurcation of the arms diminishes.

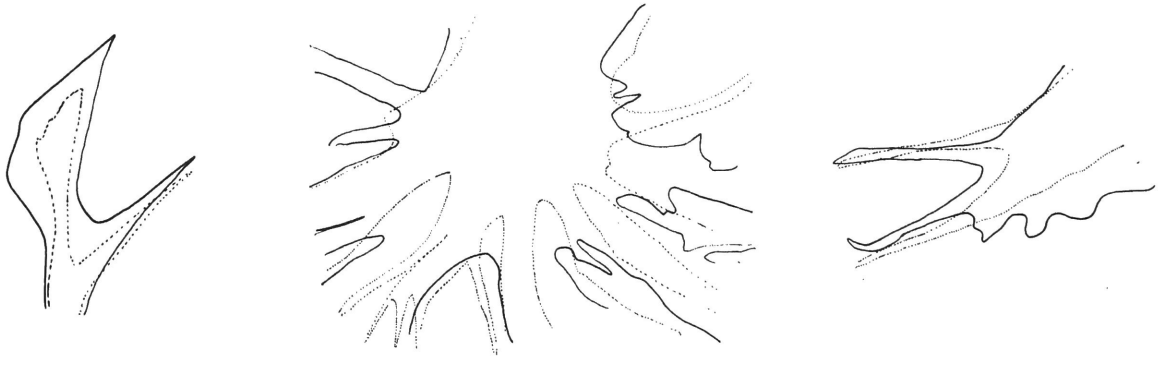


Fig. 35.

* Occasionally, this collapse may be preceded by a slight bulging of the granule-free portions of the arms.

These changes are illustrated in Fig. 35 in tracings made from time-lapse films of cultured cells. The solid lines represent the dispersed cell boundary. The dotted line represents the concentrated cell boundary.

Delayed Shape Changes. Changes in cell shape continue for some time after concentration is complete. In discoid cells, the flattened cytoplasm furrows and forms arms during the several minutes after the completion of concentration. Small attachments pull up from the coverslip, and the biradial symmetry of the cell increases. These changes can be clearly seen in the following set of overlaid tracings of a flattened, cultured cell containing only a few granules (Fig. 36).

The first tracing (1) shows the dispersed state. The cell contains only a few granules. The nuclei are towards the right-hand side of the central region, and the form of the cell is unsymmetrical.

In the second tracing (2), adrenalin has been administered and the granules are concentrated. They have all migrated into the region between the two nuclei which, in consequence, have been displaced toward the right-hand side of the cell. In the film it is clear that the upper-most nucleus is initially pushed aside, not by the granules, but by some other element. It is as if an invisible strand between the centre of the cell and the distal-most end of the upper right-hand arm had suddenly contracted and exerted a force upon the nucleus.

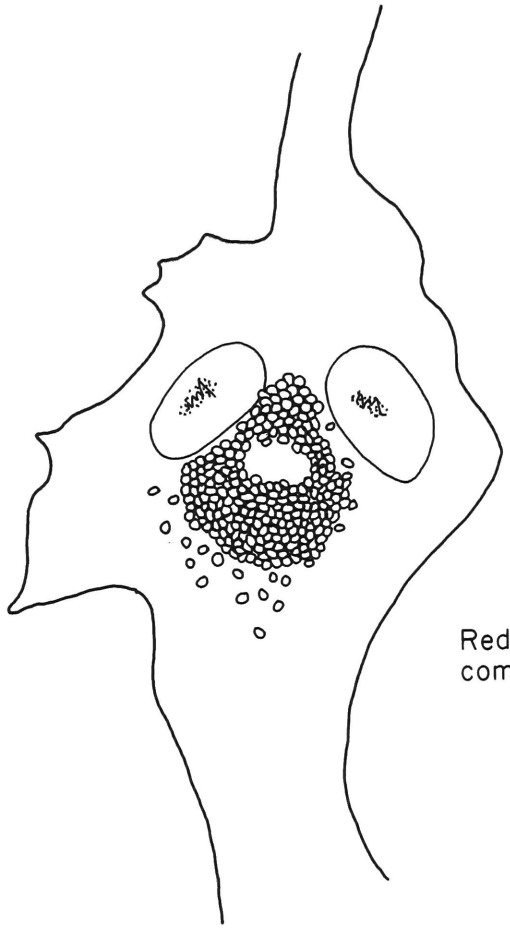
In the third tracing (3), the cell is changing shape and becoming oriented between the two major attachments towards the right-hand side. Small attachments pull up. The granule mass is becoming situated at the geometric centre of the cell, and only a small, cleared central region may be seen.

In the fourth tracing (4), the change of shape continues. The cell becomes increasingly biradially symmetrical.

In the last tracing (5), re-dispersion is commencing.

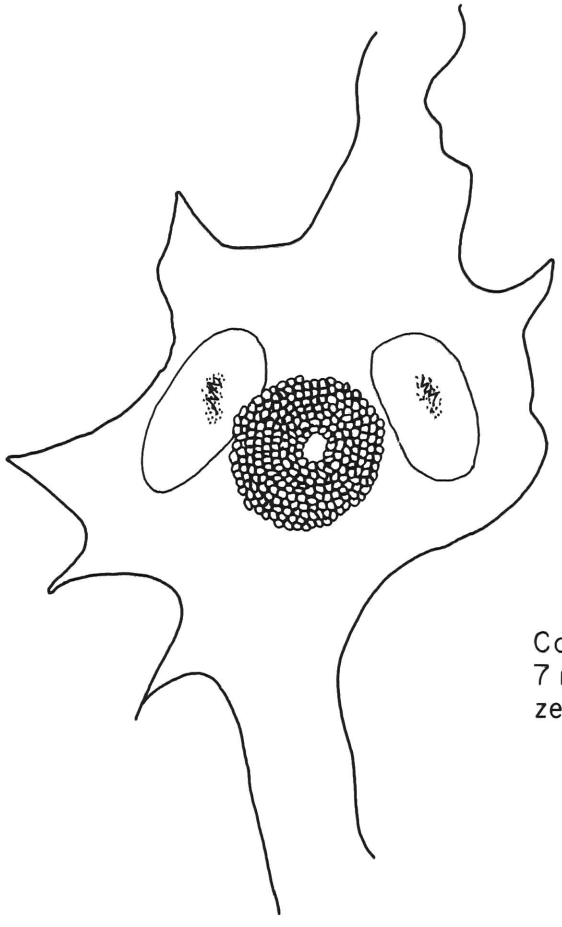
c. Behavior of Cell Parts.

The characteristics of the movements of granules, and the changes in the position of the cell boundaries which accompany the movement, have been described in the preceding sections. In this section the changes in other parts of the cell will be described.



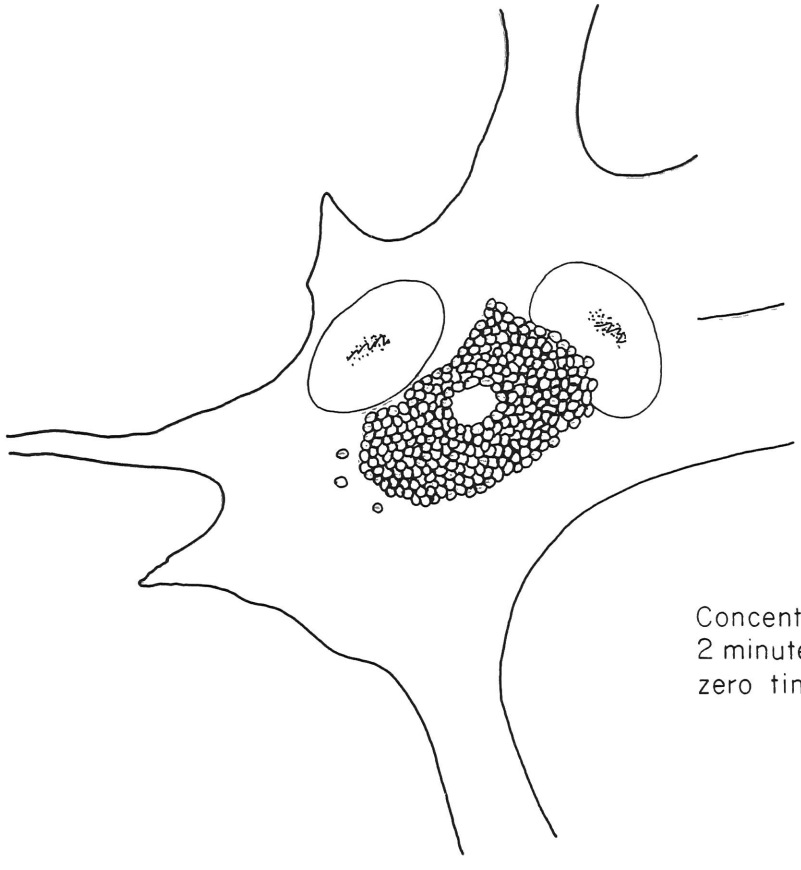
(5)

Redispersion
commencing



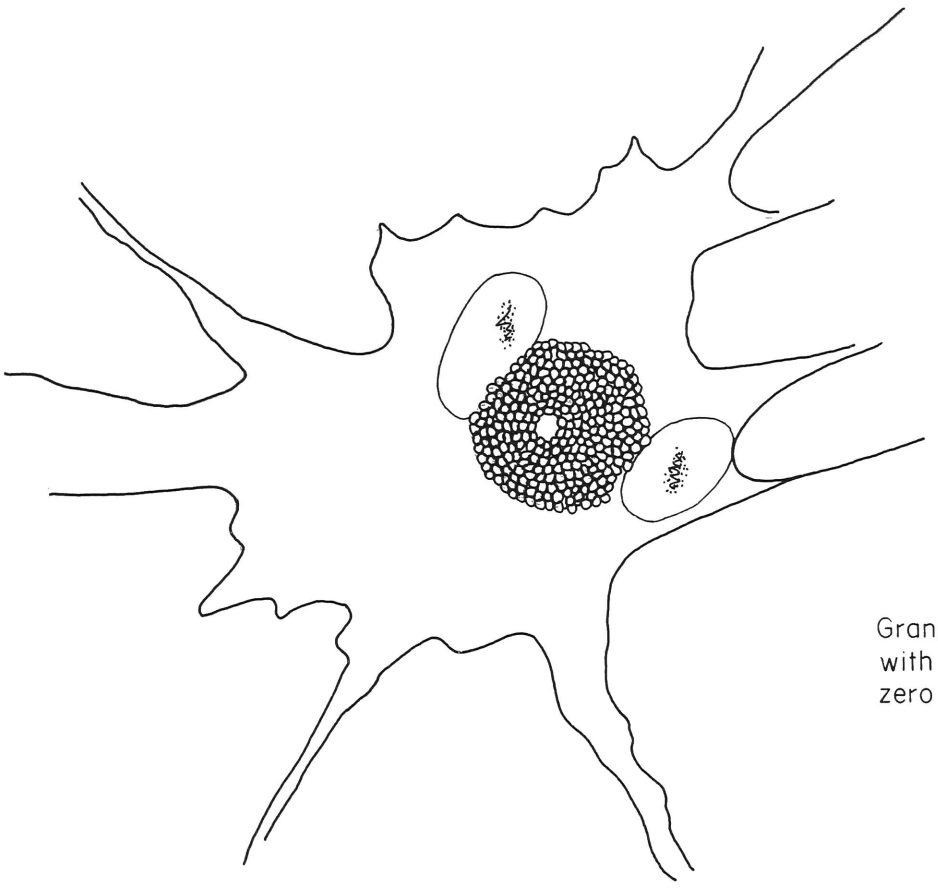
(4)

Concentrated state
7 minutes from
zero time



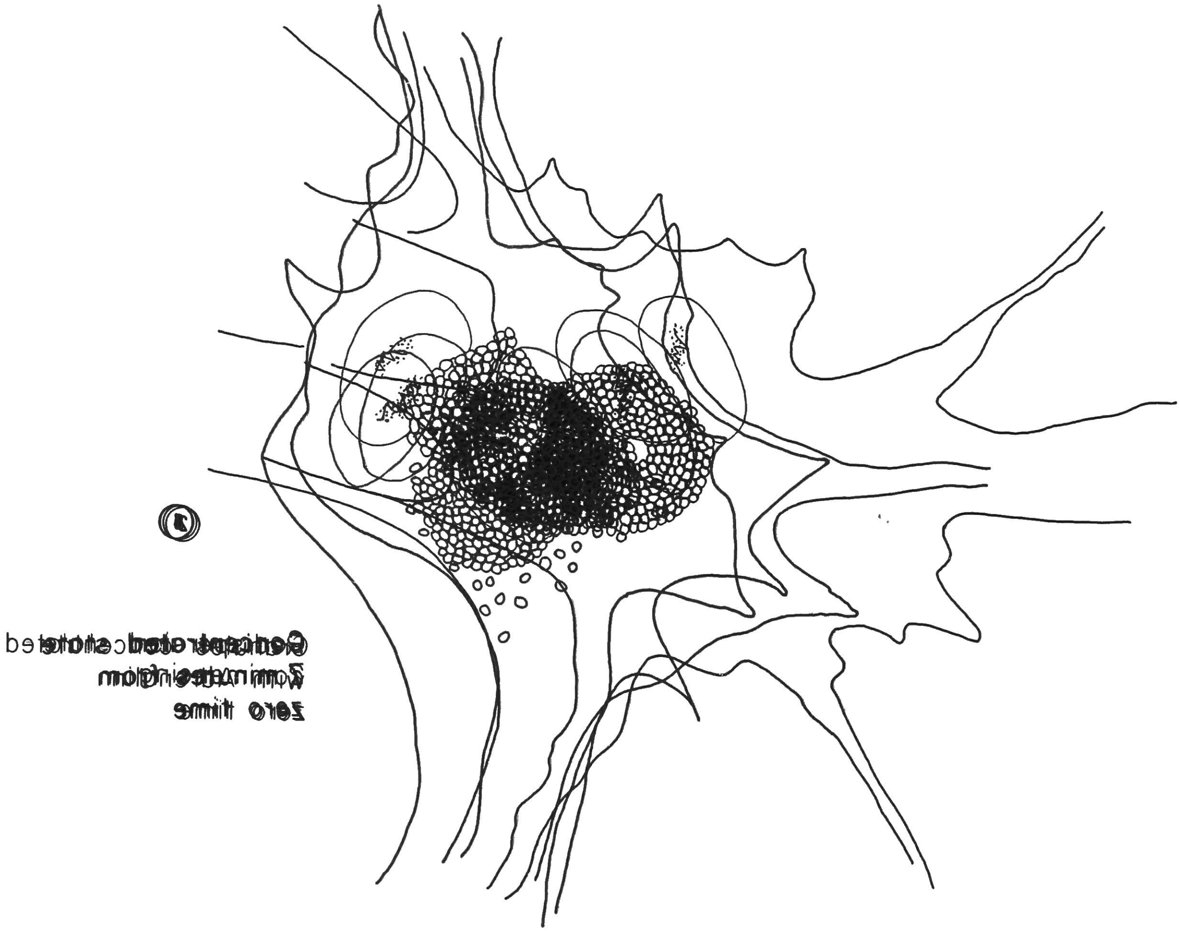
③

Concentrated state
2 minutes from
zero time



(2)

Granules concentrated
with Adrenalin
zero time


zero time

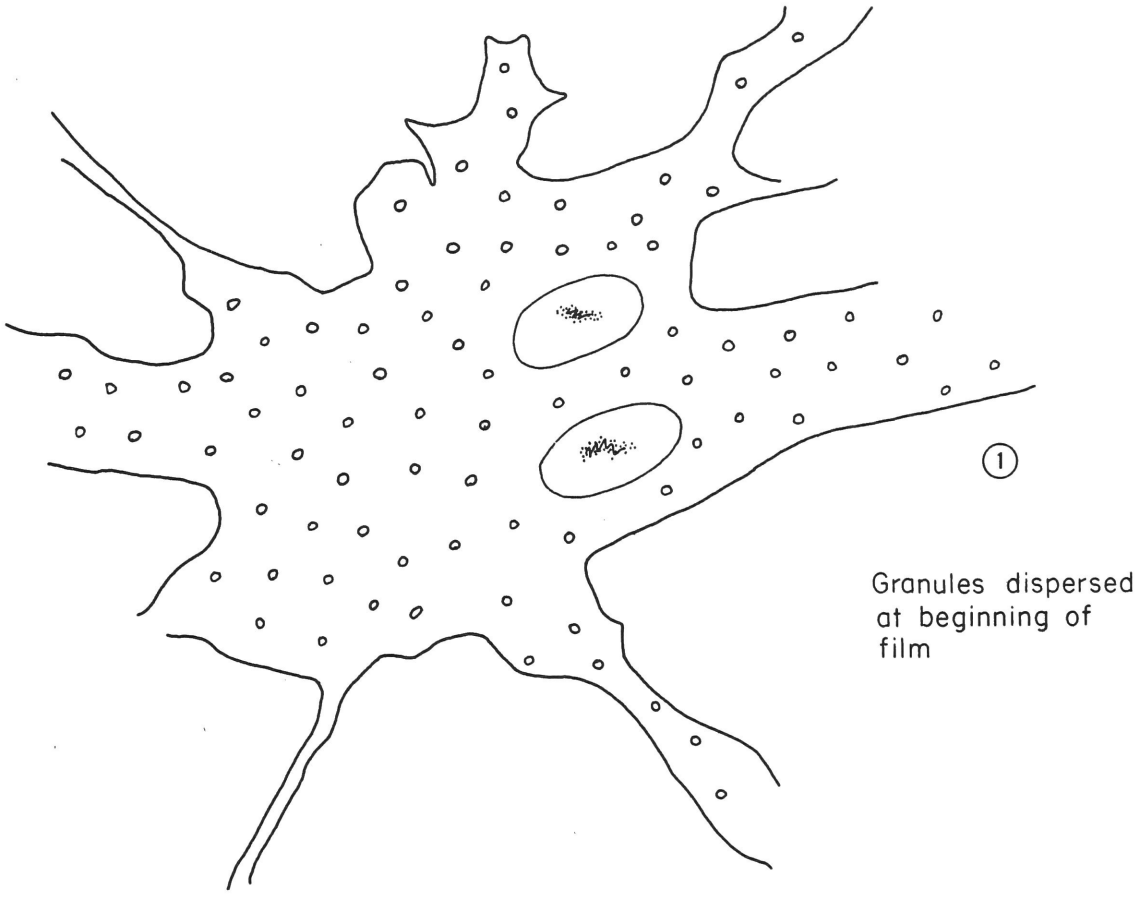
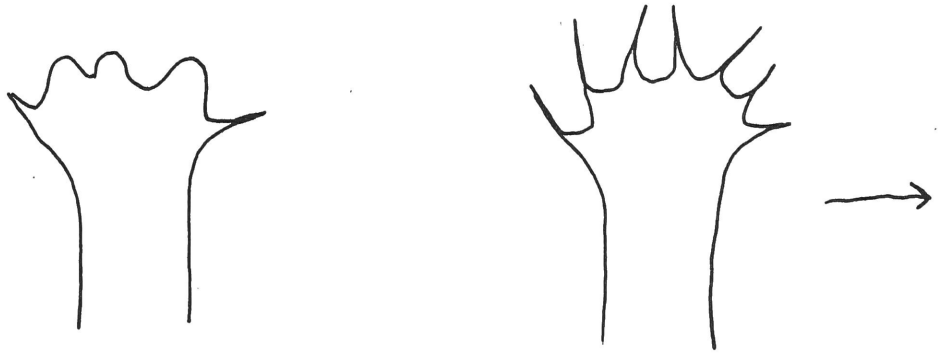


Fig. 36.

Partitions. What happens to the partitions during concentration is not known with certainty. During migration the granules maintain their columnar order in the distal regions of the arms but lose this order in the proximal regions. This loss could indicate that partitions have broken down; on the other hand, since it occurs as the granules crowd into a 3-dimensional mass, it might merely indicate that the partitions remain close to the surface of the cell and the granules are able to escape from their constraining influences. Some tubules remain extended; radiating structures can often be seen in the granule-free cytoplasm of concentrated cells when observed by phase microscopy. Electron micrographs indicate that at least some microtubules (partitions) remain surface associated during the movements of granules (see Chapter III); on the other hand, it appears from these micrographs that a considerable number of microtubules move into the central regions with the granules.

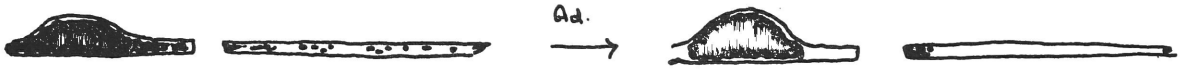
Cell Surface. No change in the activity of the cell surface can be seen during concentration. The microspikes, a characteristic protrusion of the cell surface, remain extended during granule migration and exhibit no changes in their behavior. However, shortly after concentration, the microspikes disappear, and the cell border exhibits an activity best described as a series of distensions followed by collapses.



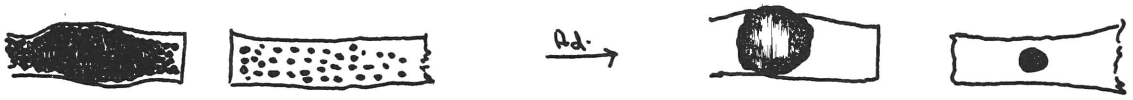
Nuclei and Mitochondria. Neither nuclei nor mitochondria appear to be a part of the cytoplasm which contains the granules. The nuclei are passively pushed aside as the granules enter the central region of the cell. The mitochondria do not migrate with the granules, but, on the contrary, remain in the distal regions of the cell where they can be seen actively moving about.

d. Ancillary Observations.

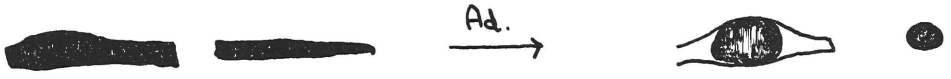
i. Microdissection. The granules in arms severed from the main body of the cell with a microneedle continue to migrate in response to adrenalin. In most severed arms, the granule migration continues to be in the distal-proximal direction.



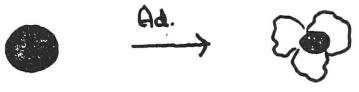
In broad, severed pieces, the granules often migrated to the central part of the severed piece.



In one cell in which granules were densely packed throughout the severed piece, the piece rounded up in response to adrenalin.



ii. All forms of melanocytes exhibit concentration. Cells dissociated from the scale and rounded up in the culture medium will undergo concentration. The granules in such cells withdraw into the centre, leaving behind them a clear, scalloped cytoplasm.



iii. Concentration is almost impossible to abolish. Severely damaged cells can still be concentrated by adrenalin. For example, cells put into potassium-Eagle's medium* respond by immediate concentration of their granules. After a period of about three hours, the granules in such cells re-disperse, and the cell is severely swollen. The granules exhibit considerable Brownian motion superimposed upon their shuttling motions, and they have cleared the centre of the cell (Fig. 37). The granules can be re-concentrated by administering adrenalin.

---

* Eagle's medium in which sodium salts have been replaced by potassium salts.

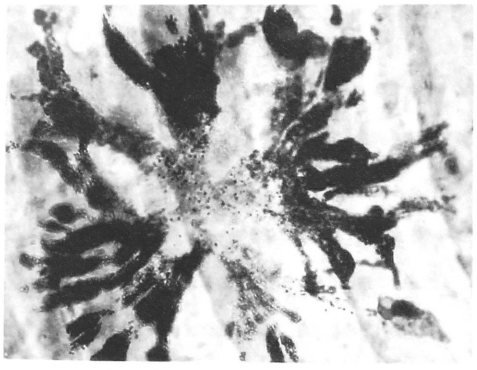


Fig. 37.

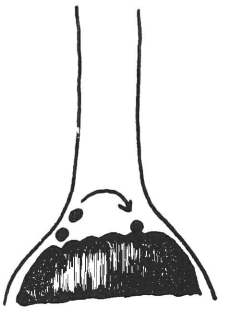
2. Dispersion.

Dispersion can be elicited by repeatedly washing a concentrated cell with fresh medium. It differs from concentration in several ways. In the first place, dispersion is invariably difficult to elicit by all agents which have been reported to trigger it (Lerner and Takahashi, 1956) and which may be considered at all physiological. In the second place, the process is much slower than concentration, requiring 2-5 minutes for completion. Furthermore, a dispersed state can seldom be maintained for any length of time and reverts almost immediately to a semi-dispersed condition. Finally, the process differs from concentration in that it exhibits no single, constant character. Whereas concentration has the same character regardless of the agents used to elicit it, the characteristics of the dispersion process vary greatly depending on the agents used. The process of dispersion described in sections a and b below is that most often observed and is produced by the administration of several types of agents--MSH, acetylcholine--or by rinsing a concentrated cell with fresh medium. Additional types of dispersion are described in section c.

a. Movement of Granules during Dispersion.

Initial Movements. Dispersion can be produced by rinsing a concentrated cell with fresh medium. It may be recalled that in a maximally-concentrated cell the granules are crowded tightly together in the central region. They exhibit a motion which is not the characteristic shuttling motion, but rather a slow movement in a single direction; they may be almost motionless. No granules break away from this central mass, and the granules appear to be highly constrained. When such a concentrated cell is rinsed with fresh medium, a few granules at the front region break free of these constraining influences and make short excur-

sions away from the mass. These excursions are, in the beginning, unidirectional; i.e., granules move continuously in a shallow arc from one side of the mass to the other. With continual rinsing, the mobilities of all granules in the front region increases, and the linear front takes on a jagged appearance. Although the first visible movements of granules during dispersion occur at the front region, careful focusing with the microscope reveals an increased mobility at all levels; the area of the mass increases and the granules move farther apart. It seems likely that some change of conditions has occurred, not merely at the front region but throughout the entire mass.



As the mobilities of granules increase, and many granules begin to move into the basal regions of the arm, the characteristic shuttling motions return. Pairs and small groups of granules move out from the centre in continual shuttling motion with a superimposed net outward drift of regions. Columns of granules may be seen to align at the distally-directed phase of the shuttling motion and to lose alignment during the proximally-directed phase.

Gradually, many granules crowd into the basal and proximal regions of the arms. They are loosely packed, and the front region is far from being linear as the separate granules within it move back and forth in independent shuttling motions.

Later Events. After many granules have crowded into the proximal regions of the arms, further dispersion may occur in one of two ways. The process described above may continue. The granules exhibit continual shuttling motions, and dispersion is accomplished by a net outward drift of granule regions. Or alternatively, the shuttling motions may become co-ordinated, dispersion then occurring by pulsation modulating a net outward drift. Pulsation may be co-ordinated over the entire mass, or different parts of the cell may pulsate in an un-co-ordinated fashion. Dispersion by pulsation is the phenomenon most frequently observed. No process of dispersion is at all the mirror image of concentration.

The dispersion process described above appears to result from

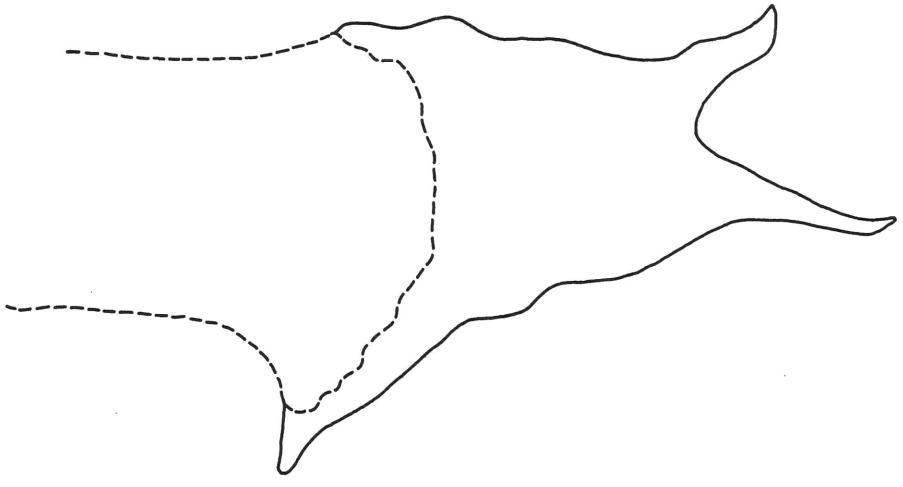
the successive outward migration of granules in the front region. It appears that the granules within the mass remain tightly packed together, and that a loosening of the granule constraints occurs only in the front regions. Actually, there is evidence that a pervasive change of conditions occurs throughout the mass. The observation has been mentioned above that mobilities of granules at all levels increase. In addition, the occurrence of pulsation indicates that co-ordinated distally-directed movement occurs among the granules both at the distal edges of the mass and deep within it.

There is another type of dispersion often observed in response to the rinsing of a concentrated preparation with fresh medium. In this type, the mass of granules moves outward apparently under the impress of forces arising deep within it. The granules in the front regions exhibit no increase in mobilities and no shuttling motions; rather, they remain in a linear front with their relative positions kept constant.

b. Relation of Cell Boundaries and Granule Mass.

The boundaries of the melanocyte cannot be seen in cells in situ. Nevertheless, they are visible in cultured cells, and changes of their positions accompanying movement of the granule mass may be observed. Two specific examples of dispersion in cultured cells are presented below:

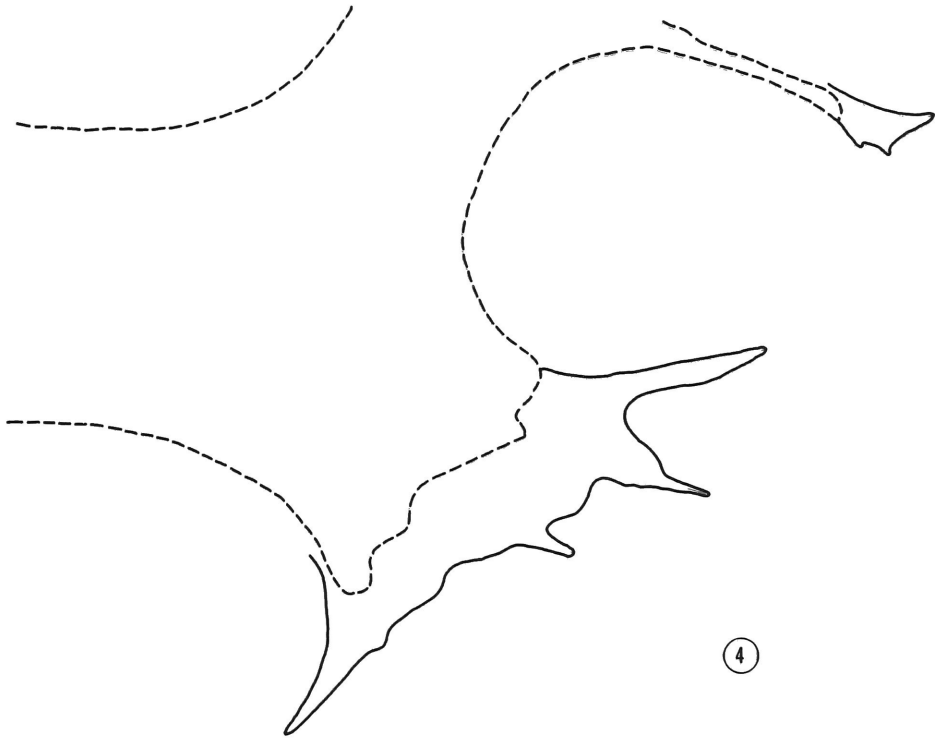
i. Dispersion produced by rinsing a concentrated cell with fresh medium. In Fig. 38 are presented the overlaid tracings of successive positions of the front region and the cell boundary in a cell dispersing in culture. Such dispersing cells--when concentrated and then rinsed with fresh medium--exhibit very strong re-dispersing tendencies originating deep within the granule mass. Initially, the granules move out as a mass from the central region of the cell (1). Their boundary is convex. In the front region, the granules appear motionless, whereas the granules deep within the mass are in violent movement. The dispersing mass gradually spreads and flattens in the lateral direction (2, 3). Due to the characteristics of the migration, one has the impression that the granules in the front region form a rigid barrier, yet strong dispersive forces from deep within the mass force the granules through this barrier at its weakest points (4, top). All of Fig. 38 summarizes well the change of shape in the cell boundary which accompanies the dispersion of the granule mass.



①

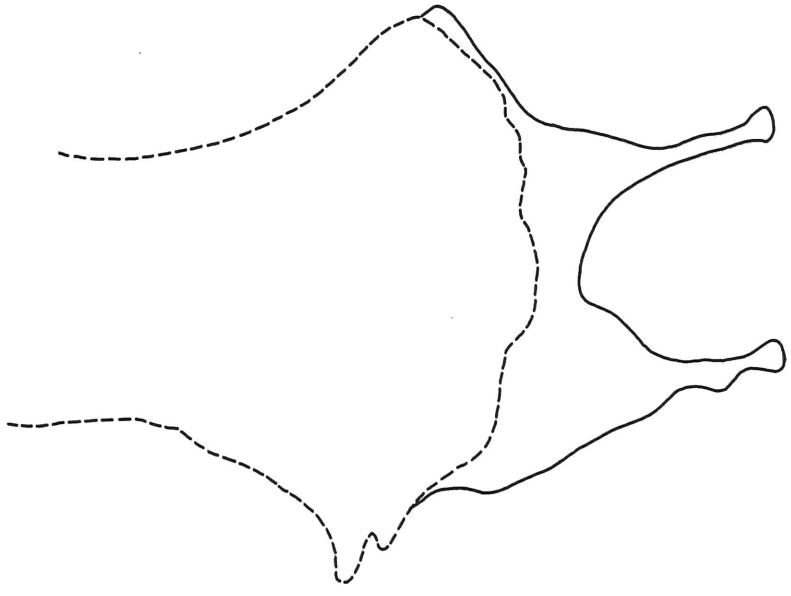
Fig. 38.

Relationship between Cell Border and Distal Edge
of Granule Mass during Dispersion

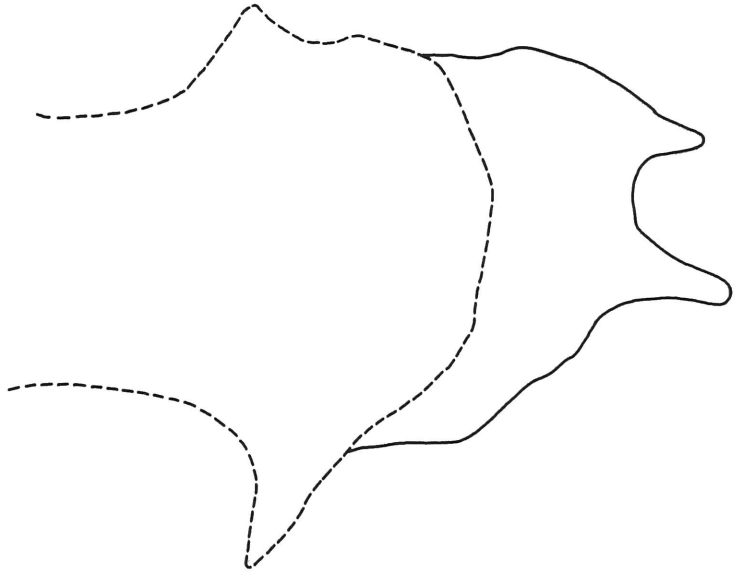


④

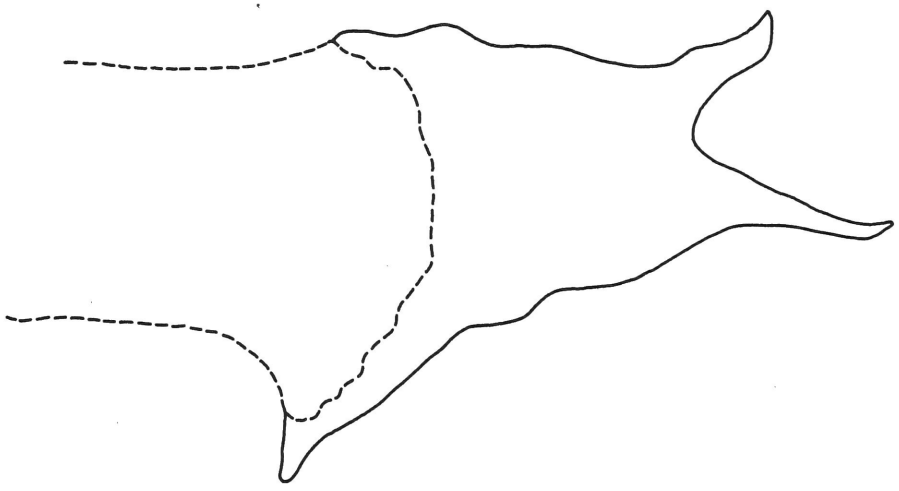
----- Distal edge of granule mass
——— Cell boundary



3



(2)

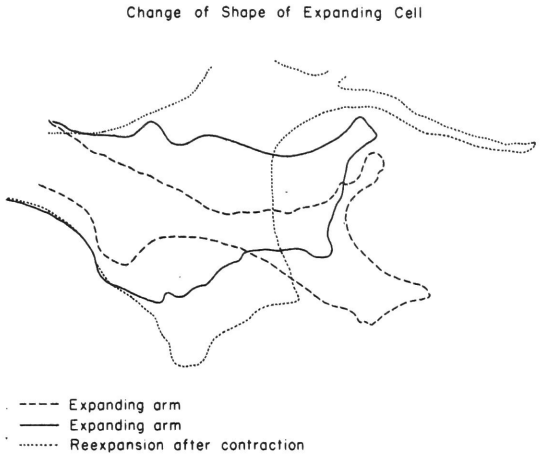


①

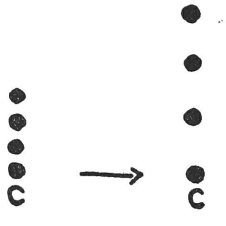
Fig. 38.

The rather striking changes in cell form which accompany the dispersion process can be more clearly seen in Fig. 39; these are the superimposed successive tracings of the cell boundary described above. The entire cell flattens and spreads.

Fig. 39.



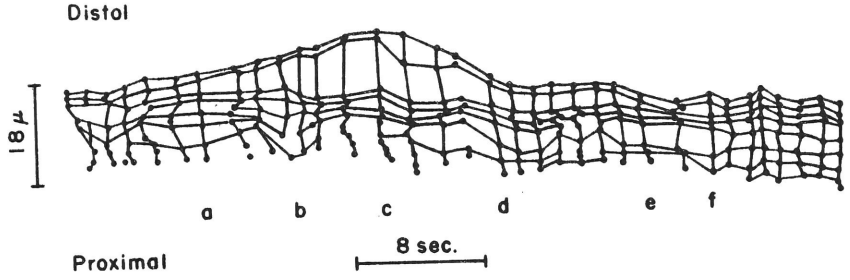
ii. Dispersion in cell with few granules. Additional characteristics of dispersion have been observed in the flattened cell containing few granules, for which concentration was recorded in tracings in Fig. 36. The first event in dispersion in such cells is a widening of the arms. Immediately thereafter, the granules move out from the central regions. In this small mass of granules can be seen granules both at the front regions and in the centre beginning to move outwards at the same time. The granules in the central part of the mass are the first to stop moving, and they remain near the central region of the cell. The granules at the front region travel right out to the distal edge of the cell. The granules between the centre of the cell and the distal end of the arms become spaced through the cytoplasm.



c. Other Types of Dispersion.

i. Rapid dispersion elicited by fat-soluble anesthetics. A very rapid dispersion can be elicited by the administration of acetone (5%), carbon tetrachloride (5%), and various other agents considered to be fat soluble anesthetics. All the granules lose contact with one another and migrate outwards rapidly and continuously. The granules are distributed throughout the arms in approximately 30-60 seconds. Within

Momentary Dispersion in Cultured Cells



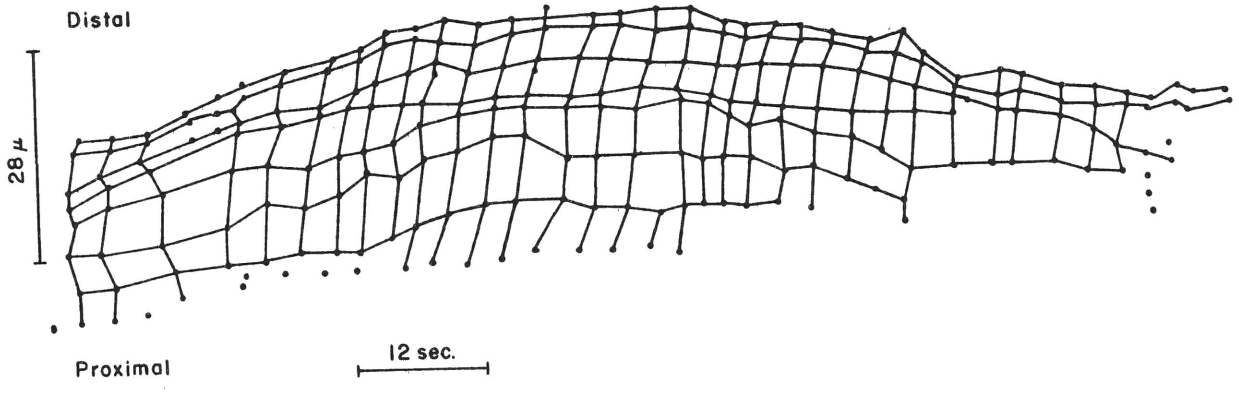


Fig. 40

a few minutes the central region of the cell becomes cleared of granules altogether. This dispersion is reversible if the cell is rinsed with fresh medium within a few minutes after completion of the process. Otherwise, the movement of the granules slows and after five minutes ceases altogether. When this occurs, the process cannot be reversed.

ii. Momentary dispersion. In addition to the dispersion described above which involved the entire mass of granules, there is very often observed a type of dispersion which involves only a few granules at the distal edges of the granule mass. These dispersions have been designated 'momentary dispersion' because the granules involved in such processes do not remain in the distal regions of the arm for long but often return immediately to the central mass. Momentary dispersion may be elicited by almost any agent and occurs most often when the granules have been very tightly concentrated by a high dose of adrenalin (1×10^{-3} M). Such treatment with adrenalin does not impair the viability of the melanocytes, and they will re-disperse if thoroughly rinsed.

Momentary dispersion occurs in the following manner: A tightly packed column of granules in the vicinity of the granule front begins to move in the distal direction. The granules lose contact with one another as they move. The central-most granules of the column cease moving first. The distal-most granules of the column may travel 3/4 of the way down the arms. The intermediate granules become spaced between these two extreme granules and oscillate back and forth with a movement that suggests they reside in definite places on an elastic which is stretched taut. The position-time relation of granules in a single column undergoing this type of dispersion is shown in Fig. 40. These tracings were made of momentary dispersion in cultured cells. Vertical lines connect granules in the same column. Horizontal lines connect the positions taken by a single granule at successive intervals of time.

3. Comparison of Concentrated and Dispersed States.

Movements of the granules. The characteristics of granule movements are different in the two cell states. In dispersed cells, the granules are scattered through the cytoplasm and often are separated from one another by considerable distances. They exhibit continual shuttling motions. In concentrated cells, the granules are located in the central re-

gions of the cell and are very tightly packed together. No shuttling motions are visible. The granules may exhibit a slow, unidirectional motion, or movement may cease altogether.

Physical properties of the two cell states. There are differences in the physical properties of concentrated and dispersed cells. Concentrated cells are less elastic and more easily broken by a very slight pressure on the coverslip than are dispersed cells.

If concentrated cells are cut with a microneedle, the granules remain fixed in place, and the cut is clean; when dispersed cells are cut, the cytoplasm invariably clings to the needle, and streams of granules pour into the medium.

4. Summary of Differences between Concentration and Dispersion.

It is clear that the processes of concentration and dispersion are by no means mirror images of each other. The major differences are summarized below:

	CONCENTRATION	DISPERSION
1.	Begins at the distal ends of the arms.	First evident at the distal part of the concentrated mass, but may not actually begin here. In cultured cells, impulses to disperse seem to arise deep within the mass.
2.	Fast: 30-60 seconds.	Slow by most agents.
3.	No shuttling motions.	Shuttling motions always present.
4.	Arms narrow towards a mid-line.	Arms flatten.
5.	Can be maintained for some time.	Cannot be maintained and usually reverts to a semi-dispersed state.

V. INTERPRETATION

The subject of this thesis has been a single cell--the melanocyte of Fundulus heteroclitus. In the preceding chapters, an account has been given of the form and fine structure of the cell; the movements of granules in living cells--both the localized shuttling motions and the mass motions of granules in response to hormones--have been described. The problem of this thesis is to explain, on the basis of this information, the intracellular migration of granules. Such an explanation entails a description of the modus operandi of a granule-moving system--that set of cell parts which move the granules, and how they function.

A. THEORIES OF PREVIOUS WORKERS

Several theories of the granule-moving system have been proposed in the past on the basis of diverse kinds of information. The granules have been postulated to be in a 'net' which contracted and expanded to move them by Zimmermann (1893) ((and later by Schmidt (1919) and Biedermann (1892))), who observed a dense, fibrous cytoplasm associated with the granules in histological preparations. Biedermann further proposed that the 'net' was attached to the centrioles which were in some way essential to the contraction. This theory of the 'net' was subsequently elaborated by Franz (1939-40), who observed rod-like structures in both fixed and living cells. He postulated that there were two sets of these rods--an exoskeletal set which remained surface-associated and an endoskeletal set which, by 'melting' and reforming, moved the granules (see p. 71).

Cell fine structure has been previously studied by Falk and Rhodin (1957), who postulated that the granules are contained in an intracellular sac which is moved by the contraction of a surrounding ring of fibres.*

The movements of granules in living cells were observed by Ballowitz (1914) who noted their columnar arrangement and their peculiar 'start-stop' motions (shuttling motions). He proposed that the granules are confined in channels and are moved by peristalsis of the channel walls.**

---

* Criticism of this work is found on p. 27.

** Ballowitz made a time-lapse film of granule movements in 1914,

An electrophoretic theory of granule movement has been proposed by a Japanese worker (Kinosita, 1953 and 1963). On the basis of his observations that the granules are negatively charged and that there exists an electrical gradient between the central region and the periphery of the cell--the sign of which is reversed in the concentrated and dispersed states--he postulated that the granules are in a relatively structureless cytoplasm and are moved by an electrical field interacting with their surface charges.

The final major theory is the sol-gel theory of migrations--the postulate that the granule-moving system of the cell consists of parts of colloidal dimensions, the granules being moved by a clumping or dispersion of these parts (a 'reversible coagulation such as commonly occurs in emulsoids') (Spaeth, 1913). The sol-gel theory is based entirely upon observed differences in the physical properties of the two cell states--the cytoplasm of concentrated cells resembles a gel, that of dispersed cells is more fluid. Such differences were first suggested by the fact that the granules of concentrated cells appear to be highly constrained and often motionless, whereas the granules of dispersed cells move freely. Later it was shown that concentrated cells are less elastic than dispersed cells (Spaeth, 1916); also, the cytoplasm of concentrated cells imposes greater resistance to the passage of a microneedle sideways than does that of dispersed cells (Matthews, 1931); furthermore, granules of concentrated cells cannot be displaced by high-speed centrifugation, whereas granules of dispersed cells can be displaced (Marsland, 1944). Thus, the cell resembled a classical colloid undergoing, in response to hormones, reversible coagulation, and the sol-gel theory was proposed on the basis of this analogy.

The theories reviewed above will be assessed later in the light of observations reported in this thesis--both the movements of granules and the cell fine structure.

B. GRANULE-MOVING SYSTEM

The movements of granules in living cells are interpreted in this section, and a theory of the granule-moving system is proposed. The parts

---

just a few years after the invention of moving pictures. This film was shown at an international congress held that year where, he reports, although the time-lapse interval was too great to permit a detailed analysis of the movements of granules, nevertheless the film was received with such great acclaim that it had to be shown twice. Selected photographs from the films are included in his 1914 paper.

of the system are ascertained in part 1 below--the granules are on a structured continuum which expands and contracts to move them. The dynamics of the system are described in part 2--the distribution of granules within the cell is the resultant of a dynamic equilibrium between concentrative and dispersive forces manifested locally in the shuttling motions (2a); the energetics of the process, and certain characteristics of the pertubations of state, are discussed briefly (2b).

1. Parts of the Granule-moving System.

a. Evidence for Structured Continuum.

No features of the cytoplasm of living cells are visible except the black markers. Yet the movement of these may give considerable information concerning the mechanism of their movement.

The first question then is whether the granules propel themselves or whether they are passively moved. The simplest observations of melanocytes indicate that the movements of granules are passive rather than active. For example, in no way do these spherical bodies resemble mitochondria which move like inchworms along a substratum. Furthermore, their lateral excursions indicate that they are not being ratcheted along a cell structure. Neither do the granules move freely through the cytoplasm, but in resting cells are confined by cellular structures to small regions; it is unlikely that the granules could propel themselves to the centre through this structured cytoplasm at the uniform rates often observed. These uniform rates, and the fact that the relative distances between granules remain constant, also indicate that concentration and dispersion do not result from an aggregation or repulsion of granules.

The granules appear, then, to be moved passively. That they are moved by electrical forces (Kinosita, 1953 and 1963) seems unlikely. Although the gradients observed by this worker--of the order of 14 mv.--are of sufficient magnitude to move the granules at the rates recorded in an aqueous medium (30-60 microns in approximately 30 secs.), that the interior of the cell is an aqueous medium seems unlikely. Electron microscopy indicates that the granules are completely immersed in cell structure that might be expected to greatly retard their movements.*

---

* There are other observations which seem not explicable by an electrophoretic theory: First, the granules are set in motion by a recruit-

The granules appear, then, to be carried in a moving cytoplasm. There are two alternative mechanisms for setting the cytoplasm in motion. Either external forces are applied or else the forces arise internally due to some change in the cytoplasmic interactions.

Several types of externally applied forces will be considered briefly. That the granules are moved by external forces is suggested by the uniform rate of granule movement in the distal regions, a uniformity which implies that the granules are being carried in a moving fluid. Peristalsis of the long arms of *in situ* cells might be suggested as a likely mechanism. However, in concentrating cultured cells, the boundaries of which are visible, peristalsis has never been observed. Moreover, both the bizarre shapes of cultured cells (see Appendix) and the fact that spherical cells dissociated from the scale and suspended in culture medium will undergo concentration, would seem to discredit this theory. Peristalsis of the channel walls (proposed by Ballowitz, 1914) would not account for the uniform flow observed in all channels.** Another possibility is that the peripheral cytoplasm gels, extruding the granules into the centre. This theory was suggested by Haurowitz (see Waring, 1963, p. 205) who thought the granules which remained stranded at the distal edges of the cell had been trapped in the gelling cytoplasm. But against this theory is the observation that such stranded granules, if observed for some time, eventually migrate into the central regions of the cell.

---

ment wave travelling at the order of diffusion velocities--10 microns/sec.; if the granules were moved by electrical forces, the onset of migrations should be simultaneous throughout the cell. Secondly, arms of cultured cells can be severed from the main cell body and oriented more than 90° away from their original position; granule migration continues in such severed arms, indicating that the gradient along which they move does not involve the centre of the cell but persists in severed pieces. (All dissections made by the Japanese worker were performed on *in situ* preparations which do not admit of this observation.)

** There is a theoretical objection to peristalsis as a mechanism for moving the granules. The granules draw close together as they concentrate, and in cultured cells, material can be seen to be extruded distally. There is a question as to whether peristalsis--a process observed in large organs, can cause the separation of granules from cytoplasm in these small dimensions of only a few microns. In spaces of such dimensions, the properties of fluids are quite different from what they are in large volumes--the Reynolds number is small and the viscous drag on the particle would be too great to permit separation of particles and fluids by contraction of the channel walls.

Nor do there appear to originate pressure differences due to changes in the central regions of the cell. Two possibilities come to mind. For example, if the central regions suddenly expanded, sucking the granules in, the first granules to move would be those in the basal regions of the arms; yet this has not been observed. Neither do the granules move to the centre to replace some extruded material since little material is extruded; a great deal of cytoplasm moves with the granules into the central region which humps up to accommodate it.

Since no externally applied forces can account for granule movement, the remaining possibility is that the forces arise within the granule-bearing cytoplasm; i.e., the movement of granules occurs because of changes in the internal interactions of the cytoplasm. In fact, all the observations can be interpreted by assuming that the granules are in a structured continuum which moves them by contraction and expansion.

Many observations indicate that the granule-bearing cytoplasm is highly structured. In the first place, despite the appearance of flow in the distal arms, the granules are not moving in a fluid. There is no velocity profile across the arms and no alteration of rate at places of bifurcation; moreover, granules move more rapidly through wide, uncongested portions of the arms than through the narrow parts; close inspection reveals that within the moving columns some granules stand still, other move backwards. In addition, confinement to local regions indicates considerable structure in the cytoplasm. Certain features of the movement attest to actual mechanical interconnections--co-ordinated movements among granules separated by some distances is often observed in cultured cells; a small group of granules moving together during momentary dispersion appear to be connected; the movements of distal granules, suggesting the 'uncoiling of a rope', attests to some pull exerted on the distal granules by means of a mechanical connection. The observations above have been interpreted to indicate that the granules are in a structured continuum.

Evidence that this continuum contracts* to move the granules is of two kinds. The first is evidence of a change in the interactions of the

---

* By 'contracts' is understood shortening or drawing together of the parts.

cytoplasm which contains the granules: The granules become tightly packed together during concentration and their movements highly constrained; in addition, there is a change in the physical properties of the cell--the cytoplasm of concentrated cells is more fluid in character. Also in support of the theory that the continuum contracts is evidence for tensions exerted by the continuum on the cell envelope--the arms narrow towards the mid-line, small hand-holds pull up, and the biradial symmetry increases. In addition, granules stranded at the distal edges of concentrated cells behave as if under constant tension from the central part and move into this region over time.

Dispersion, like concentration, is also due to forces arising within the continuum. In the first place, no major changes in cell shape precede dispersion. In the second place, dispersion appears to be forceful; e.g., a stellate cell may flatten into a disc during the process. Thirdly, these forces appear to originate deep within the mass since the granules in the central part of the mass can be seen to be in violent motion, whereas those in the front region may be motionless.

Summary. Characteristics of the movements of granules have made it possible not only to rule unlikely many theories of granule movement, but also to cite considerable positive evidence that the forces to move the granules arise within the cytoplasm which contains them.

The granule-moving system appears to be a structured continuum which expands and contracts in response to hormones.

b. Relations of Continuum to Other Cell Parts.

The relations of the continuum with other cell parts have been determined by observations of living cells:

The bulk of the continuum is in the central region of the cell. At its centre is a clear, gelled area containing the cell centrioles. The continuum of concentrated cells retains a vast number of attachments to the periphery as evidenced by tensions exerted on the cell envelope. Attachment of the continuum to the centre is not a requirement for its ability to respond to hormones (contrary to Biedermann, 1892) since granules in severed arms still migrate.

The continuum comprises, in addition to the granules, a considerable amount of cell cytoplasm, evidenced both by the collapse of arms

and by the humping up of the central region. A very small amount of material is extruded distally.* Neither the nuclei nor the mitochondria are part of the continuum since the nuclei are passively pushed aside by the granules and the mitochondria remain stranded in the distal regions of the arms. Whether or not the partitions (inferred from the alignment of granules, Chapter III) are a part of the continuum is not known.

Volume changes have not been studied.

The envelope of the cultured cell retains its stellate form during granule movements. Striking changes of cell form subsequently occur as a function of the state of the continuum; the contracted continuum exerts tension on the periphery as a result of which the cell arms narrow and small attachments pull up; the dispersing mass, on the other hand, exerts pressure on the envelope and dendritic cells flatten and spread into discoid forms.

2. Dynamics of the Granule-moving System.

a. State of the System.

The discrete granules have been shown, in the previous section, to be fixed like beads in a structured continuum of whose activity they are the passive markers. A question immediately arises concerning the significance of the shuttling motions.

The evidence is that the shuttling motions are caused by the same forces which cause the mass motions, i.e., are the manifestation of local contractions and expansions of the continuum.

In Chapter IV, three types of motions were described--shuttling, pulsation, and mass motions. There is some difficulty in distinguishing between these three types of motion since there appears to be a continual transition between them. Shuttling motions are greatly affected by net concentrating and dispersing tendencies. For example, if a net-dispersing tendency is superimposed upon the shuttling motions, the granules remain at the distal end of the distally-directed path for some time. Also, as concentration begins, the shuttling motions become rapid before giving way to continuous movements. Pulsations are similarly affected. All these

---

* By electron microscopy this material has been shown to consist of at least the membrane-bounded compartments.

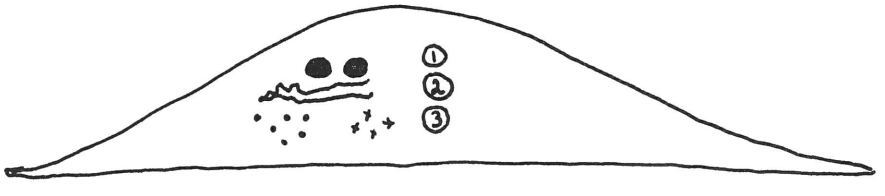
motions have similar characteristics--the proximally-directed path is traversed more rapidly than the distally-directed path. The local movements of the granules are thus a small mirror image of the mass motions, and indicate perpetual, localized changes in the properties of the continuum.

As a result of the above interpretation, a more complete definition of the granule-moving system may be given: The granule-moving system is a structured continuum whose state, represented spatially in the distribution of granules, is a function of a dynamic equilibrium between concentrative and dispersive forces, manifested locally in the shuttling motions. The equilibrium between forces may be perturbed by hormones as a result of which the system passes to a new stable state, this transition being marked by the mass motions.

b. Perturbations of State.

Two problems now arise concerning the mechanical work performed by the granule-moving system. The first is to ascertain the energy requirements of the concentrative and dispersive processes; the second concerns the triggering of these processes by hormones. In the preceding sections, the movements of granules have given information about the parts of the system and about the dynamic state of the system. It is suggested that they may also yield information about these two problems.

At the outset, the full extent of the granule-moving system may be diagrammatically represented at its several levels. The granules



are the only visible markers of the cell cytoplasm (1). Their movements are passive, the manifestations of activities of the cell structures in which they are embedded (2). The activities of these structures are themselves a function of molecular events occurring within the structures (3).

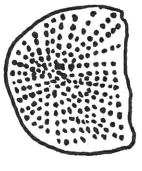
The state of the granule-moving system is ultimately a func-

tion of chemical equilibria involving the molecular structures of the parts and certain molecular species in their environment. Concentrative and dispersive forces arise as the result of perturbations of these chemical equilibria. The movements of granules may therefore give information about events occurring in the continuum at two structural levels. Whereas, certain features indicate properties of the continuum, other features give information about molecular events occurring within the continuum.

Energetics. The characteristics of granule traverse suggest that dispersion may be an energy-storage process and concentration, energy release: The distally-directed path of both shuttling and mass motions is traversed more slowly than the proximally-directed path. In addition, dispersion is difficult to elicit, can always be overcome by concentrating hormones, and the dispersed state is difficult to maintain. Concentration on the other hand is a rapid process elicited readily by many agents and very difficult to abolish; the concentrated state may be maintained for several hours. The structured continuum may be compared to an elastic. It is stretched during dispersion; concentrating hormones release it and it contracts due to entropic forces.

Perturbation of State. The shuttling motions indicate that there exist continual fluctuations in the chemical equilibrium determining the state of the parts. Related movements of granules, and the rate of movement, give information about the extent and magnitude of these perturbations.

Independence of granule motions--the fact that one granule in a channel may move while adjacent granules remain motionless--indicates that these perturbations may be highly localized.



Waves of perturbations may often be seen in the field of granules. Waves of successive distally-directed movement are occasionally seen to pass along columns from the centre to the periphery. The rate of granule traverse--affected markedly by net tendencies superimposed upon the cell--must manifest the strength of the forces. Total period is shortest when cells with strong dispersing tendencies are administered concentrating stimuli.

The mass motions are the result of perturbations of the state of the system by hormones with resulting passage of the system to a new stable state. The problems of hormone action are several--which chemicals enter the system from the environment, where they enter it, and where changes in the properties of the continuum are first initiated.

Contraction of the expanded continuum appears to be initiated at the distal tips of the arms. Some stimulus enters the cell at this point and travels through the continuum at a rate which is of the order of diffusion velocities for small molecules. The granules recede down the long arms, exhibiting a linear granule front as they crowd into the proximal regions. This linearity can be explained as a function of concentrative forces entering the continuum at its distal edge, and the resistance of the crowded granules in the proximal regions to translocation of the front. Dispersion, regardless of where stimuli enter the cell, appears to be initiated deep within the granule mass; the granules at the distal edge of the mass may be motionless and the entire mass moved out by impulses arising within it.

As for the question of what molecules enter the cell to cause these processes, the fact that so many agents elicit one or the other suggests that they may not all enter the system, but rather, affect cell permeability to certain ions.*

c. Discussion of Electron Microscopy.

The granule-moving system and its activities have been described at the level of resolution of the light microscope. This description constitutes a framework within which the system may be investigated at finer levels of resolution. A full description of the granule-moving system entails specification of the events at several levels: The parts of the continuum, and the work they do in moving the granules; the chemical equilibrium upon which the state of the parts depends; and how this equilibrium is perturbed by hormones. The first problem is to ascertain the parts of the granule-moving system.

---

* Dispersion may be elicited by MSH, acetylcholine, acidic medium, hydrostatic pressure. Concentration may be elicited by adrenalin, alkaline medium, heat, and many other agents (see Lerner and Takahashi, 1956; Fingerman, 1965).

i. Fine structure of the continuum. The fine structure of the continuum may be sought in the central region of concentrated cells where it would be expected to have contracted with the granules. In the centre of these cells lie the centrioles. Among the granules which are tightly packed around these bodies occur several tubular structures: the microtubules, the smooth endoplasmic reticulum, and some fibrils. In addition to these parts which constitute the resolvable structures of the continuum, there is an electron-light matrix--a part of the cell not resolved into structure.*

Three previous theories of the parts of the continuum have been proposed. The sol-gel theory is a postulate that the continuum consists of parts of colloidal dimensions, interacting by surface charges. Studies by electron microscopy indicate that this theory is an inadequate theory of cell structure, since it does not take account of the high degree of differentiation of the parts. Neither is there any reason to assume that the work done is by surface interactions, since these tubular compartments might instead individually extend and coil. The sol-gel theory is, moreover, based on inference from demonstrated differences in the physical properties of concentrated and dispersed cells. But such differences in physical properties would arise under conditions of increased interactions of any part, and are not evidence for any particular type of fine structure. Two previous workers have proposed theories of the structure of the continuum on the basis of direct examination of cells by light microscopy. Zimmermann observed a 'fibrous network', a network subsequently shown by Franz to consist primarily of rod-like structures. Studies by electron microscopy corroborate these findings and indicate that these rod-like structures are microtubules.

ii. Granule-moving apparatus. Within the parts of the continuum mentioned above arises the force to move the granules. Some parts may perform the mechanical work of moving the granules, others may limit the

---

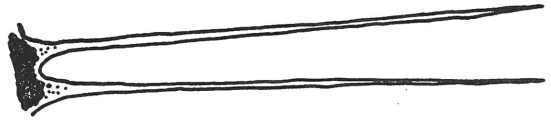
* Several structures are not a part of the granule-moving apparatus. The large, membrane-bounded compartments which may be seen among the granules in dispersed cells prove to be extruded from the mass in concentrated cells; whether these large compartments are part of a more extensive system of membranous channels which are part of the continuum is not known. Mitochondria remain in the distal regions of the arms. One group of surface-associated tubules does not appear to participate in granule movement.

path of the shuttling motions, still others may bind the structures together.

The question is: Do the visible structures do the work, or is it done by the invisible structures? There appear to be two possibilities for the granule-moving system, neither of which can be distinguished between on the basis of the electron microscopy presented in this thesis. The first possibility is that the electron-light matrix is the source of granule movement, the parts being perhaps skeletal elements or passive differentiated regions within the matrix. The second possibility is that some of the prominent cell parts--the microtubules, or the smooth endoplasmic reticulum--are the granule-moving apparatus, the matrix perhaps functioning as a lubricating fluid. These parts might extend and coil or retract to move the granules.*

Electron micrographs give no basis for distinguishing among these possibilities. No attachments between granules and any of these structures have been found; no structures exhibit any change in dimensions which might indicate their role in moving the granules. Although no discrimination between mechanisms is possible, nevertheless, evidence for and against several mechanisms will be considered briefly in the light of boundary conditions set by living cells and studies by other workers.

Three major requirements to be fulfilled by any structure are established by consideration of the living cell; the first is a function of the shape of the cell--the granules must be pushed out into long, thin, collapsed arms for distances of 60 microns during dispersion and pulled back through these long arms during concentration. Both these processes appear to occur by strong forces.



In the second place the structures must be able to cause pulsation of the entire mass of granules as a whole. Thirdly, despite the capacity

---

* A third possibility is some complex mechanism--granules moved in by one structure and moved out by another.

of this structure to cause the entire mass to pulsate, small segments of the structure must be extremely extensible; during momentary dispersion, a few granules in a column which appear to be joined together by a connecting substrate move out together and become spaced apart over distances of up to 30 microns; in addition, characteristics of the shuttling motions require the same property--a single granule within a column may make an excursion of up to 4 microns in length, the granules immediately adjacent remaining motionless. Other requirements will be mentioned in the following text.

That the matrix is the major granule-moving system of the cell does not seem likely. There is very little of it in comparison with the large number of structures which fill the cell, and it seems unlikely that it could do the work to move the granules across these long distances.*

The more probable theory is that the cell structures--perhaps the endoplasmic reticulum or the microtubules--constitute the granule-moving apparatus. Microtubules have recently attracted considerable attention. Electron microscopists have discovered them in a variety of tissues, fixed with glutaraldehyde, and have attempted to demonstrate a mechanical function for them, as yet without great success.**

---

* Fine structure of the matrix is not known. However, the matrix might be able to do the work if it consisted of densely packed, highly distensible structures. No mere swelling of the entire cytoplasm will account for dispersion since both concentrated and dispersed states may be swollen with no major displacement of granules; in addition, certain types of changes between the fluid and solid state do not result in granule movement--acidic medium (pH 5.0), for example, gels the cell (reversibly) with no net displacement of granules.

** Microtubules have recently been found in many tissues of both plant and animal origin when fixed in glutaraldehyde (Ledbetter and Porter, 1963). No clear function can be ascribed to them. Their lack of bending and their occurrence in the marginal band of red blood cells (Fawcett, 1962) suggest a skeletal function for them. Of greatest interest has been the fact that they occur in many motile systems--the spindle apparatus (Mazia et al., 1961; Kane, 1962; Roth and Daniels, 1962; Harris, 1961), the flagella of many animals (Gibbons et al., 1960; Pease, 1963), cilia (Gibbons, 1961), the microspikes of cultured cells (Taylor, 1965), and nematocysts of hydra (Slautterback, 1963). These structures, of the same form in all instances, show some variations in diameter (150-280 Å). They appear to be composed of a series of filaments arranged in parallel (Porter, 1964; Pease, 1963; Watson et al., 1964).

There have been several attempts to implicate the tubules in

The microtubules may well be the granule-moving system of melanocytes. They are among the most prominent features of the cell, appear in large numbers among the granules, and although no dimension changes can be seen during concentration, nevertheless, some loss of alignment seems evident. Franz (1939-40) previously postulated that they are, in fact, the granule-moving system of the cell, 'melting' to move the granules to the centre and reforming to move them back into the distal regions. He obtained considerable evidence that a change in the properties of these structures does occur. In fixed cells, whereas the rods of dispersed cells were straight and extended to the cell boundaries, those of concentrated cells were wavy, darkly-staining, and extended only to the border of the granule mass. In living cells the columns of granules of dispersed cells became wavy in appearance and lost alignment as the granules commenced centreward migration. The microtubules, therefore, seem a likely candidate to move the granules.

There are several objections which can be advanced against tub-

---

the performance of mechanical work. The rationale of studies on non-muscular, motile systems consists in demonstrating that they possess some of the properties of muscle--that glycerol-extracted preparations continue to perform mechanical work upon the addition of ATP (after Szent-Gyorgi, 1949), and that the system possesses ATPase activity, or myosin-and actin-like proteins. These properties have been demonstrated for many of the systems mentioned. Thus, glycerol-extracted spindles exhibit chromosome migration in response to added ATP (Hofmann-Berling, 1954). ATPase activity has been demonstrated in the spindle (Mazia et al., 1961; Hartmann, 1964). Glycerol-extracted flagella beat with a slow, undulating motion, the frequency of which varies with the ATP added (Brokaw, 1961). ATPase activity in flagella has been demonstrated (Tibbs, 1959; Daems et al., 1963) and actin-and myosin-like components have been shown in this structure (Bishop, 1958). ATPase activity has been demonstrated in cilia (Lansing et al., 1961) and recently, glycerol-extracted preparations have been made (Gibbons, 1963).

So far, in none of these systems have the 'muscle-like' properties been shown to reside in the microtubules. However, one recent study of Gibbons has implicated them (1963). He finally accomplished glycerol-extraction in cilia. His preparations exhibited both intact arms (small structures bordering the microtubules) and ATPase activity. If the divalent cations were removed by dialysis both the arms of the preparation went into solution and the ATPase activity was lost. When the dialysate was returned, the arms and the ATPase activity returned. ATPase activity appeared associated with the arms of the tubules. This is the only study to date which has at all implicated the tubules.

ules as a possible mechanism. That the columns of granules become wavy at the beginning of concentration has not been observed during present studies. The loss of alignment which occurs as the granules enter the proximal region of the arms occurs as the depth of the arms increases, and may be explained as an escape of granules from constraining tubules near the cell surface.* In addition, behavior of microtubules can be observed in another cell structure in which they occur--the microspikes of cultured cells (Taylor, 1965). Although these structures do exhibit mechanical changes--appearing to break in several places and then falling back into the membrane--no change in their behavior occurs during concentration. The last major question is whether these tubules could fulfill the requirements presented on pages 69-70. Structures of the lengths which these tubules appear to be would have to undergo a considerable change of properties as they extended to move the granules out and coiled or collapsed to bring them into the centre. This change of properties should be evidenced in a difference in the dimensions of the tubules in the two different states. But no dimension change is obvious at resolutions attained in this study. The observed lack of bending of microtubules suggests 'stiffness' and a possible function as skeletal supports--a function which this cell with its complex, stellate form may well require.

A third alternative is that the smooth endoplasmic reticulum might move the granules. Nothing is known about the mechanical functions of this structure in any tissue. Evidence that it might constitute the granule-moving apparatus is adduced solely from its appearance in electron micrographs taken with the requirements to be fulfilled (pp. 69-70). In many sections, segments of endoplasmic reticulum may entirely surround the granules; since these segments are only visible for short distances, it might be inferred that this tubular structure is flexible and in 3-dimensions coils around the granules and permeates the granule mass. In its structural features, the endoplasmic reticulum seems the most capable of any structure of fulfilling the requirements. In the first place, since it appears to permeate the mass of granules, it could cause pulsation of the entire mass. In addition, it may also fulfill the requirement for

---

* Whether Franz could distinguish between microtubules and endoplasmic reticulum on the basis of staining alone is not clear.

extreme extensibility since its coils--by extending--would permit a local movement of a single granule over considerable distance without perturbing adjacent granules. Moreover, extension of the coils of this structure, by stiffening or swelling all along their lengths, might well result in mass movement of the granules up the long arms.

In conclusion, the fine structure of the continuum has been determined by electron microscopy. Several alternatives for the granule-moving apparatus have been discussed. No decision can be made among them on the basis of present evidence.

CONCLUSION

SUMMARY

Color changes of animals are the result of an intracellular migration of pigment granules within single cells. These granule migrations have been studied in melanocytes of Fundulus heteroclitus, and on the basis of this work a theory of the granule-moving system has been proposed: The melanocyte granules are in a structured continuum which moves them by contraction and expansion; the distribution of granules is the outcome of a dynamic equilibrium between concentrative and dispersive forces, manifested locally in the shuttling motions; hormones perturb the steady-state with the resultant passage of the system to a new stable state, the transition being marked by the mass motions of granules.

Certain features of the motions suggest that dispersion is an energy-storing process, concentration, an energy-releasing process. Concentration is initiated at the distal tips of the arms, indicating perhaps that concentrating stimuli enter the cell at this place; dispersion originates deep within the continuum.

Fine structure of the continuum has been investigated by electron microscopy. Prominent in the cytoplasm are large numbers of microtubules. Several alternatives for the granule-moving apparatus are discussed. There is no way, on the basis of this work, to decide among them.

FUTURE WORK

Mechanics of the system. The movements of granules is an instance of a phenomenon of great interest to biologists--the transduction of chemical and electrical energy into intracellular mechanical work. No less interesting, perhaps, is triggering of these intracellular processes by a hormone arriving in the external environment of the cell.

Melanocytes possess several features which may make them interesting subjects in which to pursue both of these problems. The black granules, mirroring a continuum in which they are embedded, themselves form a field through which perturbations due to hormones pass in waves.

The movement of granules thus gives considerable information both about properties of the continuum and about molecular events occurring within it. The effects of many different agents on a variety of variables may be observed. The mechanical process marked by the movements of granules is slow enough to be observed by eye, unlike the contraction of muscle, which is rapid; yet the mechanical processes are not continuous, as are the movements of cilia and flagella, but the system exhibits two end states between which it can be made to pass at the will of the investigator.

Moreover, several practical considerations make the melanocyte an ideal subject in which to pursue future investigations; they may be easily dissociated from the fish scale and cultured on coverslips. Distinguished by their black pigment granules, they may be readily seen under the dissecting microscope and microdissected or studied with electrical techniques. If they are placed in culture chambers many features of the cell may be observed by phase microscopy and studied with time-lapse cinematography.

Three types of future investigations appear fruitful. The first two are an extension of the analysis presented in this thesis--the relative movements of granules should be analyzed in greater detail in cells which contain fewer granules;* high resolution electron microscopy, together with cytochemical techniques, might indicate dimension changes or otherwise implicate certain structures in the function of granule movement.

But the answer to many specific problems--the parts of the system, the energetics of the processes, the definition of the chemical equilibria which determine the state of the system--attend isolation of the granule-moving apparatus. In favor of this enterprise are the facts that specimens of Fundulus may be obtained in large numbers and melano-

---

* The great difficulty in this analysis has been that the number of granules is so great that no details of their movements can be observed. How to obtain cells with few granules was learned only recently; small, dendritic cells containing few granules are present at all times on the scale and occur in large numbers when fish are transferred to dark backgrounds. The granules of these cells concentrate in response to adrenalin, and their movements may be clearly observed in cultured preparations. One example has been included in this thesis (Fig. 3b).

cytes readily dissociated from the scales; the presence of black markers makes it possible to follow these cells through the manipulations of separation from other cells with the naked eye; this property also makes it possible to dissect individual cells on the coverslips. The difficulties of such an undertaking should not be underrated. The possibility of its success lies in the fact that the continuum may be contracted through these operations, and thereby stabilized to procedures which would disrupt the membranes; and furthermore, the granule-moving system remains intact in cells swollen in K-Eagle's medium and severely damaged.

Melanocyte as a granule-moving system. The melanocyte stands at the end of a long investigation into mechanisms of animal color changes. The natural course of future work is to explicate this problem further in terms of molecular mechanisms among cell structures. But there is another perhaps more interesting problem which arises, connected with the status of the melanocyte as an organism. That is the problem of describing the cell in terms of the integrated activities of its components, and specifying the changes in the patterns of its activity in relation to the granule movements. Even in these preliminary studies there has been evidence that certain cell activities differ in the two end states; the nuclei of dispersed cells were often found to be lobate in form, exhibiting many nuclear pores and surrounded by ribosomes, whereas nuclei of concentrated cells were more often ovoid, exhibiting few pores. In addition, small vesicles are found in dispersed cells but not in concentrated cells. It is possible that in specifying end-states of granule distribution, we have also specified two different metabolic states.

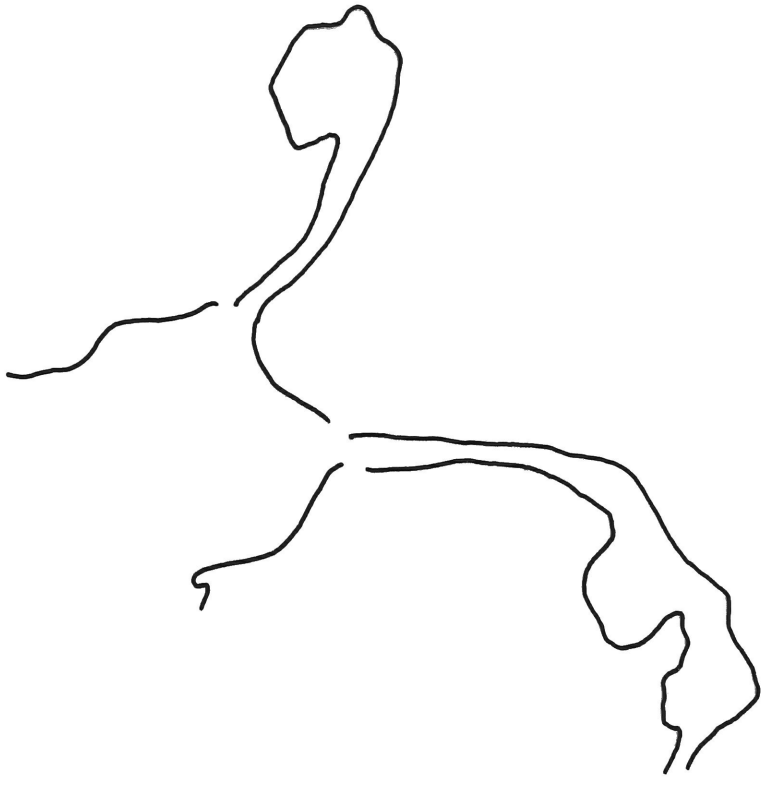
The form of description of the living cell presented in this thesis lends itself well to a description of the whole cell in terms of its activities. It would appear that the melanocyte exists in context in the fish for the sole reason of changing the color of the animal; when the fish needs to darken, melanocytes increase in number. Moreover, the granules, and the moving of these granules, are so characteristic a feature of this cell that the designation of the melanocyte as a 'granule-moving system' is not an arbitrary one. The activities of the granule-moving system may be recognized as a series of 'states' of the system, manifested spatially in the distribution of granules, determined by a balance of dispersive and concentrative forces. The activities of sub-systems within

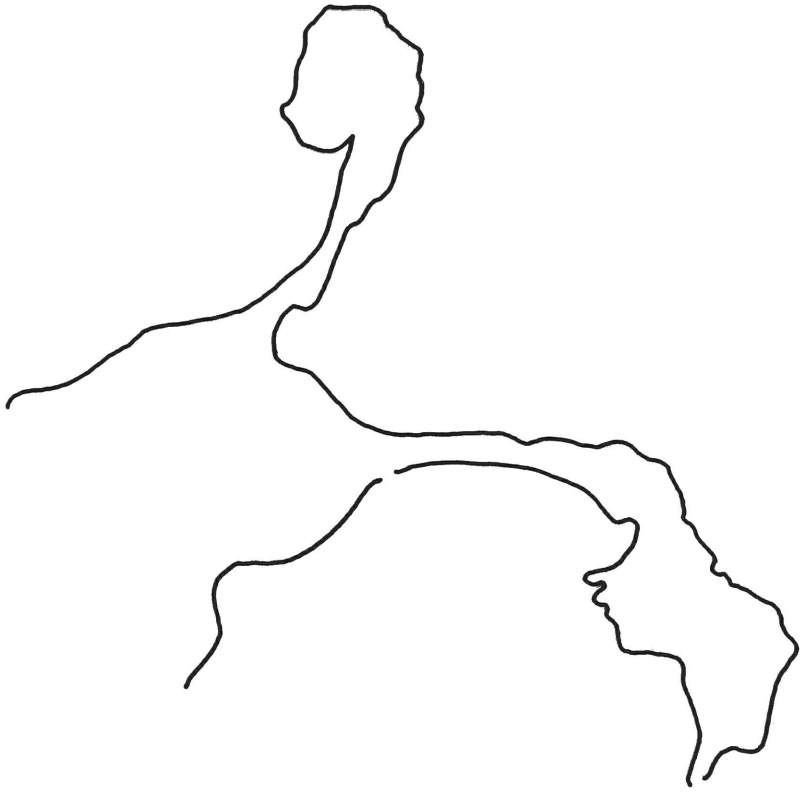
this major system--the cellular energy-producing systems, the maintance systems--would be related to the activities of the granule-moving apparatus. This form of description approximates the true state of an organism, which is the performance of constant activity by parts which are themselves continually breaking down and re-forming.

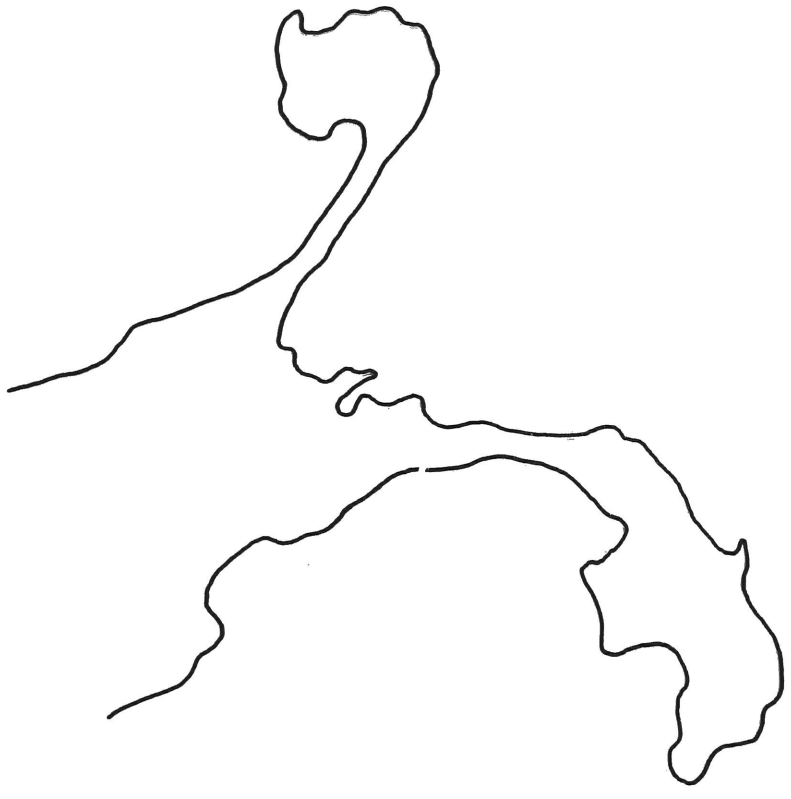
APPENDIX

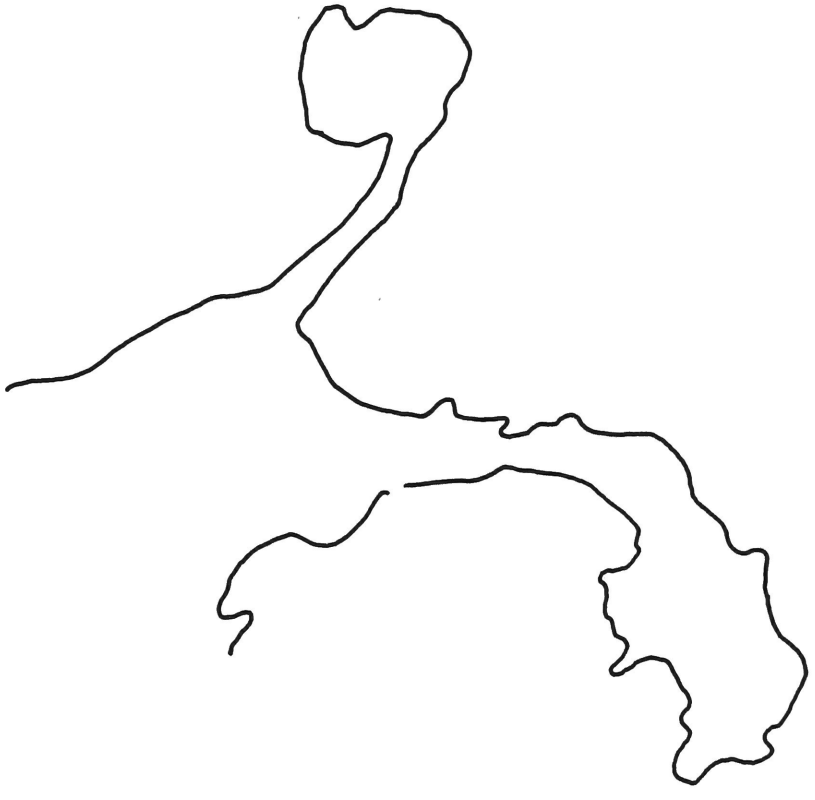
Overlaid tracings of successive changes in the shape of the granule mass in a highly frilled and flattened cultured cell undergoing contraction are included herein. The time course of the process recorded is approximately 20 seconds. These tracings convey better than any figures in this thesis the sense of the dynamics of the process. Tracing #1 is the resting cell; the distal edge of the granule mass is concomitant with the cell boundary; successive tracings show the crescent-shaped granule front. Tracing #13 is not the end of concentration, for finally the granule mass will take the shape of a sphere in the centre of the cell. If tracings #1 and #13 are superimposed, a shift in the position of the lateral boundary is evident and is interpreted as indicating tension exerted on the distal-most granules by the central part of the cell.

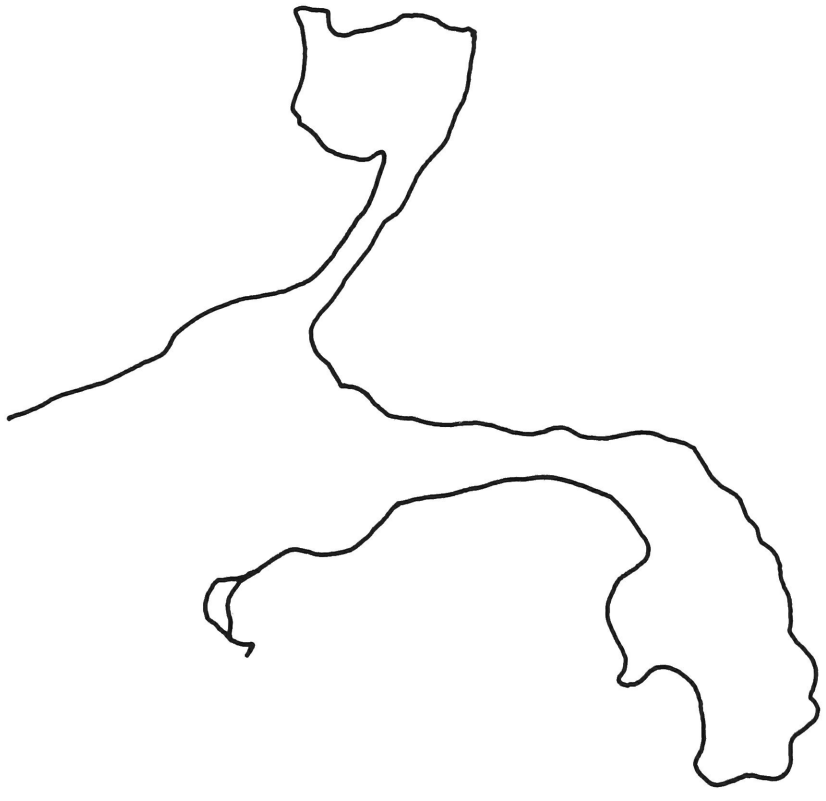
Changes in Shape
of the Distal Edge of the Granule Mass
during Process of Concentration

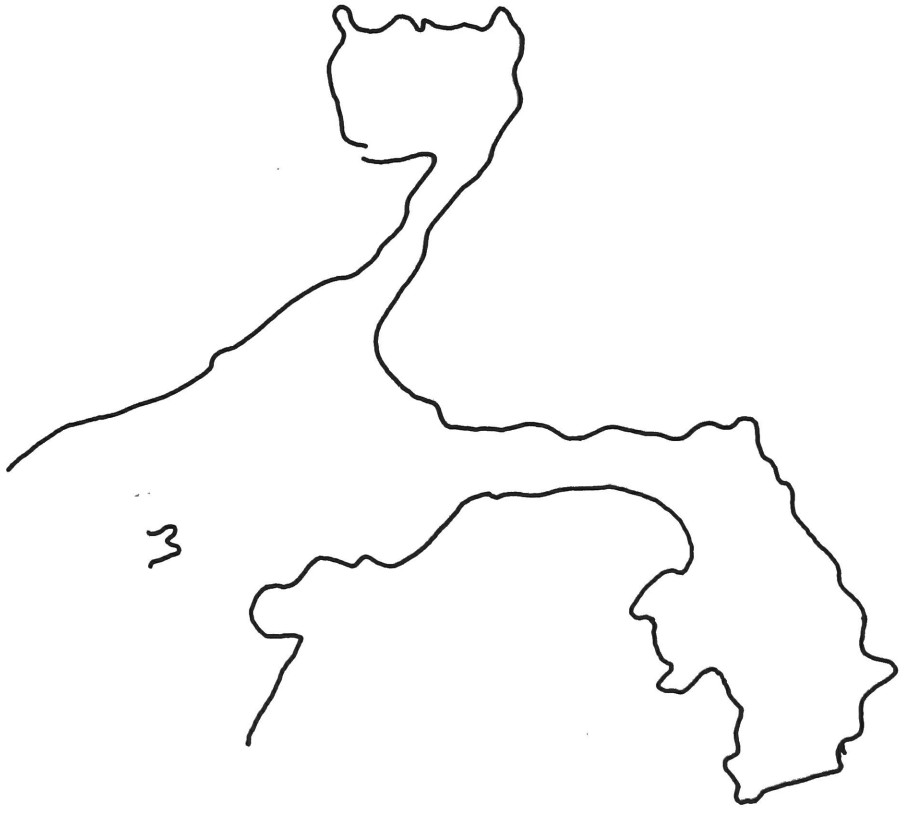


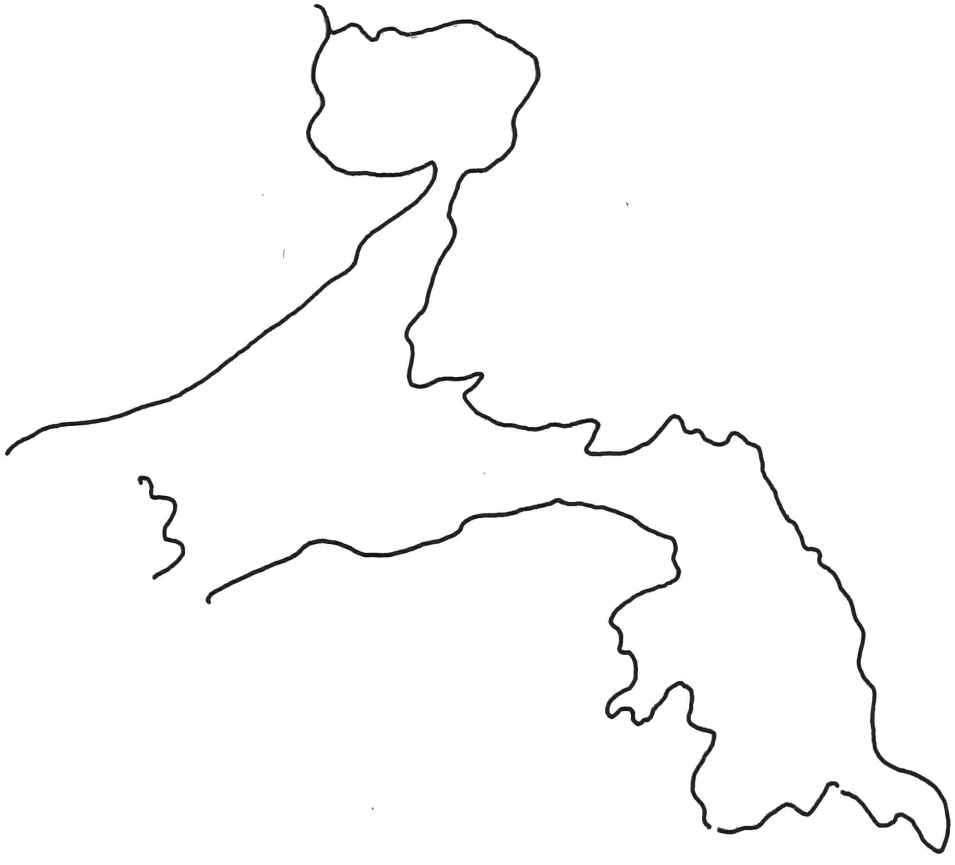


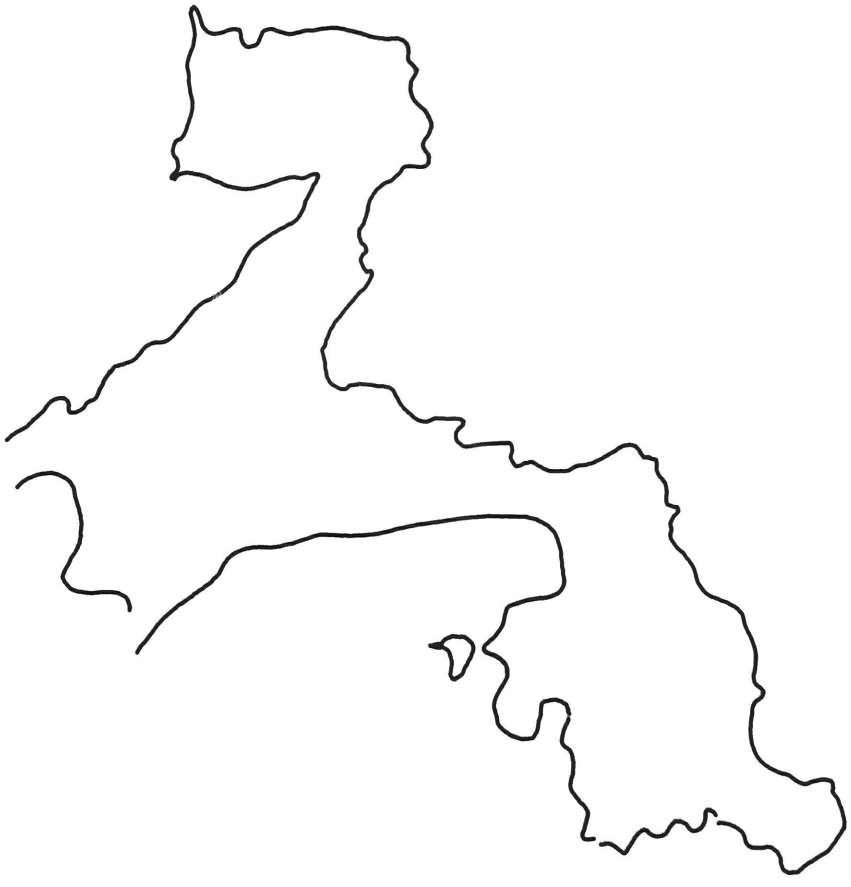




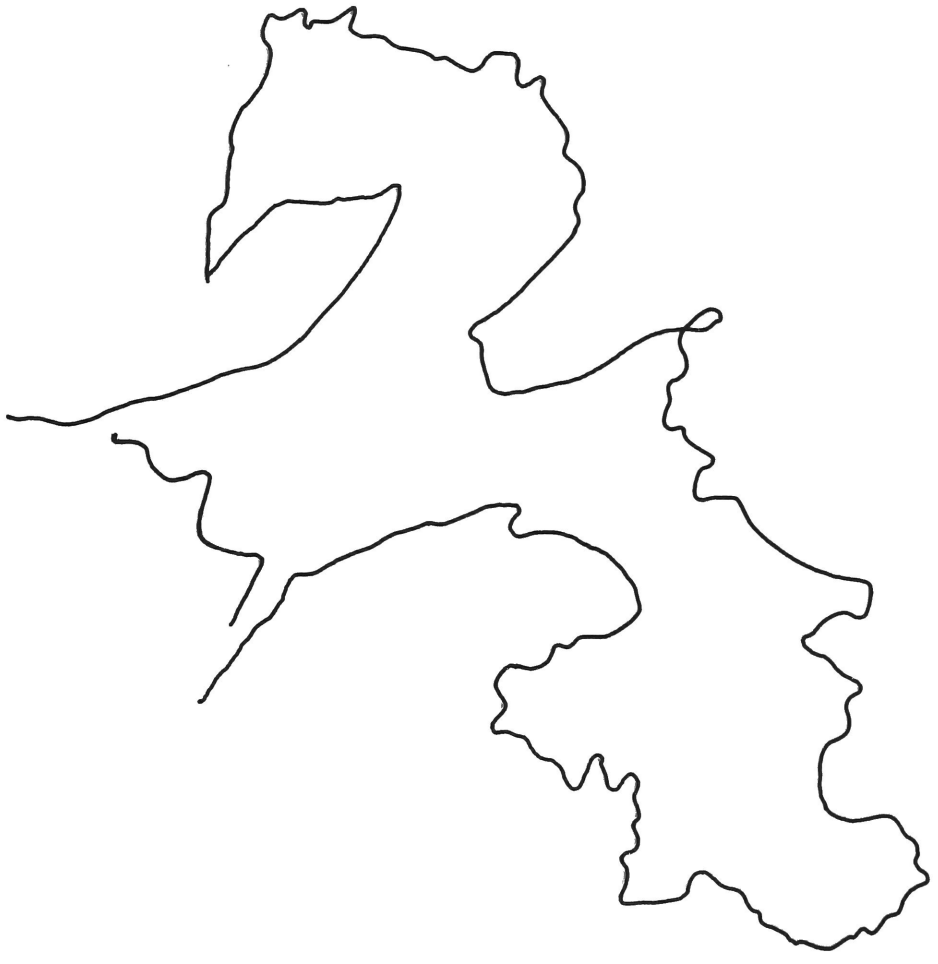


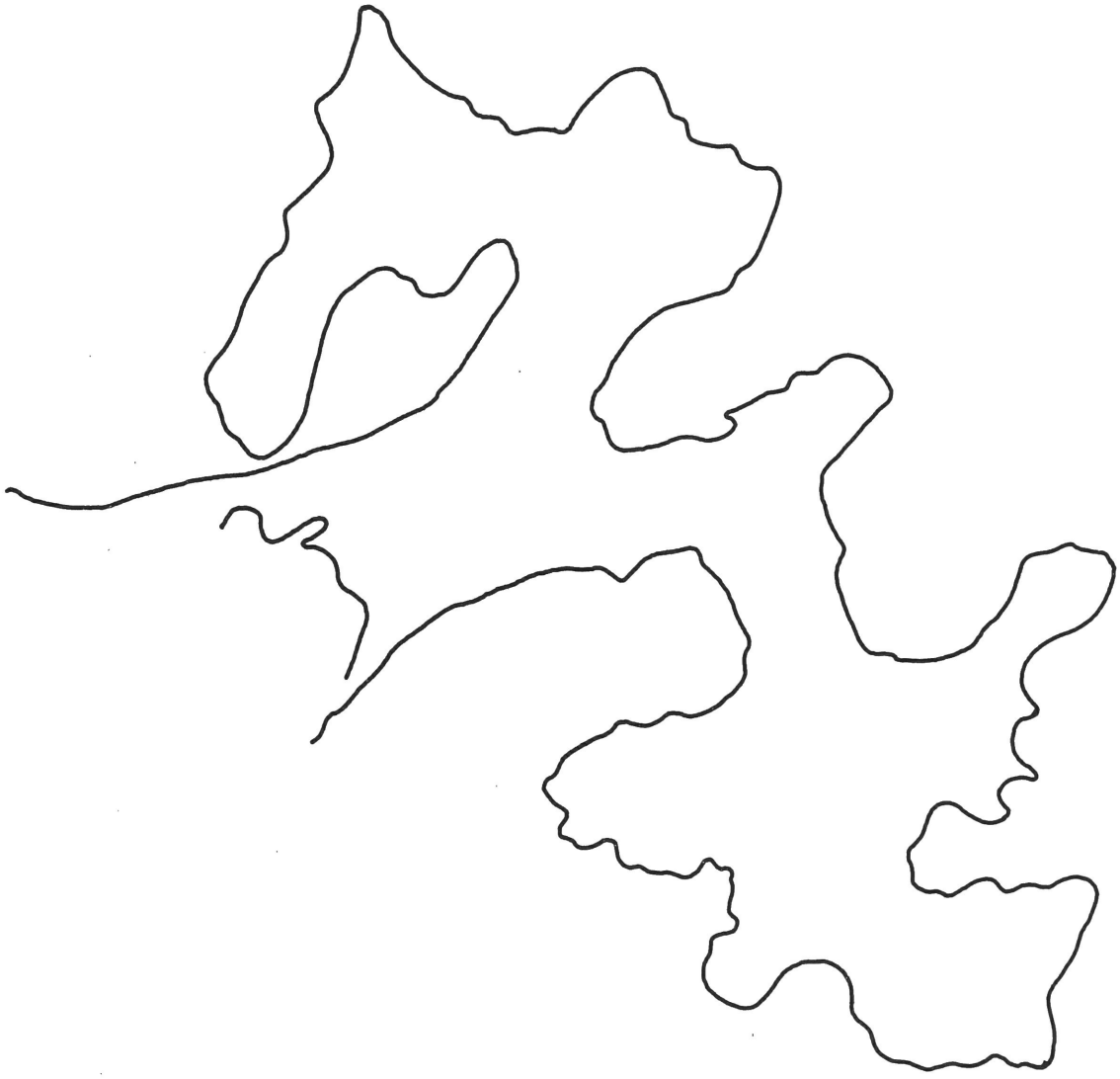

3





4





①

BIBLIOGRAPHY

Acherson, F. M. (1840) Ueber die Hautdrüsen der Frösche, Arch. Anat. Physiol. und wiss. Med., 15-23.

Ballowitz, E. (1893) Die Nervenendigungen der Pigmentzellen, ein Beitrag zur Kenntnis des Zusammenhanges der Endverzweigungen der Nerven mit dem Protoplasma der Zellen, Z. wiss. Zool., 56, 673-709.

Ballowitz, E. (1914) Ueber die Pigmentströmung in den Farbstoggzellen und die Kanälchenstruktur des Chromatophoren-Protoplasmas, Arch. ges. Physiol., 157, 165-210.

Biedermann, W. (1892) Ueber den Farbenwechsel der Frösche, Pflüger Arch. f. Physiol., 51, 455-508.

Bishop, D. W. (1958) Sperm contractile protein, Anat. Rec., 131, 533-534.

Bozler, E. (1928) Ueber die Tätigkeit der einzelnen glatten Muskelfaser bei der Kontraktion. II. Mitteilung: Die Chromatophorenmuskeln der Cephalopoden, Z. Vergl. Physiol., 7, 379-406.

Brokaw, C. J. (1961) Movement and nucleoside polyphosphatase activity of isolated flagella from Polytoma uvella, Exp. Cell Res., 22, 151-162.

Brücke, E. (1852) Untersuchungen ueber den Farbenwechsel des afrikanischen Chamäleons, Denkschr. Akad. Wiss. Wien, math.-nat. Cl., 4, 179-210.

Buchholz, R. (1863) Ueber die Mikropyle von Osmerus eperlanus, Arch. Anat. Physiol. und wiss. Med., 71-81.

Corona, A. e Moroni, A. (1898) Contributo allo studio dell' estratto di capsule surrenali, Riforma Med., 14, 433,448, 459.

Cuvier, G. (1817) Mémoire sur les Cephalopodes et sur leur anatomi, Mémoires pour servir à l'histoire et l'anatomie des Mollusques, Paris, Deterville, Mém. 1.

Daems, W. T., Persijn, J. P. and Tates, A. D. (1963) Fine structural localization of ATPase activity in mature sperm of Drosophilia melanogaster, Exp. Cell Res., 32, 163-167.

DuShane, G. P. (1944) The embryology of vertegrate pigment cells. Part I. Amphibia, Quart. Rev. Biol., 18, 109-127.

Falk, S. and Rhodin, J. (1957) Mechanism of migration within teleost melanophores, Proc. Stockholm Conference, New York, Academic Press, 213-215.

Fawcett, D. W. (1962) Physiologically significant specializations of the cell surface, Circulation, 26, 1105-1132.

Fingerman, M. (1965) Chromatophores, Physiol. Rev., #2, 45, 296-339.

Fox, D. L. (1953) Animal biochromes and structural colors, London, Cambridge University Press.

Franz, V. (1935-36) Struktur und Mechanismus der Melanophoren im Farbenkleid der Teleostier, Z. Zellforsch, und mikroskop. Anat., 23, 150-197.

Franz, V. (1939-40) Struktur und Mechanismus der Melanophoren, teil II: Das Endoskelett, Z. Zellforsch. und mikroskop. Anat., 30, 194-234.

Frisch, K. von (1910) Ueber die Beziehungen der Pigmentzellen in der Fischhaut zum sympathischen Nervensystem, In, Festschrift zum Sechzigsten Geburtstag Richard Hertwigs (München), 3, 15-28, Jena, Ferlag von Gustav Fischer.

Frohlich, A. (1910) Farbwechselreaktionen bei Palaemon, Arch. Entwicklungsmech Organ., 29, 432-438.

Fuchs, R. F. (1914) Der Farbenwechsel und die chromatische Hautfunktion der Tiere, In Winterstein, Handbuch der vergleichende Physiologie, 3, 1189-1656.

Gibbons, I. R. and Grimstone, A. V. (1960) On flagellar structure in certain flagellates, J. Biophys. and Biochem. Cytol., 7, 697-716.

Gibbons, I. R. (1961) The relationship between the fine structure and direction of beat in gill cilia of a lamellibranch mollusc, J. Biophys. and Biochem. Cytol., 11, 179-205.

Gibbons, I. R. (1963) Studies on the protein component of cilia from Tetrahymena pyriformis, Proc. Nat. Acad. Sci., 50, 1002-1010.

Gilson, A. A. (1926) Melanophores in developing and adult Fundulus, J. Exp. Zool., 45, 415-455.

Harris, P. (1961) Some structural and functional aspects of the mitotic apparatus in sea urchin embryos, J. Cell Biol., 14, 475-487.

Hartmann, J. R. (1964) Cytochemical localization of ATPase in the mitotic apparatus of Hela and sarcoma 180 tissue culture cells, J. Cell Biol., 23, 363-376.

Healey, E. G. (1951) The colour change of the minnow (Phoxinus laevis Ag.), J. Exp. Biol., 28, 298-319.

Hofmann-Berling, H. (1954) Die Bedeutung des Adenosintriphosphate für die Zellund Kernteilungsbewegungen in der Anaphase, Biochim. et Biophys. Acta, 15, 226-236.

Hogben, L. T. (1924) The pigmentary effector system, Edinburgh, Oliver and Boyd.

Hooker, D. (1914) Amoeboid movement in the corial melanophores of Rana, Amer. Jour. Anat., 16, 237-250.

Kamada, T. and Kinosita, H. (1944) Movements of granules in fish melanophores, Proc. Imp. Acad. Tokyo, 20, 484-492.

Kane, R. E. (1962) The mitotic apparatus. Fine structure of the isolated unit, J. Cell Biol., 15, 279-287.

Keeble, F. W. and Gamble, F. W. (1903) The colour-physiology of higher Crustacea, Proc. Roy. Soc. London, 71, 69-71.

Keeble, F. W. and Gamble, F. W. (1904) The colour-physiology of higher Crustacea, Phil. Trans. Roy. Soc. London, B, 196, 295-388.

Kinosita, H. (1953) Studies on the mechanism of pigment migration within melanophores with special reference to their electric potentials, Ann. Zool. Japan, 26, 115-127.

Kinosita, H. (1963) Electrophoretic theory of pigment migration within fish melanophores, Ann. New York Acad. Sci., 100, 992-1003.

Koller, G. (1925) Farbwechsel bie Crangon vulgaris, Verh. Deutsch. zool. Ges., 30, 128-137.

Kröyer, H. (1842) Monographisk fremstilling af slaegten Hippolytes nordiske arter, Dansk. Vid. Selskabet Afh., IX, 209-360.

Lansing, A. I. and Lamy, F. (1961) Localization of ATPase in rotifer cilia, J. Biophys. and Biochem. Cytol., 11, 498-501.

Ledbetter, M. C. and Porter, K. P. (1963) A 'microtubule' in plant cell fine structure, J. Cell Biol., 19, 239-250.

Lerner, A. B., Fitzpatrick, T. B., Calkins, E. and Summerson, W. H. (1950) Mammalian tyrosinase: the relationship of copper to enzymatic activity, J. Biol. Chem., 187, 793-802.

Lerner, A. B. and Takahashi, Y. (1956) Hormonal control of melanin pigmentation, Rec. Prog. Hor. Res., 12, 303-320.

Lieben, S. (1906) Ueber die Wirkung von Extrakten chromaffinen Gewebes (Adrenalin) auf die Pigmentzellen, Centralbl. Physiol., 20, 108-117.

Lorinz, A. L. (1954) Pigmentation, In Rothman, S., ed., Physiology and biochemistry of the skin, Chicago, University of Chicago Press.

Luft, J. H. (1961) Improvements on epoxy resin embedding methods, J. Biophys. and Biochem. Cytol., 7, 409-410.

Marsland, D. A. (1944) Mechanism of pigment displacement in unicellular chromatophores, Biol. Bull., 87, 252-261.

Matthews, S. A. (1931) Observations on pigment migration within the fish melanophore, J. Exp. Zool., 58, 471-486.

Mazia, D., Chaffee, R. R. and Iverson, R. M. (1961) Adenosine triphosphatase in the mitotic apparatus, Proc. Nat. Acad. Sci., 47, 788-790.

Milne-Edwards, H. (1834) Note sur les changements de couleurs du Caméléon, Ann. Sci. Nat., ser. 2 (Zool.) 1, 46-54.

Parker, G. H. (1930-31) Chromatophores, Biol. Rev. 5-6, 59-90.

Paul, J. (1961a, b) Cell and tissue culture, Baltimore, Williams and Wilkins, pp. 95(a) and 81(b).

Pease, D. C. (1963) The ultrastructure of flagellar fibrils, J. Cell Biol., 18, 313-326.

Perkins, E. B. (1928) Colour changes in crustaceans, especially in Palaemonetes, J. Exp. Zool., 50, 71-105.

Porter, K. R. (1964) Morphology of microtubules of plant cells, Science, 144, 872-874.

Porter, K. R. and Bonneville, M. A. (1964) An introduction to the fine structure of cells and tissues, 2nd ed., Philadelphia, Lea and Febiger.

Pouchet, G. (1872) Sur les rapides changements de coloration provoqués expérimentalement chez les crustacés et sur les colorations bleues des poissons, J. Anat. Physiol., 8, 401-407.

Raper, H. (1928) The aerobic oxidases, Physiol. Revs., 8, 245-282.

Rawles, M. E. (1948) Origin of melanophores and their role in development of color patterns in vertebrates, Physiol. Revs., 28, 383-408.

Redfield, A. C. (1916) The coordination of chromatophores by hormones, Science, 43, 580-581.

Reynolds, E. S. (1963) The use of lead citrate at high pH as an electron-opaque stain in electron microscopy, J. Cell Biol., 17, 208-215.

Roesel von Rosenhof, A. I. (1758) Historia naturalis ranarum nostratium, Nurnberg, The Author.

Roth, L. E. and Daniels, E. W. (1962) Electron microscopic studies of mitosis in amebae. II. The giant ameba Pelomyxa carolinensis, J. Cell Biol., 12, 57-78.

Rynberk, G. van (1906) Ueber den durch Chromatophoren bedingten Farbenwechsel der Tiere, Ergbn. Physiol., 5, 347-571.

Sabatini, D. D., Bensch, K. and Barnett, R. J. (1963) Cytochemical and electron microscopy. The preservation of cellular ultrastructure and enxymatic activity by aldehyde fixation, J. Cell Biol., 17, 19-58.

Sangiovanni, G. (1819) Descrizione d'un particolare sistema di organi cromoforo-expansivo-dermoideo e die fenomeni ch'esso produce, scoperto nei molluschi cefalopodi, Giornale enciclopedico di Napoli, anno 13, no. 9.

Sars, G. (1867) Histoire naturelle des Crustacés d'eau douce de Norvege, Christiania, The Author.

Schmidt, V. J. (1919) Vollzeit sich Ballung und Expansion des Pigmentes in den Melanophoren von Rana nach Art amoboider Bewegungen oder-durch intrazelullare Korchenstromung?, Biol. Centralb., 39, 140-144.

Seiji, M., Shimae, K., Birbeck, M. S. C. and Fitzpatrick, T. B. (1963) Subcellular localization of melanin biosynthesis, Ann. New York Acad. Sci., 100, 497-533.

Shanes, A. M. and Nigrelli, R. F. (1941) The chromatophores of Fundulus heteroclitus in polarized light, Zoologica, 26, 237-240.

Siebold, K. T. E. von (1863) Die Susswasserfische von Mitteleuropa, Leipzig, W. Engelmann.

Slautterback, D. B. (1963) Cytoplasmic microtubules. 1. Hydra, J. Cell Biol., 18, 367-388.

Spaeth, R. A. (1913) The mechanism of the contraction in the melanophores of fishes, Anat. Anz., 44, 520-524.

Spaeth, R. A. (1916) Evidence proving the melanophore to be a disguised type of smooth muscle cell, J. Exp. Zool., 20, 193-215.

Stark, J. (1830) On changes observed in the colour of fishes, Edinburgh New Philos. Journ., IX, 327-331.

Szent-Györgi, A. (1949) Free energy relations and contraction of acto-myosin, Biol. Bull., 96, 140-161.

Taylor, A. C. (1965) The centrioles and microtubules, Am. Naturalist, 99, 267-278.

Tibbs, J. (1959) The adenosine triphosphatase activity of perch sperm flagella, Biochim. et Biophys. Acta, 33, 220-226.

Vallisnieri, A. (1715) Osservazioni intorno alle rane, Venezia, Istoria del Camaleonte.

Waring, H. (1963) Color change mechanisms of cold-blooded vertebrates, New York, Academic Press.

Watson, M. L. (1958) Staining of tissue sections for electron microscopy with heavy metals, J. Biophys. and Biochem. Cytol., 4, 475-478.

Watson, M. R., Alexander, J. B. and Silvester, N. R. (1964) The cilia of Tetrahymena pyriformis, Exp. Cell Res., 33, 112-129.

Wittich, W. von (1854) Die grüne Farbe der Haut unserer Frösche; ihre physiologischen und pathologischen Veranderungen, Arch. Anat. Physiol. und wiss. Med., 41-59.

Zimmermann, K. W. (1893) Ueber die Contraction der Pigmentzellen der Knochenfische, Verh. anat. Ges., 7, 76-81.

GENERAL REFERENCES

Pigment Cell Symposia

Gordon, M., ed. (1948) Biology of melanomas, New York, New York Academy of Sciences.

Gordon, M., ed. (1953) Pigment cell growth, New York, Academic Press.

Gordon, M., ed. (1959) Pigment cell biology, New York, Academic Press.

Riley, V. and Fortner, J. G., eds. (1963) The pigment cell--molecular, biochemical and clinical aspects, Ann. New York Acad. Sci., 100.

Fish Pigments

Fox, D. L. (1953) Animal biochromes and structural colours, London, Cambridge University Press.

Animal Color Changes

Hogben, L. (1924) The pigmentary effector system, Edinburgh, Oliver and Boyd.

Parker, G. H. (1936) Color changes of animals in relation to nervous activity, Philadelphia, University of Pennsylvania Press.

Parker, G. H. (1948) Animal colour changes and their neurohumours, London, Cambridge University Press.

Waring, H. (1963) Color change mechanisms of cold-blooded vertebrates, New York, Academic Press.

End