ACTION OF COLCEMID IN SEA URCHIN EGGS

ARTHUR M. ZIMMERMAN and SELMA ZIMMERMAN

From the Department of Zoology, University of Toronto, Toronto, Canada, and the Marine Biological Laboratory, Woods Hole, Massachusetts

ABSTRACT

The effects of Colcemid, the deacetyl-*N*-methyl derivative of colchicine, on the eggs of *Arbacia punctulata* were investigated. Colcemid in concentrations of 2.7×10^{-5} M or greater blocks syngamy (the fusion of the pronuclei) in these eggs. Although a tenfold decrease in concentration of Colcemid usually permits the pronuclei to fuse, the subsequent division is blocked. In the sea urchin egg, the duration of presyngamy is about 15 min during which time there is no DNA synthesis. However, DNA synthesis is recorded in Colcemid-blocked cells prior to syngamy. Radioautographs of Colcemid-blocked cells which were immersed into thymidine-^{3}H exhibited silver grains above each of the pronuclei. The action of Colcemid on *Arbacia* eggs is reversible. Nevertheless, exposures to 2.7×10^{-5} M Colcemid for only 3 min, initiated 5 min after insemination, caused delays of 70 min in subsequent division. In general, cells are more sensitive to Colcemid prior to the time when the mitotic spindle is being assembled than at presyngamy stages. The results are discussed in terms of Colcemid action on pronuclear fusion and cell division.

INTRODUCTION

Colcemid (deacetyl-*N*-methylcolchicine) is a colchicine derivative which, although similar in action to its parent compound, is more effective and less toxic than colchicine (2, 5, 6). Sauaia and Mazia (9) found that Colcemid specifically disorganizes the mitotic apparatus in sea urchin eggs. Specific disruption of sea urchin mitotic apparatus is also known to occur as a result of high pressure treatment (17). Recently, Zimmerman and Silberman (18) found that high pressure could block pronuclear fusion in sea urchin eggs while permitting the individual pronuclei to incorporate thymidine-^{3}H although DNA synthesis does not normally occur in these eggs until after the pronuclei fuse. As a further extension of this work and in view of the recent indication by Marsland (8) that colchicine and pressure may produce similarly directed changes in the mitotic apparatus structure, we thought it would be interesting to study the effect of Colcemid on pronuclear activity in sea urchin eggs.

The present investigation has two main objectives: (1) to see whether Colcemid would block syngamy in *Arbacia* eggs and to study DNA synthesis in such Colcemid-treated cells; (2) to establish the effects of Colcemid on the division schedule of sea urchin eggs.

MATERIALS AND METHODS

LIVING MATERIAL: Eggs of *Arbacia punctulata* were obtained by intracoelomic injections of 0.5 M KCl. The eggs were washed in three changes of filtered seawater prior to use. The sperm, obtained from excised testes, were placed in a dry stender dish and stored at 4°C. Just prior to insemination one to two drops of sperm concentrate were added to 15 ml of seawater, and four to five drops of the diluted sperm suspension were added to the eggs. Only those batches of eggs in which 98% of the eggs showed fertilization membranes were used.

RADIOAUTOGRAPHIC STUDIES: 5 min after insemination, the eggs were placed into varying concentrations of Colcemid in the presence of thymidine-^{3}H (2 μc/ml) and incubated for 30- 60-min duration (at 20–21°C). The Colcemid (deacetyl-

N-methylcolchicine) was graciously supplied by Ciba Company, Dorval, Quebec. The tritiated thymidine (specific activity 6.7 C/mmole) was obtained from New England Nuclear Corporation, Boston. After incubation, the cells were fixed in Bouin's solution for 30 min; the eggs were then washed three times in 50% ethanol, dehydrated in graded alcohols, and embedded in tissue mat. Subsequently, 5-μ serial sections were placed on subbed slides, "cleared," and either covered with AR10 radioautographic stripping film or dipped into NTB2 liquid emulsion. In certain experiments the cleared slides were incubated in DNase (Worthington Co., Harrison, N. J.) solution (1 mg/ml) buffered with veronal, at pH 7.5, for 36–48 hr at 37°C, prior to being covered with radioautographic emulsion. After a 23–25-day exposure period, the emulsion-covered slides were subjected to photographic development (3, 7). The slides were analyzed and photographed under phase.

COLCEMID REVERSAL: In other experiments the eggs were placed into varying concentrations of Colcemid, at specified times after insemination, for various durations (3, 5, 10, 15, 30, and 60 min). The cells were then washed three times in filtered sea water, and the time of 50% division was recorded for both experimental and control cells. The excess division delay was calculated as the difference in time to 50% cleavage between the Colcemid-treated eggs and the control eggs, minus the time the eggs were in the Colcemid.

RESULTS

Effects of Colcemid on Pronuclear Fusion

Inseminated eggs were placed into various concentrations of Colcemid, in the presence of thymidine-^{3}H, 5 min after insemination, for 30- and 60-min durations. The time for pronuclear fusion in *Arbacia punctulata* eggs (within temperature ranges of 20–21°C) is about 10–14 min. The fusion of the pronuclei (syngamy) involves two phases: the movement of pronuclei toward each other in the center of the cell; and the mixing of the genomes. When the concentration of Colcemid was 2.7×10^{-4} M, there was no appreciable movement of the pronuclei toward fusion. When the concentration was reduced to 2.7×10^{-5} M, there was some movement of the pronuclei toward fusion, since many cells showed pronuclei at varying distances from each other. Fig. 1 illustrates the stage of pronuclear fusion following a 30-min exposure to Colcemid in the presence of thymidine-^{3}H. As shown in Fig. 1 *a* and *b*, at concentrations of 2.7×10^{-5} M or greater, Colcemid blocks the movement of the pronuclei toward fusion but does not block incorporation of thymidine-^{3}H. When the concentration of Colcemid was reduced to 2.7×10^{-6} M, serial sections of the material revealed that, in most cases, the pronuclei had fused. In some cells the pronuclei were in close proximity to each other but had not completely fused, as shown in Fig. 1 *c* in which the radioautographic pattern indicates that the male and female pronuclei are in close proximity. In this case, fusion is not completed and the silver grains remain in relatively localized areas. This finding is in contrast to that in the control cells, shown in Fig. 1 *d*, which were incubated in the presence of thymidine-^{3}H for 30 min without Colcemid; in this photomicrograph the radioautographic grains are distributed homogeneously over the fusion nucleus. In this experiment, 99% of the thymidine-^{3}H–labeled control eggs divided 50 min after insemination.

When the duration of exposure to Colcemid and thymidine-^{3}H was extended to 60 min, the general patterns were unchanged except that the number of grains over the pronuclei and over the fused nuclei was markedly increased.

In a few instances, polyspermy was observed and, as shown in Fig. 2, the radioautographic grains are localized in three masses above the pronuclei.

Colcemid Reversal

Sauaia and Mazia (9) have reported that 2.7×10^{-5} M Colcemid irreversibly blocks eggs of the sea urchin, *Strongylocentrotus purpuratus*, at metaphase, following a 5-min exposure. In order to establish the effect of Colcemid on the fistr division schedule in the eggs of *Arbacia*, the cells were treated at 5 and 30 min after insemination, for varying periods of time. Subsequently, they were removed from the Colcemid and washed three times in filtered seawater, and the time for 50% division was recorded. At least 100 cells were counted at each observation, and the cells were observed at frequent intervals (2–5 min) during the cleavage periods. At other stages, they were observed at 5-min intervals. The excess division delay is calculated as the difference in time for 50% furrowing between the Colcemid-washed cells and the control cells, minus the duration of Colcemid treatment.

The relationship between the excess division delays and the duration of the treatment is repre-

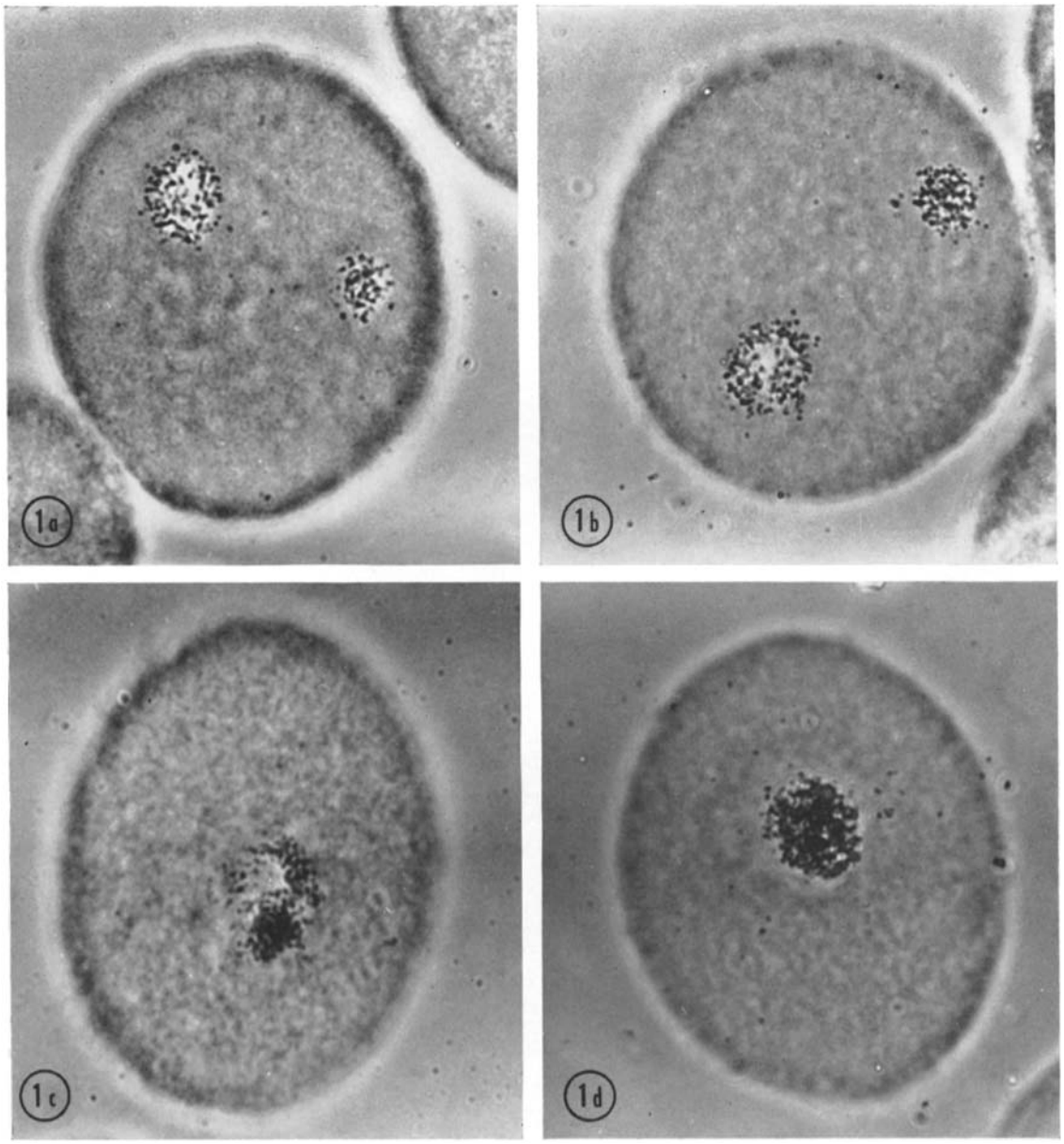

FIGURE 1 Radioautographs of *Arbacia* eggs following 30-min immersion in varying concentrations of Colcemid in the presence of thymidine-^{3}H (2 μc/ml). Colcemid and thymidine-^{3}H treatment was initiated 5 min after insemination. Figs. 1 *a–c* show eggs that were placed into 2.7×10^{-4}, 2.7×10^{-5}, and 2.7×10^{-6} M Colcemid, respectively, in the presence of thymidine-^{3}H. In Figs. 1 *a* and *b*, the silver grains are localized in two masses above the two pronuclei; however, in Fig. 1 *c*, the silver grains are localized in two masses close together. Fig. 1 *d* shows radioautographic pattern of a control cell incubated for the same duration (30 min) as the other cells, but not subjected to Colcemid treatment.

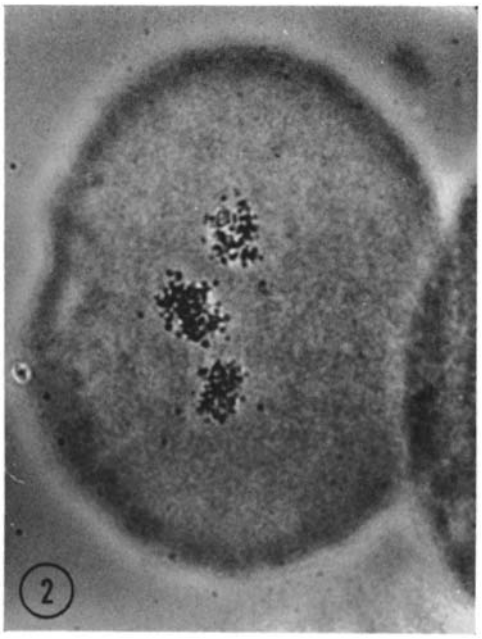

FIGURE 2 Radioautographic pattern of an egg cell in which treatment with Colcemid (1.1×10^{-4} M) and thymidine-^{3}H (2 μc/ml) was initiated 5 min after insemination and extended for a duration of 60 min. The silver grains are localized in three separate masses above the pronuclei. This illustrates the radioautographic pattern found in a polyspermic egg, in which more than one sperm has entered the egg.

sented in Fig. 3, following a treatment with 2.7×10^{-5} M Colcemid that was initiated 5 min after insemination for durations of 3, 5, 10, 15, 30, and 60 min. A delay of 70 min was found following a 3-min exposure to Colcemid; as the duration of exposure was increased, the subsequent delays were markedly increased. Although the non-treated controls divided quite synchronously (requiring 7–10 min for time between 10 and 90% division of the cell population) the Colcemid-treated cells were less synchronous (e.g. 53-60 min for the time between 10–90% division) following a 5-min exposure. After a 15-min exposure, the time between 10 and 90% division was increased to 65 min. Longer exposures to Colcemid led to more prolonged intervals of time between 10–90% division.

In other experiments, eggs were similarly treated with 2.7×10^{-5} M Colcemid for periods of 3, 5, 10, and 15 min but treatment was initiated 30 min after insemination. Since these studies were conducted later in summer, on a water table between 22° and 24°C, it is difficult to state precisely the stage at which the cells were first immersed in Colcemid. Under these temperature conditions, the 30-min time following insemination corresponds to prophase, since 50% cleavage occurred between 40 and 46 min in the controls. A comparison between treatments initiated at 5 min and at 30 min after insemination (see Fig. 4) reveals that the cells are more sensitive to Colcemid 30 min after insemination than at 5 min after insemination, for each of the individual exposure times.

When the concentration of Colcemid was reduced to 2.7×10^{-6} M, the division delays were markedly reduced. With this more dilute concentration of Colcemid, a 15-min treatment initiated 30 min after insemination caused a delay of only 12 min, whereas at the higher concentration (2.7×10^{-5} M) the division delay was 216 min.

DISCUSSION

The present study on the action of Colcemid in eggs of *Arbacia punctulata* demonstrates that this antimitotic agent is capable of blocking pronuclear fusion. Furthermore, it has been shown that the Colcemid-blocked pronuclei incorporate thymidine-^{3}H.

The extent of Colcemid inhibition on pronuclear fusion is dependent upon the concentration. In his report of studies with radioactive colchicine, Taylor (12) points out that colchicine is bound to cells at a rate proportional to its concentration. In the present experiments Colcemid at a concentration of 2.7×10^{-5} M prevented syngamy, but if the concentration was reduced by a factor of 10 then pronuclear fusion usually occurred. Recently, Dr. K. Nakamura (Kyoto University), while studying the path of the pronuclei in sea urchin eggs,[1] also found that Colcemid could block syngamy.

In the sea urchin egg (*Arbacia*), the duration of presyngamy is about 15 min and DNA synthesis normally occurs following pronuclear fusion (16). In the present study, when syngamy was blocked by Colcemid there was incorporation of thymidine-^{3}H in the absence of pronuclear fusion. These findings corroborate the results of Bucher and Mazia (1) and Zimmerman and Silberman (18) who found that sea urchin pronuclei also incorporated thymidine-^{3}H when their fusion was blocked by mercaptoethanol and hydrostatic pressure, respec-

[1] K. Nakamura. Personal communication.

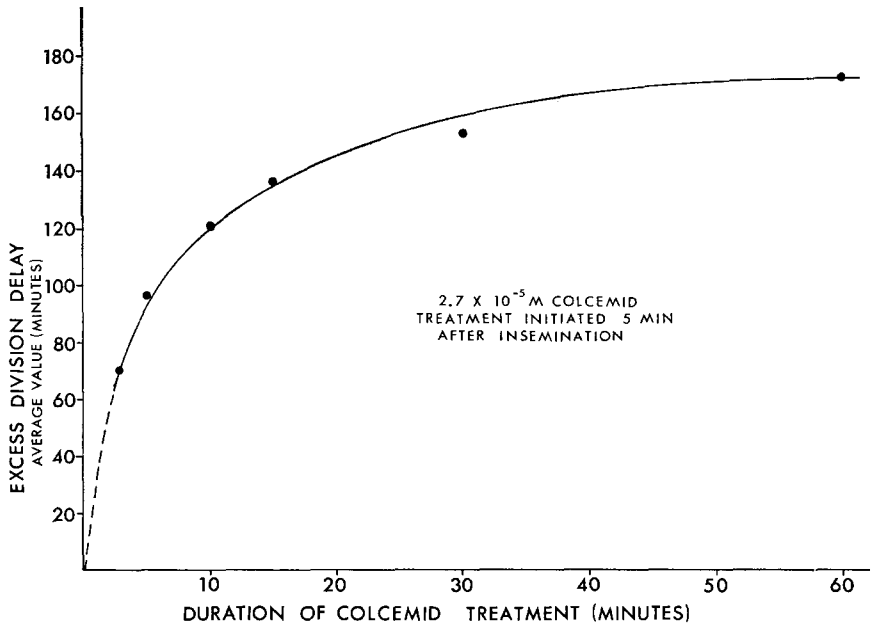

FIGURE 3 The effects of Colcemid on division delay in *Arbacia* eggs. The eggs were immersed in 2.7 × 10^{-5} M Colcemid 5 min after insemination. The average value of the excess division delay for first division is plotted as a function of the duration of Colcemid treatment.

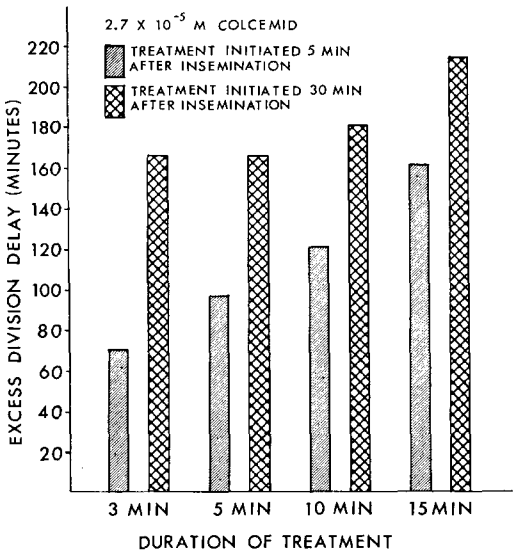

FIGURE 4 Comparison of the effects of Colcemid during the first division cycle following treatment initiated at 5 and 30 min after insemination. The eggs were treated with 2.7 × 10^{-5} M Colcemid for varying periods of time. In each experiment, the division delay is shown following the specified duration of immersion in Colcemid.

tively. Thus, in sea urchin eggs, DNA synthesis can occur in pronuclei when pronuclear fusion is blocked. However, in sand dollar eggs (10) and grasshopper eggs (11), DNA synthesis normally occurs prior to pronuclear fusion. Thus, it appears that fusion of pronuclei is not a necessary prerequisite for initiation of the biochemical events associated with the incorporation of thymidine-^{3}H.

In the present experiments, the effects of Colcemid were found to be reversible. However, Sauaia and Mazia (9) report that the action of Colcemid (at comparable concentrations) was irreversible in eggs of the sea urchin, *Strongylocentrotus purpuratus*. The mechanism of Colcemid action is complex in that the time required for reversal of blockage does not correspond directly to the time of exposure to this drug (see Fig. 3). Furthermore, the present studies indicate that brief exposures (3–5 min) to 2.7 × 10^{-5} M Colcemid resulted in extended delays of subsequent cytokinesis (see Fig. 4). Thus, it would seem that Colcemid penetrates the cell rapidly and remains bound to sites in the cell for some time after removal of the cell from the Colcemid solution. These observations are compatible with those of Taylor (12), who finds a rapid penetration of

colchicine in cultured cells and observes a slow loss of bound colchicine from these cells when they were resuspended on colchicine-free medium.

It was found that *Arbacia* eggs are more sensitive to Colcemid at prophase (when mitotic structures begin to form) than at presyngamy stages, as indicated from division delay studies (see Fig. 4). Taylor's work (12) also suggests that cells in different stages of mitosis were differentially sensitive to colchicine inhibition. The increased sensitivity to Colcemid at prophase as compared with syngamy may reflect an increased intracellular binding of Colcemid or it may reflect an increase in cell permeability to Colcemid at prophase. Sauaia and Mazia (9) believe that Colcemid disorganizes the mitotic spindle structure in the sea urchin, *Strongylocentrotus purpuratus*, by weakening molecular bonding. Their preliminary data do not indicate any interference of ATPase activity in mitotic apparatus isolated from Colcemid-treated cells. Inoué and coworkers (4), using 10^{-5} M Colcemid, report a loss of birefringence in the mitotic spindle fibers in the egg of the marine annelid, *Pectinaria gouldi*. Furthermore, the recovery of spindle birefringence did not appear to be dependent upon protein synthesis. In this connection, it is perhaps germane to note that Taylor (12) reports no measurable effect of colchicine on rates of synthesis of DNA, RNA, and protein after mitosis was completely inhibited. In the present study, we have also been able to demonstrate DNA synthesis in Colcemid-blocked cells. Recently, Zimmerman and Silberman (19) have demonstrated that high pressure affects the division schedule of *Arabacia* eggs to a greater degree when applied at prophase than at any other time during the division schedule, presumably through interference with the newly forming mitotic spindle processes.

Marsland (8), investigating the combined effects of colchicine and hydrostatic pressure on eggs of the sea urchin, *Lytechinus*, found that these agents act synergistically in inhibiting mitotic function. These results were expected since colchicine (13) and high pressure (15) have produced similar disruptive effects on microtubule structure (in the helizoan, *Actinosphaerium*). In view of the similar effect of Colcemid (present experiments) and high pressure (18) in preventing pronuclear fusion and in disrupting microtubule structure and in view of the proposal that microtubules may be involved in cell motion and cell elongation (14), it is interesting to consider that Colcemid and high pressure may affect the movement of pronuclei by affecting microtubule structure. This view is compatible with the concept that either colchicine or Colcemid exerts its effect through interference with polymerization of molecular subunits, which are essential for the formation of fibrous protein structures used in cellular activity.

We gratefully acknowledge the technical assistance of Mr. Terry Rosen.

This work was supported by a grant of the National Cancer Institute of Canada and the National Research Council of Canada.

Received for publication 11 January 1967.

REFERENCES

1. Bucher, N. L. R., and D. Mazia. 1960. *J. Biophys. Biochem. Cytol.* **7**:651.
2. Dustin, P., Jr. 1963. *Pharmacol. Rev.* **15**:449.
3. Fitzgerald, P. J., E. Simmel, J. Weinstein, and C. Martin. 1953. *Lab. Invest.* **2**:181.
4. Inoué, S., H. Sato, and M. Ascher. 1965. *Biol. Bull.* **129**:409.
5. Kobayashi, N., and K. Nakamura. 1957. *Zool. Mag.* (*Dobutsugaku Zasshi*). **66**:258.
6. Kobayashi, N., and K. Nakamura. 1957. *Zool. Mag.* (*Dobutsugaku Zasshi*). **66**:377.
7. Korpriwa, B. M., and D. Leblond. 1962. *J. Histochem. Cytochem.* **10**:269.
8. Marsland, D. 1966. *J. Cell. Comp. Physiol.* **67**: 333.
9. Sauaia, H., and D. Mazia. 1961. *Pathol. Biol.* **9**:473.
10. Simmel, E. B., and D. Karnofsky. 1961. *J. Biophys. Biochem. Cytol.* **10**:59.
11. Swift, H., and R. Kleinfeld. 1953. *Physiol. Zool.* **25**:301.
12. Taylor, E. W. 1965. *J. Cell Biol.* **25**:145.
13. Tilney, L. G. 1965. *Anat. Record.* **151**:426.
14. Tilney, L. G., and K. R. Porter. 1965. *Protoplasma.* **60**:317.
15. Tilney, L. G., Y. Hiramoto, and D. Marsland. 1966. *J. Cell Biol.* **29**:77.
16. Zimmerman, A. M. 1963. *Exptl. Cell Res.* **31**:39.
17. Zimmerman, A. M., and D. Marsland. 1964. *Exptl. Cell Res.* **35**:293.
18. Zimmerman, A. M., and L. Silberman. 1964. *Biol. Bull.* **127**:355.
19. Zimmerman, A. M., and L. Silberman. 1965. *Exptl. Cell Res.* **38**:454.