The Multiplicity of the Mitotic Centers and the Time-Course of Their Duplication and Separation*

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ABSTRACT

In this study, the reproduction of the mitotic centers in the eggs of a sea urchin, *Strongylocentrotus purpuratus* and a sand dollar *Dendraster excentricus* has been studied by means of experimental designs that do not depend on the actual visualization of centrioles. The centers are defined in operational terms as potential poles. Blockage of mitosis by *mercaptoethanol*, it was found, inhibits the duplication of the centers, but does not inhibit the splitting and separation of centers that have already duplicated and thus potential poles could be realized as actual poles in multipolar divisions. At all times, the center is at least a duplex structure; that is, it contains two potential poles. The actual duplication process is the earliest event in a given mitotic cycle, taking place at very early interphase or in late telophase of the previous division. The splitting of the centers following duplication is a distinct process, dissociable from the duplication as such. Duplication and splitting normally occur at about the same time in the mitotic cycle, with a precession of the former. That is, as the two members of a pair of "old" centers split, each one gives rise to a new one, which remains associated with it until the next phase of splitting and duplication occurs. The results are consistent with what is termed a "generative" model of the self-reproduction of an intracellular body. According to this, the body does not immediately produce a full-fledged copy of itself, with simultaneous fission, but the primary duplication event involves only a part of the parent structure. This gives rise to a "germ" or "seed" which then grows to be equivalent to the parent body, and finally splits from it.

I. INTRODUCTION

In animal cells, the *reproductive* events of mitosis are focalized in the chromosomes, the kinetochores (viewed as regions of the chromosomes specialized for certain mitotic functions) and in the centers. Formally, the process of mitosis can be described completely in terms of the following acts. (1) The centers duplicate and sister centers separate to form the poles. (2) The chromosomes duplicate and sister kinetochores connect to sister centers. (3) Sister chromosomes move to sister centers. Whatever the pathways or mechanisms involved, these are the only rules that need be observed.

The term *center* is used in a functional sense and will be given an operational meaning by the experiments to be described; the essential property of a center in our usage is that it determines a *pole* during mitosis. There are some objections (*e.g.* Cleveland, 1953) to such usage in preference to a morphological term such as "centriole." If we prefer the functional term in the present context, it is only because we are employing operational tactics for obtaining information about the structure and development of the mitotic centers, and

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have not yet referred our results to morphological entities.

II. Material and Methods

The eggs of the sea urchin *Strongylocentrotus purpuratus*, collected from intertidal sites in the San Francisco area, and of the sand dollar *Dendraster excentricus*, collected by "aqualung" diving at La Jolla or in Mission Bay, San Diego were the experimental material. Essentially the same results were obtained with both of these echinoid species, the only differences being in absolute times of various events at the appropriate temperatures. The *Dendraster* egg is the more transparent and more favorable for observation in the living condition. It operates more reliably at about 20° and divides more rapidly and more synchronously. The experiments with *S. purpuratus* were done at 15°. Some of the details of the handling of the material are given in two earlier papers (Mazia, 1958; Mazia and Zimmerman, 1958.) Since the experimental design hinges on the effects of *beta mercaptoethanol*, it may be mentioned that the reagent used was obtained from Eastman Organic Chemicals, Distillation Products Industries, Rochester, New York. Although the solutions are reasonably stable for ordinary purposes, they were made up fresh for each experiment. The oxidation product of mercaptoethanol, dithiodiglycol, has characteristic biological effects at low concentrations. Therefore, precautions with mercaptoethanol solutions are demanded not so much by the lowering of its concentration as by the appearance of dithiodiglycol.

III. QUADRIPARTITION. THE MULTIPLICITY OF THE CENTERS

A. Experimental Design:

The chief tool in the design of these experiments was mercaptoethanol. In two earlier papers, the blocking of division by mercaptoethanol has been discussed in some detail (Mazia, 1958; Mazia and Zimmerman, 1958). For sea urchin and sand dollar eggs, the effective concentration is in the range 0.075–0.1 M, and in the present experiments 0.075 or 0.08 M was used. In the earlier work, the essential finding was that a division could be blocked if the mercaptoethanol was introduced at any time before a "point of no return" which has been located early in metaphase. The blockage was fully reversible, and the delay in division was exactly equal to the time spent in mercaptoethanol. It was observed, in both of the published studies, that if the eggs were blocked just at metaphase and were removed from the block at the time when the controls were in their second division, they would divide directly from one cell to four cells. This *quadripartition* in *S. purpuratus* was illustrated by Mazia and Zimmerman (1958, Fig. 1 of that paper). Various stages of the four-way division of *Dendraster* are shown in Fig. 1.

The above procedure, in which the eggs are blocked until the controls have divided for a second time, will be referred to as the "standard" quadripartition experiment. The design of such experiments is diagrammed in Fig. 2 *B*. A later section will deal with the dependence of quadripartition on the duration of the blockage with mercaptoethanol.

The division of cells into more than two daughters is not a rare phenomenon. In plant and animal tissue it is reckoned as a pathological manifestation of mitosis (*e.g.* Politzer, 1934). In eggs it is a common consequence of polyspermy and has been seen after exposure to chemicals, ether, for example (Wilson, 1901; Swann, 1954). Immers and Runnström (1959) have recently described four-way divisions following treatment of sea urchin eggs with cyanide.

Let us reemphasize the fact that the mercaptoethanol is being used here solely as an analytical tool. In the following discussions, no part of the argument depends on any stipulation as to the mechanism of action of the mercaptoethanol, a question that has been the subject of the previous papers. Nor is there any reason either to affirm or to doubt that other and even unrelated chemicals might be used for the same purpose.

Our experimental questions are motivated by the simple observation that a cell which is normally destined to divide into two can be made to divide into four under defined conditions. At the very least, the system should give information about the reproduction and separation of the centers and it obviously raises questions as to the divisibility of the chromosomal material.

B. Descriptive Aspects of Quadripartition:

1. Mitotic Apparatus.—If a cell just entering metaphase is blocked with mercaptoethanol, the mitotic apparatus loses its highly oriented structure. In the living egg it is seen as a large clear volume in the center of the cell, more of less spherical. As has been shown earlier (Mazia and Zimmerman, 1958), the apparent disorganization takes the form of a "loosening" or disordering of the well polarized fibrous structure. This is quickly reversed when the mercaptoethanol is removed as

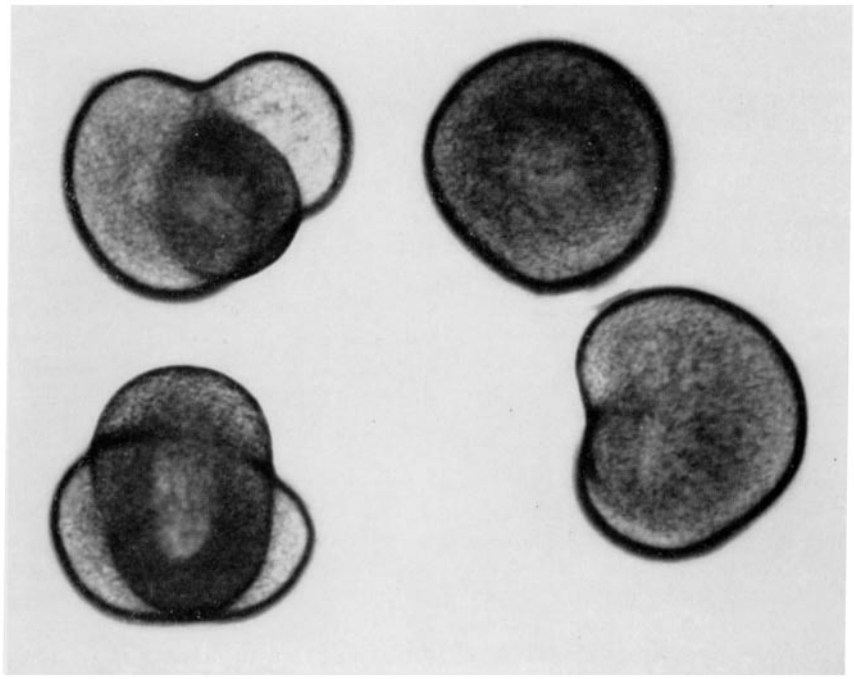


FIG. 1. Four-way division of *Dendraster* eggs during recovery from blockage with mercaptoethanol.

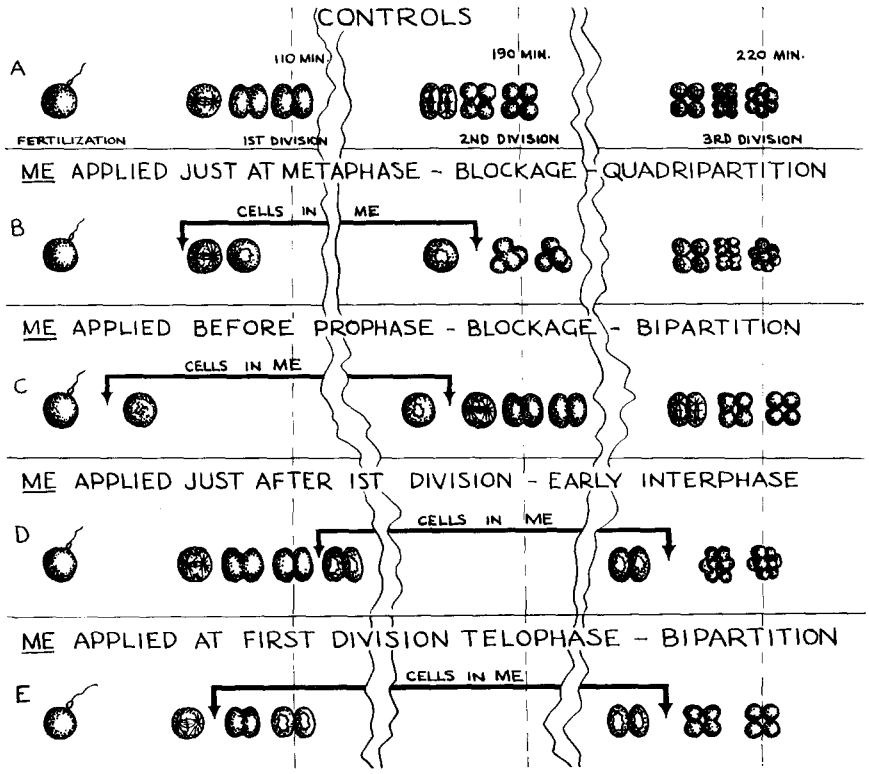


FIG. 2. Design of experiments to determine the number of potential poles present at a given time and thus to establish the time of duplication of the centers. Mercaptoethanol is introduced at various times before a given division and removed after the second following division of the controls. If the cells divide into four upon recovery, it is concluded that the centers have already duplicated at the time of exposure to mercaptoethanol. Experimental data are given in Tables I and II, and an interpretation of the events is given in Figs. 9 to 11.

though the structure were "pulling itself together" (Figs. 3 to 6, Mazia and Zimmerman, 1958).

As was described by Mazia (1959, Fig. 13), experiments done in collaboration with Dr. A. M. Zimmerman had shown that when the block was prolonged until the controls were going through their second division, the mitotic apparatus emerged as a tetrapolar figure upon reversal of the block. In Fig. 3, we see several stages in the recovery or "pulling together" of the blocked mitotic apparatus. One extraordinary feature of the blockage and recovery—the scattering of the chromosomes from their metaphase positions and the restoration of the metaphase plate—will be discussed in a later publication.

In the *Dendraster* egg, the disordering of the bipolar figure and its emergence as a tetrapolar figure may be observed *in vivo*.

It must be emphasized that the chromosomes remained condensed during the period of blockage and did not pass through an interphase stage. This was checked carefully and is important for the later discussion.

Thus, the quadripartition experiments involve the generation of a tetrapolar mitotic apparatus from a bipolar one during a prolonged period of blockage.

2. Cytokinesis.—The superficial sign of quadripartition is the commencement of four-way furrowing. Under the best conditions, this is completed; four fully separated cells are formed. However, in a number of experiments, the furrows were observed to abort before completion. They suddenly regressed and were observed only as shallow and sometimes very crooked grooves on the egg surface. In some other cases, one furrow was completed while the other aborted. Nuclear processes proceeded normally at first and quite often four nuclei were formed whether or not the furrows were completed. In some cases, there was fusion of the nuclei left in a single cell by failure of cytokinesis. Where the furrows regressed, they generally resumed their activity at a later moment, and now the same furrows completed the division of the cell into four. It is premature to present hypotheses concerning the meaning of the abortive cytokinesis and, indeed, we have been more interested in avoiding it. We have found that the regression of furrows is avoided if we work at temperatures lower than those used in our earlier experiments, hence the temperatures specified in the previous section.

Not infrequently, a number of the eggs in the population divided into three rather than into four.

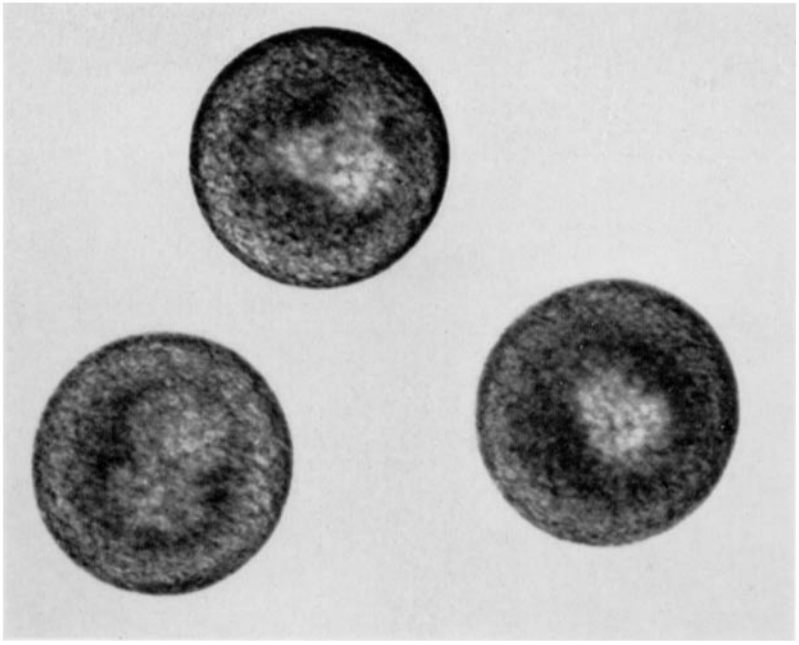


FIG. 3. Recovery from blockage by mercaptoethanol as observed in living *Dendraster* egg. Egg at top of photograph is forming a tetrapolar figure.

These, it turned out, provided valuable internal controls in certain experiments.

C. Quadripartition at the Second Division:

Because the first division cycle of echinoid eggs is somewhat complicated and unusual, involving a number of steps peculiar to fertilization and syngamy, we undertook to test whether the quadripartition procedure was applicable to the second and more typical division cycle. Eggs in the two-cell stage were placed in mercaptoethanol at the beginning of the second-division metaphase and were blocked until the controls had entered their third division. When the eggs were then restored to sea water, they divided directly from two cells to eight cells (Fig. 4). No difference was observed in the behavior of the two species used. The two blastomeres did not always undergo quadriparti-

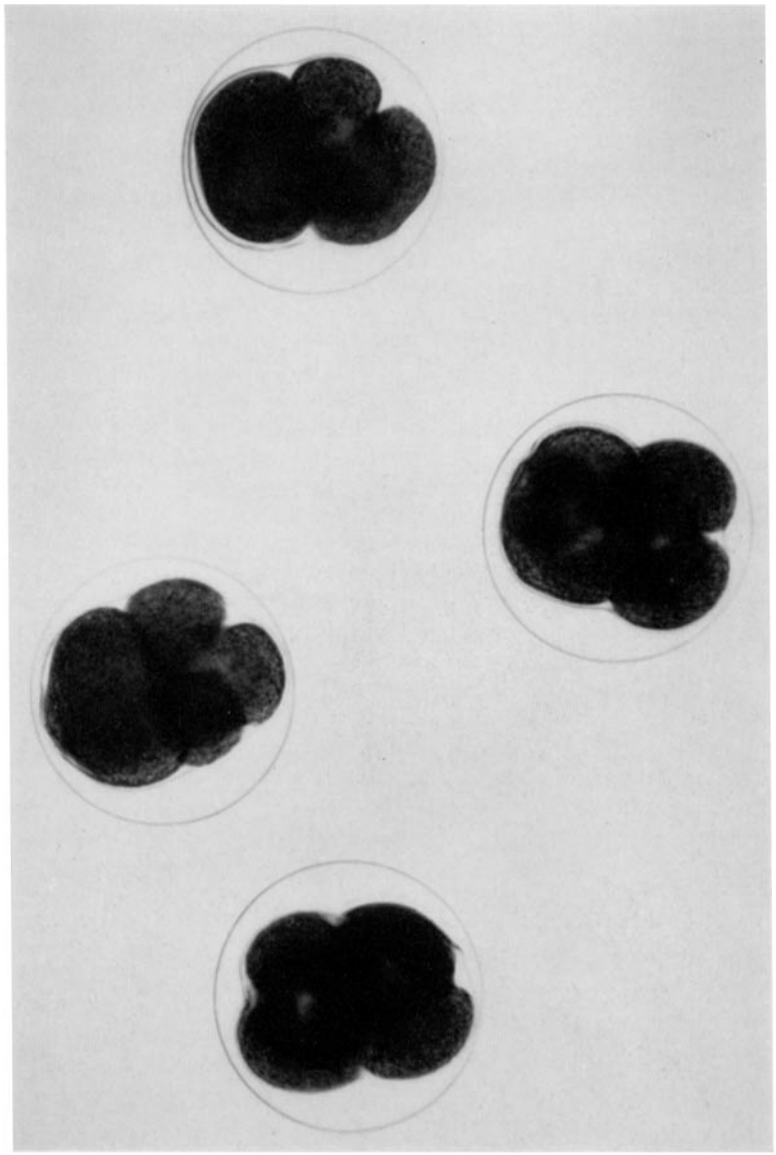


FIG. 4. Division of 2-cell stages of *S. purpuratus* into 8 cells, typical of cases where mercaptoethanol was introduced between the time of the first cleavage and metaphase of second cleavage.

tion in perfect synchrony; in the course of the recovery from mercaptoethanol, embryos consisting of one undivided blastomere and one which had divided to four were commonly seen.

Thus, it is possible, by the same procedure to induce the division of a one-cell stage to four cells or of a two-cell stage to eight cells.

D. The Division Following Quadripartition:

If we imagine that quadripartition involves the distribution among four cells of centers (or their subunits) normally destined for two cells, we might expect to find some defect in the centers received by each of the four cells. By superficial observation alone, these cells are capable of proceeding through further divisions, therefore the defect could not be irreparable. What actually happens was discovered by one of us (T. B.) in observations on living and fixed *Dendraster* eggs after quadripartition. It was seen that the cells formed by quadripartition formed a *monopolar figure* when they entered the next mitotic cycle. This figure is illustrated in Fig. 5; it is clearly a "half-spindle", perfect in the details of the achromatic figure. These cells obviously could not divide with half a mitotic apparatus, and they did not. Rather, they re-entered interphase, presumably went through another cycle of reproduction of the centers, and then entered mitosis again with a normal bipolar figure and divided.

Clearly, the quadripartition does involve the four-way distribution of centers that would normally be partitioned between two cells, rather than an extra duplication of the centers while in mercaptoethanol. If the latter had happened, the daughter cells would be expected to form normal bipolar figures. In fact, having received only half of their normal share of the parental centers, they respond in a mathematically exact way, by making just half of a mitotic apparatus!

In a small number of individuals, the cells did form bipolar figures at the first mitosis after quadripartition, after which they divided directly into eight. We do not know whether the extra centers in these were made before, during, or after exposure to mercaptoethanol.

E. Discussion; Multiplicity of the Centers:

In this discussion, we are defining the multiplicity of the centers in an operational way, in terms of potential poles. The standard quadripartition experiments have been designed to elicit the separation of the potential poles, and hence to count them in terms of the number of cells produced or the number of poles actually observed in the mitotic apparatus at division. For the moment, no hypotheses need be made concerning the chemical mechanism of the action of mercaptoethanol. It becomes merely an instrument capable of blocking the duplication of the centers but permitting the separation of the existing units once duplication has taken place. One reason for believing this to be the case—rather than the alternative that the centers go through an extra duplication step during the block—has already been given: the fact that the daughters produced by quadripartition form a mitotic apparatus with only one pole. There could hardly be better evidence that they received centers having only half the normal "valence." Additional evidence will be given below, when we consider the time of duplication of the centers.

It is a little difficult to find appropriate terminology for describing the degrees of multiplicity of the centers. The suffix "ploidy" has been preempted for a relationship in which it is less appropriate. The concept of "valence" would be appropriate, but it is generally taken to refer to meiotic conditions of chromosomes. Here we shall merely use a "-plicity" scale to refer to the numbers of identical units present in a cell. In our context, "-plicity" is defined functionally; *e.g.*, a center is duplex if it is capable of forming two functional poles.

Our present conclusion is that the centers are normally duplex at all times, though it is conceivable that they may be single for a small portion of their cycle.

Each observed center is capable of being split into two fully functional centers, but unless it is so split by experimental means it exists and is propagated as a double entity. The normal duplication of the centers is viewed as a process whereby two units give rise to four.

The idea of a duplex center is not a new one. Amidst all the descriptions of the morphological variations of the centers, bodies containing two stainable particles have often been figured. However, as Schrader (1953) points out in his summary of the literature on the morphology of centrioles, attempts to identify stainable granules as centrioles may lead to considerable confusion. The most detailed studies of the most favorable material, certain flagellates with very large centrioles, have been made by Cleveland (1957). He has observed the stages in the reproduction of the centri-

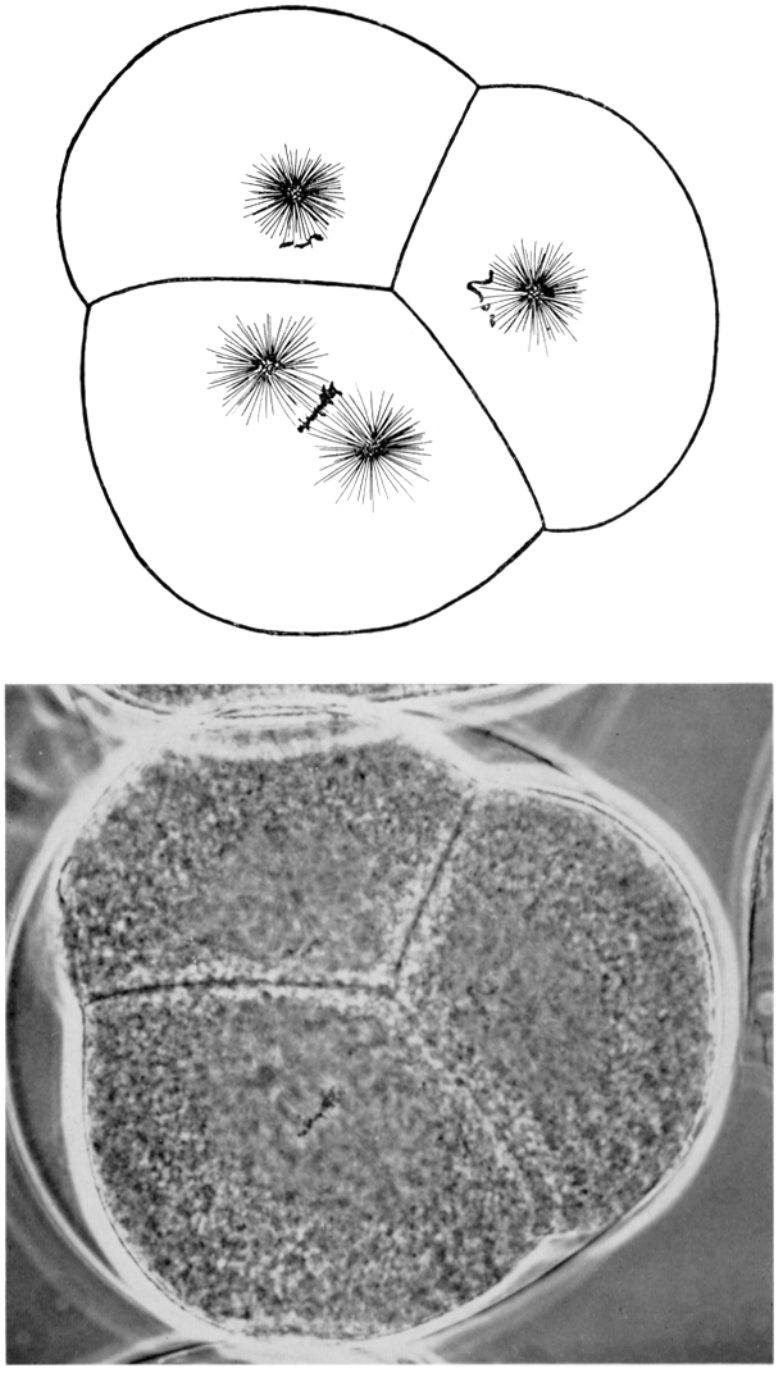


FIG. 5. Formation of monopolar mitotic figures following "quadripartition" of *S. purpuratus* eggs. In this case, the egg had divided into three; one furrow having failed. The cell on the left serves as a control, receiving a normal complement of centers and forming a bipolar figure at the next mitosis. The two cells on the right are typical of the products of quadripartition. They receive only half the normal complement of centers and form monopolar figures. Photograph: phase contrast view of egg fixed in 3:1 ethanol-acetic acid and flattened in 45 per cent acetic acid.

oles in a number of species. In every case, he observes that the daughter cells come out of division with two centrioles, one "old" and one visibly "new," and enter division with four, the two having produced new ones, still attached to them, during the intervening time.

The centrioles of a variety of vertebrate cells have been studied by means of the electron microscope (*e.g.* de Harven and Bernhard, 1956; Porter, 1957; Amano, 1957; Bessis *et al.*, 1958; Sotelo and Trujillo-Cenóz, 1958), and in all of these cases two units are observed, each resolvable as a cylinder made up of fine tubules. We expect that there will be a time in the division cycle when four units are present. By our criteria, these would not have to be identical in appearance; the "old" and the "new" might differ morphologically; all that is required is that a decisive event has taken place which provides the cell with four potential poles. Relevant electron-microscopic observations have now been reported by de Harven and Dustin (1959). Their beautiful photographs show pairs of full-sized centrioles, from which two smaller units having the basic cylindrical structure of the mammalian centriole appear to be "growing."

In the quadripartition experiments, the duplicity of the centers is determined without resort to visual resolution or to assumptions as to what particles will be called centrioles. Indeed, the echinoid egg is one case where distinct compact centrioles are not always seen by microscopic methods; their existence and behavior has been inferred from the presence of asters, and this alone would give no indication that the centers are duplex.

However, it is more important that the experimental analysis of the multiplicity of the centers yields functional information than that it serves as a *tour de force* whereby one can do microscopy without a microscope, so to speak. The functional information is that the two parts of which a center is normally composed are separable and are individually competent to form a complete and active pole. Thus the functioning of the center under normal conditions does not depend on its doubleness. On the contrary, the doubleness only seems to signify that four functional units are clustered in two pairs, thus providing for a normal division of the cell and its chromosomes into two.

If the total number of potential poles in a normal cell at metaphase is four, then a cell that began with half the normal number should possess two at the time of the next following metaphase. Yet it forms only one pole. We may not say that the centers can function only in pairs, because the quadripartition experiment itself tells us that this is not so. The apparent paradox is resolved by observations to be discussed later; in effect, a potential pole can become an actual pole only after it is "split" from its parent. This demands time and the function of the mercaptoethanol block is to provide that time. The complete interpretation of the formation of a monopolar figure, which depends on facts that have not yet been presented, is diagrammed in Fig. 10.

Obviously, one of the most interesting questions arising from experiments on quadripartition is: What happens to the chromosomes? This has been investigated by T. Bibring and will be presented by him in a following publication.

IV. THE TIME OF DUPLICATION OF THE CENTERS

A. Experiments:

The simplest conclusion from the quadripartition experiments, so far as the centers are concerned, is that the cells enter the first metaphase containing four units capable of forming poles, even though only two poles are formed normally. Likewise, when they enter the second metaphase, each of the blastomeres contains four such units. It follows from the latter observation that a duplication step (actually a multiplication from two to four units) has taken place in each cell between first metaphase and second metaphase and it is equally reasonable to suppose that at least one such step has occurred before the first division.

If the ability to form four cells after blockage is a sign that the multiplication of potential centers from two to four has already taken place by the time the block is imposed, what would happen if we introduce the mercaptoethanol before the duplication has taken place? Either the duplication would be blocked, in which case the cells could only divide into two, or else the duplication would be unaffected, in which case we could learn nothing more. If the former alternative holds, we could identify the time when the duplication event is completed, in a functional sense, without being subjected to any of the uncertainties of microscopic recognition. Fortunately, this is the case.

The design of the experiments on the time of duplication of the centers was essentially like that already described, except that now the eggs were put into mercaptoethanol at various intervals fol-

lowing fertilization. In practice, all of those that were blocked before the first division were removed at the time the controls went into second division. Those that had passed the first "point of no return" and were blocked before the second division were restored to sea water at the time the controls entered the third division (Fig. 2). It might seem that one variable, the total time of immersion in mercaptoethanol, was uncontrolled, but it was ascertained experimentally that the same results were obtained if successive samples of eggs were removed from mercaptoethanol at the same intervals (beginning at the time of the division of the controls) at which they were immersed, so that the total time of exposure was constant. To this extent, the time of exposure is a matter of indifference.

Samples for observation were fixed in ethanol-acetic acid at the time of immersion in mercaptoethanol, in order to relate the time of duplication of the centers to the mitotic stage.

Table I gives the results of one of a series of experiments on *Dendraster* eggs.

Table II is an example of the more complete data that are now available for the egg of *S. purpuratus*. The essential point is that the egg goes through a transition from a stage at which it can only divide into two to one in which it can divide into four. From this it passes to a stage where it can divide from two to four and on into one where it can divide from two to eight. The span of time during which the transition takes place is merely a measure of the asynchrony of the population.

TABLE I

Time of Completion of First Duplication of Centers Following Fertilization in Dendraster excentricus

Cells were put into 0.08 M mercaptoethanol at times indicated and were removed at 88 minutes after fertilization.

Time of immersion into mercaptoethanol expressed as:		Type of division after recovery
Minutes after fertilization	Minutes before division of controls	
20	35	1 → 2
22.5	32.5	1 → 2, few 1 → 4
25	30	1 → 4
27.5	27.5	1 → 4
30	25	1 → 4
32.5	22.5	1 → 4
35	20	1 → 4

TABLE II

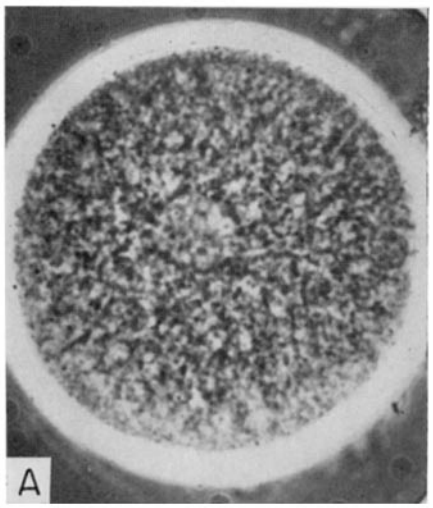
Experimental Data on S. purpuratus Eggs from which the Times and the Mitotic Stages at which the Centers Complete their First and Second Duplications Are Deduced

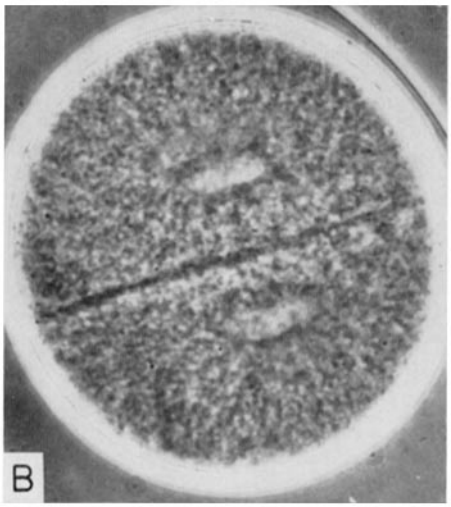
Experimental design is given in Fig. 2. The times of duplication, as judged from the times at which the majority of the population becomes capable of quadripartition, are indicated by broken lines in right-hand columns.

	Cells put into mercaptoethanol at:		Cleavage pattern after recovery			
	Minutes after fertilization	Stage	1 → 2	1 → 4	2 → 4	2 → 8
	20	Pronuclear fusion	90%	Some polyspermy		
	30	Clear fusion nucleus	>50	<50		
	40	Elongated fusion nucleus	20	70		
A	45	Same		70		
	50	Same		80		
	60	Earliest prophase		80		
	70	Prophase		80	Passed block	
	75	Prometaphase		80	5	
	85	Metaphase		80	10	
	95	Anaphase			90	
	105	Beginning division			80	20
	110	Reconstitution of nuclei			70	30
	115	Just divided-elongated nuclei			40	60
B	120	Nuclei rounding up			30	70
	130	"Resting"				80
	140	"Resting"				
	150	Earliest prophase				
	160	Prophase				
	170	Metaphase				
	180	Anaphase				
	190	Division				

Removed from mercaptoethanol: A, after controls divided to 4 cells. B, after controls divided to 8 cells.

Let us consider the interpretation of this rather intricate experiment more closely. Quadripartition depends on the fact that mercaptoethanol does not block the splitting and separation of existing centers, even though it blocks the formation of new ones and blocks division. During the block, two pairs of centers are given time to separate into four independent centers while other mitotic events are arrested. If the block is imposed before the time of duplication of the centers, only two centers are present when mercaptoethanol is removed. These, we assume, do duplicate when





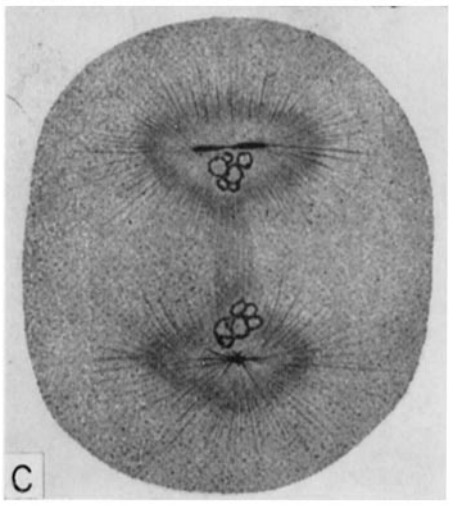


FIG. 6.

A. Stage at which the first duplication of the centers in *S. purpuratus* eggs is completed, 30 minutes after fertilization. This is the stage at which fusion of pronuclei is completed. Material fixed in 3:1 ethanol-acetic acid, flattened in 45 per cent acetic acid, and photographed in phase contrast.

B. Stage at which the second duplication of the centers in *S. purpuratus* eggs is completed, 120 minutes after fertilization. Furrow just completed and nuclei just reconstituted.

C. A drawing by Boveri of the division of the centers in the egg of the sea urchin (Boveri, 1900). This drawing shows quite clearly the events taking place at the stage just preceding that shown in photograph *B:* the centers are splitting just as the interphase nucleus begins to reconstitute. This drawing may also be compared with Fig. 8 *B* of the present paper.

progress toward division is resumed, but now the other processes are keeping pace, and the cell arrives at division in the normal way, with a pair of centers at each of two poles.

The interpretation of these experiments is diagrammed in Figs. 9 to 11, but this may not be intelligible until we have considered the relation of duplication to division.

In drawing conclusions from Table II, we may take the points at which 50 per cent of the cells become capable of dividing into four as the critical stages at which the centers have completed their duplication. We may first consider what these points represent in terms of mitotic stages. The images of eggs fixed at these times are given in Figs. 6 *A* and *B*. For the first division, the critical stage is that of completion of pronuclear fusion, while for the second it is the time of transition from telophase into interphase. Thus the time of duplication of the centers corresponds to the period when the interphase nucleus is forming, perhaps describable as a terminal telophase. Relative to cytokinesis, the time of doubling of the centers corresponds to the time of completion of the furrow. Since "interphase" is so brief in these eggs, perhaps the best reference point is the forthcoming prophase. As is seen in Table II, the event we are interpreting as the time of duplication of the centers occurs about 40 minutes before prophase, in both the first and second division cycles of *S. purpuratus*.

The appearance of the centers at this time is most interesting, and we can illustrate it no better than by drawing on one of the descriptions by the Old Masters (Fig. 6 *C*). The center of the aster contains a compact, often elongated mass, presumably of centriolar material, which appears to be in the act of division. The same stage is shown in a different context in Fig. 8 *B*.

If, as so much evidence indicates, the reproduction of the chromosomes begins during interphase, we can say that the reproduction of the centers is an earlier event. Taking place during the terminal period of the previous division, it is perhaps the earliest event assignable to a given division. The fact that it takes place during this phase has further significance which will be considered in a later section of this paper.

B. Discussion; the Duplication of the Centers:

We can now conclude that quadripartition is not the result of an extra duplication of the centers while in mercaptoethanol, a possibility proposed earlier (Mazia, 1959), but depends on the fact that the centers must have duplicated (going from two to four) before the block is imposed. This conclusion is reinforced by the observations of the monopolar mitotic apparatus in the progeny of quadripartition (Fig. 5) for which there is no other obvious explanation. We are then permitted to identify the stage *at which the duplication is completed*, as has been described. By our criteria, it comes very early with respect to the next division. In the echinoid eggs we have used, we place it at the transition from telophase to interphase.

Again, we must stress the functional character of our criteria, which has important advantages but now also has some serious disadvantages when unsupported by morphological details. The obvious advantage is that we can say when the daughter centers become competent to form future poles, or at least have reached a critical point where mercaptoethanol can no longer head off their development. The drawback is that we cannot relate their competence to their structural growth, without parallel morphological information.

The term "growth" is used advisedly, with reference to the observations of Cleveland (1957). He finds that the reproduction of the large centrioles of flagellates is by no means a division or fission process. Rather, the parent centriole proliferates a minute body, first observed as a dot connected to it by a fiber, and this germ of a "new" centriole grows into a full-fledged one like the "old." The growth takes place at a distance from the old centriole, but old and new remain connected by the fiber while it is taking place. If one counted his old and new centrioles together, we would conclude that the flagellates always contained two centers at the end of a division, and these had multiplied to four quite early in the preparations for the next division. This would be in agreement with our findings, except that we conclude that the cells we have studied have four centers at an even earlier time: about the stage when the preceding nuclear telophase and cytokinesis are just completed.

As has been mentioned, de Harven and Dustin (1959) have observed what is interpretable as the proliferation of two "young" centrioles from two mature ones in their electron-microscopic studies of mammalian material. Their photograph places

the time of the growth of the "young" centrioles in the prophase period.

The duplication of the centers, as studied experimentally by us and microscopically by others, prompts some infrequently asked questions concerning the meaning of the "duplication" of a complex cytoplasmic particle. The first is a matter of definition. Since geneticists have done most of the thinking on the subject of "self-duplication," it is inevitable that they will have been responsible for most of the dogma associated with the concept. For example, they insist on mutability as a criterion of "self-duplication" (*e.g.* Pontecorvo, 1958). This has real meaning (which is defined by operations performed by geneticists) for a particle having some genetic expression, but has no meaning for the centriole, which either forms a mitotic pole or does not form a mitotic pole. The centers are regarded as self-duplicating (perhaps Pontecorvo's term "self-reproducing" is more appropriate) because they normally arise from existing centers; this is a matter of observation. If they are not "self-reproducing" another term is needed to describe these real cases where a particle is responsible for the appearance of a second one just like it. We shall not discuss here the alleged *de novo* generation of centers in certain eggs because an investigation of this question in our laboratory is still incomplete.

Until recently, the image of the self-reproduction of a particle has tended to be restricted to what we may call the "fission model." In this, the process of material duplication of a body is coupled with its division into two equal daughter units. This image has seemed to correspond with what is seen in the case of the chromosomes, and may well be correct for that case. The alternative model, which we may call a "generative model," is that a body as complex as the centriole contains a reproducing "germ" or "seed" of molecular dimensions. This gives rise to its like, which in turn directs the growth of a replica of the original body. Only recently have these two models been differentiated in anything like a theoretical way (Penrose, 1959). Superficially, the "generative" model might seem to be excessively complex. Once we think of reproduction in molecular terms, the "fission" model is the more complex if it calls for the use of the large, complex, and 3-dimensional centriole such as has been described by the electron microscopist as a template for the production of another such unit. A good example of "generative" reproduction is given by bacteriophage; DNA units of molecular dimensions reproduce and the progeny direct the organization of the complex phage particles around themselves. Any form of "fission" of a complete phage particle is now unthinkable.

In any event, what direct evidence we do have available does suggest that new centrioles grow from old, and there is every reason to postulate that the basic reproductive event involves a much smaller unit than the complete centriole. Our experiments establish the critical "point of no return" of this event, after which it is no longer influenced by mercaptoethanol. It is not surprising that we place the time of duplication, thus defined, at an earlier stage in the cell cycle than that at which visible "new" centrioles have been seen by microscopists.

In establishing this critical time, our experiments do not say whether the duplication process itself is a sudden one or a gradual one, nor do they tell us when we shall expect to see two full-grown centrioles as the ultimate products of the duplication.

V. THE SPLITTING AND SEPARATION OF THE CENTERS

A. Experimental:

We have seen that there are generally twice as many potential poles as actual ones at metaphase, and that the potentialities are realized under such conditions as are given by the quadripartition experiments. In general, these conditions provide a delay in the mitotic proceedings during which the splitting and separation of centers, which appears to be insensitive to mercaptoethanol, can take place. We are thus provided with an experimental design for studying the splitting and separation events apart from the duplication events. The experiments ask the following question: given a cell with four potential poles, what must happen before we realize four actual poles? The experiments to be described deal with the time-course of these events.

S. purpuratus eggs are put into mercaptoethanol at various times following the completion of the duplication of the centers as defined in the previous section. They are put back into sea water after various times of blockage in mercaptoethanol. The experiment asks: when do the cells reach a stage when the four centers become independent, as measured by the realization of four poles?

One group of eggs was put into the mercapto-

ethanol 20 minutes earlier than a second group, in order to determine whether it was the absolute duration of exposure to mercaptoethanol, or the "clock time" of some process that was unaffected by mercaptoethanol that determined the event which permitted the cells to divide into four.

The experimental design and the observed results are given in Fig. 7.

In some respects, the results given in Fig. 7 are not unambiguous. In both groups, four-way division appeared only if the cells were permitted to remain in mercaptoethanol until the time when the controls were dividing. To this extent, we conclude that the "signal" for what we term the "splitting" of the duplex centers is given at about the time of normal division. The term "splitting" refers to some event which permits the members of a pair of centrioles to separate and function independently. By speaking of a "signal" at a certain time that is related to the division of the control cells, we merely mean to indicate that some processes are proceeding at a normal rate independent of the effects of mercaptoethanol, and these, at a certain stage, are responsible for the "splitting."

The ambiguity of the results lies in the fact that the frequency of four-way divisions—in contrast to the time at which they first appear—does seem to depend on the total time of immersion in mercaptoethanol. This we may attribute to the fact that the cell will form two furrows only if the centers, once split, have separated sufficiently far from each other before cytokinesis sets in. We are, then, distinguishing between a "splitting" event, as defined above, and the actual movement apart of the centers once they have split, which is an observable phenomenon.

The visible aspects of these experiments are shown in Fig. 8. This represents the mitotic apparatus in eggs which have been removed from mercaptoethanol after various times of immersion and, permitted to recover for 8 minutes in sea water before immersion into 30 per cent ethanol at −10°

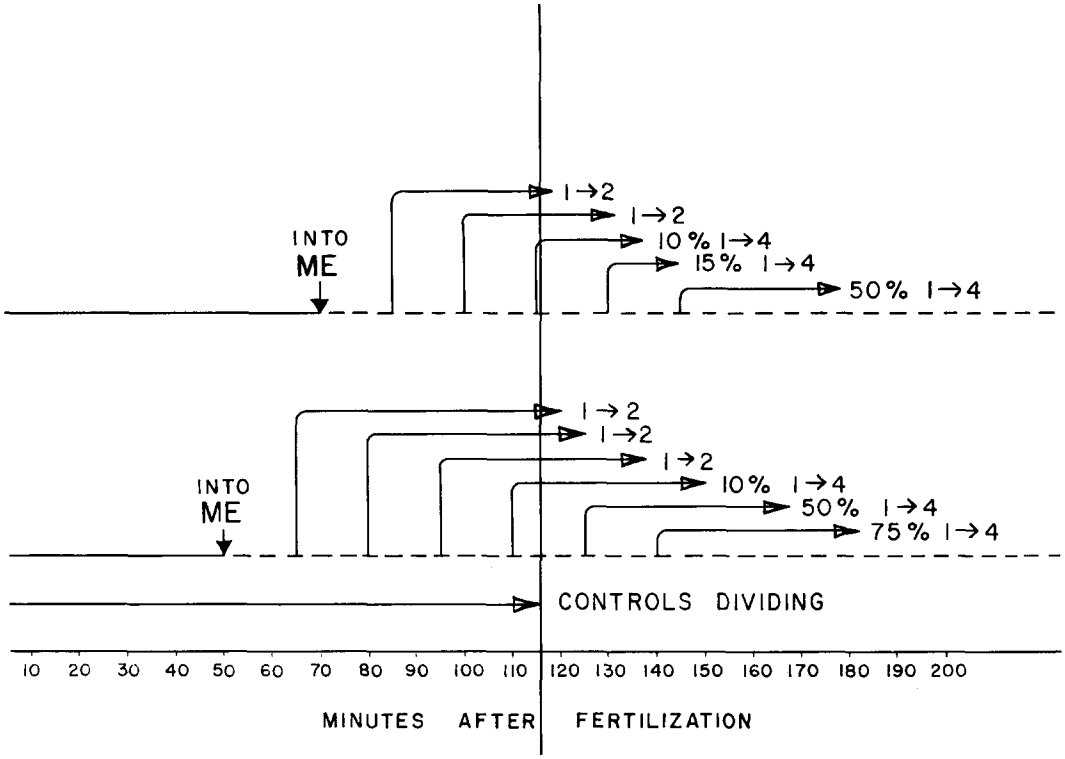
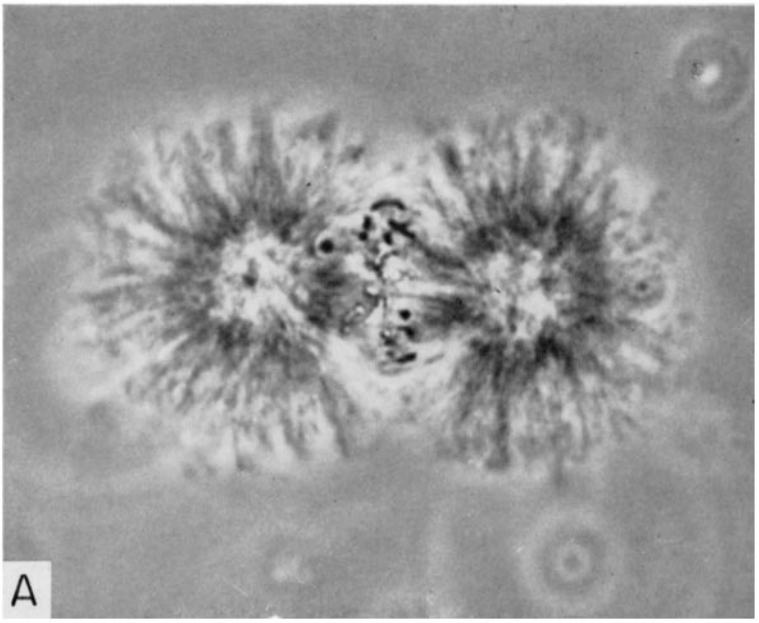
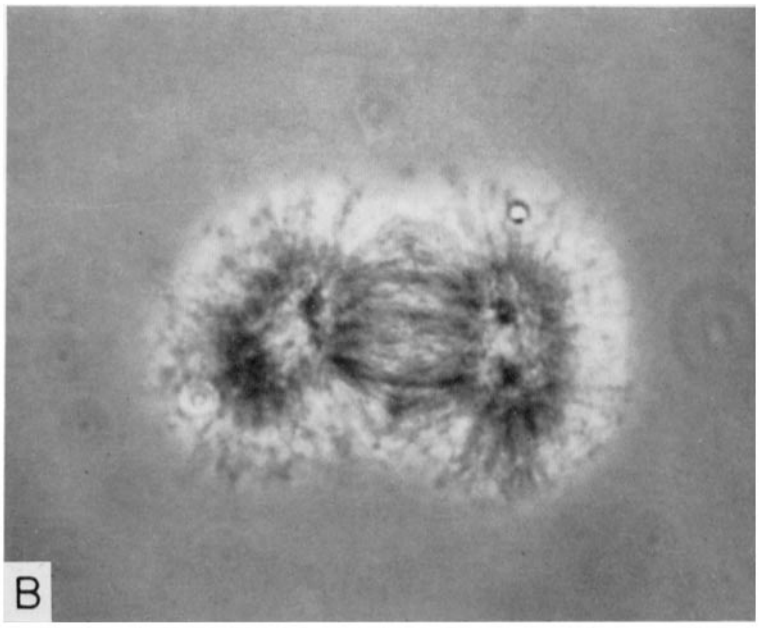


FIG. 7. Experimental data on time of splitting of the centers in *S. purpuratus*. Eggs were immersed in 0.075 M mercaptoethanol at times shown. Samples were returned to sea water at times indicated by bases of arrows, and divided at times shown by points of arrows. The essential finding is that 1 → 4 division (quadripartition) is possible only if the cells are blocked by mercaptoethanol until the time when the controls are dividing, or later.

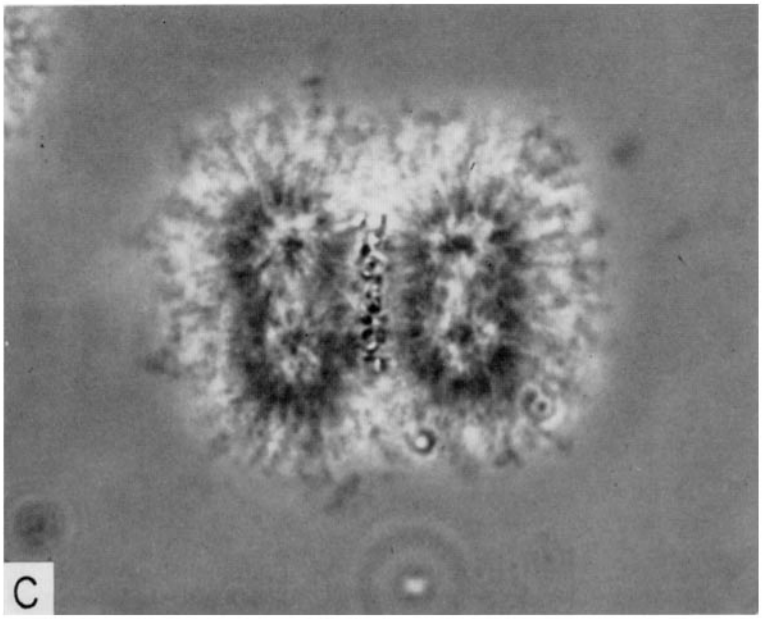


A. Removed from mercaptoethanol after 15 minutes, and before the controls have divided. The centers have not split.

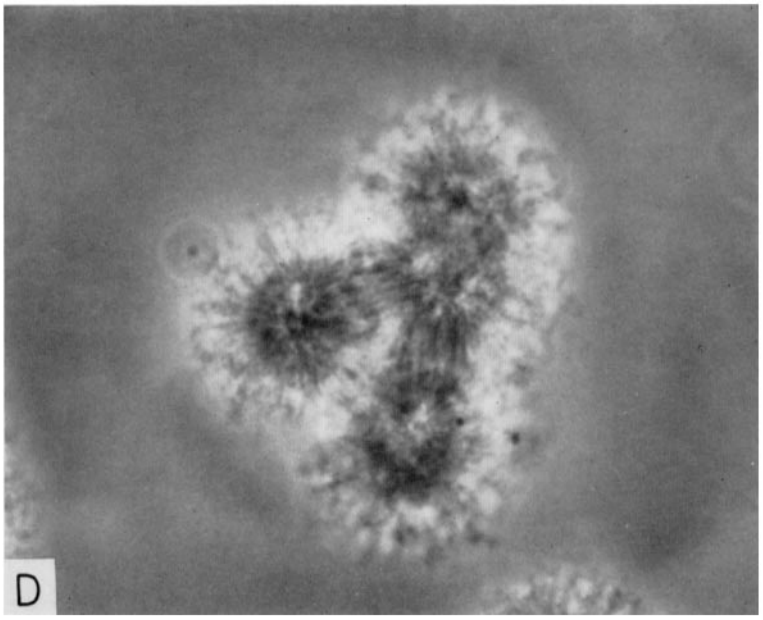


B. Removed from mercaptoethanol after 30 minutes, which is well after the controls have divided. The centers have just split. At this stage, compact bodies are seen in the centers (compare with Fig. 6 *C*).

FIG. 8. Splitting and separation of the centers during blockage by mercaptoethanol. *S. purpuratus* eggs were placed in 0.075 M mercaptoethanol at 75 minutes after fertilization. Samples were removed at various times, permitted to recover in sea water for 10 minutes, then immersed in 30 per cent ethanol at −10°. The mitotic apparatus was isolated by the digitonin method and photo-



C. Removed from mercaptoethanol after 45 minutes. Centers have split and are well separated.



D. Removed from mercaptoethanol after 60 minutes. Centers are fully separated and arrayed in characteristic terahedral formation.

graphed with the aid of phase contrast. The centers would have been more difficult to observe in mitotic apparatus isolated directly from mercaptoethanol, but can be observed readily after 8 minutes of recovery in sea water, which is sufficient for the reorganization of the fibrous structure (Mazia and Zimmerman, 1958).

During these 8 minutes, the mitotic apparatus had recovered its fibrous structure, as described by Mazia and Zimmerman (1958), but we presume that the separation of the centers was not much greater than it had been at the time of removal of the mercaptoethanol. After exposure to cold ethanol, the mitotic apparatus was isolated for observation by the digitonin method (Mazia, 1955).

It is seen that the mitotic figure removed from mercaptoethanol before the time of cleavage of the controls is normally bipolar. But if it is removed shortly after this time the centers have clearly split, though the two units are not very far apart. As time in mercaptoethanol increases, they move further apart until they form a perfect tetrahedral figure. Clearly, mercaptoethanol has no effect on the movement of the centers, once they have duplicated and split.

The diagrams presented in Figs. 9 to 11 will serve later as a guide to the interpretation of these experiments and their relation to the preceding ones.

There is no difficulty in understanding why the frequency of quadripartition increases with the length of time spent in mercaptoethanol, for although they continue to move apart during the "recovery" period, the onset of division soon overtakes them.

What is more difficult to establish is whether there is actually a limiting event which we call the "splitting" of the pairs of centers; that is, a distinct event which permits them to move apart. Observations of the time of the first visible signs of separation (Fig. 8) and the data plotted in Fig. 7 considered in terms of the earliest time at which quadripartition can be observed, suggest that such an event exists, timed by the flow of processes that are not affected by mercaptoethanol. In the following discussion, we shall pursue the implications of this suggestion while recognizing that it is consistent with, but not unequivocally established by the data.

The timing of the "splitting" event is important in relation to the earlier evidence on the timing of the duplication of the centers. If we interpret Table II and Fig. 7 correctly, the duplication event (defined as the doubling of the number of potential poles) and the splitting event (defined as the time when sister centers become independent) take place during the same period of the cell cycle, around the time of completion of mitosis and the onset of cytokinesis.

SUMMARIZING DISCUSSION; RELATIONS BETWEEN DUPLICATION, SPLITTING, AND SEPARATION IN THE REPRODUCTION OF THE CENTERS

This analysis of the reproduction of the centers has resolved the over-all multiplication of the number of mitotic poles into three processes: (1) Duplication in the sense of the determination of a copy of the original body. It is possible that this may be further resolved (section IV, *B*) as the reproduction of a part of the body, a "germ" or "seed," which then determines the growth of a complete center; (2) splitting, an event whereby the original unit and its product become separable and capable of functioning independently to form mitotic poles; and (3) the physical separation of the centers following splitting.

The interpretation of the normal course of events and of all of our experimental modifications is given in Figs. 9 to 11. This is an internally consistent representation of the number of units composing the centers, their "generative" method of duplication, their splitting, their separation, and their relation to mitosis and cell division, based on the experimental designs given in Figs. 2 and 7, and on the experimental results presented in other tables and figures. There is no doubt that the duplication and the separation of the centers are actual and dissociable events, one a problem in molecular replication and growth, the other a baffling problem of a highly oriented movement of large bodies over long distances. Our interpretation of the data given in Fig. 7 leads to the hypothesis that "splitting" is also a real event, quite accurately timed, and distinct from the other two, but the evidence is not as compelling. It could be argued as well that "splitting" is merely the beginning of the movement-apart of the sister centers, which is initiated by a "signal" given at the clock-time of telophase whether or not the cells are blocked by mercaptoethanol. Let us admit that the latter is the simpler hypothesis, and that "splitting" and the actual migration of the centers are distinguished because they have somewhat different implications.

The problem of "splitting" is clearly related to the question of the mechanism of the reproduction of intracellular bodies. In the "fission" models of reproduction, as in certain formulations of the replication of DNA (reviewed by Delbrück and Stent, 1957), the synthesis and splitting-apart of daughter units are inseparable events. This is clearly not the case with the centers. Our analysis

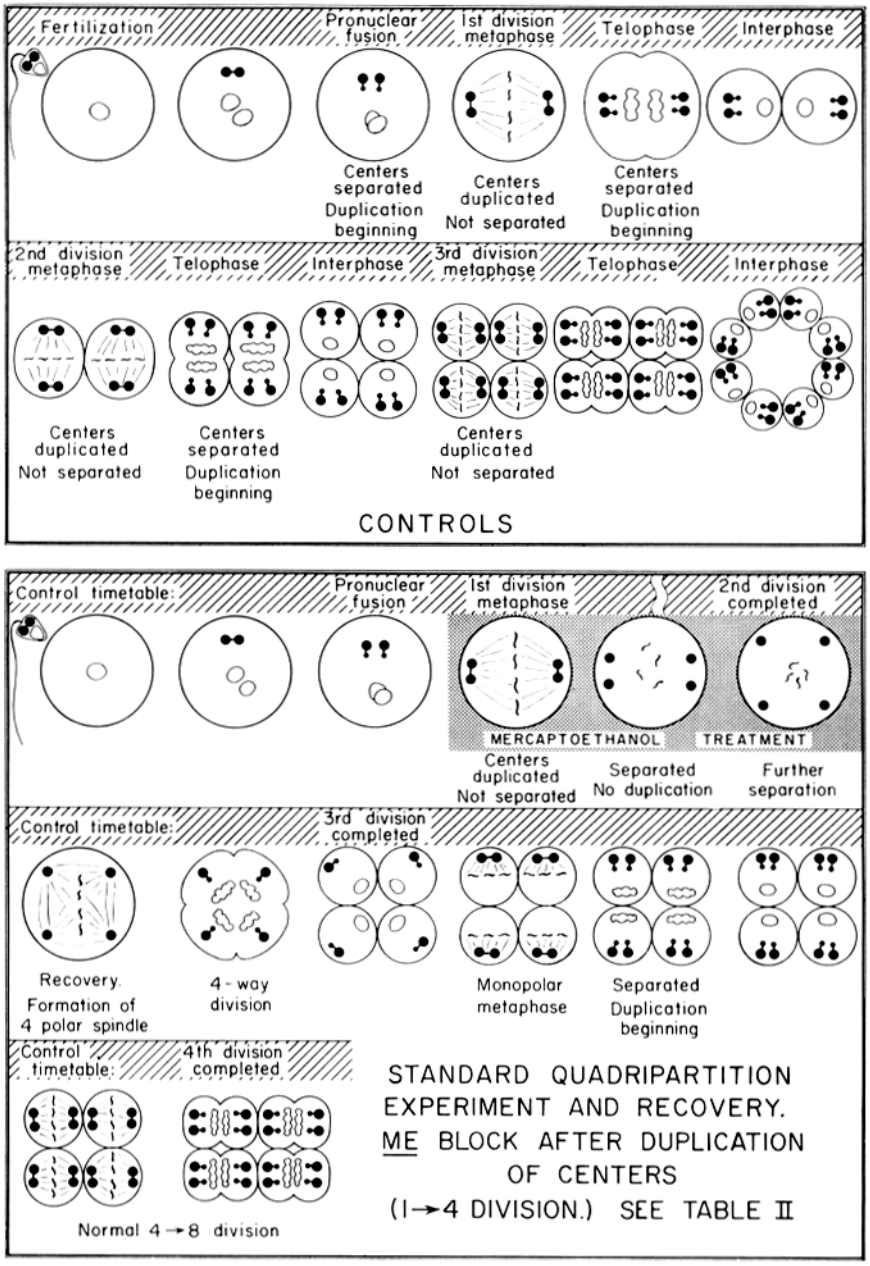


FIG. 9. *Upper frame.* A diagrammatic interpretation of the reproductive cycle of the centers. All nuclear figures shown are purely symbolic, and are introduced merely to identify mitotic stages. Functional units of the centers are represented by solid dots. Small dots indicate newly formed unit. Bars connecting the units indicate that they have not yet split from each other.

Lower frame. An interpretation of the basic quadripartition experiment, in which it is assumed that the mercaptoethanol block is imposed after the duplication of the centers but before they have split. During blockage, the centers split and separate, forming four poles each of which is composed of half the normal number of units. The following mitosis is monopolar.

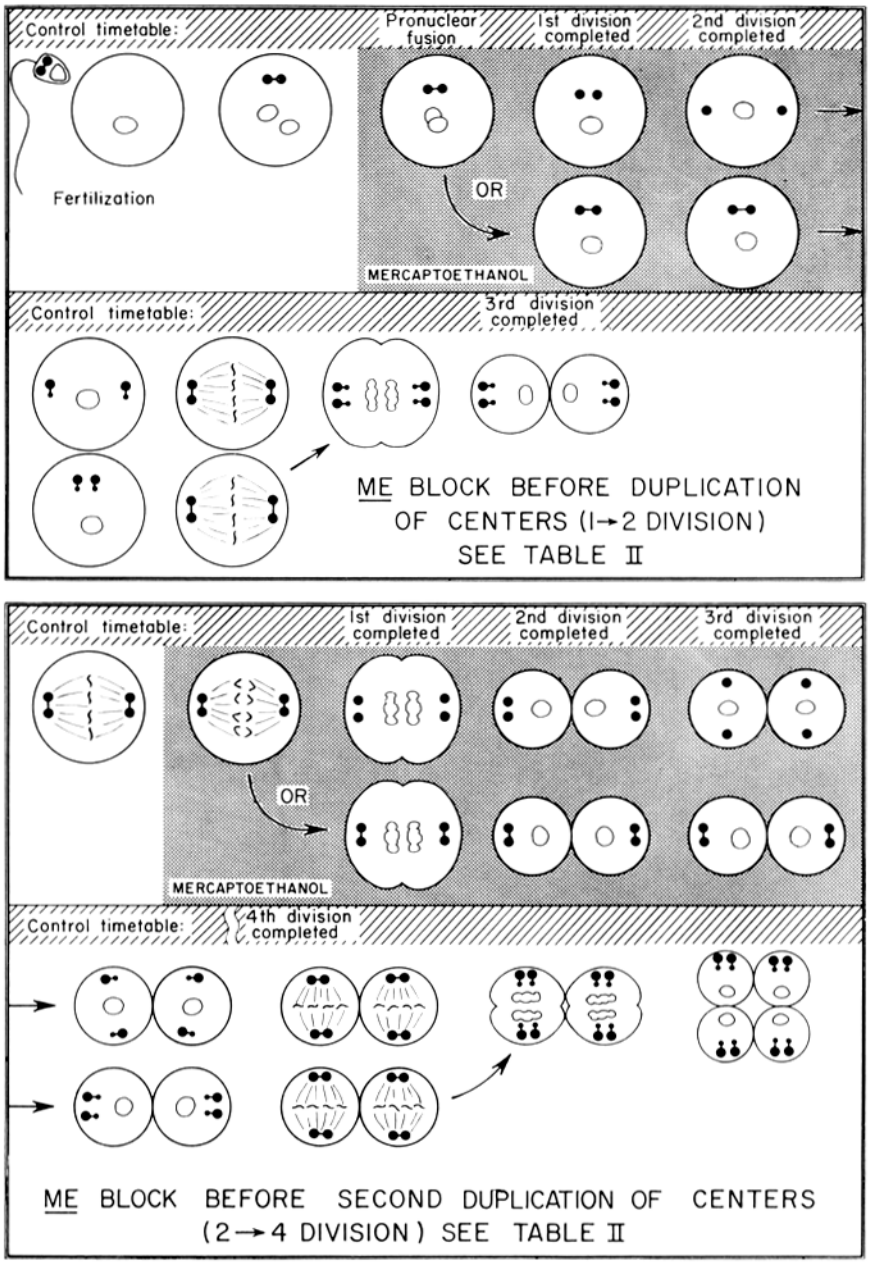


FIG. 10. *Upper frame.* Interpretation of the observation that cells divide into two upon recovery from mercaptoethanol block if the block is imposed early. The interpretation depends on the hypothesis that mercaptoethanol inhibits the duplication of the centers. Alternative schemes are given because it is not known whether centers which have not duplicated split during the time spent in mercaptoethanol.

Lower frame. This is essentially the same as the upper diagram, but represents the course of events when the mercaptoethanol block is imposed before the second duplication of the centers, which is thought to take place during the time the controls are going through their first division.

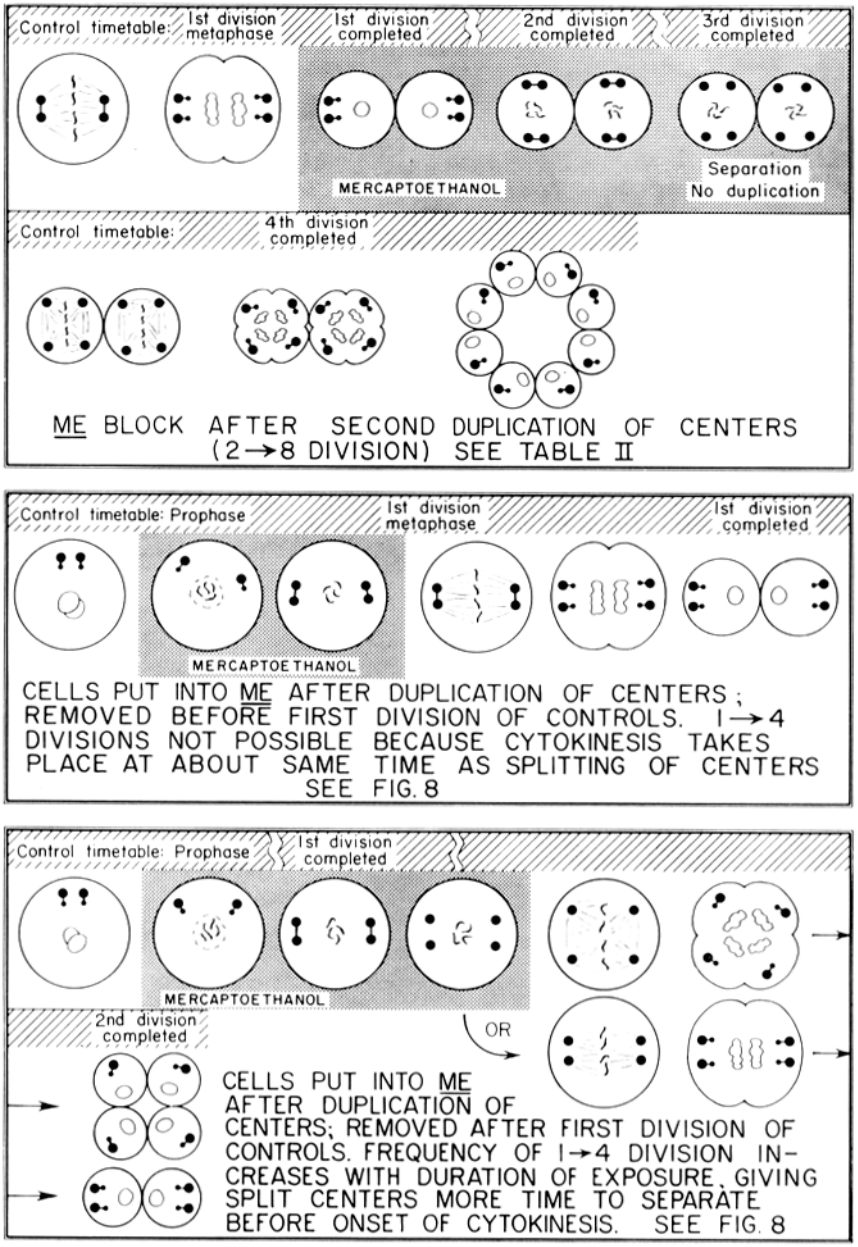


FIG. 11. *Upper frame.* This is equivalent to the lower frame of Fig. 10, but applies to the cases in which 2 → 8 divisions result from blockage by mercaptoethanol at any time from the end of the first division to the metaphase of the second division.

Middle frame and lower frame. Interpretation of experiments given in Fig. 7. The time at which quadripartition becomes possible is interpreted as the time at which the centers split.

of quadripartition experiments shows that splitting may take place—and at the normal time—without concurrent duplication, yielding units of half the normal "valence" (section III D). Nevertheless, it is seen, by comparing Table II and Fig. 7, that the two *normally* take place at the same time, or at least within the same period of the cell cycle. This may be a coincidence, but it may also mean

that the "old" units cannot produce "new" ones until they are separated. In brief, duplication could depend on splitting even though splitting clearly does not depend on duplication. The proposition that splitting follows one cell-cycle behind duplication is a simple consequence of what has been said. As sister centrioles are splitting from each other at telophase to permit the polarization of the next division, each is generating a daughter from which it can be split at the telophase of that next division to provide poles for the next following division (Fig. 9). Such a precession makes little sense in formal terms, and can only be a consequence of the actual mechanisms of the reproduction and splitting of the centers. For example, if the daughter centers require a period of growth before they are equivalent to their parents, as the "generative" model requires, we could understand why simultaneous duplication and splitting is impossible for a given unit.

The present experiments tell us nothing about the mechanism of the actual movement-apart of the centers, except that it is unaffected by mercaptoethanol, but they do provide a useful experimental system for studying this mysterious process independently of all other mitotic events.

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