THE DIRECT ISOLATION OF THE MITOTIC APPARATUS

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ABSTRACT

A method for isolating the mitotic apparatus from dividing sea urchin eggs without the use of ethyl alcohol or of detergents is described. In the present method, the eggs are dispersed directly in a medium containing 1 M (to 1.15 M) sucrose, 0.15 M dithiodiglycol, and 0.001 M Versene at pH 6, releasing the visibly intact mitotic apparatus. The method is designed for studies of enzyme activities, lipid components, and the variables affecting the stability of the apparatus.

INTRODUCTION

The development of a method of isolating the mitotic apparatus (1) provided a demonstration that it "exists" as a distinct body, and work based on this finding (8) revealed certain features of its chemistry, particularly its protein chemistry. The first group of methods revealed their own limitations, as will be seen, and led to a search for a method of direct isolation of the mitotic apparatus from dividing cells, one which would eliminate the obvious sources of artifactitious error in the older methods even though it admittedly might introduce others.

THE EARLIER METHODS

A family of methods, similar in principle, was developed from the original method of Mazia and Dan. The essential steps were (*a*) arrest of the cell and stabilization of the mitotic apparatus by immersion in 30 per cent ethanol at $-10°C$, and (*b*) selective solubilization of the cell surface and cytoplasm, preserving the apparatus in a morphologically normal form. All the methods required the use of the cold alcohol as a first step, but they have varied with respect to further procedures (2–4). The version most used in this laboratory calls for the use of digitonin as the agent of selective solubilization.

A certain amount of descriptive chemical information has been obtained by the use of mitotic apparatus isolated by the alcohol-detergent method: information about composition (2, 5); morphology after various treatments (*e.g.* 6, 7); cytochemistry of various stages of mitosis (8); immunochemical features (9); and structural aspects of the colchicine effect (10), etc.

On the other hand, we were disappointed in attempts to demonstrate the presence of ATPase in the isolated mitotic apparatus, which seemed to be a reasonable expectation. While the negative results could have been meaningful, one could hardly avoid apprehension concerning the possible denaturing effects of the alcohol, the detergent, or both, in spite of the argument that some enzymes had been expected to survive these treatments in other material. Thus, one desideratum of an improved technique of isolation would be the preservation of activities that were absent from the earlier preparations.

A second desideratum arose as information on

the submicroscopic structure of the mitotic apparatus began to accumulate, beginning with the work of Porter (11) and de Harven and Bernhard (12). These and later works have suggested that the traditional "fibrous" structure of the mitotic apparatus included tubular filamentous elements which might very well have a lipoprotein character. If we even suspect that this is so, a method of isolation involving the use of detergents is almost certain to modify or to eliminate the lipid components and thus to give a grossly incomplete chemical picture. In addition, we were increasingly concerned by the contrast between the striking instability of the mitotic apparatus in the cell and the stability of the isolated material, and this source of misinterpretation was pointed out by others (*e.g.* 13). Though the extreme stability of the original preparations, which was the stability of a system "vulcanized" by S—S bonds, was reduced as greater precautions against temperature and oxidation effects were introduced, it remained a fact that deliberate stabilization by cold alcohol introduced a variable that could not be assessed accurately when making hypotheses concerning the bonding of the native mitotic apparatus.

RATIONALE OF THE NEW METHOD FOR DIRECT ISOLATION

The requirements of the preservation of enzymes and of lipids thus have set some of the criteria for the development of a method for the direct isolation of the mitotic apparatus. In addition, the stabilization by alcohol was to be avoided. However, all our experience demonstrated that some contribution must be made to the stabilization of the mitotic apparatus once it was outside the cell. When dividing sea urchin eggs, in which the mitotic apparatus could clearly be seen, were homogenized in various simple media, such as isotonic sugar solutions, the apparatus could be seen to vanish.

In a long series of studies, an important participation of —SH groups in the structure of the mitotic apparatus had been indicated. In earlier work, it was suggested that the apparatus was an S—S bonded structure (1, 2). Experimental results on blockage and disorientation of the apparatus conformed to predictions from this hypothesis (6). Later improvements in the handling of the apparatus isolated by alcohol-digitonin, however, suggested that while the bonding might be through —SH, we were not necessarily dealing with a simple —S—S— bonded structure (5; discussed in 14, 15); the mitotic apparatus could now be dissolved in *p*-chlormercuribenzoate and Salyrgan. At the same time, the important cytochemical studies by Kawamura and Dan (16) and Kawamura (17) revealed a build-up of stainable protein SH, rather than S—S, in the mitotic apparatus before metaphase, and a decline of protein SH during the later stages of division. The hypothesis that the mitotic apparatus is a sulfur-bonded system has sought support in the fluctuations of soluble thiol groups in the dividing cell as well as in the properties of the mitotic apparatus. The existence of cyclic fluctuation of "glutathione" concentration, first proposed by Rapkine (18), was confirmed by Sakai and Dan (19), who found, however, that the substance involved was not glutathione itself, but a TCA-soluble protein of polypeptide. These developments (reviewed in 15) have some bearing on the following speculations.

The hypothesis on which the present method is based is, in its present form, a good deal less definite than one calling for simple S—S bonding. It imagines that a major part of the intermolecular bonding responsible for the stability of the mitotic apparatus does involve linkages through thiol groups. One way of picturing these is as H bonds or cationic bridges through —SH groups. It has been pointed out, and we are indebted to Mr. Thomas Bibring for this idea, that the extreme weakness of such bonds need not be a damaging argument in this case, where the total number of bonds might be very great. A thermodynamic analysis by Inoue (20, 21) suggests that the orientation and perhaps the total stability of the mitotic spindle may in fact be the result of the cooperative action of bonds having a ΔF of only -0.65 kcal/mole.

Another way of imagining the situation is in terms of a *statistical* sulfur bonding. The premise is that the system contains pairs of SH groups in close proximity; this is supported by the ease with which the mitotic apparatus can be oxidized to a state of extreme stability, where it can be dissolved only by strong reducing agents under alkaline conditions (5). One supposes that, under given conditions, there is a probability that a certain proportion of the pairs of SH groups will be in the fully oxidized S—S form. The groups are

imagined to be in a constant flux of oxidation and reduction, so that the over-all stability is only a statistical reflection of the number of pairs that are linked at any given instant. It is now proposed that a certain number of pairs may even be half oxidized, since the complete oxidation is a two electron process. We have no basis for judging the lifetime of any one half oxidized pair, but if the premise is correct there is bound to be a number of them in the system at any given time. This picture would image the mitotic apparatus as a gigantic free radical with a constant virtual flow of electrons within it, and between it and surrounding systems capable of participating in the flow.

The stability of the mitotic apparatus at any one time would depend, then, on the number of pairs of linked thiol groups, and this theoretically could be poised by a comparable redox system in the medium surrounding the mitotic apparatus. The poising effect would consist of a "buffering" by the surrounding system of the flow of electrons to and from the thiol groups of the mitotic apparatus. It might be proposed that the system described by Rapkine (18) and identified by Sakai and Dan (19) served this function in the cell. In any case, the present method was based on a procedure of stabilization which, while still artificial, purported to mimic the conditions of stability within the cell, substituting for a totally unnatural stabilizing treatment such as exposure to alcohol.

The hypothesis as developed above represents the afterthoughts of one of us and was proposed in a recent publication (15). The practical development of the method was based on exploratory experiments in which it was reasoned that we might exploit the converse of the effects of mercaptoethanol which had already been studied (5). It was thought that if mercaptoethanol disoriented the mitotic apparatus, its oxidized form, dithiodiglycol, might stabilize it by increasing the number of S—S bonds through a reversible oxidation. Preliminary experiments did seem to bear this out; when dithiodiglycol was present, the mitotic apparatus did remain whole after the cell was broken in an otherwise inadequate medium. The use of this principle was described in several earlier publications (22, 14), and the purpose of the present paper is to describe the details of the method as it is now used. The reasons for the various steps are described along with the procedures.

PREPARATION OF CELLS

Thus far, only sea urchin eggs, mainly those of *Strongylocentrotus purpuratus*, have been used. In addition to the fact that eggs are still the only good source of large quantities of cells in synchronous mitosis, a good deal of information about the surface characteristics of these cells (reviewed in 23) may be exploited in finding the best conditions for homogenization.

The gentle homogenization technique used in this procedure requires that the eggs be freed of their fertilization membranes and of other surface layers. The following procedure is in use. A dense suspension of eggs in sea water is inseminated at 15°C, at which temperature the eggs will be maintained. As soon as the fertilization membranes have begun to lift (in about 30 seconds), the suspension is mixed with 2 volumes of Ca-free sea water containing about 1 mg/ml of mercaptoethylgluconamide and about 0.01 M Versene. The pH of this mixture must be about 7.5–8.5. Mercaptoethylgluconamide serves as an—SH agent which does not penetrate the cells but which prevents the hardening of the fertilization membrane in accordance with the hypothesis (23) that this hardening involves disulfide formation. (The substance was brought to our attention by Dr. David Doherty, of the Oak Ridge National Laboratory, who supplied the first samples we used. It can be obtained from the Cyclo Chemical Corp., Los Angeles 1, California.) The purpose of the Versene in relatively high concentration is to remove the divalent ions at the time the hyaline layer is forming on the surface of the egg, reducing stickiness and aiding the preparation of "naked" and fragile eggs. After 15 minutes, during which time the suspension is stirred for purposes of oxygenation, it is passed several times through a layer of fine silk (25 mesh) to strip off the fertilization membranes. If the eggs agglutinate, they are handled very gently, as they tear and break if they are stirred violently when clumped. The eggs are allowed to settle, the supernatant containing fertilization membranes and sperm is drawn off, and the eggs are gently suspended in Ca-free sea water. This step is repeated. In Ca-free sea water the eggs appear naked, with no visible hyaline layer, and are not sticky. As naked eggs, they are very fragile and it is hazardous to centrifuge them. Hence one risks the two periods of settling under gravity, during which time there is some danger of anoxia. After the second washing with Ca-free sea water the eggs are maintained at 15°C (a temperature appropriate for marine eggs on the northern Pacific coast) with gentle stirring. A slow stirrer, just adequate to prevent any settling of the eggs, provides sufficient aeration.

ISOLATION

At the time of transition from prophase to metakinesis, when the nuclear membranes have just broken down, the eggs are collected by low speed centrifugation, and washed once with a mixture consisting of 90 per cent 1 M dextrose and 10 per cent Ca-free sea water. The purpose of this isotonic mixture is to bring down the ionic strength at which the isolation will be made. In our ordinary procedure, the eggs will have reached metaphase by the time they are centrifuged from the mixture of dextrose and Ca-free sea water (about 85 minutes after fertilization.) If they have not, they will proceed to metaphase or any later stage that is desired while in this medium. When they have reached the desired stage, they are suspended in 10 volumes of the isolation medium (SVD, discussed below), allowed to remain in this medium at 15°C for 2 minutes, then shaken moderately. Hand shaking of the suspension in a flask has been found to be adequate. The suspension is then put into an ice bath and all further steps take place at 0°C. The eggs appear to crumble in the SVD, and the mitotic apparatus, usually surrounded by a layer of yolk particles in the asters, is set free (Fig. 1). A more rapid dispersal of the eggs can be achieved by forcing them through a rather coarse (0.5 mm or so) glass capillary with a Luer syringe. This will tend to break some anaphase spindles into half spindles and break off some asters from spindles, but this may not matter if the object is to collect the material for chemical analysis.

This, then, is the isolation procedure itself. The procedure for concentrating the mitotic apparatus with a minimum of contamination will be described below. Let us now consider the character of the medium (SVD) in which the isolation is done. It consists of 1.0–1.15 M sucrose; 0.15 M dithiodiglycol ($OHCH_2\ CH_2—SCH_2\ CH_2OH$); 0.001 M Versene; pH carefully maintained between 6.0 and 6.2, with best results at 6.0.

It is necessary to use a near-isotonic medium because the mitotic apparatus itself shows quasi-osmotic behavior, as mentioned below. The eggs did not break well in the presence of appreciable concentrations of electrolytes. Of the non-electrolyte media tested, sucrose was the most satisfactory because its density is favorable for keeping the large particles—especially yolk, which is the most conspicuous contaminant—in suspension when the mitotic apparatus is centrifuged down. Otherwise, dextrose is quite

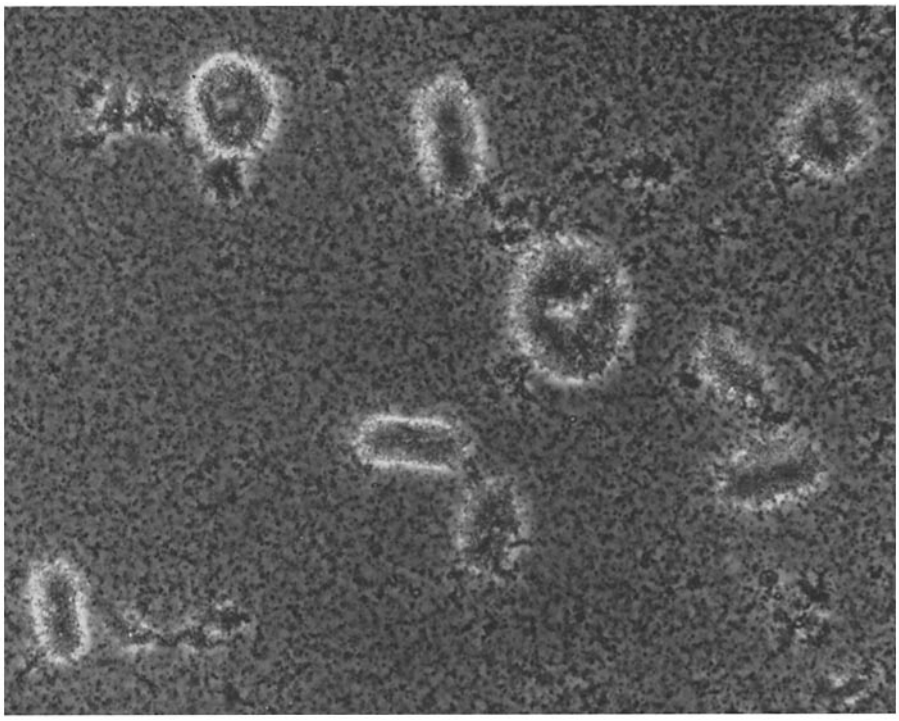

FIGURE 1

Low magnification phase contrast view of homogenate immediately after eggs are broken in isolation medium. Note mitotic apparatus. × 240.

adequate. Though most of the work has been done in a medium based on 1.0 M sucrose, it has recently been found that 1.1 M or 1.15 M sucrose provides for somewhat greater mechanical stability of the mitotic apparatus during centrifugation. Presumably the slight dehydration, which is visible as a sharpening of the fibers, makes for greater strength.

The Versene contributes to the smoothness of the dispersion of the cytoplasm; if it is eliminated, the eggs do not break so well and the particles are not so well dispersed.

The pH control is essential to the stability of the isolated mitotic apparatus, and represents a condition that may be artificial. If the isolation is carried out at pH 6.5 or higher, the mitotic apparatus is initially pale and flaccid and soon disperses.

The reasons for using dithiodiglycol have been discussed fully above. If it is left out, the mitotic apparatus is not recovered. This does not mean that the reason for using it was the correct one, or that other stabilizing agents will not be found which work as well. Other compounds—dihydric alcohols without S—S, other S—S compounds, and other oxidizing agents—have been tried with varying success, but as yet none has been as satisfactory as dithiodiglycol in our experience.

CONCENTRATION AND WASHING

The mitotic apparatus is freed from the particle suspension, in which it was isolated, by low speed (*ca.* 200 *g*) centrifugation at 0°C and repeated washing in the sucrose-Versene-dithiodiglycol (SVD) medium. At this centrifugal force, it sediments in about 30 minutes in 40 ml conical glass tubes in a horizontally swinging centrifuge, while the yolk particles, mitochondria, fat droplets, and smaller particles tend to remain suspended in the dense medium. The yield is increased if the homogenate is centrifuged twice under these conditions. Heavy debris carried over from the original egg suspension—fine sand, detritus from the body cavity of the sea urchin, etc.—packs in the conical tip of the tube as do unbroken eggs, and if the overlying mitotic apparatus is resuspended with care, these contaminants will be left behind when the suspension of mitotic apparatus is drawn off. The worst potential contaminants are unbroken eggs and fertilization membranes which may have

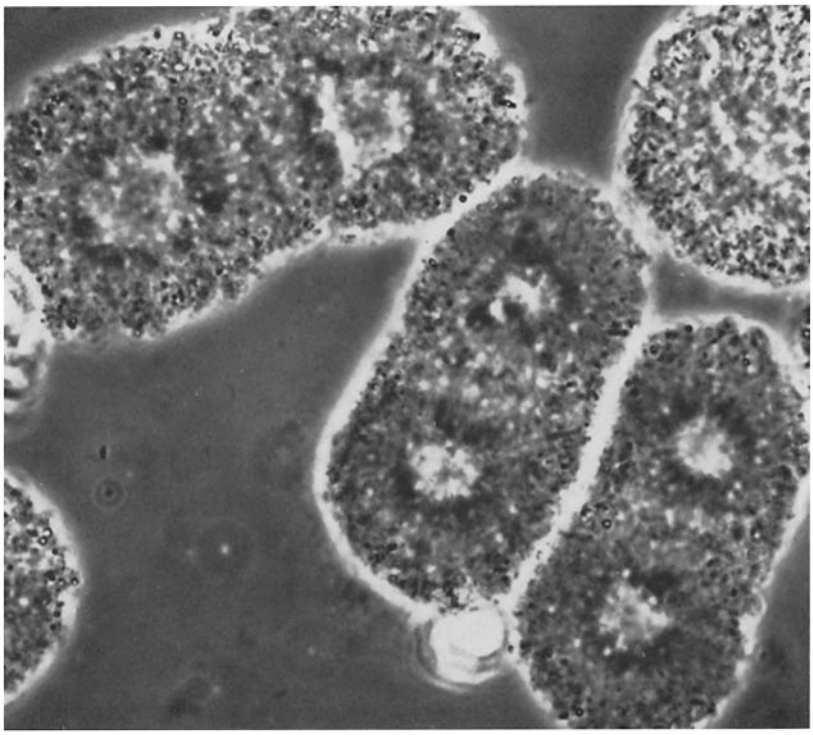

FIGURE 2

Phase contrast view of mitotic apparatus after washing in SVD medium. Chromosomes are not clearly visible but would become so if the ionic strength were raised. Note that the asters still contain granules at the periphery. × 950.

been carried over from the initial steps; these are negligible in the best isolations. Three washings generally suffice to remove suspended contaminants, leaving a pellet of concentrated mitotic apparatus (Fig. 2). Realistically speaking, the maximum yields have been in the range of several hundred milligrams, although there is no reason, other than the conservation of sea urchins, why very much larger batches could not be prepared.

PROPERTIES OF THE ISOLATED MITOTIC APPARATUS

The mitotic apparatus isolated by the above method looks very much as it does in the living cell; its interior is clear and hyaline appearing, and distinct fibers are not seen. It is complete with spindle, asters, and chromosomes, although the chromosomes are not very visible, even with phase contrast, while in the SVD medium. They become visible when the ionic strength is increased. In one sense, the preparations are not very clean; there are always yolk particles in the asters. In another sense, these yolk particles are to be expected in a "natural" apparatus, for it is evident from both light microscopy and electron microscopy that yolk particles are actually embedded between the astral rays in the living egg. Thus, a "whole" aster is bound to include such particles and a "whole" mitotic apparatus should include all particles contained within it in the cell. In this respect, the present method based on direct mechanical isolation of the whole mass represented by the mitotic apparatus is fundamentally different from the older one, which was based on a solubility difference between the stabilized fiber structure and the other constituents of the cell.

It is easy enough to make cosmetically "purer" preparations, though at some sacrifice of desirable or meaningful properties. For example, the material may be put through a brief exposure to a very dilute medium, in which the yolk particles ex-

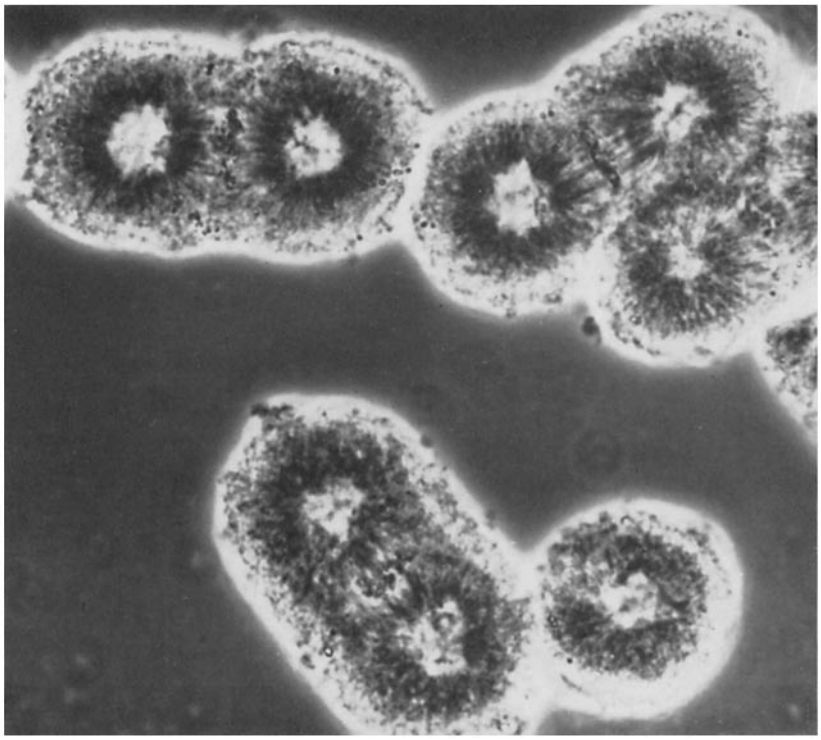

FIGURE 3

Phase contrast view of mitotic apparatus isolated in SVD and exposed to 5×10^{-4} M $CaCl_2$. The same picture is obtained with appropriate concentrations of Mg salts. Note the chromosomes, the sharp "fibers," and the nearly complete elimination of the large particles at the periphery of the asters. × 950.

plode according to their expected osmotic properties (25). The apparatus survives if it is quickly restored to its normal SVD medium, but one will then be uncertain what the residue of the yolk is contributing as a contaminant. One of the most interesting properties of the mitotic apparatus isolated by the present method is that it is irreversibly stabilized by Mg^{++} and Ca^{++} solutions at low concentrations. Thus, if the apparatus is washed in 5×10^{-4} M $CaCl_2$, the yolk is exploded and the apparatus becomes very resistant to solution. (Fig. 3).

STABILITY AND SOLUBILITY

The contributions of the several factors in the isolation medium to the stability of the isolated mitotic apparatus are reversible; it is by no means "fixed." If the sucrose concentration is varied, the behavior of the apparatus follows an "osmotic" pattern, although this has not been tested quantitatively. At higher concentration of sucrose, the isolated apparatus shrinks; at low concentration, it swells and appears to dissolve. If the dithiodiglycol is removed, the apparatus tends to dissolve or at least to become very fragile in the isotonic sucrose alone. If the pH is raised, the stability is lowered. Versene is not necessary for stability, but it is retained in the medium to prevent clumping of particles during the isolations.

As has been mentioned, exposure to Ca^{++} or Mg^{++} irreversibly stabilizes the over-all structure. It can be seen that the contents of yolk particles and other particles are removed by treatment with dilute solutions of salts of these ions. These treatments have been employed in later work to isolate a fibrous "skeleton," but they do not permit the later dissolution of this skeleton. Even strong solutions of Versene or highly allkaline conditions will not reverse the effects of Ca^{++} or Mg^{++}.

The preparations are readily soluble in salt solutions, leaving behind various particles: chromosomes, which are seen to float free, and the central cores of the asters, which may ultimately provide a means for isolating centrioles. If the apparatus is dissolved in *isotonic* KCl (0.53 M) at pH 7.5–8.5, the yolk particles remain visibly intact, and it can be assumed that other osmotically sensitive particles will remain intact. After centrifugation at high speed, the supernatant is regarded as a solution of the fibrous components of the apparatus, while the sediment is viewed as representing the particles trapped in or participating in the structure of the mitotic apparatus. Since the dissolution of the apparatus in isotonic KCl was preceded by repeated washing in SVD, which would remove diffusible components, the one source of misinterpretation in the operation would be the presence, in the particles, of molecules which were not soluble in SVD but became soluble in esotonic KCl. The isotonic KCl soluble fraction has been used for the study of the ATPase and the lipids of the mitotic apparatus to be described in subsequent reports.

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