THE MITOTIC APPARATUS

Identification of the Major Soluble Component of the Glycol-Isolated Mitotic Apparatus

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ABSTRACT

Mitotic apparatuses (MA) isolated from metaphase sea urchin eggs in 12% hexylene glycol at pH 6.4 can be dissolved rapidly in 0.6 M KCl, and more than one-half of the total protein of the MA is soluble under these conditions. In the phase-contrast microscope, the fibrous structure of the MA can be seen to disintegrate in KCl solution, leaving only granular material which, in the electron microscope has been seen to be largely vesicular, with no evidence of microtubules or other fibrous elements. The KCl-soluble material thus must contain the soluble components of the microtubules and consists of one major, homogeneous component with a sedimentation coefficient of 22 Svedbergs, and a much smaller amount of more heterogeneous material sedimenting at 4–5S. A component similar to the 22S component can be identified in extracts of unfertilized eggs, where it forms approximately 8% of the total cell protein. The amount of this protein present in the cell is considerably in excess of that involved in the MA, as can be shown by its presence in the soluble supernate from a mitotic apparatus isolation. This protein must form part of, or be associated with, the fibrous structure of the MA in some fashion that allows its release only upon the dissolution of the mitotic apparatus.

INTRODUCTION

One of the basic problems concerning the mitotic apparatus (MA) that should be easily answered by isolation methods is the nature of its macromolecular components. It is obvious that the identification of these components must precede any investigation of their origin within the cell or the mechanism of their assembly into the fibrous structure of the mitotic apparatus. Electron microscope studies of dividing cells (5, 7, 12, 25, 36, 39, 40) have shown that the spindle and asters are formed of 200-A tubular elements, now termed microtubules, and electron microscope observations of isolated mitotic apparatuses (19) have shown that these elements are preserved by current isolation techniques. Since similar microtubules have been identified in a wide variety of cells under

various conditions (4, 8, 10, 26, 41, 43), information gained about the components of the mitotic apparatus is of considerable general interest. In fact, the isolated mitotic apparatus may prove to be one of the most convenient sources of relatively pure and concentrated microtubules.

The experimental attack on this problem would appear to be quite straightforward—the mitotic apparatus has only to be isolated and dissolved, and its soluble components identified. Until recently, however, such investigations have been limited by the relative insolubility of the isolated MA. Identification of the constituent macromolecules of the mitotic apparatus is possible only if the MA can be brought into solution under conditions which will not modify the physical properties of these components.

The composition of the mitotic apparatus has been of interest since the beginning of isolation experiments. In the first report of the successful isolation of the mitotic apparatus from synchronously dividing sea urchin eggs (33), Mazia and Dan dissolved the alcohol-digitonin–isolated MA in 0.5 M NaOH and ran the resulting soluble material in the analytical ultracentrifuge. They found one major protein component, with a sedimentation coefficient of 4S. The highly alkaline conditions required to solubilize such MA would be expected to reduce the proteins present to their smaller subunits, and the component seen very likely resulted from such a splitting of a larger molecule.

Zimmerman (53) later found that such alcohol-digitonin–isolated MA could be dissolved under less drastic conditions in the presence of the organic mercurial Salyrgan. The MA were dissolved in 0.1 M Salyrgan, and ultracentrifugation of such solutions showed the presence of two components, one having a sedimentation coefficient similar to that seen by Mazia (3.7S) and the other a smaller amount of a more rapidly sedimenting material (8.6S). This latter material disappeared from the solution on dialysis. A measurement of the molecular weight of such dissolved MA by the Archibald approach to equilibrium method gave a value of 315,000.

Since it was relatively easy to stabilize the mitotic apparatus with the methods then available but much more difficult to dissolve it, Kane and Hersh (23) attempted to approach the problem in reverse. If the protein or proteins involved in the mitotic apparatus are present in the unfertilized egg, it might be possible to identify this "precursor" protein by comparing extracts of such cells with those of metaphase cells in which the mitotic apparatus had been stabilized by treatment with the isolation medium. If the amounts of protein involved are sufficiently large to be identified by the methods used, one would expect that the protein involved in the mitotic apparatus would decrease in amount or disappear from extracts of metaphase eggs made under such conditions. Kane and Hersh found two major components present in extracts of unfertilized eggs, which were identified by their unextrapolated sedimentation coefficients of 7 and 20S. Some indication of a fluctuation in the amount of 7S protein present in the eggs of *Arbacia punctulata* was found, but could not be confirmed in the eggs of *Strongylocentrotus purpuratus*.

An investigation of the problem of protein precursors to the mitotic apparatus was made by Went (50), using immunological methods. Antibodies to the dissolved MA were prepared and these antibodies were found to react with extracts of unfertilized eggs, indicating that some "precursor" material was present in such cells. Further experiments showed, however, that this material was not the 7S protein of Kane and Hersh. Fluorescent antibody techniques (52) demonstrated this material throughout the cytoplasm of the dividing cell, indicating that it was present in amounts considerably in excess of that required for the formation of the mitotic apparatus.

The development of methods for the direct isolation of the mitotic apparatus, first using dithiodiglycol and believed to depend on the presence of disulfide (34), but later generalized to a wide variety of simple glycols and alcohols (18, 20), has made possible a direct attack on the proteins of the mitotic apparatus, since MA isolated by these methods can be dissolved under mild conditions. Dirksen (9) first reported on the protein components of dithiodiglycol-isolated parthenogenetic asters dissolved in 0.53 M KCl at pH 9. She found two ultracentrifugal components, with sedimentation coefficients of 14 and 21S, plus a large amount of low molecular weight material. Most recently, Sakai (42) has studied the mitotic apparatus isolated in the related compound dithiodipropanol and dissolved in 0.53 M KCl. He found components similar to those seen by Dirksen (9), with sedimentation coefficients of 13 and 22S, and, in addition, identified a more slowly sedimenting 3.5S component. Comparative studies on the soluble fraction of mitotic apparatuses isolated by the original alcohol-digitonin method (33) and dis-

solved in *p*-chloromercuribenzoate showed only the 3.5 and 13S components to be present, leading Sakai (42) to conclude that these were constituents of the fibrous structure of the mitotic apparatus. His studies were concerned primarily with these components and he observed that both these components were cleaved to a 2.5S subunit by sulfite, indicating that the 13S component might be a polymer of the 3.5S. Since the 22S component was unaffected by his reagents and not present in the soluble fraction from the alcohol-digitonin MA, he did not definitely identify this as a component of the mitotic apparatus, although he did mention that it was not a ribosomal particle.

The purpose of the experiments reported here is to identify the soluble proteins of the gylcol-isolated mitotic apparatus and make them available for physical chemical study. Since stabilization of the mitotic apparatus has been shown to be controlled by the concentration of nonelectrolyte and pH (20), and not to depend on the presence of added disulfide, hexylene glycol was used for the isolation of these MA. This avoids the complication of adding disulfide to a system in which one is attempting to determine the presence and role of these bonds. It has also been shown that changes in the structure and properties of the MA occur after isolation (22), and this information was utilized in these experiments to obtain MA which had undergone a minimum of modification during or after isolation. The problems to be considered include the amount of soluble protein in the MA, the major components present in the soluble fraction, their origin in the MA, and their relation to soluble proteins of the cell. This investigation leads directly to a detailed study, which follows this paper (47), of the physical properties of the major soluble mitotic apparatus protein and its subunits. Brief reports of this work have appeared in abstract form (21, 46).

MATERIALS AND METHODS

The gametes of the sea urchins *Arbacia punctulata* and *Strongylocentrotus purpuratus* were used as experimental material. Gametes of *A. punctulata* were obtained by the application of 10 v AC across the test (13), and gametes of *S. purpuratus* were obtained by the injection of 0.53 M KCl (48). The eggs were washed several times in sea water before fertilization and only those showing 95% or better fertilization were used.

The mitotic apparatus was isolated by methods previously described (18, 20). Briefly, the method consists of the removal of the fertilization membrane by treatment of the eggs, immediately after fertilization with a 1 M solution of urea. The eggs are allowed to develop in calcium-free sea water until the desired stage is reached and then they are washed twice in a mixture of isotonic sodium potassium chlorides. The cells are washed and then lysed in 12% hexylene glycol, buffered at pH 6.4 with 0.01 M potassium phosphate. The temperature is reduced to 0°C immediately after lysis, and all succeeding operations are carried out at this temperature. The isolated MA are separated from the lysate by differential centrifugation and washed three times in the same glycol medium. They are then pelleted by centrifugation and dissolved in 0.6 M KCl, buffered at pH 7.5 with Tris-HCl. After dissolving for periods of 1–12 hr, the solution is centrifuged at 25,000 *g* for 30 min and the supernatant solution is dialyzed against a large volume of the same KCl solution for at least 12 hr.

Extracts of unfertilized eggs were prepared by removing the jelly and membranes in 1 M urea, washing in two changes of a mixture of isotonic sodium and potassium chlorides, and lysing in the same 12% hexylene glycol solution used for isolation. After lysis, the solution was centrifuged at 25,000 *g* for 30 min and the clear supernatant was dialyzed against a large volume of 0.6 M KCl, buffered at pH 7.5 with 0.01 M Tris-HCl.

Protein concentrations were routinely measured by 280/260 mμ absorption, by using the data of Warburg and Christian (49) in nomogram form. Values obtained by this method were comparable to those obtained by means of the Lowry procedure (29), which used a serum albumin standard. All solutions were dialyzed before measurement in order to eliminate interference by the relatively large amount of low molecular weight absorbing material present. The volume of packed, jellyless eggs present in the last step before lysis was used as an estimate of egg quantities. This eliminates the variability due to differing jelly volumes and gives quite reproducible results. Assuming a diameter of 74 μ for the eggs of *A. punctulata*, 1 ml of packed cells, without spaces, contains 4.7×10^6 eggs. The same volume of *S. purpuratus* eggs, with a diameter of 80 μ, contains 3.7×10^6 eggs.

Analytical ultracentrifugation was carried out at 20°C in a Spinco Model E centrifuge, with the use of a phase plate in the Schlieren optical system. Comparative measurements of concentration were made by the paper weight method and were not corrected for radial dilution or the Johnston-Ogston effect (17).

Electron microscope studies were made on pellets fixed for 30 min in 1% OsO_4 in 12% hexylene glycol at 0°C. After fixation, the pellets were dehydrated in a graded ethanol series and embedded in Araldite by the method of Luft (30). Sections were cut on an LKB ultrotome and picked up on formvar- and carbon-coated grids. They were stained with lead citrate

(38) and uranyl acetate (45) and examined in a Siemens Elmiskop I at 60 kv with a 200 μ condenser and 50 μ objective aperature.

RESULTS

Preparation of the MA Protein

The last step of the usual glycol isolation procedure involves the lysis of the metaphase cell in a 12% hexylene glycol solution at pH 6.4. The resulting lysate contains the intact MA suspended in a medium containing the soluble proteins of the cell. The mitotic apparatuses can be washed free from these soluble proteins, while retaining their native structure and solubility properties, by passing them rapidly through several changes of fresh glycol medium maintained at 0°C. Since only a few tenths of a milliliter of packed MA are obtained in the usual isolation experiment, these MA can easily be washed with 50 times their volume of glycol solution. After three such washes, the concentration of soluble protein in the last wash is reduced to a negligible value. The volume of the pellet of packed MA recovered from the last wash is then measured and an equal volume of 1.2 M KCl is added, giving a final concentration of 0.6 M. As described previously (18), freshly isolated MA dissolve immediately in such a solution, with the entire fibrous structure of the MA breaking down. Samples of this solution examined in phase contrast show no intact MA or recognizable MA fragments after a few minutes; dissolution is usually allowed to proceed for at least 1 hr at 0°C before centrifuging to remove undissolved material.

The pellet from this centrifugation contains the KCl-insoluble material of the mitotic apparatus, and the nature of this material has been determined by electron microscopy. Previous observations with the phase-contrast microscope on the breakdown of the MA in salt solutions had shown that fine granular material remains after dissolution (18), and electron microscope studies had demonstrated the presence of large numbers of vesicles, presumably lipoprotein, scattered among the filaments of the isolated MA (19). As might be predicted from these observations, the KCl-insoluble pellet consists largely of such vesicles (Fig. 1), quite uniform in size and similar in appearance to those seen in the MA. These vesicles make up the bulk of the insoluble material, while a small area at the centrifugal end of the pellet contains a number of other cytoplasmic components (Fig. 2)) which are occasionally found as contaminants of the isolated MA. Of major importance for the present work, however, is the fact that no evidence of microtubules or other filamentous material can be found at any level in the pellets. Treatment of the MA with solutions of high ionic strength thus dissolves the microtubules, causing the breakdown of the organized structure of the MA and freeing the vesicles present in the interior of the spindle and asters.

An estimate of the distribution of the total protein of the MA between the KCl-soluble and KCl-insoluble fractions can be made by dissolving the KCl-insoluble pellet in 0.1 M KOH. In a series of five experiments, the KCl-soluble fraction made up 50–70% of the total protein, with an average value of 60%. In terms of the yield from 1 ml of packed cells, approximately 0.1 ml of MA is obtained, containing 0.75 mg of total protein, of which 0.45 mg is KCl soluble and 0.30 mg is KCl insoluble.

Since no evidence of microtubules or other filamentous structures can be found in the KCl-insoluble pellet, the KCl-soluble fraction must contain the dissolved components of these elements. When run in the analytical ultracentrifuge, this solution gives a relatively simple schlieren pattern (Fig. 3). One major, rapidly sedimenting peak is present, with an unextrapolated sedimentation coefficient of 22S, plus a smaller amount of more slowly sedimenting, heterogeneous material in the range of 4–5S. The 22S material makes up approximately 80% of the protein present in the KCl-soluble fraction (and thus one-half of the total protein of the MA) and it is this relatively homogeneous component, which forms the bulk of the soluble protein of the glycol-isolated mitotic apparatus, that is of major interest in this and the following report (47). This is not to imply that the more slowly sedimenting material may not also be of importance in the MA, but the small amount present and its heterogeneity make it much more difficult to isolate any well-defined component for investigation.

This pattern is simpler than that found by Dirksen (9) and by Sakai (42) for dithiol-isolated MA. In addition to much larger amounts of slowly sedimenting material, both of these authors found that a 14S component was present, while the 22S component made up a relatively small fraction of the soluble material. However, Sakai's results (42) indicate that the 14 and 3.5S components are

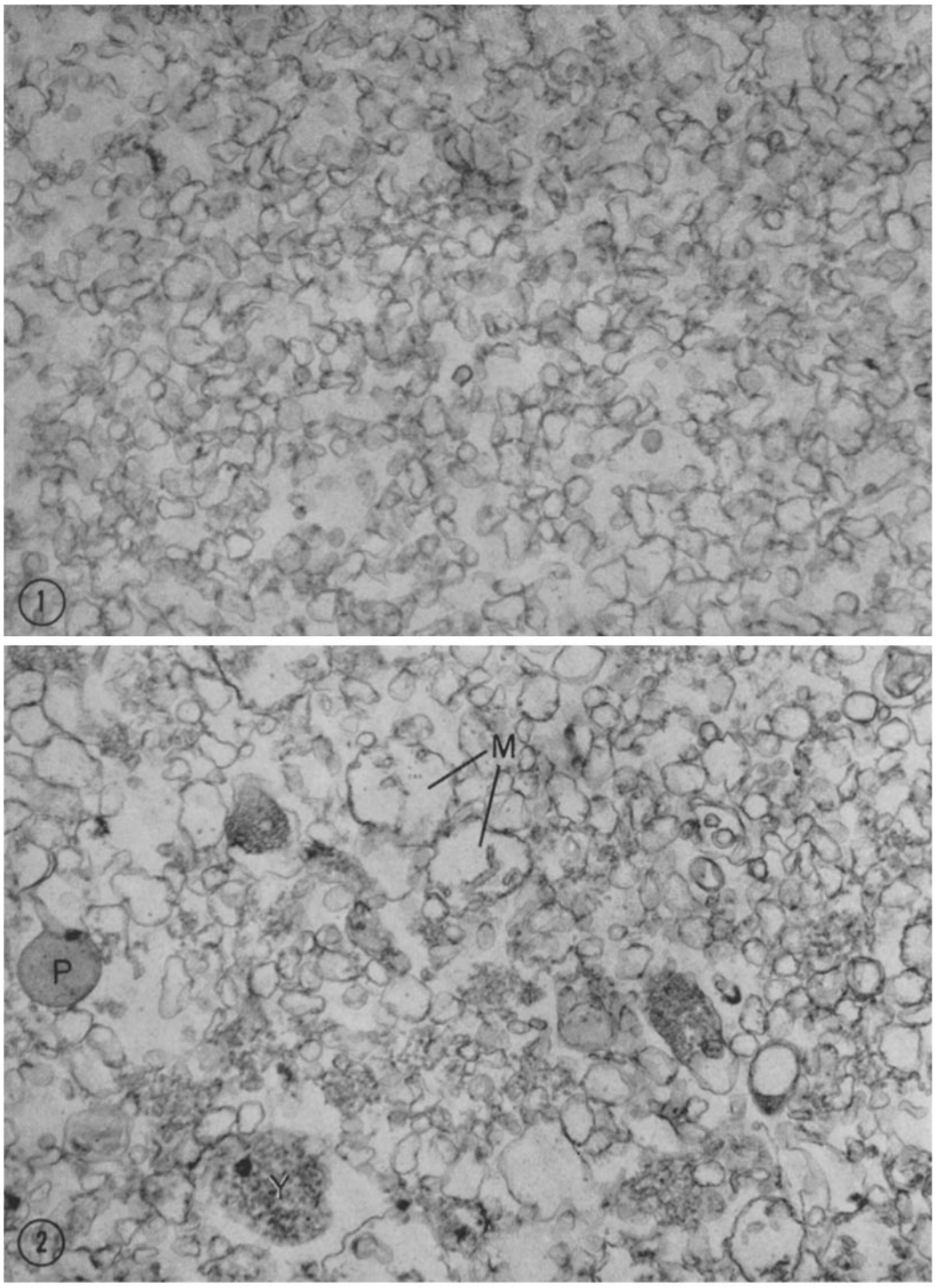

FIGURE 1 Centripetal part of the pellet which remains after dissolving glycol-isolated mitotic apparatuses in 0.6 M KCL. × 23,500.

FIGURE 2 Centrifugal end of the same pellet. Swollen mitochondria (*M*) and lysed yolk granules (*Y*) can be identified and an intact granule, possibly pigment, is present at *P*. × 23,500.

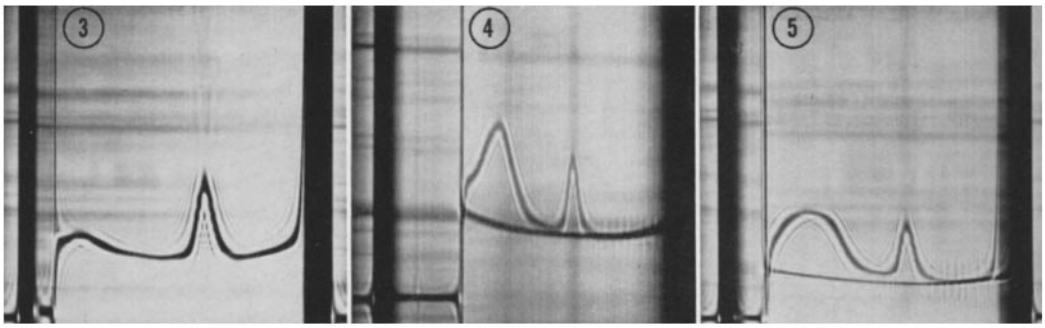

FIGURE 3. Soluble fraction of the glycol-isolated mitotic apparatus dissolved in 0.6 M KCl. 48 min after reaching speed of 42,040 rpm, bar angle 50°.

FIGURE 4 Supernatant solution from glycol isolation, concentrated and dialyzed into 0.6 M KCl. 16 min after reaching speed of 59,780 rpm, bar angle 60°.

FIGURE 5 Glycol extract of unfertilized eggs, dialyzed into 0.6 M KCl. 48 min after reaching speed of 42,040 rpm, bar angle 60°.

related, and in the following report (47) it will be demonstrated that a 14S component and a variety of smaller components can be obtained from a homogeneous preparation of 22S protein under suitable conditions.

Distribution of the 22S Component in the Cell

The extracts of metaphase eggs prepared by Kane and Hersh (23) contained a component similar to this 22S protein which makes up the bulk of the soluble protein of the glycol-isolated MA. These extracts contained a relatively large amount of this component, even though the eggs had been treated with the low temperature ethanol solution then used to render the MA insoluble, thus eliminating this protein from consideration at that time as a possible MA constituent. However, this result would also be obtained if the 22S component is present in the egg in amounts considerably in excess of that which is involved in the MA. This possibility was investigated in the present experiments by retaining the supernatant solution from an isolation procedure, removing the particulate material by centrifugation, and concentrating the soluble protein present. The schlieren pattern of an ultracentrifuge run on the resulting solution is shown in Fig. 4. An easily identifiable 22S peak precedes the large amount of more slowly sedimenting material present. In a following report (47) it will be shown that such 22S material, prepared from eggs by a number of methods, is identical in its physical properties and amino acid composition with that present in the KCl-soluble fraction of the isolated MA.

However, the concentration relations of the components in the KCl-soluble MA fraction and the cell lysate are reversed. The 22S protein forms about 80% of the KCl-soluble fraction of the glycol-isolated MA, whereas this component makes up only 25% of the protein present in the lysate. This difference in composition is of considerable significance since it indicates that the protein obtained from the dissolved MA does not originate from soluble cytoplasmic material trapped in the interstices of the apparatus and not removed by washing, as this would have the same relative composition as the supernate.

Measurements of the soluble protein present in the lysate from a number of isolation experiments gave an average value of about 50 mg of soluble protein per milliliter of packed cells, under the conditions of this experiment. If the 22S component makes up one-quarter of this soluble protein, approximately 12 mg of 22S protein are present in 1 ml of cells. This amount of 22S protein is much in excess of that recovered from the isolated mitotic apparatus; the measured values indicate that only 3–4% of the total 22S is found in the MA. The value for the percentage of 22S in the MA may be somewhat low, as a result of less than 100% synchrony, yield, or recovery of MA, but it is clear that only the order of 5% of the total 22S protein present in the cell is involved in the glycol-insoluble structure of the isolate mitotic apparatus.

Kane and Hersh (23) had also found that a similar protein is present in extracts of unfertilized eggs, and the experiments of Went (50, 52) showed that a component immunologically similar to the protein of the MA could be detected in the unfertilized egg. This observation was confirmed in the present experiments by preparing extracts of unfertilized eggs with a procedure identical with that used for isolation. Such extracts (Fig. 5) are similar in composition to those from metaphase eggs, with the 22S protein again making up approximately one-quarter of the soluble protein present. Measurements of the total protein soluble in 12% hexylene glycol give the same value as for metaphase eggs, thus giving the same result for the quantity of 22S protein per milliliter of cells. The amount of protein involved in the mitotic apparatus is, of course, too small to make a detectable difference in these amounts.

Relation to Total Cell Protein

The finding that only a small fraction of the total 22S protein present in the egg is involved in the structure of the first cleavage mitotic apparatus is of considerable significance with regard to such problems as the origin of mitotic apparatus protein and the mechanism and control of the process of assembly of the MA from smaller units, and this relation of MA 22S to cell 22S would appear to be the most useful comparison. However, it is of some interest to relate the total MA protein to total cell protein, since such measurements have been made in other investigations (33, 35). This relation is probably not of general significance, since it includes all the proteins of the cell, which in the case of egg cells involves a large amount of storage protein. Also, the variety of materials present in a preparation of whole eggs renders any value for total protein somewhat ambiguous, as the definition of protein depends on the method of measurement used.

An estimate of the total cell protein was obtained by dissolving in KOH the residue remaining after the extraction of unfertilized eggs with glycol solution. The protein present in this solution was then added to that present in the glycol extract, to give a value for total protein. An average of 150 mg of protein per milliliter of packed cells was obtained, in good agreement with the values for Kjeldahl nitrogen per milliliter of *A. punctulata* eggs reported by Ballentine (2). With the use of a factor of 6.25 mg protein per milligram of nitrogen, Ballentine's results give an average value of 168 mg of protein per milliliter of cells.

The value for the total cell protein can then be used to calculate a value for the total mitotic apparatus protein as percentage of the total cell protein, which in this case is approximately 0.5%, considerably lower than the values previously reported (33, 35). The total cell protein measurement can also be used to calculate that the 22S protein makes up about 8% of the total protein of the cell.

DISCUSSION

Origin of the 22S Protein in the Isolated MA

These experiments demonstrate that the 22S protein is the major soluble component obtained by dissolving the glycol-isolated mitotic apparatus in solutions of high ionic strength. In addition, it will be shown in a following report (47) that many of the differing components obtained by previous workers from other dissolved MA can be derived from this one protein.

The evidence presented here indicates that this protein originates from, or is associated with, the filamentous elements of the mitotic apparatus. The following observations support this conclusion:

1. When observed with phase contrast, the breakdown of the MA in salt solution is seen to begin with a collapse of the organized fibrous structure of the MA, freeing the chromosomes and small granules, which then disperse. Since the microtubules form the only continuous elements in the MA, their dissolution should produce precisely this breakdown of the organized MA structure.

2. Electron microscope examination of the pellet of KCl-insoluble material shows that it contains the vesicular components of the MA, with no evidence of microtubules or other filamentous structures, whereas these microtubules are easily visible in electron micrographs of the isolated MA (19). Thus, unless the microtubules are considered to be present in some insoluble but invisible form in the pellet, their soluble components must be present in the supernatant solution.

3. This solution contains one major ultracentrifugal component, the 22S protein, plus a smaller amount of more heterogeneous material sedimenting at 4–5S. Both of these components may have arisen from the breakdown of the microtubules, but the 22S protein is of primary interest

here, since it makes up the largest fraction of the soluble material. The total amount of slowly sedimenting material is much less than that of the 22S component, and, as it is quite heterogeneous, no one component of this material is likely to be present in sufficient amount to account for the total protein of the tubules. This fraction may include other MA components, such as those of Sakai (42), but the relatively small amount present in the glycol-isolated MA and the large amount of material in this sedimentation range present in the egg make such identification difficult.

4. If the 22S protein does not originate from the fibrous structure of the MA, its presence in extracts of the MA would require some alternative explanation. It is unlikely to have originated from soluble lysate material trapped in the interstices of the MA, since such trapped material would be expected to have a protein composition similar to that of the lysate, while the KCl-soluble material of the MA contains much more of the 22S protein than of the more slowly sedimenting material, which is the reverse of the composition of the lysate solution. Yolk granules attached to the MA are also an unlikely source, as they are few in number and are apparently already osmotically lysed by the glycol solution and unaffected by the KCl extraction, since they have the same appearance in electron micrographs of the freshly isolated MA (19) as they have in the KCl-extracted pellet shown here.

The possibility of either of these sources for the 22S protein is rendered even more improbable by the observation that the 22S protein of the MA becomes insoluble in KCl with time, and this loss of solubility of the MA is accompanied by changes in the fine structure of its microtubules (22). These effects are temperature dependent, and in each case the loss of solubility of the 22S protein is correlated directly with the structural changes. These results are difficult to explain on the basis of trapped lysate material or extracted granular protein.

One alternative explanation for the origin of the 22S protein, which cannot be eliminated on the basis of the experiments reported here, is that it exists in the MA in bound form, associated with the microtubules, rather than forming part of their structure. Since the 22S protein of the MA cannot be removed by extensive washing with glycol solution, in which it is known to be soluble, but is immediately released upon the breakdown of the structure of the MA in solutions of high ionic strength, this hypothesis would require that the protein be associated directly with the microtubules in such fashion that it becomes available only upon their dissolution. The possibility that the 22S protein exists in such bound form in the MA must remain until the structural organization of the microtubules of the MA is clarified.

Role of the 22S Protein in the Cell

The presence of an excess of 22S protein in the unfertilized egg relates directly to the problem of possible "precursors" to the mitotic apparatus, which has been investigated with immunological methods by Went (50, 52). The results obtained here confirm Went's observation that a major component of the isolated mitotic apparatus could be detected in the unfertilized egg. In later studies (51) a second component was identified and also found in the unfertilized egg. The presence of some protein related to the mitotic apparatus in the unfertilized sea urchin egg is hardly surprising, as these cells have already completed two maturation divisions. The number of cleavages that might be carried out with this preexisting protein is difficult to compute, for, although it is obvious that the the volume of the cells must halve at each division, there is little information on the relation of MA size to cell size in later divisions, and the observation of Harris (12) that later divisions may be anastral adds an additional complication. These questions are also related to the problem of the synthesis of mitotic apparatus proteins, which has been investigated by a number of workers (6, 11, 32, 44), but, until the number of proteins involved in the mitotic apparatus and their roles in its structure are known, the data presented here cannot contribute directly to the solution of this problem.

Following the preliminary work of Stephens (46) on the characterization of 22S protein, Malkin et al. (31) reported on a protein of similar properties which was found to be localized, at least in part, in the granule fraction of the sea urchin egg. This is in agreement with the observations of Went (51), who found that both of the immunologically identifiable precursors of the mitotic apparatus were associated with the granule fraction of the egg. This material thus may be present in both a soluble pool and an insoluble pool, with only a fraction of the total protein present available in soluble form at any time. Went (51)

has also suggested the possibility that the concentration of soluble precursor in the cell might be held constant by mobilization from the insoluble pool.

The presence of excess spindle-associated protein in the cell is also relevant to the work of Inoué (14–16), who has postulated, on the basis of temperature-birefringence measurements, that a dynamic equilibrium might exist between oriented material and unoriented material in the spindle and has suggested that this equilibrium might be involved in chromosome movement. The determination of whether the excess 22S protein in the cell is involved in such an equilibrium must await further clarification of its role in the mitotic apparatus, but such a possibility remains as an interesting speculation.

The relation of the 22S protein to the structure of the mitotic apparatus will be discussed in the following report (47), after the presentation of the physical characteristics of the protein. It is obvious, however, that the protein as isolated from the mitotic apparatus and the cell is too large to form a subunit of the microtubule as presently visualized (1, 3, 27, 37). The fact that this protein is obtained in the form of a 22S particle from the dissolved MA does not require, of course, that it exist in this form in the intact mitotic apparatus. It may be reduced to one of its subunits (47), undergo other conformational changes, or require the addition of other protein components before incorporation into the MA. The more slowly sedimenting material found in the KCl-soluble fraction may be of importance in the latter case. If this protein is associated with, rather than forming a subunit of, the microtubular structure of the MA, these geometrical restrictions would no longer apply. In any case, the fact that this protein makes up the largest part of the soluble protein of the MA after dissolution of its microtubules and can account for most of the other components seen in the soluble fraction by previous workers (47) indicates that it must have some basic role in the structure and function of the mitotic apparatus.

Relation to Total Cell Protein

The value of 0.5% obtained here for the relation of total mitotic apparatus protein to total cell protein is considerably lower than previously reported values, and this discrepancy will be considered briefly. No exact value for this relation can be expected, since the size and degree of contamination of the mitotic apparatus vary with the exact stage of division used and with rather small changes in the isolation conditions (20). Mazia and Dan (33) originally reported that 2% of the total cell protein was found in the MA, but this was revised upwards in the experiments of Mazia and Roslansky (35) to 12%, a value very much larger than that found here. Since this relation involves a value for the total cell protein and the total MA protein, these will be considered in order.

The value of 150 mg of total protein per milliliter of packed cells found here is in agreement with the values in the literature. Most measurements are given in terms of protein per cell, which in the present case would be 3.2×10^{-5}. The value obtained by Ballentine (2) for the egg of *A. punctulata* in these terms is 3.7×10^{-5} mg protein per egg. Data in the literature allow the calculation of at least two independent values for the eggs of *S. purpuratus*. These eggs have a diameter of 80 μ, as compared with 74 μ for *A. punctulata*, and have a correspondingly larger amount of protein per egg, since the number of eggs in a given volume is smaller. Leitch (28) reported two values from determinations of Kjeldahl nitrogen, which average to 4.3×10^{-5} mg of protein per egg, and Kavanau's extensive measurements on the free and protein-bound amino acids in this egg (24) can be recalculated to give another measure of protein per egg. Excluding the values for free amino acids and using an average residue weight of 115, his results give a value of 4.7×10^{-5} mg of protein per egg. Thus it seems reasonable to assume that the value for *A. punctulata* lies in the range of 3–4 $\times 10^{-5}$ and that for *S. purpuratus* in the range of 4–5 $\times 10^{-5}$ mg of protein per egg. In the first measurement of the relation of MA to egg protein, Mazia and Dan (33) estimated a value for total egg protein of 14×10^{-5} mg, but later measurements of Mazia and Roslansky (35) gave a value of 6.2 $\times 10^{-5}$ mg, in general agreement with the values summarized above.

In the present experiments, 1 ml of packed *A. punctulata* eggs yielded approximately 0.1 ml of MA, which contained 0.75 mg of total protein. Dividing by the number of eggs per milliliter gives a value for protein per MA of 0.016×10^{-5}, more than an order of magnitude smaller than those reported by previous workers. Mazia and Dan (33) gave a value of 0.34×10^{-5} and Mazia and

Roslansky (35) reported 0.72×10^{-5} mg of protein per MA. Differences in the value for the percentage of total cell protein contained in the MA are thus primarily due to the difference in the figures for protein per MA, suggesting that different isolation methods might be responsible. However, Mazia and Dan (33) also reported that 10 ml of eggs gave approximately 10 mg of MA protein, or 1 mg of MA protein per milliliter of cells in fairly good agreement with the value of 0.75 mg of MA protein per milliliter of cells obtained in these experiments. The yield of total MA protein per volume of cells is thus independent of the isolation method used, and this yield is incompatible with a value of 12% for the total cell protein in the MA. If 1 ml of eggs yields approximately 1 mg of MA protein, this MA protein cannot form 12% of the total cell protein, for the total protein per ml of cells would then be less than 10 mg, whereas all available data give a value of approximately 150 mg of protein per milliliter of eggs. Thus, the value for the percentage of total egg protein present in the MA must be at least an order of magnitude smaller than 12% and lie in the range of 1%.

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REFERENCES

1. ANDRÉ, J., and J. P. THIÉRY. 1963. *J. Microscopie.* **2:** 71.
2. BALLENTINE, R. 1940. *J. Cellular Comp. Physiol.* **15:** 121.
3. BARNICOT, N. A. 1966. *J. Cell Sci.* **1:** 217.
4. BEHNKE, O. 1964. *J. Ultrastruct. Res.* **11:** 139.
5. BERNHARD, W., and E. DE HARVEN. 1958. *Intern. Kongr. Elektronenmikroskopie.* **2:** 217.
6. BIBRING, T., and G. H. COUSINEAU. 1964. *Nature.* **204:** 805.
7. DALES, S. 1963. *Proc. Nat. Acad. Sci. U. S.* **50:** 268.
8. DE-THÉ, G. 1964. *J. Cell Biol.* **23:** 265.
9. DIRKSEN, E. R. 1964. *Exptl. Cell Res.* **36:** 256.
10. GONATAS, N. K., and E. ROBBINS. 1964. Protoplasma. **59:** 25.
11. GROSS, P. R., and G. H. COUSINEAU. 1963. *J. Cell Biol.* **19:** 260.
12. HARRIS, P. 1962. *J. Cell Biol.* **14:** 475.
13. HARVEY, E. B. 1952. *Biol. Bull.* **103:** 284.
14. INOUÉ, S. 1959. *Rev. Mod. Phys.* **31:** 402.
15. INOUÉ, S. 1964. *In* Primitive Motile Systems in Cell Biology. (R. D. Allen and N. Kamiya, editors). Academic Press Inc., New York. 549.
16. INOUÉ, S., H. SATO, R. E. KANE, and R. E. STEPHENS. 1965. *J. Cell Biol.* **27:** (No. 2). 115A. (Abstr.)
17. JOHNSTON, J. P., and A. G. OGSTON. 1946. *Trans. Faraday Soc.* **42:** 789.
18. KANE, R. E. 1962. *J. Cell Biol.* **12:** 47.
19. KANE, R. E. 1962. *J. Cell Biol.* **15:** 279.
20. KANE, R. E. 1965. *J. Cell Biol.* **25** (1, Pt. 2)**:** 137.
21. KANE, R. E. 1965. *Biol. Bull.* **129:** 396.
22. KANE, R. E., and A. FORER. 1965. *J. Cell Biol.* **25:** 31.
23. KANE, R. E., and R. T. HERSH. 1959. *Exptl. Cell Res.* **16:** 59.
24. KAVANAU, J. L. 1953. *J. Exptl. Zool.* **122:** 285.
25. KRISHAN, A., and R. C. BUCK. 1965. *J. Cell Biol.* **24:** 433.
26. LEDBETTER, M. C., and K. R. PORTER. 1963. *J. Cell Biol.* **19:** 239.
27. LEDBETTER, M. C., and K. R. PORTER. 1964. *Science.* **144:** 872.
28. LEITCH, J. L. 1934. *J. Cellular Comp. Physiol.* **4:** 457.
29. LOWRY, O. H., N. J. ROSENBROUGH, A. L. FARR, and R. S. RANDALL. 1951. *J. Biol. Chem.* **193:** 1965.
30. LUFT, J. H. 1961. *J. Biophys. Biochem. Cytol.* **2:** 409.
31. MALKIN, L. I., J. MANGAN, and P. R. GROSS. 1965. *Develop. Biol.* **12:** 520.
32. MANGAN, J., T. MIKI-NOUMURA, and P. R. GROSS. 1965. *Science.* **147:** 1575.
33. MAZIA, D., and K. DAN. 1952. *Proc. Natl. Acad. Sci. U. S.* **38:** 826.
34. MAZIA, D., J. M. MITCHISON, H. MEDINA, and P. HARRIS. 1961. *J. Biophys. Biochem. Cytol.* **10:** 467.
35. MAZIA, D., and J. D. ROSLANSKY. 1956. *Protoplasma.* **46:** 528.
36. MURRAY, R. G., A. S. MURRAY, and A. PIZZO. 1965. *J. Cell Biol.* **26:** 601.
37. PEASE, D. C. 1963. *J. Cell Biol.* **18:** 313.
38. REYNOLDS, E. S. 1963. *J. Cell Biol.* **17:** 208.
39. ROBBINS, E., and N. K. GONATAS. 1964. *J. Cell Biol.* **21:** 429.
40. ROTH, L. E., and E. W. DANIELS. 1962. *J. Cell Biol.* **12:** 57.
41. SANDBORN, E., P. F. KOEN, J. D. MCNABB, and G. MOORE. 1964. *J. Ultrastruct. Res.* **11:** 123.
42. SAKAI, H. 1966. *Biochim. Biophys. Acta.* **112:** 132.
43. SLAUTTERBACK, D. B. 1963. *J. Cell Biol.* **18:** 367.

44. STAFFORD, D. W., and R. M. IVERSON. 1964. *Science.* **143:** 580.
45. STEMPAK, J. G., and R. T. WARD. 1964. *J. Cell Biol.* **22:** 697.
46. STEPHENS, R. E. 1965. *Biol. Bull.* **129:** 396.
47. STEPHENS, R. E. 1967. *J. Cell Biol.* **32:** 255.
48. TYLER, A. 1949. *Collecting Net.* **19:** 19.
49. WARBURG, O., and W. CHRISTIAN. 1941. *Biochem. Z.* **310:** 384.
50. WENT, H. 1959. *J. Biophys. Biochem. Cytol.* **6:** 447.
51. WENT, H. 1960. *Ann. N. Y. Acad. Sci.* **90:** 422.
52. WENT, H., and D. MAZIA. 1959. *Exptl. Cell Res. Suppl.* **7:** 200.
53. ZIMMERMAN, A. M. 1960. *Exptl. Cell Res.* **20:** 529.