Published May 1, 1969

IMMUNOCHEMICAL STUDIES OF 22S PROTEIN FROM ISOLATED MITOTIC APPARATUS

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ABSTRACT

Evidence is presented that the "22S protein" of mitotic apparatus isolated from sea urchin eggs is not microtubule protein. An antibody preparation active against 22S protein is described, and immunochemical studies of the distribution of 22S protein in various cellular fractions and among morphological features of mitotic apparatus are reported. The protein is ubiquitous in the metaphase egg fractions that were tested but is not found in sperm flagella. It is immunologically distinct from proposed microtubule protein isolated from mitotic apparatus by the method of Sakai, and from proposed microtubule protein obtained after extraction with mild acid. It exists in nontubule material of isolated mitotic apparatus but is not detectable in microtubules.

INTRODUCTION

In various studies (1–9), mitotic apparatus isolated from sea urchin eggs has been extracted to obtain proteins thought to be instrumental in its function. Among these proteins is the "22S protein" particularly considered in this paper; it is the major soluble component of mitotic apparatus isolated by the method of Kane (10) and extracted with 0.6 M potassium chloride (5).

"Mitotic apparatus" is used in this paper to mean the cohesive unit which can be isolated by known procedures. Electron microscopy has shown that isolated mitotic apparatus is in general a highly complex structure (7–9, 11, 12). The focus of recent work has therefore been to identify a protein as the structural protein of the microtubules that appear to make up the spindle fibers. The microtubules evidently play a role in the traction of the chromosomes and are the morphological feature of isolated mitotic apparatus to which a function in mitosis is most readily assigned. The 22S protein has been proposed to be microtubule protein (5), as have other proteins differently obtained. It has been further suggested that such other proteins may in some cases correspond to subunits of the 22S protein (6, 8).

Among the data used to support the contention that a given protein represents structural protein of microtubules is the demonstration that the microtubules disappear morphologically under extraction conditions which recover the protein. This necessarily ignores the possibility that microtubules disappear for some reason other than the solubilization of their molecular components. In addition, the fact that more than one protein is generally extracted forces an additional choice among extracted proteins for the favored candidate. Physicochemical similarities between a protein and known structural microtubule proteins

from other sources can be convincing, but it is sometimes difficult to know whether the properties compared are specific to microtubule protein alone. It appears that the two most direct means of identifying an isolated protein with microtubules are either the reconstitution of microtubules from the protein (which has so far proved elusive for proteins from mitotic apparatus) or the direct localization of the protein in microtubules by means of labeled antibodies (13). We have employed the latter approach with respect to the 22S protein. Also, using the criterion of immunochemical specificity, we have determined the distribution of 22S protein (or cross-reacting proteins) in unfertilized eggs, in sperm tail, in fractions of metaphase eggs other than the mitotic apparatus, and in soluble fractions derived from mitotic apparatus and proposed to contain the microtubule protein. These latter studies generally follow the immunochemical studies of mitotic apparatus previously undertaken by Went (14–16), but it has been our emphasis to use as antigen a protein previously defined by its physicochemical properties and thus avoid a definition that is to any extent immunochemical.

MATERIALS AND METHODS

Biological Material

Sea urchins were *Strongylocentrotus purpuratus*, obtained from Pacific BioMarine Supply Co., Venice, California. They were stored in Instant Ocean Model 110-C aquaria (Aquarium Systems, Inc., Wickliffe, Ohio). Gametes were obtained by injection of 0.5 M potassium chloride. Handling of the gametes and embryos was carried out at 15–17°C; artificial seawater was used.

Preparation of 22S Protein

The protein was prepared from acetone powder of unfertilized eggs by the procedure of Stephens (17). The preparation used in obtaining an antiserum was subjected to four cycles of purification by centrifugation and analyzed in the analytical ultracentrifuge. Later preparations were tested by immunodiffusion against an antiserum to total unfertilized eggs, and any preparation giving more than one precipitin band was subjected to further purification by an additional centrifugation cycle.

Preparation of Anti-22S Immunoglobulin

The preparation of 22S protein used for immunization was dialyzed against physiological buffered saline (0.15 M sodium chloride in 0.01 M phosphate buffer, pH 7.3) immediately before use. About 1 mg was injected in the cornea of rabbits, then 3 mg were injected intramuscularly six times at 2-day intervals. After 6 wk, another milligram was injected in the cornea, and the first bleeding was 1 wk later, by cardiac puncture. 5 days before subsequent bleedings, 5 mg of antigen were given subcutaneously. The IgG fraction was obtained from the whole serum by precipitation at a final concentration of 1.6 M ammonium sulfate, was washed in 2.0 M ammonium sulfate, and redissolved, with dialysis, in physiological buffered saline. (In keeping with current nomenclature, the symbol IgG will be used here to denote 7S gamma globulin.) In order to ensure antibody activity against a maximum number of antigenic determinant sites, the IgG fractions from three immunized rabbits were pooled.

Removal of Fertilization Membranes

These were removed from eggs, for later isolation of mitotic apparatus, by a modification of a method of Mazia et al. (18). Unfertilized eggs were washed three times in aerated seawater before use and were then suspended in a 0.1% solution of mercaptoethylgluconamide (Cyclo Chemical Corp., Los Angeles, Calif.) in aerated seawater and fertilized in this solution, which prevents the fertilization membranes from hardening after elevation. 15 min after fertilization, membranes were removed by passing the eggs several times through silk bolting cloth of an appropriate size; the hole size was approximately 40 × 65 μ. (A 0.045% solution of reduced glutathione in aerated seawater, titrated to pH 7.9, may be used instead of the mercaptoethylgluconamide solution.) After the membranes were stripped, the eggs were washed twice by settling and resuspension in a wide, shallow dish containing a solution of 19 parts of 0.5 M sodium chloride and one part of 0.5 M potassium chloride (10). These washes remove stripped membranes and excess sperm and also wash away lysis products so that little or no adhesion of eggs results. Finally, the eggs were resuspended to seawater and alowed to develop with gentle stirring.

The advantage of this method is that the medium used to soften the membranes does not inhibit fertilization; fertilization percentages may even be slightly improved by the medium. Using media that must be added after fertilization, we have found that in many egg populations some membranes have hardened before others have elevated, so that 100% removal of fertilization membranes is often impossible. With the method described here, 100% fertilization and 100% stripping of membranes is routinely possible with minimal egg lysis.

Isolation of Mitotic Apparatus

The apparatus was isolated at first metaphase by the method of Kane (10), using molar 2-methyl-2,4-pentanediol in 0.01 M phosphate buffer at pH 6.4 in the isolation step. After isolation the mitotic apparatus was washed four times at 4°C by centrifugation and resuspension in isolation medium.

Preparation of Sperm Tail Fractions

All procedures were carried out at 0–4°C. Sperm tails were cut in a Waring blendor (19) in sodium citrate isotonic with seawater at pH 6.5 (0.25 M sodium citrate titrated to pH 6.5 with 1.0 M citric acid). Sperm heads were separated by centrifugation for 20 min at 750 *g*; tails were sedimented for resuspension at 14,000 *g* for 20 min. Two cycles of these centrifugations were performed to obtain the final sperm tail preparation. A similar procedure (20) with seawater containing 10^{-4} M EDTA as the medium was also used.

Preparations of sperm tail outer fiber protein were made according to Stephens et al. (20), except that the sodium mersalyl dissolution medium was replaced by 0.01 M meralluride sodium solution (Mercuhydrin) in 0.01 M borate buffer at pH 9.0 (9). Preparations containing inner fiber protein (as well as dynein and "matrix") were made according to the method devised by Gibbons for cilia (21). The procedures were monitored throughout by phase microscopy and electron microscopy, as appropriate.

Immunodiffusion

Ouchterlony's immunodiffusion technique (22) was used except that in many cases the agar was dissolved in 0.6 M potassium chloride in 0.005 M phosphate buffer at pH 7.3, a medium in which 22S protein is known to be soluble. Antisera and antigens, if not already in this medium, were dialyzed against the medium before use. In cases in which the antigen content of two preparations was to be compared, the preparations were placed in wells at the same concentration of total protein. Incubation was at 37°C.

Immunoelectrophoresis and Agar Electrophoresis

Electrophoresis was carried out on microscope slides in 0.5 or 1% agar in barbital buffer at pH 8.6 (1.66 g acid + 10.50 g base per liter for agar, and 1.38 g acid + 8.76 g base per liter for electrode chamber). After electrophoresis, the slides were either subjected to the standard immunodiffusion procedure or stained in 0.6% Amido Black in 90% methanol:20% acetic acid, 1:1.

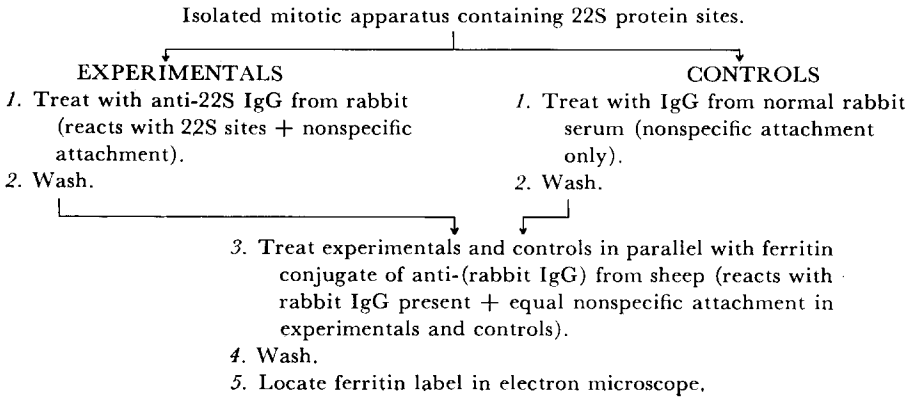

FIGURE 1 Plan of labeling experiments.

Preparation of Ferritin-Conjugated Immunoglobulin

The study with ferritin-labeled antibody was carried out by the two-layer method (23), in which the ferritin-labeled antibody employed is against rabbit IgG, and rabbit IgG of appropriate specificity is initially used to locate the antigen (Fig. 1). An antiserum to rabbit IgG was made in sheep (24), and its IgG fraction was conjugated to cadmium-freed (25) ferritin by the technique of Singer and Schick (26), using toluene 2,4-diisocyanate as the coupling agent. The conjugate was purified of most of the uncoupled immunoglobulin and ferritin by centrifugation and continuous flow paper electrophoresis (24). In order to ensure that conjugation had occurred, the conjugate was tested by immunoelectrophoresis against an antiferritin serum and an antiserum to sheep IgG. Precipitin bands were formed against the major

electrophoretic component by both these antisera, indicating that ferritin and IgG were migrating together and were therefore conjugated. The immunological reactivity of the conjugate was tested in the electron microscope on a known system (23) of unfertilized eggs treated with an antiserum to unfertilized eggs, and with nonimmune serum as control. The conjugate gave specific localization in this system. This conjugate was used for all localization experiments.

Ferritin-Labeling of Isolated Mitotic Apparatus (see Fig. 1)

PREFIXATION LABELING: Washed unfixed mitotic apparatus was allowed to react in isolation medium at 4°C for up to 45 min with rabbit IgG previously dialyzed against the isolation medium. IgG from antiserum to 22S protein was used in experimental samples, and IgG from normal rabbit serum was used in controls. Mitotic apparatus was then washed four times in cold isolation medium by centrifugation and resuspension, and allowed to react, under the same conditions, with a ferritin conjugate of antibody obtained from sheep serum against rabbit IgG. The mitotic apparatus was then washed nine times in isolation medium and once in sodium acetate buffer isotonic with seawater, pH 6.1, before fixation in 2% osmium tetroxide in the same buffer (27).

POSTFIXATION LABELING: Washed isolated mitotic apparatus was fixed for 2–12 min in 3% glutaraldehyde or 10% formaldehyde in physiological buffered saline, or 4% formaldehyde in the acetate buffer used for osmium tetroxide fixation, or 3% glutaraldehyde in sodium cacodylate buffer made isotonic with seawater, pH 6.0. After fixation the samples were washed in cold physiological buffered saline five times by centrifugation and resuspension. The rest of the procedure was carried out at 15°–17°C. The mitotic apparatus was allowed to react with experimental or control rabbit IgG for 45 min, washed four times, reacted for 45 min with the ferritin conjugate of antibody to rabbit IgG, and washed eight times in physiological buffered saline. After one wash in sodium acetate buffer, the samples were fixed in 2% osmium tetroxide as above.

Electron Microscopy

Samples were fixed or postfixed in cold 2% osmium tetroxide in acetate buffer isotonic with seawater at pH 6.1 (27). Dehydration was in graded alcohols and propylene oxide, and embedding was in Epon 812 (28). Sectioning was carried out on an LKB Ultrotome III. Where necessary, 3-μ sections were cut and examined by phase microscopy in order to select mitotic apparatus oriented longitudinally for subsequent thin sectioning. The sections were stained with 0.1% lead citrate (29) and were examined in a Hitachi HU-11B electron microscope.

RESULTS

Properties of the Immunizing Antigen and the Antibody

The immunizing antigen used in this work was 22S protein prepared according to R. E. Stephens (personal communication and reference 17) from

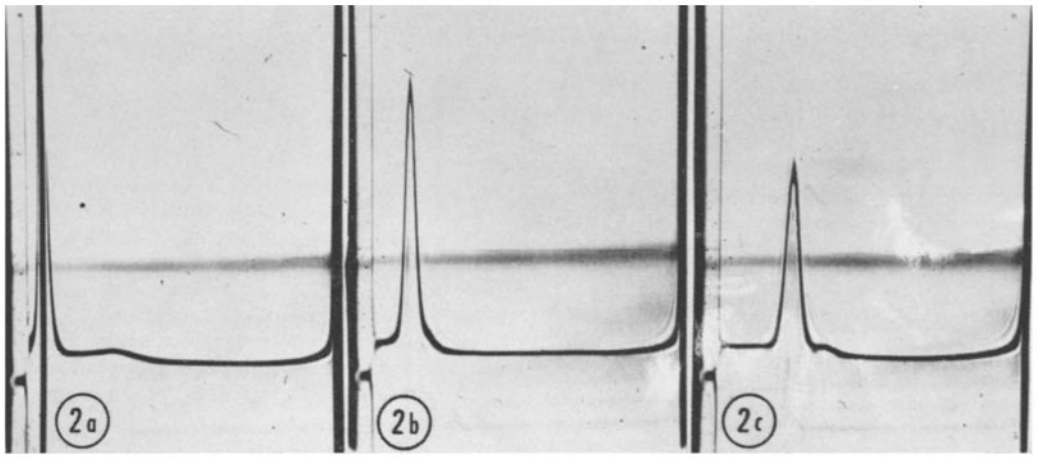

FIGURE 2 Sedimentation pattern of the preparation of 22S protein from unfertilized eggs used to obtain anti-22S serum. Centrifugation in Beckman-Spinco Model E ultracentrifuge. Sample concentration 6 mg/ml in 0.6 M potassium chloride + 0.005 M phosphate buffer, pH 7.3. $S_{20,w}$ for the major boundary = 23S. Two impurities are shown, which may be aggregates of 22S protein.

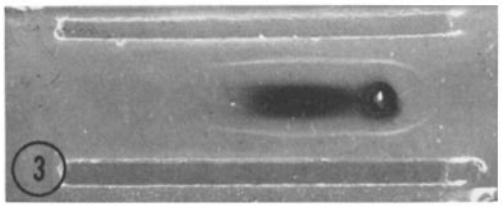

FIGURE 3 A preparation of 22S protein from unfertilized eggs after electrophoresis in agar for 5 hr at 170 v on twin slides. One slide was stained with Amido Black, the other slide was reacted with antiserum to 22S protein placed in two troughs. In this figure photographs of the two slides have been superimposed. The precipitin band formed with the antiserum encompasses the major migrating protein (black area), known to be 22S protein.

unfertilized eggs, rather than mitotic apparatus, because of their easier availability. We will show below that 22S protein from mitotic apparatus gives a reaction of immunochemical identity with 22S protein from unfertilized eggs. Upon analytical ultracentrifugation, the preparation used for immunization (Fig. 2) was found to contain a major component with $S_{20,w}$ = 23S (6 mg/ml). Two minor components are also seen. The heterogeneous, rapidly sedimenting material shown in Fig. 2 *a* is almost certainly an aggregate of 22S protein; it correlates with an opalescence which inevitably increases in a 22S protein preparation upon standing. The other contaminant may or may not be an aggregate of 22S protein. When tested by double diffusion in agar (22), the antibody against the preparation gave a single precipitin band.

Immunoelectrophoresis was used for verifying that the antigen giving rise to this band was in fact 22S protein and not a minor component of the immunizing preparation. Duplicate samples of 22S protein were subjected to electrophoresis in agar on twin slides, in parallel. After electrophoresis, one slide was stained with Amido Black to localize the distributed protein, while in the other slide antibody was added to two troughs cut near the sides of the slide, and diffusion and reaction of antigen and antibody were allowed to proceed at 37°C. A single precipitin band developed, and a comparison of this band with the stained slide showed that the band center corresponds to the region of densest protein staining. Fig. 3 shows such an immunoelectrophoresis slide with its corresponding stained electrophoresis slide superimposed. The result indicates that the antibody is against the major component of the immunizing antigen, which is known from analytical ultracentrifugation (Fig. 2) to be 22S protein. A material showing a reaction of immunochemical identity with the major component remains in the well during electrophoresis. We assume that this material is a reversible aggregate of 22S protein, the

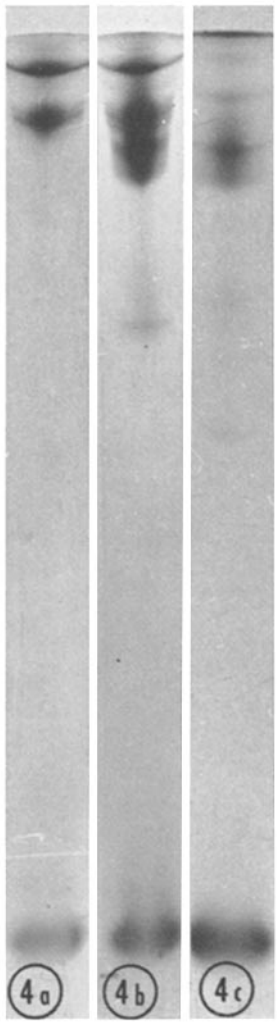

FIGURE 4 Electrophoresis in polyacrylamide gel of (*a*) preparation of 22S protein alone, (*b*) redissolved antigen-antibody precipitate obtained by reaction of 22S protein preparation with IgG fraction from its antiserum, (*c*) IgG fraction alone. All three bands detectable in the preparation of 22S protein are present also in the precipitin.

same opalescent material that is responsible for the boundary in Fig. 2 *a*.

Further evidence on the specificity of the antibody was obtained by examining the components of an antigen-antibody precipitate by electrophoresis in polyacrylamide gel, using the acid-urea system of Takayama et al. (30) as modified by Zahler et al. (31). A preparation of 22S protein was reacted at 37°C with the IgG fraction from its antiserum, with the use of proportions which were on the antigen-excess side of equivalence, but still gave a visible precipitate. After 90 min the precipitate was centrifuged out, washed by resuspension and centrifugation in physiological buffered saline, and redissolved in the acid-urea solution used for the sample in polyacrylamide gel electrophoresis (30). Electrophoresis was carried out on the redissolved precipitate, along with samples of the antigen alone and of IgG alone. The gels were stained with Coomassie Blue, and the patterns obtained were compared. As can be seen in Fig. 4, the antigen alone gave a major and two minor bands (R_f values 0.041, 0.087, and 0.097, with respect to a ribonuclease marker). Although 22S protein is known to have a complex pattern of subunits, which often exist in equilibrium (17), we cannot say whether all three bands derive from 22S protein. All three bands, however, are represented in about the same proportions as in the antigen, and, with unmodified mobilities, in the redissolved antigen-antibody complex. Other bands can be attributed to IgG, although in this case the mobilities appear to have been slightly modified. These results confirm that 22S protein is represented in the antiserum by a specific antibody.

Our indirect evidence on the subject is totally consistent with the conclusion that the 22S antigen-antibody system is responsible for our characteristic single precipitin band. Thus, all preparations of soluble protein to be described which give the precipitin band are known from sedimentation studies to contain a component with a sedimentation coefficient of 22S. When the 22S component is known to be a major one, the band is strong and located centrally between antigen and antibody wells. When the 22S component is known to be minor, the band is weak and located near the antigen well. Preparations known not to contain a 22S component do not give the precipitin band, even when they are derived from preparations which do so by procedures which remove the 22S component.

On the basis of the above results, we conclude that our antibody preparation may be used in immunochemical assays for 22S protein, or proteins sufficiently similar to be cross-reactive. It may be noted also that the 22S protein is homogeneous by the immunodiffusion test.

Distribution of 22S Protein

MITOTIC APPARATUS: Kane (5) and Stephens (17) have shown that 22S protein extracted from unfertilized eggs and that extracted from mitotic apparatus are alike in sedimentation coefficient and amino acid composition. We have extended this comparison to their immunological properties. Preparation of 22S protein from isolated mitotic apparatus was carried out according to Kane (5) to obtain potassium chloride–soluble mitotic apparatus proteins which, according to Kane (5) and Stephens (17), consist almost entirely of 22S protein. When preparations of 22S protein from unfertilized eggs and from isolated mitotic apparatus were run against anti-22S serum by immunodiffusion in agar, a single converging precipitin band was obtained (Fig. 5), indicating their immunological identity. Since the concentrations of total protein in the two antigen wells were equal, the symmetry of the precipitin band shows that 22S protein is a major component of the preparation from mitotic apparatus, as it is of the preparation from unfertilized eggs. This result is consistent with the results of Kane (5) and Stephens (17).

FRACTIONS OBTAINED BY MITOTIC APPARATUS ISOLATION: The several fractions from metaphase eggs which are discarded during normal preparation of isolated mitotic apparatus (32) were tested for the presence of 22S protein by immunodiffusion. The whole egg rinse (WER) and the supernatant (S) which remains after removal of mitotic apparatus from dispersed eggs were centrifuged for 10 min at 500 *g* to remove contaminating mitotic apparatus. Centrifugation for 30 min at 30,000 *g* yielded supernatants (WER-S and S-S) containing components soluble in the medium used to isolate the mitotic apparatus. The pellets from this centrifugation (WER-P and S-P) contain components other than mitotic apparatus which remain particulate in isolation medium. As a test for the presence of 22S protein in these particulates, they were subjected to the same procedure which is used to extract 22S protein from mitotic apparatus, after which material remaining particulate was discarded. The four fractions thus ob-

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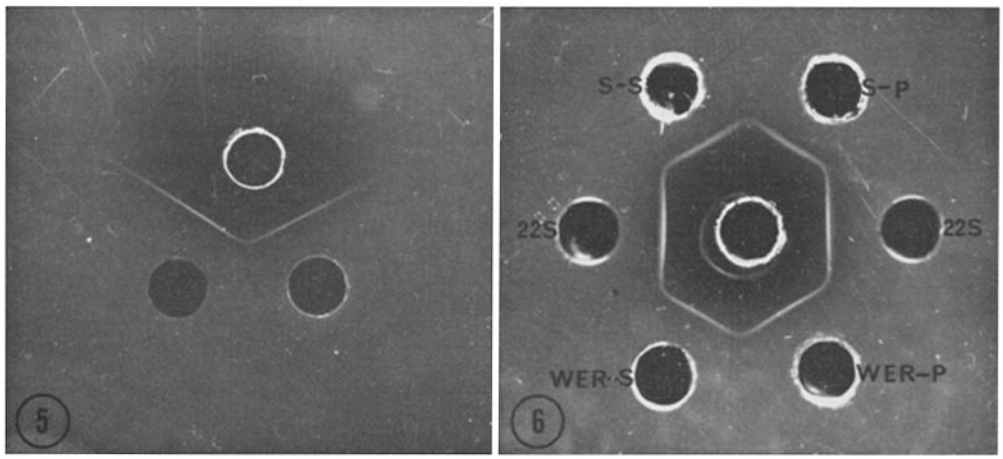

FIGURE 5 Immunodiffusion test of immunochemical identity of 22S protein from unfertilized eggs and from isolated mitotic apparatus. Top well, antiserum to 22S protein; left well, antigen from unfertilized eggs; right well, antigen from dispersed mitotic apparatus. Antigens were placed in the wells at the same concentration. The converging precipitin band indicates that the two proteins are immunochemically identical.

FIGURE 6 Immunodiffusion plate of nonmitotic apparatus fractions obtained from the isolation procedure, run against antiserum to 22S protein in the center well. For description of antigen well labels, see text. 22S protein was from unfertilized eggs. Antigens were placed in the wells at the same concentrations of total protein. The characteristic 22S protein band is strong in all fractions.

tained, along with a 22S protein sample from unfertilized eggs, were run by immunodiffusion against anti-22S serum. Fig. 6 shows that the 22S protein is present in all of these fractions.

SPERM: On the assumption that 22S protein might be associated with microtubules, and because Gibbons (33) has reported the presence of a 21S protein in the axoneme of cilia, attempts were made to detect 22S protein, or a protein cross-reacting with it immunochemically, in microtubule preparations from sperm tail. Samples of solubilized outer fibers of *S. purpuratus* sperm tail (see Materials and Methods) were dialyzed against the potassium chloride medium before being run on immunodiffusion plates. Preparations containing inner fibers (as well as dynein and "matrix") were treated the same way. Preparations of sperm heads, acetone powder of whole tails, and frozen-thawed whole tails were additionally homogenized in the potassium chloride medium and run against anti-22S serum. The experiments were run for 3 days under conditions where the 22S precipitin band appeared in 1 day. In some experiments, sperm protein was also used in considerably higher concentration than 22S protein. There was no indication of 22S protein in these preparations.

OTHER PREPARATIONS OF SOLUBLE PROTEIN FROM ISOLATED MITOTIC APPARATUS: Isolated mitotic apparatus was extracted in various ways to obtain extracts that have been proposed to contain microtubule protein, and the presence of 22S protein was assayed by immunodiffusion (Figs. 7 and 8).

Mitotic apparatus was isolated and extracted as described by Sakai (4). This method is known to give an extract showing three peaks in the analytical ultracentrifuge; a single 3.5S peak, proposed to represent microtubule protein, can then be isolated by precipitation with calcium (4, 6). Immunochemical comparison of the total extract with 22S protein shows that 22S protein is present in the extract. The ultracentrifuge pattern of the whole extract in fact contains a minor peak sedimenting at approximately 22S. The protein remains in the supernatant fluid, however, when precipitation with calcium is carried out as described by Sakai (Fig. 7). The calcium precipitate, redissolved in 10 mM EDTA at pH 8.5 and dialyzed against 0.6 M potassium chloride, shows no cross-reactivity with 22S protein (Fig. 7). The 3.5S protein of Sakai, proposed to be microtubule protein,

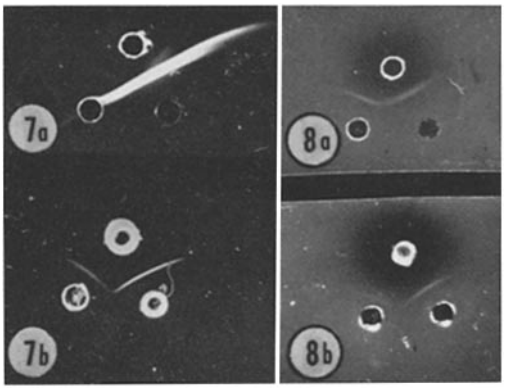

FIGURE 7 Immunodiffusion plates of Sakai's extract from dithiodipropanol-isolated mitotic apparatus compared with 22S protein. Antiserum to 22S protein in top wells. Right well, 22S protein from unfertilized eggs. Left wells contain (*a*) calcium-insoluble and (*b*) calcium-soluble fractions of the extract. The calcium-insoluble fraction does not contain 22S protein.

FIGURE 8 Immunodiffusion test of the acid extract from isolated mitotic apparatus with 22S protein from unfertilized eggs as a standard (right wells). Antiserum is in top wells. Left wells contain (*a*) soluble proteins obtained after extracting the isolated mitotic apparatus with dilute hydrochloric acid, pH 3.0, and (*b*) the same extract after centrifugation for 5 hr at 100,000 *g*. The cross-reactivity of the extract with 22S protein is considerably diminished by this procedure, which largely removes a minor component sedimenting at 22S.

is therefore immunochemically distinct from 22S protein.

Mitotic apparatus isolated by the method of Kane was also extracted with hydrochloric acid at pH 3.0, a procedure which makes the microtubules disappear morphologically without dispersing the mitotic apparatus (9). This extract also shows cross-reactivity with 22S protein (Fig. 8) and contains a minor component which sediments at approximately 22S. As in the previous procedure, the minor component can be removed as a calcium-soluble supernatant, leaving a fraction proposed to contain microtubule protein (9). Removal of the minor component by centrifugation, however, results in almost complete loss of cross-reactivity with 22S protein (Fig. 8). The proposed microtubule protein obtained by acid extraction is therefore also distinct from 22S protein.

Ultrastructural Localization of the 22S Protein in Mitotic Apparatus

Localization of the 22S protein with ferritin-labeled antibody was performed on isolated mitotic apparatus, rather than intact metaphase eggs, in order to facilitate the passage of antibody and conjugate into and out of the mitotic apparatus region. For minimizing the possibility of redistribution of mitotic apparatus components during the labeling procedure, two approaches were used (see Fig. 1).

(*a*) The mitotic apparatus was allowed to react with anti-22S while still in isolation medium; before use, the antibody was dialyzed against the same medium. The subsequent washes, the reaction with ferritin-conjugated antibodies to rabbit IgG, and the final washes were all carried out in isolation medium. As this medium is a very unusual one for carrying out an antigen-antibody reaction, we tested whether the reaction could take place by an immunodiffusion experiment, with an agar plate made up with isolation medium, and 22S protein and anti-22S immunoglobulin dialyzed against the same medium. A normal precipitin band was obtained.

(*b*) Isolated mitotic apparatus was fixed in various glutaraldehyde and formaldehyde fixatives and washed in cold physiological buffered saline (0.15 M sodium chloride in 0.01 M phosphate buffer, pH 7.3). The reactions with antibodies and washes were then carried out in physiological buffered saline.

The localization experiments gave essentially the same results, regardless of the method used. Isolated mitotic apparatus, as described by several workers (9, 11, 12), contains chromosomes, vesicles, ribosomes, and microtubules. There is also an amorphous component which runs in strands through the structure, embedding ribosomes and coating most of the vesicle and microtubule walls. The amorphous component has not been emphasized in previous descriptions of isolated mitotic apparatus, but we have observed it in all our preparations; indeed, we suppose that it is responsible for retaining the otherwise unattached ribosomes and vesicles in mitotic apparatus. After treatment of mitotic apparatus to localize 22S protein, the ferritin label appears to be associated with this amorphous component (Fig. 9, region *A*). In regions of naked microtuble wall (Fig. 9, region *B*), there is no ferritin label, suggesting that the 22S protein is not associated with

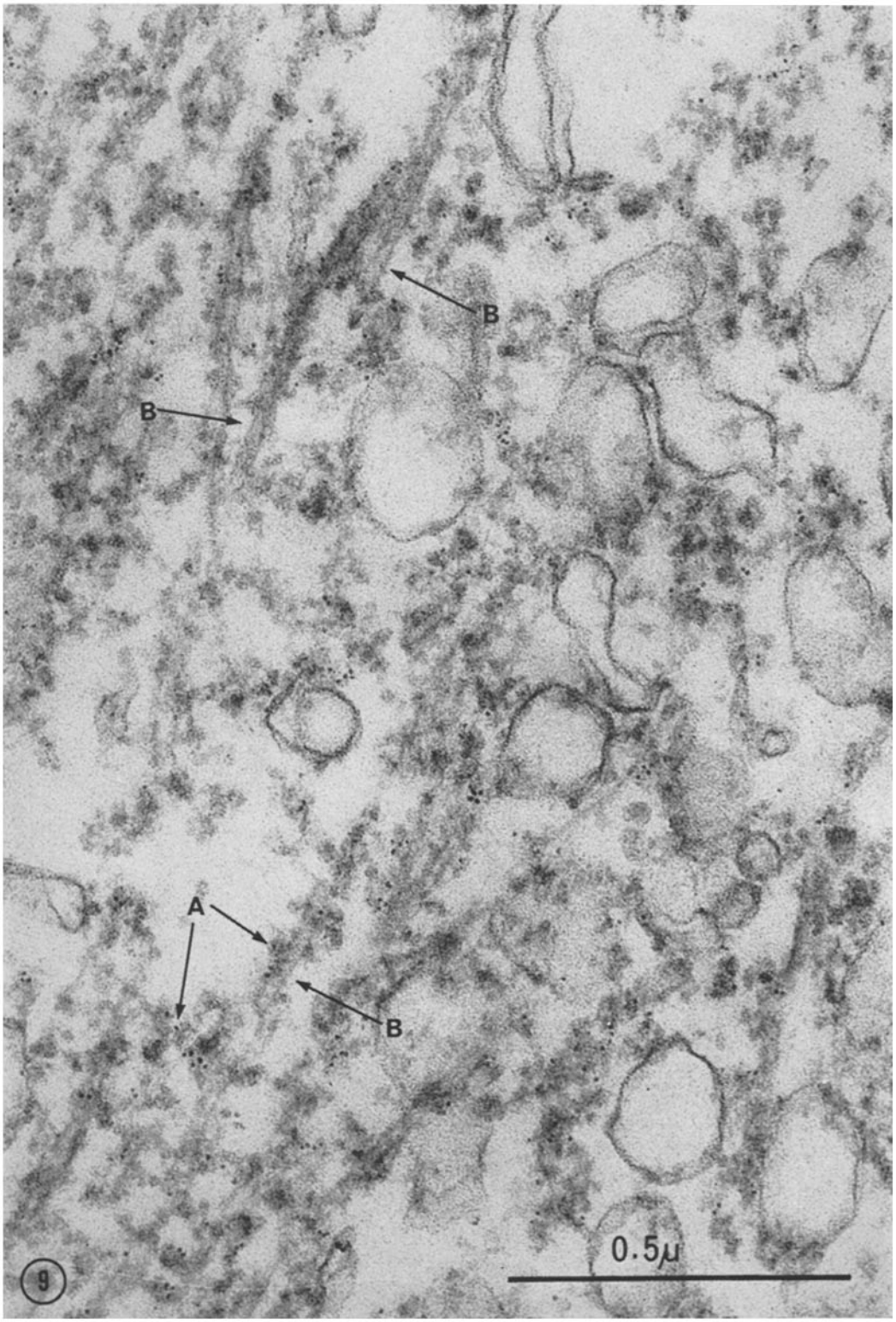

FIGURE 9 Electron micrograph of a region of mitotic apparatus after treatment for localization of 22S protein by ferritin-labeled antibody, using the two-layer method. The majority of ferritin label is associated with an amorphous component (*A*) embedding microtubules, ribosomes, and vesicles. Stretches of naked microtubule wall (*B*) do not show ferritin label. × 107,000.

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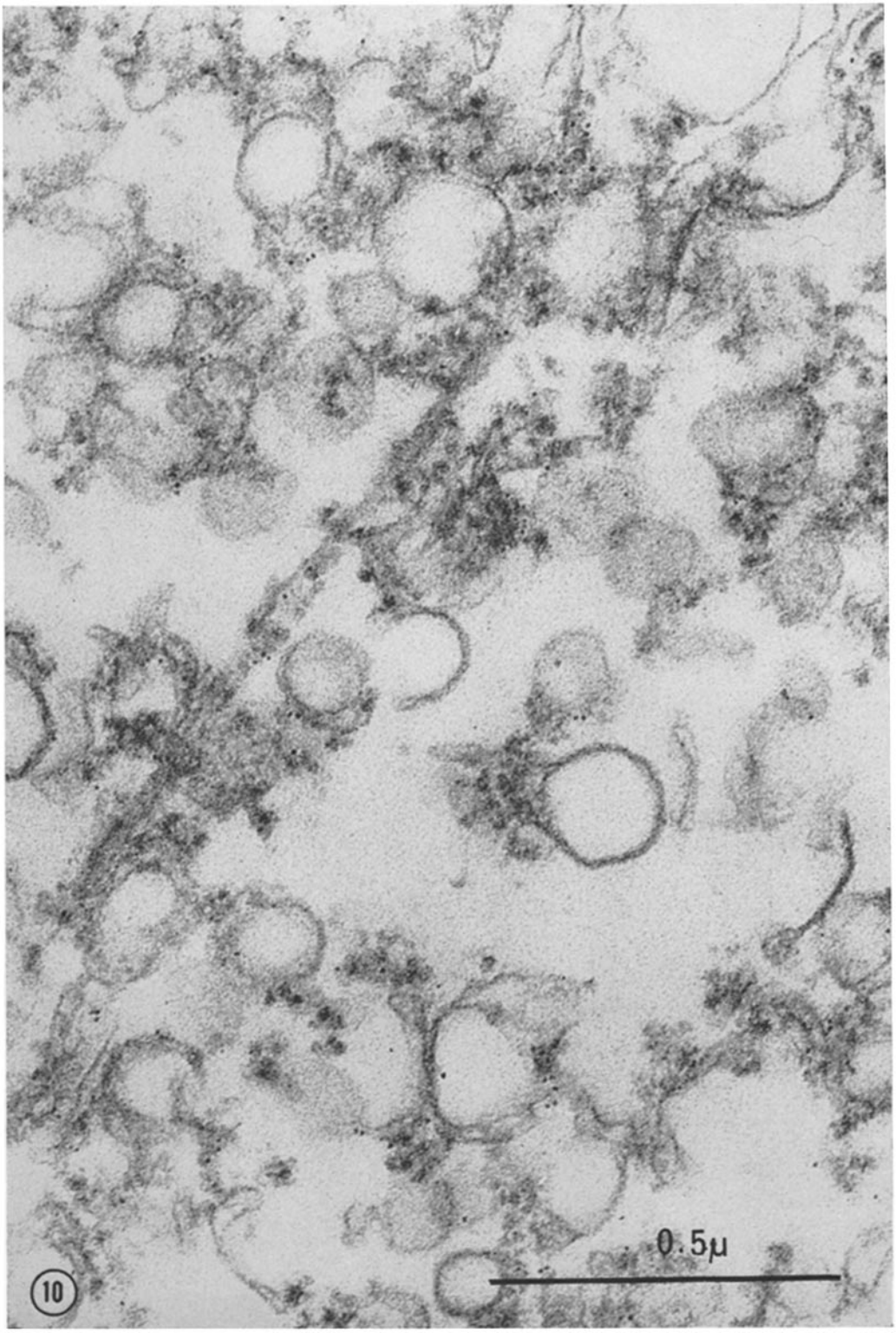

FIGURE 10 Electron micrograph of control group treated with IgG fraction from nonimmune rabbit serum and then with the same ferritin conjugate as in Fig. 9. Ferritin is still present in a distribution similar to that in the experimental group. Quantitatively, however, there is a distinct difference in labeling between the two groups. × 107,000.

the microtubules but rather with the amorphous component.

Some of the ferritin label observed, however, was unspecific, since control samples treated with nonimmune IgG also showed some ferritin (Fig. 10). Indeed, there is no difference in *distribution* of label between experimental samples and controls, since evidently the amorphous material is also responsible for nonspecific labeling. Specific labeling is, however, present, in that a difference in *amount* of label (Figs. 9 and 10) shows the dependence of label on the use of anti-22S specific antibody. In order to quantify the relative amount of labeling in experimentals and controls, counts were made on electron micrographs of the number of ferritin molecules per square micron of the original section. For avoiding discrepancies due to a diffusion gradient of ferritin molecules into the mitotic apparatus or to differing proportions of structures in different regions, the same areas of mitotic apparatus were compared in experimental and control micrographs. This was done by cutting thick sections of mitotic apparatus for phase-contrast examination before proceeding to thin sectioning for electron microscopy, and choosing for further sectioning only mitotic apparatus which were sectioned medially and longitudinally in the final thick section. After preparation of an electron micrograph, the central spindle region of each mitotic apparatus (between the chromosomes and centrospheres, but containing neither of these structures) was marked out; the number of ferritin molecules was counted, and the area was calculated in square micra of the original section. Counts on nine different samples (covering over 100 μ^2 in all) on anti-22S–treated and normal immunoglobulin-treated mitotic apparatus gave values of 133 $\pm$ 22 and 47 $\pm$ 8 ferritin molecules/μ^2, respectively (mean $\pm$ standard deviation). The distribution of the counts is shown in Fig. 11.

The fact that the same ferritin conjugate was used on both groups excludes error from differing proportions of unconjugated ferritin. Section thickness was also compared in experimental and control micrographs, by interference colors and by using the exposure meter on the electron microscope to compare the electron beam transmitted by collodion mounting film alone with that transmitted by tissue-free parts of the Epon section. The mean section thickness was quite similar in the two groups and does not account for the difference in labeling.

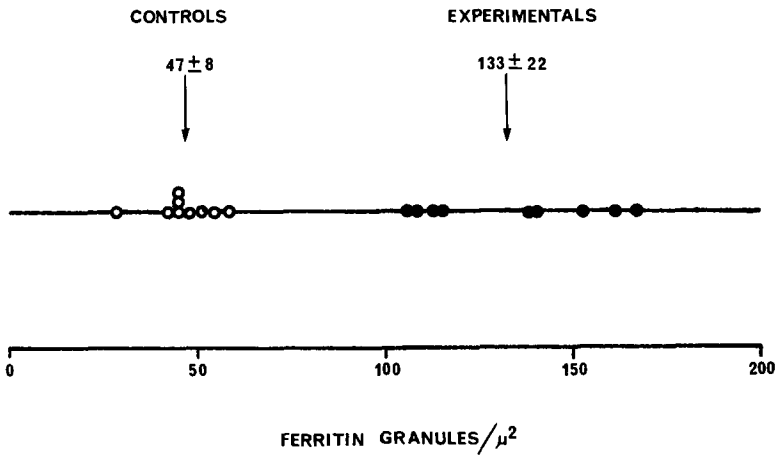

FIGURE 11 Distribution of counts of the density of ferritin labeling in experimentals and controls. Open circles, controls; closed circles, experimentals.

We conclude, first, that there is 22S protein located in the amorphous material which runs through isolated mitotic apparatus, and second, that within the sensitivity of the method there is no 22S protein in microtubules.

DISCUSSION

We have employed a direct approach, that of immunochemical assay for 22S protein, to determine whether 22S protein or a protein cross-reacting with it can be detected in the microtubules of isolated mitotic apparatus, in known preparations

of microtubule protein from sperm tail, and in protein preparations from mitotic apparatus which have been proposed to contain microtubule protein and which could contain 22S protein in the form of subunits of the 22S species. The result is in all cases negative; using an antibody preparation known to be reactive with 22S protein, under conditions where in each case the antigen-antibody reaction is known to be retained, we cannot detect 22S protein in any of these cases.

This finding is consistent with indirect evidence which strongly suggests that 22S protein is not a structural constituent of microtubules. The fine structure of microtubules revealed by negative staining, which is taken to show the construction of microtubules from their protein monomer, is quite similar for microtubules of mitotic apparatus (6, 34), sperm flagella (35, 36), and other sources. The corresponding monomer protein has been rather clearly identified in the case of inner pair microtubules of sperm flagella (37, 38) and outer doublet microtubules of sperm flagella (20, 36, 39) and cilia (33, 38, 40), These proteins have a monomer molecular weight of about 60,000 (36, 38, 40), corresponding reasonably with the monomer unit seen by negative staining, while the molecular weight of the smallest reported subunit of 22S protein is about 110,000 (17).

Moreover, the sperm tail and ciliary proteins resemble each other, and resemble actin of striated muscle, in amino acid composition (36, 40, 41), in precipitability by low concentrations of calcium (9, 42), and in binding 1 mole of nucleotide per mole (20), as well as in molecular weight. Shelanski and Taylor have further shown that the proteins from inner pair microtubules of sperm flagella bind one molecule of colchicine per protein dimer (38); actin probably also binds colchicine, in that colchicine affects its polymerization (43). The 22S protein has contrasting properties in these respects. It has an amino acid content with no resemblance to that of actin (17), it is soluble in calcium, it contains no nucleotide (17), and it does not bind colchicine (7). As described above, it is immunochemically distinct from microtubule proteins of sperm flagella. However, proteins have been extracted from mitotic apparatus under conditions considered to extract microtubule protein which have, from available data, various patterns of resemblance to actin and the ciliary and sperm tail proteins, including resemblances in molecular weight (4, 36), amino acid composition (36, 44) or sulfhydryl content (4), precipitability by calcium (4, 9), and a pattern of colchicine binding similar to that of the proteins from sperm flagalla (7). Moreover, Ruby (19) has shown that a protein of a fraction from sperm flagella having actin-like properties cross-reacts immunochemically with protein from isolated mitotic apparatus.

In the case of the proposed microtubule protein obtained from mitotic apparatus by Sakai, the suggestion has been made, on the basis of similarities in sedimentation coefficients, that it may correspond to a subunit of 22S protein (6, 8). Our immunochemical findings do not support this possibility, consistent with the fact that the Sakai protein is precipitable by calcium while the 22S protein is not, and with large discrepancies in the reported molecular weights for the species proposed to be identical (4, 17). A protein obtained from isolated mitotic apparatus by Miki-Noumura (8) also shows the recurring sedimentation coefficient; in this case it is not yet clear whether the protein is a subunit of 22S protein or derived from the protein obtained by Sakai.

The protein obtained by us (9) after mild acid extraction of mitotic apparatus, which causes the selective morphological disappearance of the microtubules, is precipitable by calcium like Sakai's protein and is immunochemically distinct from 22S protein.

Went (14–16) has previously reported immunochemical studies of two mitotic apparatus proteins, P1 and P2. Both P1 and P2 are present in an extract of mitotic apparatus protein obtained by an earlier version of the method used by Sakai; our findings indicate that 22S protein is present in this extract. P1 was the only antigen detected by various antisera in an extract of mitotic apparatus isolated by the alcohol-digitonin method; it is not known whether 22S protein is present in this extract. The point has been discussed by Sakai (4) and by Stephens (17) for a somewhat different extract that was studied physicochemically by Zimmerman (2).

It is tempting to identify P2 with 22S protein on the basis of the above correlations. However, Went's P2 protein has the distinctive property that it is no longer detectable immunochemically after exposing it briefly to high pH. We have repeated this experiment for 22S protein both from mitotic apparatus as prepared by Sakai and from unfertilized eggs, and find no detectable diminution in precipitin-forming activity due to this treatment.

On this basis, it does not appear that P2 can be the 22S protein. We have no particular reason for equating 22S protein with P1, however. The possibility remains that neither P1 nor P2 is 22S protein, and that 22S protein, which is possibly no more than a minor component in either preparation studied by Went, was not detected by his antisera.

Our finding that 22S protein is present in all tested fractions of metaphase eggs is consistent with previous findings of Kane (5) and Malkin et al. (45). Kane found 22S protein in the soluble fraction obtained during isolation of mitotic apparatus, and Malkin et al. concluded that a presumably identical protein was present in high concentration in yolk of intact eggs. We find that it is present also in nonmitotic apparatus particulate fractions obtained during isolation. It should be mentioned that the yolk particles lyse in Kane's hypotonic isolation medium. If 22S protein has only marginal solubility under these conditions, it might adsorb strongly to various particulates that are present. In the isotonic procedure of mitotic apparatus isolation used by Sakai, in which yolk particles remain intact, much less 22S protein is obtained from mitotic apparatus under similar extraction conditions. Alternatively, the presence of mitotic apparatus protein in other fractions has been interpreted as indicating appropriate precursor relationships (5, 14–16).

The location of 22S protein in the amorphous matrix of isolated mitotic apparatus does not give any definite indication as to its function. Roth (46) has proposed that a similar material observed in amebae might represent a microtubule precursor. Our labeling studies do not of course exclude the possibility that components other than 22S protein are located in the amorphous material, but the data do not support the idea that the role proposed by Roth is filled by 22S protein.

We are grateful to Drs. L. W. Cunningham, J. Davies, S. Harshman and G. Mosig of Vanderbilt University for advice on various procedures and for loan of equipment, and to Mrs. Darlene Hale and Mrs. Willenor Eaton for skilled technical assistance. This work was supported by grants No. GB-4588 and GB-7221 from the National Science Foundation.

Received for publication 30 October 1968.

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