THE MECHANISM OF ACTION OF COLCHICINE

Colchicine Binding to Sea Urchin Eggs and the Mitotic Apparatus

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ABSTRACT

Colchicine forms a complex in vivo with a protein present in fertilized or unfertilized sea urchin eggs; similar binding was obtained in vitro with the soluble fraction from egg homogenates. Kinetic parameters and binding equilibrium constant were essentially the same in vivo and in vitro. The binding site protein was shown to have a sedimentation constant of 6S by zone centrifugation. The protein was present in extracts of the isolated mitotic apparatus at a concentration which was several times higher than in whole-egg homogenates. It was extracted from the mitotic apparatus at low ionic strength under conditions which lead to the disappearance of microtubules. No binding could be detected to the 27S protein, previously described by Kane, which is a major protein component of the isolated mitotic apparatus. The properties of the colchicine-binding protein, (binding constant, sedimentation constant, Sephadex elution volume) are similar to those obtained with the protein from mammalian cells, sea-urchin sperm tails, and brain tissue, and thus support the conclusion that the protein is a subunit of microtubules.

INTRODUCTION

Previous work demonstrated that colchicine-^{3}H binds reversibly to cellular sites in human carcinoma cells strain KB (1). Similar binding takes place in soluble extracts incubated with colchicine (2). The binding is reversible, appears to depend on the macromolecule's being in its native state, and does not involve chemical modification of colchicine. A survey of colchicine binding in various cell types showed that the binding sites were not unique to mitotic cells but could be correlated with the presence of microtubules.

The most extensive information on mitosis has been obtained with sea urchin eggs, which are the only material from which the mitotic spindle has been isolated and analyzed chemically. Therefore we have investigated the binding of colchicine to intact sea urchin eggs, to cell-free extracts, and to the isolated mitotic apparatus. The purposes of the investigation were to show that the binding and inhibition of mitosis in sea urchin eggs is similar to that found in somatic cells in exponential growth and to demonstrate that the binding sites are located in the mitotic apparatus.

MATERIALS AND METHODS

Sea urchins, *Stronglyocentrotus purpuratus* and *Lytechinus pictus*, were obtained from Pacific Biomarine, Venice, Calif. Gametes were shed by injection of 0.5 ml of 0.53 M KCl. *Arbacia punctulata* were collected at Woods Hole, Mass. and were spawned by applying 10 v ac current across the test with lead electrodes. Eggs were collected into 250 ml synthetic sea water; sperm were collected "dry." Batches of

eggs which gave less than 95% fertilization were rejected. The composition of synthetic sea water for the Pacific urchins was 0.404 M NaCl, 8.9 mM KCl, 53 mM $MgCl_2$, 27.3 mM Na_2SO_4, 9.7 mM $CaCl_2$, 2.2 mM $NaHCO_3$; for *Arbacia*, synthetic sea water was prepared according to recipe of the Marine Biological Laboratory, Woods Hole (3).

Mitotic apparatus were isolated by a slight modification of the method of Kane (4). The isolation medium was 1 M hexylene glycol, 0.01 M sodium-phosphate buffer, 0.1 mM EDTA, pH 6.4. All phosphate buffers referred to in the text were at a concentration of 0.01 M.

Cell counts were determined with a Coulter counter. The counter efficiency was determined by direct count with a dissecting microscope.

Homogenates of whole eggs were prepared by washing three times in 0.53 M NaCl, resuspending in four volumes cold phosphate buffer, pH 6.5 and homogenizing in a Potter glass-teflon hand homogenizer. Supernatants were prepared by centrifuging at 35,000 rpm in a Spinco No. 40 rotor at 3°C for 30 min. All operations with extracts were carried out out at 0°–4°C.

Colchicine-binding assays, gel-filtration, sucrose-gradient sedimentation, and protein determinations were performed as described previously (2). For radioactivity measurements, 0.5 or 1.0 ml aliquots were placed in 10 ml Bray's solution and counted in a Packard Tri-carb Spectrometer. Radioactivity measurements on whole cells were made as described earlier (1). ATPase activity was determined according to the method of Martin and Doty (5).

The protein originally described by Kane (6) and characterized by its high sedimentation constant (variously reported as 22–27S) was prepared as follows. Unfertilized eggs were homogenized as described, and the homogenate was centrifuged 1.5 hr at 35,000 rpm in a Spinco 40 rotor at 3°C. The resulting supernatant was centrifuged 6 hr at 35,000 rpm in a Spinco SW 39 rotor at 3°C to pellet the 27S protein, and the pellet was redissolved in 0.1 M KCl in phosphate buffer. A 2 ml aliquot of the redissolved pellet was layered on a sucrose step-gradient consisting of 2 ml of 5% sucrose and 1 ml of 30% sucrose in 0.1 M KCl phosphate buffer and was centrifuged 8 hr at 35,000 rpm in a Spinco SW 39 rotor at 3°C. The bottom 1 ml was collected and dialyzed against 0.1 M KCl phosphate buffer overnight. A 0.5 ml sample of the dialysate was layered over a 30 ml volume 5–20% linear sucrose gradient in 0.1 M KCl phosphate buffer and centrifuged 18 hr at 24,000 rpm in a Spinco SW 25 rotor at 3°C. Fractions containing 27S material were pooled and the sedimentation constant was checked by velocity sedimentation in the Spinco model E.

To prepare negatively stained samples for electron microscopy, a drop of solution was placed on a carbon-coated fenestrated Formvar film on a copper-mesh grid for 2 min, excess solution was blotted away, and the grid was inverted and allowed to float on staining solution for an additional 2 min. Operations were carried out in a 4°C cold room.

Isolated mitotic apparatus were fixed 15 min in 2.5% glutaraldehyde, 0.1 M KCl, 0.01 M $MgCl_2$, 0.01 M phosphate buffer pH 6.4 at 0°C, postfixed in 1% OsO_4, pH 7, dehydrated through a graded series of alcohols, embedded in Araldite, sectioned with a Porter-Blum Ultramicrotome, and stained 1 hr in 1% uranyl acetate. All preparations were viewed in an RCA EMU 3 G microscope which was made available through the courtesy of Dr. William Bloom.

RESULTS

Colchicine Binding to Intact Sea Urchin Eggs

Sea urchin eggs, washed three times with 10 volumes synthetic sea water, were incubated with

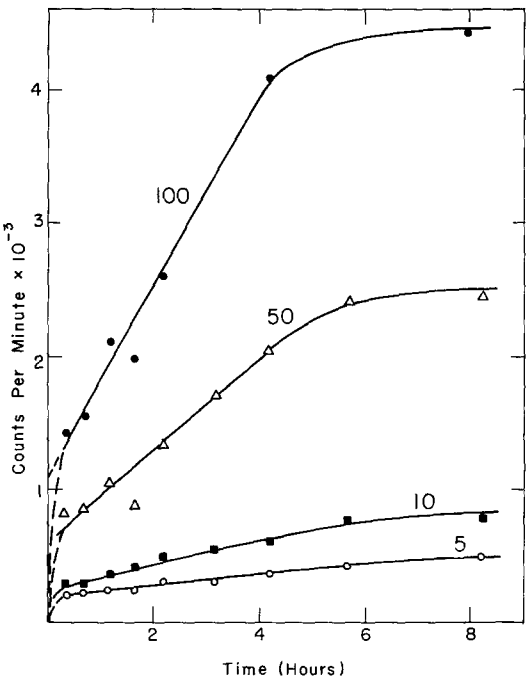

FIGURE 1 Uptake of colchicine-^{3}H by unfertilized *Lytechinus pictus* eggs at colchicine concentrations of 5, 10, 50, and 100 × 10^{-8} M at 23°C. Cell density was 25,000 cells per ml. Cells were washed three times by sedimentation with a hand centrifuge in synthetic sea water to remove external colchicine-^{3}H.

colchicine-^{3}H over a range of concentrations at a temperature appropriate to the species. At intervals, aliquots were withdrawn, were washed three times by resuspension in synthetic sea water, and were processed for determination of total radioactivity. The amount of radioactivity removed from the pellet by the fourth wash was only 1–5% of the total radioactivity of the pellet, depending on the extent of uptake. Therefore, three washes were considered adequate to free the eggs of external colchicine-^{3}H and the radioactivity found in the pelleted egg after three washes is referred to as total uptake. In some experiments 10^{-5} M unlabeled colchicine was included in the sea-water washes, but the results were identical.

An uptake experiment with unfertilized eggs of *Lytechinus pictus* at 23°C at colchicine-^{3}H concentrations of 5×10^{-8} to 10^{-6} M is illustrated in Fig. 1. Cell density was 25,000 cells per milliliter. There is (*a*) an early phase of rapid uptake; (*b*) a period of linear uptake extending over several hours; and (*c*) a plateau which represents an equilibrium state.

The uptake is reversible. Fig. 2 shows uptake of radioactivity by unfertilized *Strongtyocentrotus purpuratus* eggs at 10°C at 5×10^{-8} M colchicine-^{3}H and subsequent release into sea water containing 10^{-5} M unlabeled colchicine. In this experiment the eggs were washed free of the external label at 0°C. At the times indicated by the arrows, aliquots were washed at 0°C and resuspended in unlabeled colchicine–sea water either at 10° or 0°C. At intervals an aliquot was withdrawn, the eggs were sedimented, and the supernatant was used to determine released radioactivity. From the known radioactivity taken up by the cells at the time of resuspension, the radioactivity remaining in the cells was determined by subtraction.

The cells resuspended at 10°C lost radioactivity in a biphasic process. The initial rapid loss is the converse of the initial rapid uptake, and presumably represents a free, internal colchicine pool.

However, the loss of radioactivity from cells resuspended at 0°C was much slower and lacked the initial rapid phase. Passage of colchicine into and out of the cell, therefore, cannot be a simple diffusion process. From the rate of release at 0°C and from the time required to wash the cells (10 min) it was estimated that about 3–10% of the radioactivity would be lost in washing cells which had been harvested during the linear phase of the uptake curve. Therefore the bulk of the internal pool is retained in the washing procedure and its

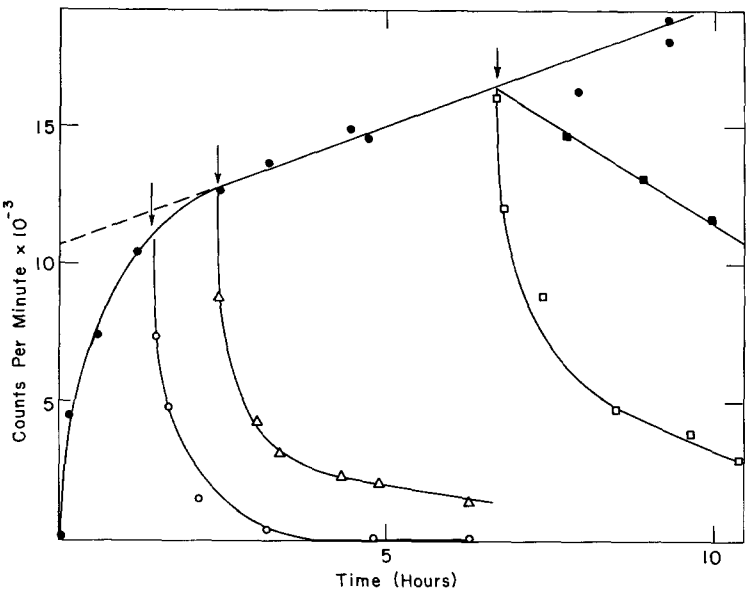

FIGURE 2 Reversibility of colchicine-^{3}H uptake by unfertilized *S. purpuratus* eggs at 16°C. Colchicine-^{3}H, 50×10^{-8} M, was added at zero times; at the times indicated by the arrows, aliquots of the population were washed three times in synthetic sea water at 0°C and resuspended in synthetic sea water containing 10^{-5} M unlabeled colchicine at 10°C (○, △ □,) or at 0°C (■). The dashed line is the extrapolation of the linear region back to zero time, which provides an estimate of the internal colchicine pool.

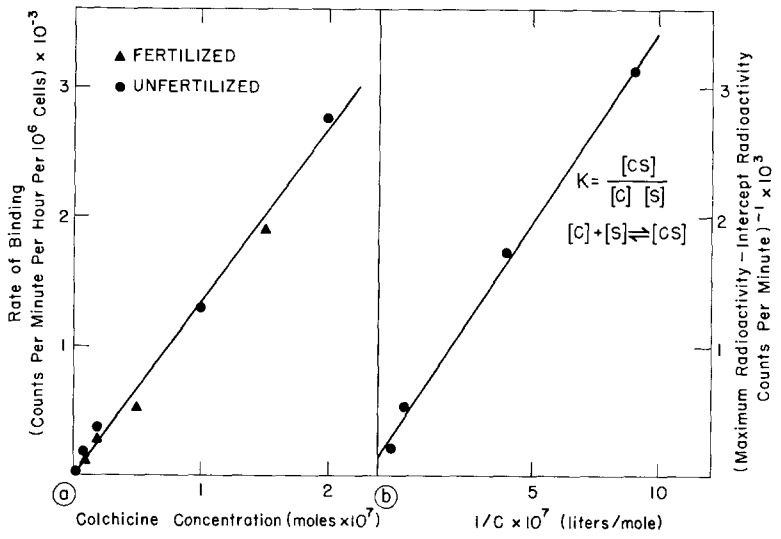

Fig. 3

FIGURE 3 Analysis of colchicine-^{3}H uptake: (*a*) Initial rate of binding determined from the linear region of the uptake curves (Fig. 1) plotted against colchicine concentration for (●) fertilized or (▲) unfertilized eggs. (*b*) The reciprocal of (plateau radioactivity-intercept radioactivity) plotted against the reciprocal of the colchicine concentration, per 10^6 eggs.

size was estimated by extrapolating the linear region of the uptake curves (Fig. 2) to zero time.

The ratio of free internal radioactivity in packed cells to the radioactivity of an equal volume of medium varied from 0.25 to 0.4 for a 20-fold change in external concentration. Although the numbers are less than one, it is probable that a moderately large fraction of the cell volume is not accessible to colchicine. Thus the interpretation of the fast uptake and release phase as the behavior of a free pool appears to be consistent with the data.

By employing a method of analysis described previously (1) quantitative agreement with the experimental data can be obtained by expressing the binding in terms of a second order chemical reaction.

$C + S \rightleftharpoons CS$ where C is the colchicine concentration, S is the free site concentration, and CS the concentration of bound sites.

(*a*) A plot of the slope of the linear portion of the uptake curves was proportional to colchicine concentration over the range 5–100 × 10^{-8} M. (Fig. 3 *a*) The same initial rate of uptake per cell was obtained with fertilized eggs and the results are also included in Fig. 3 *a*.

(*b*) The bound radioactivity is the difference between the total uptake and the free internal pool. The pool size is given by the intercept of the linear region of the curve extrapolated back to zero time. If the system is in equilibrium, a plot of (cpm at plateau − cpm at intercept)$^{-1}$ versus (C)$^{-1}$ should be a straight line. The data are plotted in this way in Fig. 3 *b* and are seen to approximately fit a straight line. The kinetic and equilibrium con; stants determined from these plots are given in Table I. Similar uptake curves and binding constants were found in a previous study with mammalian cells, strain KB (1).

As was the case with KB cells, the radioactivity was located in the soluble fraction. Unfertilized

TABLE I

Values of The Binding Parameters for Unfertilized Sea Urchin Eggs and Egg Extracts

	In vivo*	In vitro‡
k_1 liters moles^{-1} hours^{-1}	0.12 × 10^6	0.34 × 10^6
k_{-1} hours^{-1}	0.10	0.15
K liters moles^{-1}	1.2 × 10^6	2.3 × 10^6

* Values obtained using double reciprocal plot, e.g. Fig. 3 *b*, for data on *L. pictus* eggs at 23°C.

‡‡ Values obtained using log versus tine plot, e.g. Fig. 7 *b*, for data on *A. punctulata* eggs at 37°C.

Lytechinus eggs were incubated for 8 hr at 23°C with colchicine-^{3}H at 10^{-6} M. The eggs were collected, washed three times in 0.53 M KCl to remove free colchicine, and homogenized in 0.1 M KCl, 0.01 M phosphate, pH 6.5. The homogenate was fractionated by differential centrifugation. More than 95% of the radioactivity was found in the fraction which did not sediment in 1.5 hr at 100,000 *g*.

In order that the fraction of the radioactivity that was macromolecularly bound could be determined, aliquots of the supernatant were subjected to gel-filtration on G-100 Sephadex and to zone centrifugation. A typical gel-filtration profile from an experiment showing 45% binding is shown in Fig. 4. The void volume of the column was previously determined with a blue dextran marker. In a number of experiments, bound radioactivity varied from 20 to 50% of total radioactivity. This figure is comparable to that obtained from KB cells and is probably an underestimate of the binding in whole cells because of dissociation which occurs while the system is being processed. Also the size of the internal pool in sea urchin eggs relative to the bound colchicine was considerably larger than in KB cells and the pool was not removed during the washing procedure.

Zone centrifugation through a linear 5–20% sucrose gradient of the leading radioactivity peak from a gel-filtration experiment typically gave the profile shown in Fig. 5 *a*. A bovine-serum albumin marker was included to calibrate the gradient. A major peak at approximately 6S is observed pulling away from a distribution of activity leading down from the meniscus. Since the duration of the sedimentation was 14 hr, the observed activity pattern is consistent with the slow dissociation of a single 6S species of colchicine-site complex. The data, however, do not rule out the possibility of some binding to a site with a sedimentation constant of 1–3 S. It is concluded that most of the colchicine in washed eggs as in KB cells is bound to a 6S macromolecule which appears in the soluble fraction after homogenization.

Colchicine Binding to Extracts of Sea Urchin Eggs

In a previous study (2) it was shown that colchicine binding could be demonstrated in vitro with soluble supernatants from mammalian cells, and that the kinetic constants were comparable to those of whole cells. Similar results are here described with extracts of unfertilized as well as fertilized sea urchin eggs.

Soluble extracts of unfertilized eggs of the available species of sea urchins are deeply colored due to released echinochromes, especially in *Arbacia punctulata*. Most of the pigment in extracts of *Arbacia* eggs can be removed by subjecting the extract to gel-filtration over G-25 Sephadex equilibrated with 0.01 M phosphate buffer pH 6.5 at 0°C. The echinochrome absorbed to the G-25 while the fraction voided by the column retained most of its binding activity and was the starting point for the in vitro binding experiments. This

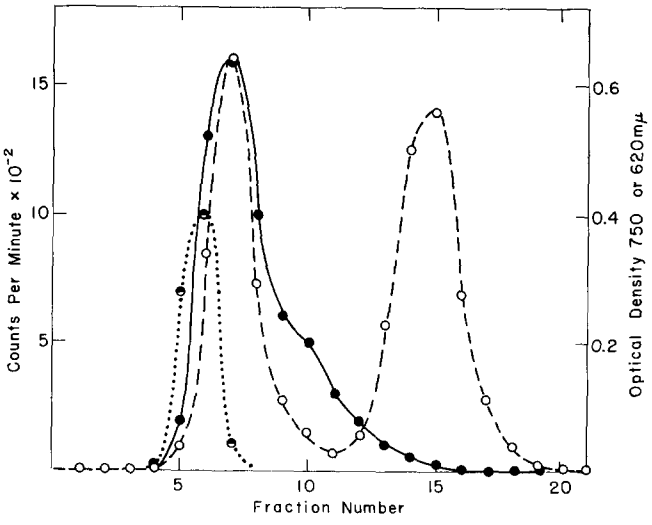

FIGURE 4 Gel filtration of colchicine binding in vivo. *S. purpuratus* eggs were incubated with 5×10^{-6} M colchicine and 1 ml of the soluble fraction from a homogenate of washed eggs was applied to a 1 × 15-cm G-100 Sephadex column. 1 ml fractions were collected. (○) radioactivity, (●) optical density at 750 mμ for Lowry protein determinations (◑) optical density at 620 mμ for blue dextran void-volume marker. Radioactivity and protein maxima were adjusted to coincidence.

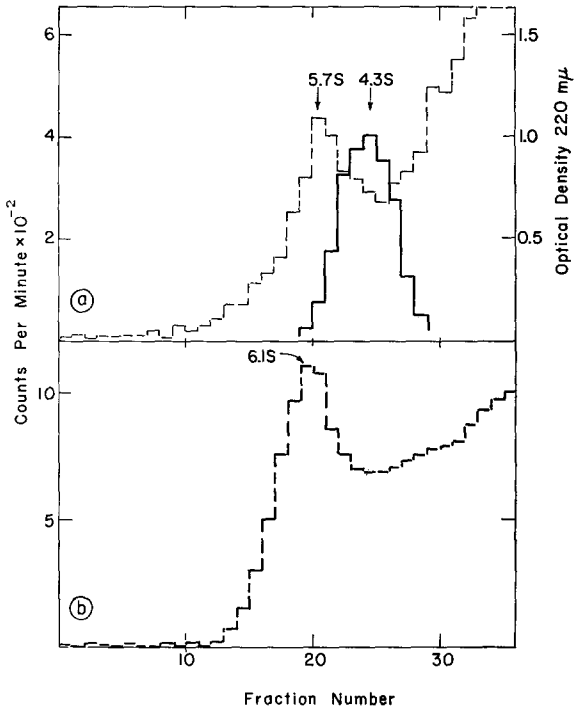

FIGURE 5 Zone centrifugation analysis of in vivo and in vitro colchicine-binding activity. (*a*) *L. pictus* eggs were incubated with 5×10^{-6} M colchicine at 23°C for 6 hr. The bound radioactivity from gel filtration of the soluble fraction was layered on a 5–20% sucrose gradient containing 0.1 M KCl in phosphate–magnesium solution. (*b*) Soluble fraction from *L. pictus* eggs, incubated with 2.5×10^{-6} M colchicine for 1 hr at 37°C. Bound radioactivity from gel filtration applied to sucrose gradient and run under same conditions as in (*a*). (-----) radioactivity, (——) optical density at 220 mμ for a bovine-serum albumin-sedimentation marker run in a parallel gradient. Sedimentation was for 14 hr at 38,000 rpm in the Spinco SW 39 rotor at 3°C.

prefiltration also showed that a low molecular-weight cofactor was not required for the binding reaction.

Bound radioactivity obtained by incubation of the soluble fraction with 2.5×10^{-6} M colchicine migrated as a 6S peak in zone sedimentation (Fig. 5 *b*).

The kinetics of binding in vitro was assayed as described previously for KB cells (2). The extract was incubated with colchicine-^{3}H at 37°C, 1 ml aliquots were removed at intervals, cooled to 0°C to stop the reaction, and applied to a 1 × 15-cm G-100 Sephadex column for the determination of bound count. The mechanism proposed for binding requires that the reaction be first order in the concentration of colchicine and binding sites. Plots of the initial rates of binding as a function of the concentration of binding sites and of colchicine are shown in Figs. 6 *a* and 6 *b*, respectively, for extracts of unfertilized *S. purpuratus* eggs. It can be seen that the required linear relations are obtained.

Determination of an equilibrium constant was hampered by the lability of the binding sites at 37°C. To avoid the large errors in measuring the equilibrium constant from a double reciprocal plot, a different method was used; the equations given in the appendix were employed. A plot of log $[(CS)_m - (CS)]$ versus t, where $(CS)_m$ is the maximum binding at concentration C, should be linear with slope $k_1C + k_d$, where k_d is the rate con-

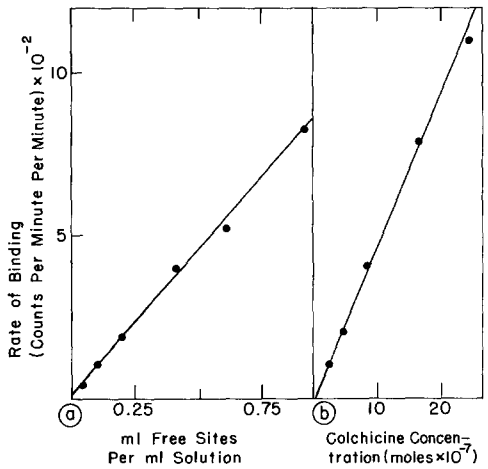

FIGURE 6 Initial rate of colchicine binding to extracts of *S. purpuratus* eggs in vitro. (*a*) Various dilutions of soluble egg extract were incubated with 2.5×10^{-6} M colchicine-^{3}H at 37°C. Binding was assayed after 5 and 10 min incubation to determine initial rates. (*b*) Initial rate of binding at constant extract concentration but variable colchicine concentration.

stant for loss of free sites. If the free, radioactive colchicine is removed, a plot of log CS versus t should be linear with slope $k_{-1} + k_d'$ where k_d' is the rate of denaturation of bound sites. Since colchicine binding stabilizes the sites, k_d' is relatively small. Thus the ratio of the slopes for large C is approximately $Ck_1/k_{-1} = CK$. Based on approximate values for k_d and k_d', the error in K should be less than a factor of two. The time course of binding and release of radioactivity is shown in Fig. 7 *a*. At the time indicated by the arrow an aliquot was withdrawn and passed over a Sephadex column to remove free colchicine, and the pooled radioactivity from the bound peak was dialyzed against excess unlabeled colchicine. The rate of dialysis of free colchicine is about 100 times that for dissociation of bound colchicine. Thus the amount of radioactivity remaining inside the dialysis tubing is essentially equal to the amount of bound complex. The equilibrium constant was obtained from the plots shown in Fig. 7 *b*. The values are given in Table I and are similar to the results obtained in vivo.

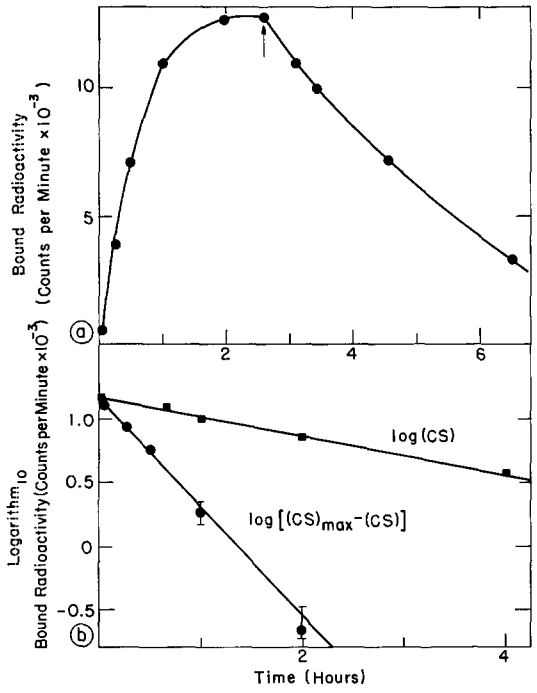

FIGURE 7 Time, course of binding, and dissociation in extracts of *A. punctulata*. (a) Binding assays were made at intervals after incubation with 2.5×10^{-6} M colchicine at 37°C. At the time indicated by the arrow, an aliquot was withdrawn, passed over a G-100 Sephadex column to remove free colchicine, and the pooled radioactivity from the bound peak was dialyzed against 10^{-5} M unlabeled colchicine at 37°C. (*b*) Logarithmic plots of bound radioactivity, CS versus time for association and dissociation. For details see text.

Colchicine Binding to Isolated Mitotic Apparatus

Having shown that binding activity in vitro is a valid measure of that in vivo, we are now in a position to test whether the binding macromolecule can be identified with a subunit of the spindle.

The mitotic apparatus (MA) was isolated from populations of *A. punctulata* and *S. purpuratus* according to the method of Kane (4). Fig. 8 shows a typical preparation of MA prepared from *S. purpuratus* as seen with phase microscopy.

Addition of colchicine in concentrations as high as 10^{-2} M to MA in the isolation medium (1 M hexylene glycol, 0.01 M phosphate, 0.1 mM EDTA, pH 6.4) does not affect the morphology as observed by phase or polarized light microscopy. Removal of the hexylene glycol in the pH range from 6.5 to 7.5 leads to immediate swelling, loss of birefringence, and partial solution of the MA, but the residue remains insensitive to colchicine. Since hexylene glycol inhibits colchicine binding and produces irreversible changes in the solubility of the MA (7), it was not possible to determine the effect of colchicine on the isolated MA under conditions which are comparable to the in vivo experiments.

Colchicine binding to soluble proteins of the MA was assayed as follows. The MA were washed three times with isolation medium at 0°C and then were sedimented to give about 0.1-ml packed volume. The MA were immediately resuspended in 0.6 M KCl in 0.01 M phosphate, 0.1 mM EDTA, pH 6.5 at a final volume of 0.5 ml. Kane has shown that under these conditions about one-half of the MA protein, including that of the microtubules, is taken into solution (6). After the preparation was examined under the phase microscope for the absence of intact MA, the solution was diluted with an equal volume of phosphate buffer to reduce the KCl concentration to 0.3 M and the hexylene-glycol concentration to below 0.1 M. This was necessary to insure optimal binding activity since it was found that addition of salt or hexylene glycol to protein extracts reduces colchicine binding. The solution was then clarified and tested for binding activity.

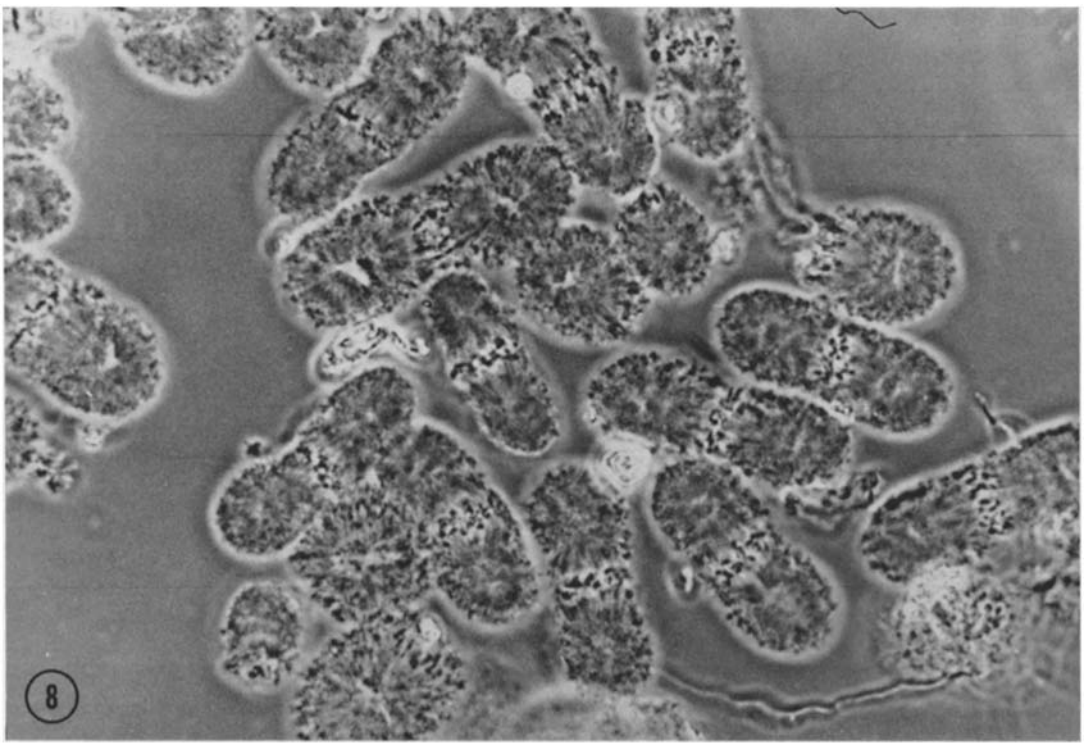

FIGURE 8 Freshly isolated mitotic apparatus from *S. purpuratus* prepared according to the method of Kane; phase contrast. × 1250

The binding activity of (*a*) the KCl extract of the isolated MA was compared with (*b*) a total lysate of the metaphase egg prepared in the MA isolation medium and treated in the same manner as the MA, (*c*) an extract of the MA brought to 0.5 M in hexylene glycol. The specific activities are summarized in Table II.

The specific activity of binding by the MA in this experiment was three times higher than that for whole eggs homogenized in phosphate buffer. However, a better comparison is provided by the lysate prepared in hexylene glycol since this compound reduces colchicine binding. The activity of the MA is six times higher than the total lysate. That there was no binding to the material extracted in the last wash of the MA could indicate that contamination by cytoplasmic proteins is small. However, in these experiments, as in most experiments with the MA, it can always be argued that any protein found in the egg cytoplasm as well as in the MA is present in the MA only as a strongly bound contaminant.

TABLE II

*Colchicine Binding Activity in Extracts of the Isolated Mitotic Apparatus**

Test sample	Specific binding activity‡
	cpm/mg protein × 10^{-3}
(*a*) Isolated MA extracted in 0.6 M KCl	10.5
(*b*) Total lysate of metaphase egg in MA isolation medium	1.7
(*c*) Isolated MA extracted in 0.6 M KCl and brought to 0.5 M in hexylene glycol	1.0

* MA were isolated from *A. punctulata*.

‡‡ Binding activity assayed by incubating the extract with 2.5 × 10^{-6} M colchicine-^{3}H for 1 hr at 37°C and determining bound-count by gel filtration.

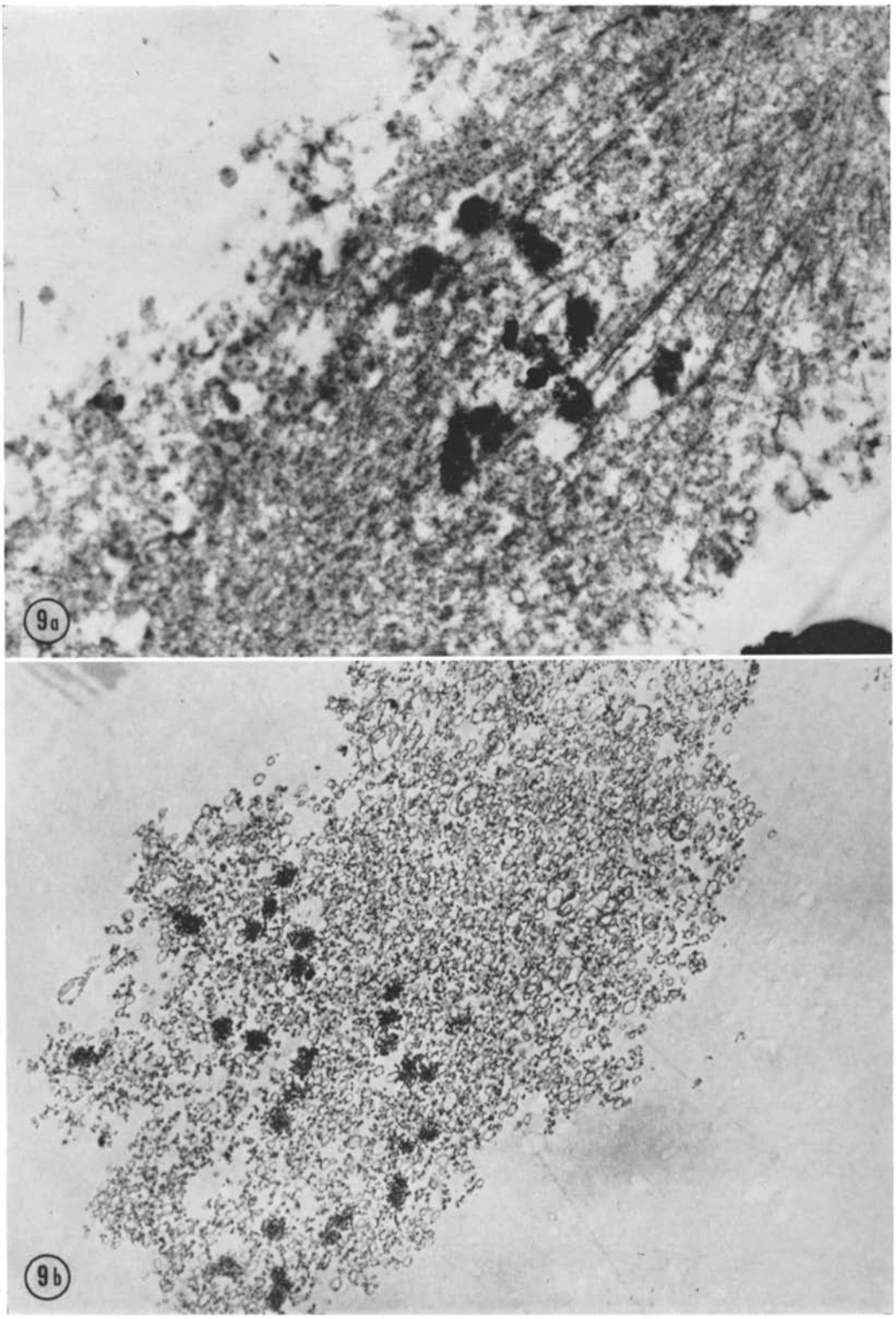

FIGURE 9 (*a*) Freshly isolated mitotic apparatus from *S. purpuratus*, note chromosomes and spindle fibers, and dense vesicular matrix. × 15,600. (*b*) Freshly isolated mitotic apparatus from *S. purpuratus* exposed to 0.01 M tris thioglycollate buffer, 0.1 M EDTA, pH 7.5 at 0°C for 10 min before fixation. Spindle fibers are absent but vesicles and chromosomes are retained. × 15,600.

TABLE III

*Differential Extraction of Colchicine-Binding Activity from Isolated Mitotic Apparatus**

Description	Protein‡‡	Total bound radioactivity§	Specific binding activity
	mg	*cpm* × 10^3	*cpm/mg* × 10^3
(*a*) MA extracted in tris-thioglycollate buffer pH 7.5 for 10 min at 0°C	0.44	10.6	24.0
(*b*) Residue from (*a*) re-extracted in 0.6 M KCl, pH 6.5 for 10 min at 0°C	0.47	1.5	3.2
(*c*) Residue from (*b*)	0.19	—	—
(*d*) MA extracted in 0.6 M KCl, pH 6.5 for 10 min at 0°C	0.86	8.9	10.0

* MA were isolated from *S. purpuratus*.
‡‡ Protein determined using method of Lowry (23).
§ Bound radioactivity determined by gel filtration after incubating the extract with 2.5×10^{-6} M colchicine-^{3}H for 1 hr at 37°C.

It is important to know what per cent of the total spindle protein is responsible for the binding. The lability of the binding sites as well as the adverse effect of hexylene glycol make it difficult to provide more than a rough estimate. Based on the known rate of decay of binding sites and a molecular weight of 10^5, the sites are not likely to be more than 10–15% of the total extractable protein and could be as low as 5%.

Correlation of Microtubules and Colchicine-Binding of Isolated MA

Isolated spindles suspended in low ionic strength buffer in the range of pH from 7.0 to 7.5 can be seen in the light microscope to swell, lose their birefringence, and slowly disintegrate. Fig. 9 *a* is a low-power electron micrograph of freshly isolated spindles from *S. purpuratus* while Fig. 9 *b* shows the same material after exposure to 0.01 M tris-thioglycollate buffer, 0.1 mM EDTA, pH 7.5 for 10 min at 0°C. Examination of a large number of sections showed that the chromosomes and much of the vesicular material remained after the low ionic strength treatment, but the microtubules were absent. Therefore brief treatment with low ionic strength buffer and controlled pH provides a means for differentially extracting the isolated MA. An extract prepared with low ionic strength buffer was clarified by centrifugation at 10,000 *g* for 15 min. The residue was reextracted with 0.6 M KCl, 0.01 M phosphate, 0.1 mM EDTA for 15 min, then diluted with an equal volume of phosphate buffer, and clarified to give a second soluble fraction and a final residue. Protein determinations were made on the supernatants and pellets, and colchicine-binding assays were performed on the low ionic strength and 0.6 M KCl soluble fractions as well as on the total soluble protein from spindles taken into solution initially with 0.6 M KCl. The results are summarized in Table III.

More than 80% of the colchicine-binding activity is removed along with the microtubules by the low ionic strength extraction while less than 40% of the protein is solubilized under these conditions. The total binding-activity obtained by extraction either with 0.6 M KCl or tris-EDTA pH 7.5 is comparable. These data are consistent with the hypothesis that the colchicine-binding sites are the microtubule proteins but more protein is extracted by the pH 7.5 treatment than can be accounted for by the binding protein alone.

Zone centrifugation profiles of the binding to extracts of MA protein prepared in 0.6 M KCl and in tris-EDTA pH 7.5 are given in Fig. 10 *a* and 10 *b* respectively. The extracts were incubated with colchicine and run over G-100 Sephadex columns. Aliquots from the bound peak were then layered on linear, 5–20% sucrose gradients in 0.1 M KCl, 0.01 M phosphate, 0.01 M $MgCl_2$ pH 6.5. A 6S peak was obtained with the tris-EDTA extract as well as a distribution of radioactivity at low S values, while the KCl extract showed only a distribution of radioactivity at low S values. Thus a 6S binding-protein is obtained from the MA under the same conditions of ionic strength and pH that were used to extract the protein from whole cells, sperm tails, or brain tissue. The complication introduced by the behavior after exposure to high ionic strength was not investigated further with this material because of the small quantities of protein available. A study of the sedimentation properties of the binding-site protein was undertaken with the purified protein from brain tissue and will be reported elsewhere.

Colchicine Binding to 27S Protein

Kane has reported that the protein which is extracted from isolated spindles by 0.6 M KCl

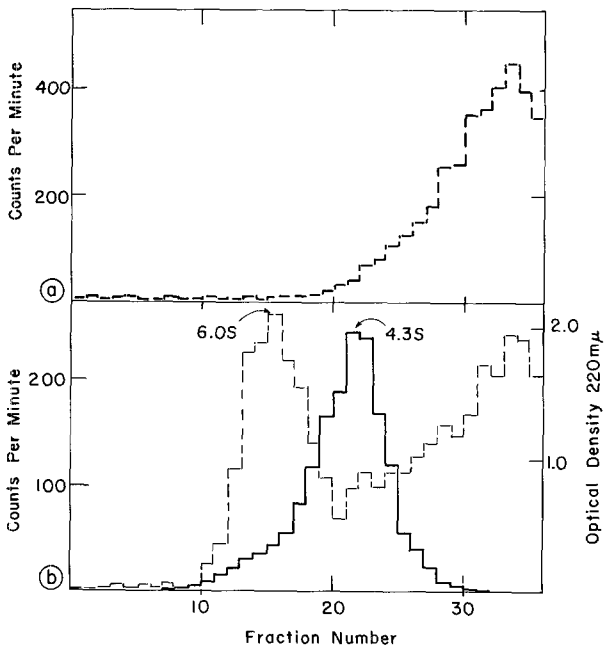

FIGURE 10 Zone sedimentation profiles of colchicine binding to extracts of isolated MA. *a* Freshly isolated MA were extracted in five volumes 0.6 M KCl at pH 6.5 for 10 min, at 0°C and then diluted with phosphate buffer to reduce the KCl concentration to 0.2 M. (*b*) Freshly isolated MA were extracted in 0.01 M tris-thioglycollate buffer, pH 7.5 for 10 min at 0°C. Extracts were clarified and incubated with 2.5×10^{-6} M colchicine-^{3}H for 1 hr at 37°C, bound radioactivity separated by gel filtration and 0.2 ml aliquots of the bound radioactivity applied to 5–20% sucrose gradients containing 0.1 M KCl in phosphate-magnesium solution. Gradients were run for 14 hr at 50,000 rpm in the Spinco SW 65 rotor at 2°C, and fractions were collected from the bottom (-----) radioactivity, (——) optical density 220 mμ of a bovine-serum albumin sedimentation marker run in a parallel gradient.

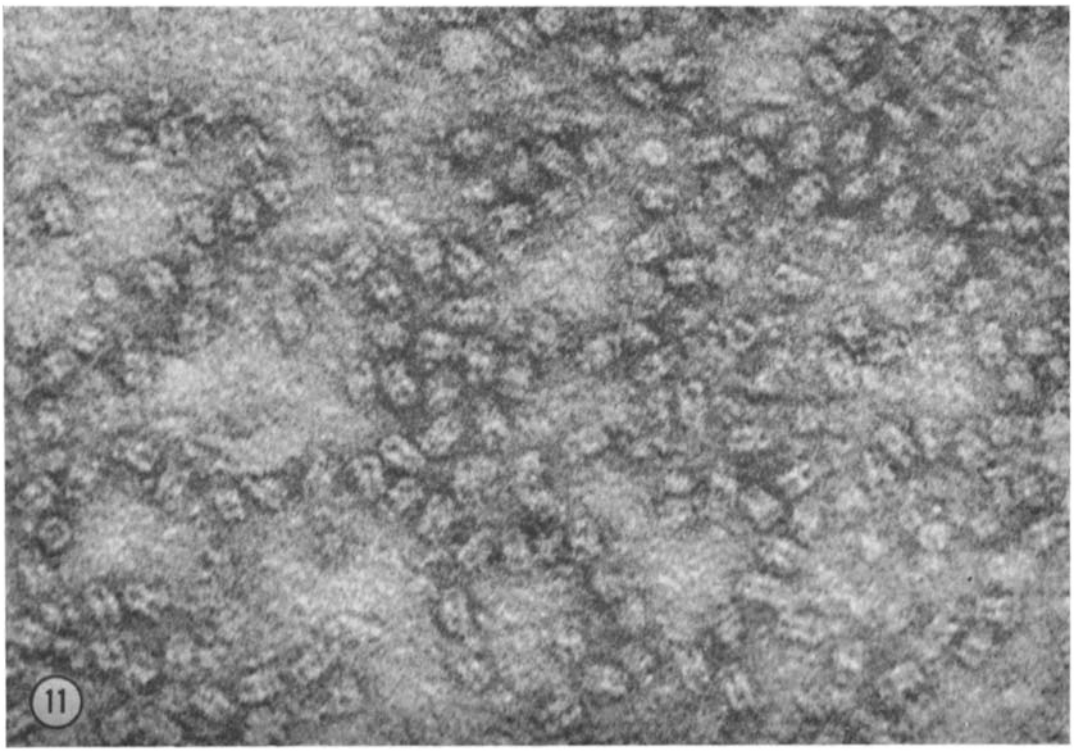

FIGURE 11 Electron micrograph of 27S protein negatively stained with 1% phosphotungstic acid at pH 5.3. Particle dimensions are approximately 250 × 150 A. × 220,000.

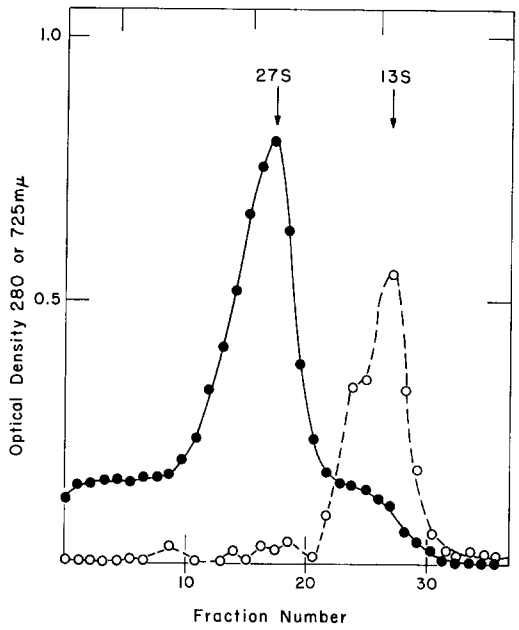

FIGURE 12 Zone sedimentation of partially purified 27S protein. A 0.5 ml sample was layered on a 30 ml volume, linear 5–20% sucrose gradient in 0.1 M KCl, 0.05 M tris-maleate buffer, pH 6.4 and spun in the Spinco SW 25 rotor for 16.5 hr at 24,000 rpm at 3°C. (——) optical density at 280 mμ, (----) ATPase activity determined after 30 min incubation at 37°C in tris-maleate buffer containing 4 mM ATP and 4 mM $CaCl_2$.

(including all the microtubule protein and colchicine-binding activity), consists primarily of one component with a sedimentation coefficient of 22S (uncorrected). Examination of MA extracts on sucrose gradients and with the analytical centrifuge confirmed that this protein was also a major component of our preparations. In order to obtain larger quantities of material the protein was purified from homogenates of whole eggs (Methods). Fig. 11 is an electron micrograph of the protein negatively stained with phosphotungstic acid. The particles have a cylindrical shape with approximate dimensions of 250 × 150 A. Their appearance is similar to the structure reported by Malkin et al. (8). The level of colchicine binding to this protein was nearly unmeasurable. Since Malkin (8) reported a corrected sedimentation constant of 27S which agrees with our values obtained on sucrose gradients, we have referred to the material as the 27S protein. The sedimentation constant at zero concentration has not been determined in this laboratory and this terminology is used only because an accepted name has not been assigned to the protein.

A sucrose-gradient profile of a partially purified preparation of 27S protein which had been incubated with colchicine is shown in Fig. 12. Colchicine binding was not detectable on the gradient. The protein did not show ATPase activity although a 13S ATPase, activated by calcium or magnesium, was present. Therefore the 27S protein, which is the major protein component of the MA as isolated by the Kane procedure, is neither the colchicine-binding protein nor the ATPase described in spindles by Mazia et al. (9).

CONCLUSIONS

Colchicine forms a complex in vivo with a protein present in fertilized or unfertilized sea urchin eggs; similar binding was obtained in vitro with the soluble fraction from egg homogenates. The kinetic parameters and binding constant both in vivo and in vitro were essentially the same as those found with KB cells; further the colchicine-macromolecule complex as isolated from eggs, KB cells, sperm tails (10), or brain tissue gave essentially the same elution volume in gel-filtration and a sedimentation coefficient of 6S in zone centrifugation; this indicates that the same class of macromolecule is responsible for the binding in all cases.

That in sea urchin eggs the initial rate of binding to fertilized and unfertilized eggs was the same, indicates the presence of the protein in similar amounts. This is consistent with the observations that the mitotic-apparatus proteins are present in the unfertilized egg (11, 12). A similar situation occurs with exponentially growing cultures of KB cells since the kinetics of binding could only be explained if the binding protein were present throughout much of interphase (1).

The specific binding activity was greater for extracts of the mitotic apparatus than for the whole egg; this indicates a higher concentration in the MA. Uncertainties introduced by lability of the binding site and the inhibitory effect of hexylene glycol prevents very accurate estimates of the distribution of the protein. It is not more than 10–20% of the protein extractable from the isolated MA and the whole egg contains two or three times as much of the protein as the MA.

The low value in the MA is not surprising since the main component is the 27S protein first described by Kane. The relationship of the 27S

protein to the 6S and 4S proteins obtained from the MA and cilia (10, 13–16) is not clear. The 27S particle has not been found in *Tetrahymena* cilia and sea-urchin sperm tails, even though these organelles should provide a purer source of tubule protein than the MA. No colchicine binding was detectable with the 27S protein although the assay would have detected the presence of a small amount of colchicine-binding sites in equilibrium with the 27S unit. Since both proteins are released from the MA under mild conditions while there is no evidence to support their interconversion, under the same conditions it is unlikely that the 27S protein is an aggregate of the 6S protein.

The finding by Malkin et al. (8) that the 27S protein is largely present in a granule fraction would be consistent with the role of the 27S protein as a storage material whose presence in the MA is not related to the mechanism of mitosis or the formation of microtubules. However, the possibility remains that the 27S particle could be a storage form of the tubule protein which is peculiar to eggs and which is not an intermediate polymer in the formation of microtubules.

The present experiments do not lead to an unequivocal answer to the question of the mechanism of inhibition of mitosis by colchicine. Treatment of mitotic cells with colchicine leads to a disorientation and possible disaggregation of the mitotic apparatus. In the electron microscope the microtubules, which are the main structural feature of the spindle, are either absent or reduced in number and lack the regular orientation characteristic of the intact spindle (16). Colchicine binding has been demonstrated to a protein prepared from the isolated mitotic apparatus and from cilia which appears to be the microtubule subunit. A plausible explanation of the mechanism of action is provided by assuming that binding of colchicine prevents assembly of the subunit into a microtubule.

The hypothesis is supported by the synergistic action of colchicine with temperature, pressure, and D_2O (17–22). These effects imply that the same target site is involved, and the results can be explained by supposing that the polymerization of subunits into microtubules is an equilibrium process possibly nucleated by centrioles and kinetochores. The equilibrium can be shifted to favor monomer by lowering temperature, increasing pressure, or complexing monomers with colchicine.

A direct test of this hypothesis would require the demonstration in a purified system that colchicine prevents polymerization of the subunits. Mitotic cells are not a convenient starting material for purification of the binding sites and for this purpose brain tissue has been used. A purification of the binding sites from brain will be described in a subsequent report.

The authors wish to express their thanks to Mrs. Michelle Chassagne and Mrs. Chantal Boyd for their technical assistance. Mr. Hiro Tonaki provided valuable assistance and advice in electron microscopy.

This work was supported by United States Public Health Service Grant GM 10992. Dr. Borisy wishes to acknowledge support by the National Science Foundation and National Aeronautics and Space Administration as well as United States Public Health Service Training Grant 5 T1 GM 780.

This work was submitted in partial fulfillment for the requirements of the Ph.D. in Biophysics.

Received for publication 9 January 1967.

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APPENDIX

KINETICS OF BINDING DENATURABLE SITES

Binding of a molecule C to independent sites S is described by the equation

$$C + S \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} CS \qquad (1)$$

where CS is the bound complex and k_1 and k_{-1} are the specific rate-constants for the association and dissociation steps. $K = k_1/k_{-1}$. At equilibrium

$$(CS)_e = k_1 CS_0/(k_1 C + k_{-1}) \qquad (2)$$

where $(CS)_e$ is the equilibrium concentration of bound sites, S_0 is the initial concentration of free sites and C is the initial concentration of the binding molecule. C is assumed to be much greater than S_0 and can, therefore, be treated as a constant.

Since experiments on colchicine binding showed that the free sites are labile but activity is stabilized by binding colchicine, two extra reactions must be included.

$$S \xrightarrow{k_d} S_d \qquad (3)$$

$$CS \xrightarrow{k_d'} S_d + C \qquad (4)$$

where k_d and k_d' are the specific rate constants for the denaturation of free and bound sites. $k_d' \ll k_d$.

Equations 1, 3, and 4 lead to a set of simultaneous differential equations, which can be solved by standard methods. $CS = \alpha \exp r_1 t + \beta \exp r_2 t$. The effect of site denaturation will be important if $k_d \gg k_{-1}$. The maximum binding is given by $(CS)_m = k_1 CS_0 \gamma/(k_1 C + k_d)$ where $\gamma = \exp [(r_1/r_2) \log (r_1/r_2)] - r_1/r_2$, $r_1 \simeq -[k_d(k_{-1} + k_d') + k_1 C k_d']/(k_1 C + k_d)$, $r_2 \simeq -(k_1 C + k_d)$, γ depends on C but even if k_d is as large as $10k_{-1}$, the variation is from 0.6 to 0.9 over the concentration range from 10^{-8} to 10^{-5} M in C.

Since $1/(CS)_m = 1/(S_0\gamma) + 1/(S_0\gamma K')\ 1/C$, where $K' = k_1/k_d$ a double reciprocal plot will be approximately linear. The error in S_0 will be small for the parameters occurring in the colchicine experiments but the apparent equilibrium constant would be k_1/k_d rather than k_1/k_{-1}.

To obtain a better value for the binding equilibrium constant we consider the progress curves for association and dissociation of the complex at a high colchicine concentration, so that $\gamma \simeq 1$. Since $r_2 \ll r_1$

$$(CS) \simeq (CS)_m/\gamma)[1 - \exp -(k_1 C + k_d)t]$$

for association and

$$(CS) = (CS)_0 \exp -(k_{-1} + k_d')t$$

or dissociation.

Plots for $\log [(CS)_m/\gamma - (CS)]$ versus t for association and $\log (CS)$ versus t for dissociation (after removal of free colchicine) should be linear and the ratio of the slopes is

$$\frac{k_1 C + k_d}{k_{-1} + k_d'}$$

Since k_d' is relatively small and C can be chosen such that $k_1 C \gg k_d$ then for a high colchicine concentration the value is approximately $k_1 C/k_{-1} = KC$.