Journal of Supramolecular Structure 2:466–485 (1974)

REGULATION OF SIZE AND BIREFRINGENCE OF THE IN VIVO MITOTIC APPARATUS

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Long chain glycols augment in size and birefringence the in vivo mitotic apparatus (MA) of marine eggs. Dinitrophenol and caffeine antagonize the effect but they can be balanced by glycols. Caffeine inhibits the phosphodiesterase for cyclic AMP (CAMP) and CAMP levels increase in its presence. However, added dibutyryl CAMP does not affect MAs or cleavage, but is taken up by eggs. Oxygen uptake studies show that caffeine depresses oxidative metabolism but does not affect ATP levels. Action through the pentosephosphate shunt is suggested.

Glycols influence the assembly of tubulin. Optical ultracentrifuge patterns of tubulin polymerized without glycol show a 6S and 30S peak. Similar patterns of tubulin polymerized at pH 6.4 in glycol and depolymerized in its absence show 6S, 8–18S, and 30S, peaks. The 8–18S peak appears in equilibrium with the 6S peak. If glycol is added to cold tubulin polymerized without glycol, only 6S and 30S peaks occur. Preparations with no 30S peak do not show 450 Å rings in the EM. Calcium depolymerizes microtubules. In the absence of glycols 450 Å rings are seen. In the presence of glycol, much higher concentrations of calcium are necessary for depolymerization, and few 450 Å rings occur.

We suggest that glycols prevent formation of the stable 30S peak, favor an intermediate structure in equilibrium with the 6S peak, and antagonize calcium depolymerization. Their in vivo effects may arise from these interactions.

INTRODUCTION

The replication of cells terminates in an event of startling rapidity and simple beauty. Sometime following the duplication of DNA, the chromosomes condense to form visible structures, the nuclear envelope disappears, and a birefringent structure, the mitotic apparatus (MA) (1) appears whose ultimate function is to guarantee the distribution of a proper set of chromosomes to daughter cells. The mechanism by which the mitotic apparatus accomplishes this task (the correlated movement of many chromosomes) is the subject of considerable discussion (see 2–5). Of equal interest is the question of how the cell assembles the MA, what controls its size, and what determines when and where the MA will form. The answers to these questions are not evident from current work but considerable progress has been made in areas where understanding is necessary before some of the more profound questions can be approached. The structure of the MA has been studied in many laboratories and it is clear that a major component of all MAs is the microtubule (6–10). All major theories of chromosome movement involve microtubules either as elements supporting structures which actually generate the motive force for

movement (11) or as elements whose assembly and disassembly directly moves chromosomes (12, 13). A theory of the latter type has been proposed by Inoué (12) on the basis of quantitative measurements of the birefringence of MAs of various marine eggs as a function of temperature. The data suggest that the birefringent material in the MA is constructed from a pool of subunits with which it is in equilibrium, the birefringence and size of the MA being a monotonic increasing function of temperature over a limited range. Qualitative (14) and quantitative (15) studies of microtubule orientation and number support the conclusion that oriented microtubules in the MA give rise to the major part of its birefringence. The quantitative data on birefringence vs temperature have been analyzed thermodynamically with a simple model which assumes that the polymerization of birefringent material (microtubules) in the cell is first order and that the depolymerization reaction is also first order (12). The assumption allows an equilibrium constant to be derived whose logarithm, when plotted against the inverse of the absolute temperature, yields a straight line the slope of which is proportional to the enthalpy of the polymerization reaction [from utilization of the van't Hoff relations (16)]. The entropy of the reaction is derived from the point at which the equilibrium constant is equal to 1 (equal amount of polymer and subunit) since at this point the free energy of the reaction is 0. The possibility that polymerization and depolymerization reactions may be other than first order was examined by Stephens (16) and rejected. Recent work on in vitro polymerization of brain tubulin suggests that microtubules tend to grow in a undirectional manner (17, 17a) and can add on to either homologous or heterologous pieces of microtubules. In addition, experiments with egg homogenates (18) show that microtubules in this system preferentially polymerize onto "organizing centers." In vivo polymerization is undoubtedly directed and oriented by special cytoplasmic structures which the cell can utilize to determine where and when microtubules will form.

The preceding discussion contains most of the ideas which will be used in the following report. The cell contains a pool of polymerizable microtubule protein (tubulin) which is polymerized in relation to cellular nucleating centers; this pool shows some form of equilibration with the microtubules of the MA and the amount of polymer actually formed depends under unperturbed conditions upon the environmental temperature. We shall discuss the effects of a number of agents on the in vivo MA which can augment the size and birefringence of that body or cause it to disappear in a characteristic manner; the results are most easily interpreted in terms of the ability of subunits to "cycle" through the MA. We will then discuss the effects of some of these agents on isolated tubulin and will interpret the in vivo effects based on the in vitro observations. This will allow greater insight into the problem of the regulation of the size and form of the in vivo MA and will throw some light on the nature of the subunit pool and the subunit-microtubule equilibrium.

OBSERVATIONS AND PRELIMINARY DISCUSSION

Augmenting Effects of Glycols on the Birefringence of the In Vivo MA

In the development of techniques for the isolation of the MA, Mazia et al. (19) introduced certain disulfide reagents in the belief that they might stabilize –S–S– bonds in the MA and so allow its direct isolation from living cells. It was shown that introduction of dithiodiglycol in dilute buffer allowed cells to be gently lysed in such a manner that the

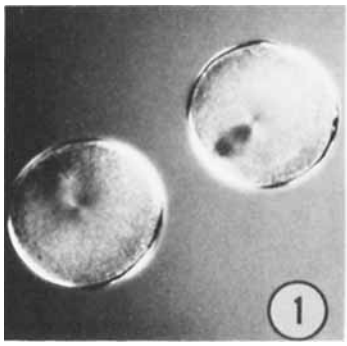


Fig. 1. Egg of the clam Spisula solidissima at late metaphase of the first meiotic division, observed in polarized light with compensator set for maximum extinction for MA in the egg at upper right (X 330).

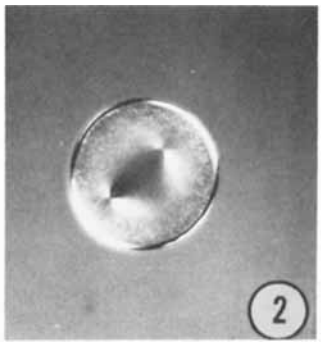


Fig. 2. Spisula egg from same batch as that in Fig. 1 treated with 0.1 M hexylene glycol (HG) in sea water for 5 min. Volume increase (excluding increase in aster size which is difficult to estimate) is four times. Retardation increase, measured with a Brace-Koehler compensator in the region of the MA immediately poleward of the chromosomes, is twice (X 330).

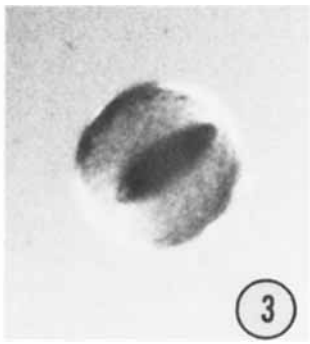


Fig. 3. Spisula egg from a different batch in 0.2 M HG in sea water for 6 min. Volume increase over untreated control is approximately ten times excluding aster. Retardation increase is two and one-half times. (X 350).

MA was liberated into the solution and could be subsequently obtained in relatively pure form by differential centrifugation. Subsequently, Kane (6) found that disulfide agents were not required for isolation but that substances such a 1, 6 hexanediol (derived from dithiodiglycol by substitution of carbons at positions 3 and 4 for the sulfers) or any six carbon glycol could be used equally as well, although the compositions of the resulting MAs when examined in the ultracentrifuge were different (4). In any event, it was felt that the function of the organic solvent was to stabilize the MA so that it could subsequently survive outside the cell. We investigated the in vivo "stabilization" of MAs by these agents and found that sulfur containing agents such as dithiodiglycol or dithiodipropanol caused a rapid disappearance of the in vivo MA when added to sea water, whereas agents such as hexanediol or hexylene glycol caused a rapid increase in both size and birefringence of the in vivo MA, an effect we termed augmentation (20). The original suggestion, therefore, that agents which can be successfully used to isolate MAs from living cells *must* stabilize the MAs was not supported and the observations provided a number of agents which could be utilized to modulate the size and appearance of the in vivo MA. We shall explore some of the details of the effects of augmenting agents on the in vivo MA of marine eggs.

Description. The basic phenomenon can be seen from Figs. 1–3 in which an egg of the surf clam, Spisula solidissima, has been placed in sea water to which 0.1 M dimethylhexanediol has been added. The egg was in metaphase of the first meiotic division prior to addition of the glycol. In the presence of the glycol, MAs enlarge both in width and in length and can attain a length up to twice that of the original MA, a width about twice the original, and a retardation measured with the polarization microscope of about double the original. Since the MA is a cylindrically symmetrical structure, this means that

MAs can increase approximately 8 times in volume. Further, since the retardation increases as well as the thickness, this is not a simple swelling of the MA but, because of the direct relationship of birefringence to microtubule number and orientation (15), it implies an increase in the number of microtubules in the augmented MA. Similar phenomena occur when eggs are immersed in D_2O in sea water and in that case counts of microtubules in the isolated augmented MAs directly verify the increase in microtubule number (12). We emphasize that the increase in MA volume involves an increase in *both* the length and the width of the MA so that any proposed mechanisms for augmentation must involve formation of new microtubules as well as increases in their length. The correlated change of length and width is general regarding most MA volume changes; it occurs in eggs in which the MA is observed during increase in environmental temperature although emphasis is usually placed on width changes as they relate to changes in retardation measured with the polarization microscope (12, 16).

Increase in glycol concentration to 0.2 M can further increase the volume of the MA and the length can increase to such an extent that the MA pushes the ends of the egg out into small projections. Volume increases up to 20-fold may occur with increases of retardation of 3 to 4 times. The augmentation effect starts within 1 min of application of the glycols and attains its maximum in 5 to 10 min depending upon the concentration of the glycol used. Elongation rates are about 1 to 2 microns per minute. There does not appear to be any specificity of the glycol with respect to where the hydroxyl groups are located on the molecule; 1, 6 hexanediol, 2, 5 hexanediol, hexylene glycol (HG) (5 methyl, 2, 4 pentanediol), and dimethyl hexanediol (2, 5 dimethyl-2, 5-hexanediol) all have approximately the same effect in terms of concentration. Glycols with smaller numbers of carbons are less effective; to obtain an augmenting effect with ethylene glycol equivalent to that of 0.1 M hexanediol requires about 1.5 M. The effect increases in potency as the chain length progresses through propane, butane, and pentane diols.

The effect is not restricted to one species of egg but has been found in eggs of three species of sea urchins, two species of annelids, one species of insect, and one species of clam and is, therefore, likely to be general throughout egg material. Sisken (21) has seen similar effects on in vivo MAs of cells in culture and in a study of secretion in the thyroid, which involves microtubules (22), it was shown that hexylene glycol and D_2O both affect secretion in a similar manner. It would appear, therefore, that glycol effects on microtubule systems are general.

The glycol effects are reversible so that if the glycol is removed the MA returns to its former size and the egg continues to cleave. Usually, however, the MA does not merely shrink but disappears (within 1 or 2 min) upon glycol removal and a new MA forms which is normal in size for the temperature of growth. Development of the egg can then proceed unabated and with no ill effects due to glycol treatment. If the glycol remains in the egg for too long a period of time, irreversible changes occur. Morphologically, the spindle shortens while the egg asters enlarge, the ultimate result being a large monaster in the center of the egg which may occupy 1/8 of its volume. If glycols are removed from eggs with such monasters, the monasters collapse but the eggs do not develop any further. Thus, the augmentation effect requires the continued presence of glycols (asters persist for up to 8 hr while the lifetime of the first meiotic MA of clams is about 20 min) and augmentation is reversible at any stage while developmental reversibility requires removal of glycols at relatively short intervals (20 to 30 min) after application.

The events in sea urchin eggs are similar to those in clam eggs in terms of increased potency of glycols with chain length up to six carbons, production of monasters, reversibility,

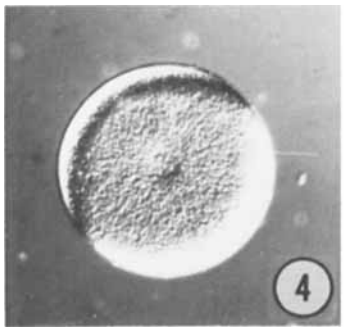


Fig. 4. Egg of the sea urchin Lytechinus pictus, 20 min after fertilization. Sperm aster radiates from nucleus. It is weakly birefringent and of retardation low enough to make measurements unreliable. Viewed with Nomarski differential interference optics (× 250).

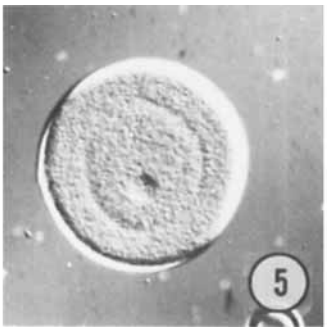


Fig. 5. Augmented sperm aster in Lytechinus egg treated with 0.2 M HG. In Nomarksi optics, the aster is visualized as a dense ring surrounding zygote nucleus (× 250).

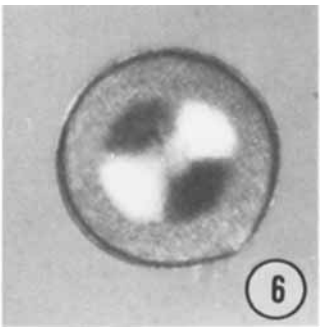


Fig. 6. Augmented aster in Lytechinus egg viewed with polarization optics. Retardation near the astral center varies from egg to egg but is at least three times that of retardation of first cleavage MA and is at least 50% greater in diameter than the interpolar length of the first cleavage MA (× 250).

etc., except that the augmentation rarely yields MAs more than 8 times the original volume. In sea urchins, however, we can examine the effects of glycols on the sperm aster, a body which forms soon after fertilization (23) and which appears to function in the movement of the male pronucleus toward the female pronucleus prior to syngamy (24). This body is normally a very weakly birefringent structure which, however, can be seen well with Nomarski differential interference optics in sufficiently transparent eggs such as those of the urchin Lytechinus pictus. Figure 4 shows a normal sperm aster in a Lytechinus egg and Fig. 5 shows an augmented aster. In the polarization microscope the birefringence of the augmented aster is about three times that of the first mitotic MA (measured near the center of the aster) and the augmented sperm aster occupies up to 1/8 the egg volume (Fig. 6). Such sperm asters can be isolated from eggs using usual isolation techniques (6) and electron micrographs show that they contain many microtubules.

Thus, glycols can induce large volume changes in MAs or asters which are already present. We may ask whether glycols can induce asters to form as can other classical cytaster inducing substances (25). We have applied glycols to unfertilized eggs and to fertilized eggs before aster or MA formation or after MA disappearance and have seen no evidence that glycols cause microtubule formation in the absence of an activated center. Augmentation always occurs subsequent to appearance of a center of formation of microtubules.

Augmentation and Protein Synthesis. Given the large increase in oriented microtubules induced by glycols, one may ask whether new protein must be synthesized by the cell either to supply the extra protein or supply a "catalytic" protein (26) required for such massive assembly. While this appears unlikely, reports have appeared which suggest that D_2O effects require a prior protein synthesis (27) and there is in fact synthesis of some tubulin prior to first cleavage (28). Further, Puromycin inhibits cleavage in sea urchin eggs (29), halting progress at the streak stage (30) just prior to nuclear envelope break-

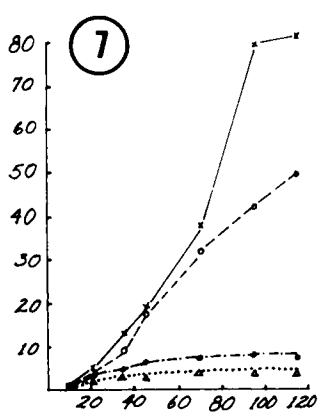


Fig. 7. Incorporation of C^{14} -valine into eggs of the sea urchin Arbacia punctulata. HG was added at 15 min after fertilization. Abscissa represents time (in min) after fertilization, ordinate represents CPM/mg protein $\times 10^{-3}$. (X——X) control; (O——O) 3×10^{-4} M. Puromycin (complete inhibition of cleavage occurs at 10^{-4} M Puromycin); (•—•—•) HG; and (△· · ·△) Puromycin plus HG. 1 μCi/ml isotope was added to sea water. Eggs were removed from sea water at the indicated times, sedimented with a hand centrifuge, washed twice with sea water containing excess cold valine, and dissolved in 1 N NaOH. Proteins were precipitated by addition of equal volumes of 35% ice cold TCA. The precipitate was gathered by centrifugation at 10,000 rpm for 10 min, redissolved in 1 N NaOH, and counted in a scintillation cocktail (33). First cleavage was at 50 min after fertilization.

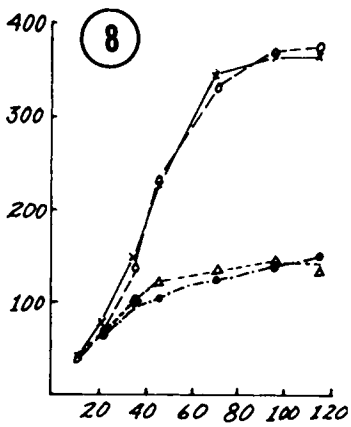


Fig. 8. Conditions are similar to those in Fig. 7 except that an aliquot of the initial NaOH digest was used for total uptake of valine. Abscissa and ordinate are as in Fig. 7.

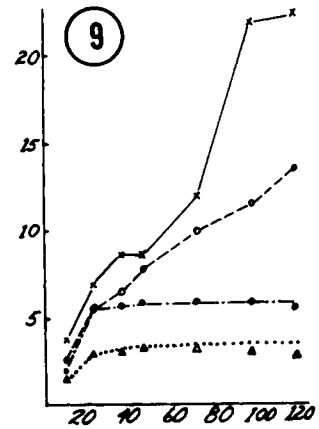


Fig. 9. The data from Figs. 7 and 8 were treated as described by Berg (34) to assess the contribution of amino acid uptake on incorporation data. The ordinate represents % incorporation of TCA precipitable protein compared to total amount of amino acid in the egg at a given time.

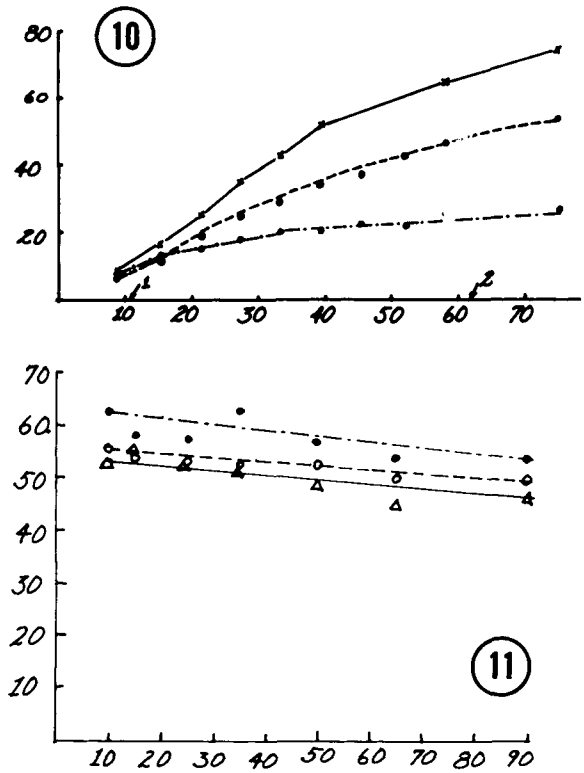


Fig. 10. A prelabeling experiment using C^{14} -valine with Arbacia punctuata eggs. Eggs were incubated for 10 min in 0.25 μCi/ml and were then washed free of excess isotope with four high volume washes with sea water (32). They were then fertilized and handled as described in the legend of Fig. 7. At 12 min (arrow 1) HG was added. Cleavage occurred at 62 min (arrow 2) on the abscissa.

Fig. 11. Total isotope from a prelabeling experiment. The slight decrease in isotope with time is due to a small amount of valine which appears to enter general metabolism and is lost as CO_2 in these eggs (34). The data indicate that the decreased incorporation seen in HG-treated eggs cannot be due to leakage of C^{14}-valine from the egg but is due to a true decrease in incorporation rate. Abscissa, ordinate, and incorporation curves coded as in Fig. 7.

down. Puromycin, however, does not inhibit sperm aster formation at high doses even if eggs are treated with the drug for several hours prior to fertilization (Fig. 6). Hexylene glycol still induces massive augmentation in the presence of Puromycin, suggesting that protein synthesis is not necessary for augmentation. However, since the glycol could act through inhibition of the effects of Puromycin on protein synthesis it was necessary to investigate the effects of these agents on protein synthesis directly.

For this purpose we performed two kinds of incorporation experiments: continuous labeling experiments (31) and prelabeling experiments (32). In each case, eggs were incubated in sea water to which C^{14} valine was added. In the case of continuous labeling experiments eggs were fertilized and then centrifuged out of sea water at proper times after fertilization, were washed with an excess of cold valine, dissolved in 1 N NaOH, and the proteins precipitated with cold 35% TCA (trichloroacetic acid) (33). An aliquot of

supernatant was removed and both supernate and NaOH redissolved TCA pellet were counted in a scintillation counter. Figures 7, 8, and 9 show the results on both incorporation of label into TCA precipitable protein and on uptake into the cell. Puromycin depresses incorporation (29) but not uptake of C^{14} valine, but hexylene glycol itself reduces uptake and suppresses incorporation. In the presence of both agents incorporation can be less than 10% of controls. In prelabeling experiments similar results can be seen (Fig. 10). In such experiments uptake suppression by glycol is not a factor since the cell uses a pool of C^{14} valine already present. Since, however, it is possible that HG not only causes inhibition of in vivo amino acid uptake into eggs, but causes loss of amino acid from the eggs, we looked at total label (TCA precipitate plus supernatant radioactive valine) and Fig. 11 shows that there are no significant differences in HG-treated eggs compared to controls or Puromycin-treated eggs, although all slowly decrease with time (34). Since some conversion of valine to other products occurs during development (32), the decreased incorporation into TCAprecipitable protein could be a reflection of the differential conversion of valine to non-amino acids by HG. This was shown not to be so by chromatographing the TCA supernate on paper. No differences between control, HG-, and Puromycin-treated eggs were seen.

The results so far strongly indicate that protein synthesis is not necessary for the glycol augmentation results. Final potential criticism can be eliminated by utilization of autoradiography. Thus, one might argue that HG, while inhibiting protein synthesis in general, nevertheless stimulates synthesis of tubulin analogous to situations in which actinomycin D stimulates synthesis of some proteins (35). If so, augmented asters should have considerably more radioactivity than the rest of the cell since HG would not only stimulate tubulin synthesis but segregate it into the aster. Eggs in Puromycin and HG were fixed and prepared for autoradiography. As can be seen in Figs. 12 and 13, no increase in grains over the augmented aster occurs although there is some tendency of grains to line up (36).

The totality of observations strongly suggests that glycols promote augmentation by mobilizing preexisting tubulin pools to polymerize in relation to activated nucleating centers. Since the MA may attain volumes many times those normally occurring under natural conditions, it appears that glycols tap a pool of tubulin for polymerization not normally available for this purpose. The independence of MA formation from protein synthesis was also inferred by Rinaldi (36).

Dispersing Effects of Colchicine, Dinitrophenol (DNP), and Methyl Xanthines (MX) [Caffeine, Aminophylline, Methylisobutylxanthine (MIX)]

In vivo, MAs will disappear when eggs are treated with the agents mentioned above (37). Phenomenologically, all the agents act in a similar manner although mechanistically they are unlikely to inhibit by the same means. Application of any of the agents to eggs possessing a MA results in a diminution of the MA so that length, width, and retardation decrease. Thus, the MA shrinks while maintaining approximately the same shape: that is, decrease in volume is a process involving shortening of microtubules *and* decrease in their number. However, with colchicine the MA always eventually disappears. The lower the dose of the alkaloid, the longer the time required for disappearance. With either DNP or MX, doses can be found at which a smaller MA is formed but does not disappear. Cleavage is slower but may eventually occur. At sufficiently high doses (5×10^{-4} M DNP, 1 mM MIX or 10 mM caffeine) complete disappearance of the MA occurs.

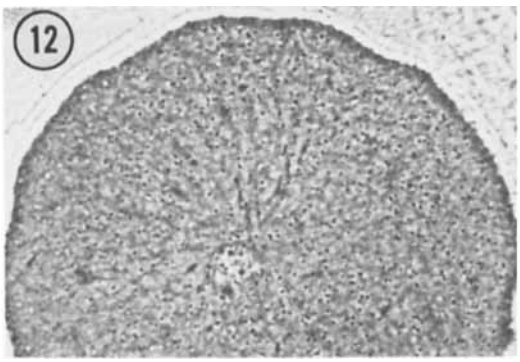


Fig. 12. Radioautograph of an egg of Lytechinus pictus. Eggs were fertilized and then incubated in C^{14}-valine as described in Fig. 7. 0.2 M HG was added to an aliquot of eggs after the sperm aster had formed and augmentation was then allowed to proceed. Eggs were fixed in 2% glutaraldehyde, briefly treated with dilute alkali to remove non-specifically bound isotope, and then embedded in Araldite. Two-micron sections were obtained and radioautographs made by standard means using liquid emulsions. Micrographs are made through a phase microscope. Grains are in dark contrast.

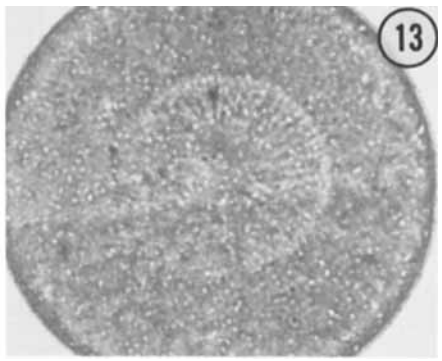


Fig. 13. Eggs from HG-treated aliquots were handled as described in Fig. 12. Grains appear in light contrast (out of focus image) since the microscope was focused on the section itself. Orientation of the grains within the augmented aster is clear.

The morphological effects of the inhibitors can be understood in terms of an Inoué (12) equilibrium model in which subunits are incorporated into the polymerized MA microtubules and can return to the pool. If these units are prevented from entering the microtubules but subunits can still return to the pool from the MA microtubules, the effect should be to proportionately decrease the MA as is actually observed. Thus, the effects of the inhibitors do not appear to be on the organized MA but on the building blocks from which it is constructed. In the case of colchicine the interaction with the MA undoubtedly is based on the known binding of the alkaloid to tubulin and the consequent inhibition of microtubule polymerization (17a). In the equilibrium model this should gradually tie up subunits as they leave the MA so that the structure eventually disappears since colchicine binding is tight. The other inhibitors appear to act differently. First, they do not directly inhibit polymerization of microtubules from brain (see below) and thus must act indirectly on microtubules. In the case of DNP, an uncoupler of oxidation phosphorylation in eggs (38) as well as other tissues, ATP levels in eggs have been shown to decrease (39) and the effect could involve ATP or potentially some other product of mitochondrial metabolism such as calcium (40) or hydrogen ion. With methyl xanthines the effect is unlikely to be through the mitochondrial system as we shall now discuss.

Methyl Xanthines and Egg Metabolism. Since methyl xanthines are inhibitors of the phosphodiesterase for cyclic AMP in higher organisms (41) and since CAMP is an inhibitor of cell division in many cells in culture, it was of interest to examine this system in sea urchin eggs. It was shown that both cyclic AMP and phosphodiesterases for CAMP do

occur in these eggs (42). Further, methyl xanthines do inhibit the phosphodiesterases and do cause an increase in the intracellular level of CAMP [see also (43)]. However, exogenously added CAMP or its dibutyryl derivative (which is often used in place of CAMP) has no effect on cell division to late larval stages at concentrations five orders of magnitude greater than those occurring in the egg (31). Uptake experiments show that the compounds enter the egg and chromatographic identification of the intracellular products show that CAMP or N^6-monobutyrylCAMP (the active form to which the dibutyryl compound is converted by an intracellular acylase) can attain levels three orders of magnitude greater than is present in the egg to begin with (44). These results strongly suggest that methyl xanthines do not inhibit cell division through activities involving the CAMP system.

In pursuing this problem we reinvestigated an observation due to Cheney (45), who showed that inhibition of cleavage in sea urchin eggs by methyl xanthines was proportional to inhibition of oxygen uptake. Complete inhibition of cleavage occurred at about 50% depression of oxygen uptake [similar depression of O_2 uptake and ATP content to about 50% of normal is associated with cleavage inhibition by carbon monoxide (46)]. Eggs of the sea urchin Strongylocentrotus purpuratus were fertilized and their O_2 uptake studied with a Warburg apparatus in the presence and absence of caffeine and MIX. With both methyl xanthines inhibition of O_2 uptake was obtained and, as reported by Cheney (45), complete inhibition of cleavage was found at approximately 50% of O_2 uptake control cultures. This suggested to us that the methyl xanthines worked through oxidative phosphorylation in a manner similar to that of DNP. Measurements of ATP levels were then undertaken and to our surprise *no* change in ATP level was found although in control cells inhibited by sodium azide [which acts in a manner similar to that of DNP (37)] approximately 50% of the ATP was lost. This result was obtained many times before we accepted it.

This clearly suggested that we look to some other process for understanding the effects of methyl xanthines and since eggs possess a very active pentose phosphate shunt which can account for half the O_2 uptake (47), we are investigating enzymes in this pathway and also the enzyme NAD-kinase which is activated within seconds of fertilization and induces a massive formation of NADPH from NADH (48). Interestingly enough Dikstein (49) has suggested that redox potentials in cells, as reflected in the ratio of reduced to oxidized pyridine nucleotide, may regulate intracellular transport ATP-ases and particularly calcium transport. He has shown the agents which "discharge" (i.e., oxidize) reduced pyridine nucleotides can cause contraction in protozoa (50) which are known to require calcium for contractility. One such agent, phenazine methosulfate, which transfers H^+ from reduced pyridine nucleotides to O_2, does inhibit cleavage in marine eggs in a manner similar to that of methyl xanthines.

Another potential site of action of methyl xanthines is a calcium uptake system which has been described in sea urchin eggs by Kinoshita (51). If this system responds to methyl xanthines in a manner similar to that of muscle, namely by release of calcium (52), we again are brought to the possibility that calcium is involved in the inhibitory action of methyl xanthines on MAs. Since calcium causes a depolymerization of microtubules both as individual tubules in suspension (53) and when built into the isolated mitotic apparatus (54) this possibility is suggestive.

In Vivo Interaction of Glycols with Colchicine, DNP, and MX

Since glycols appear to act by shifting the equilibrium in the MA toward the poly-

merized state, whereas the other agents act (although possibly indirectly) in the reverse manner, it was of interest to examine the interaction of augmenting agents and dispersing agents on the MA. In the first set of experiments glycols were utilized to increase the size of the MA in clam eggs. After maximum augmentation, eggs were perfused with colchicine plus glycol (at the same concentration utilized for augmentation). In all cases, the augmented MA or central monaster (if the augmentation has been allowed to proceed to monaster stages) disappeared within 10 to 15 min. Even at suboptimal concentrations colchicine eventually caused complete disappearance of the birefringent structure. On an equilibrium model these results suggest that colchincine binds to subunits as they cycle through the MA preventing their reincorporation into the MA even in the presence of glycol. What happens, however, if we reverse the order? In other words, what happens if we allow clam eggs to develop an MA, cause it to disappear with colchicine, and then add colchicine together with an augmenting dose of glycol? To our surprise, we found than an MA formed which was normal in size or slightly augmented but that it then disappeared within 5 to 10 min. After this, glycols had no further effect if added with colchicine. It would appear, therefore, that glycols can release a pool of tubulin which is not normally available for polymerization or colchicine binding and that these subunits can assemble into microtubules onto centers in the egg. It is already known from work by Weisenberg (55) in clam material that microtubule organizing centers form in the presence of colchicine. Thus, the tubulin pool unveiled by glycols becomes available to colchicine binding only after it "cycles" through the MA. Once this pool is used up no further "hidden" tubulin pool is revealed by glycols.

The effects of DNP and MX differ from that of colchicine in that augmented MAs may be balanced with combinations of augmenting agent and dispersing agent, an effect especially seen in monasters. At earlier stages in augmentation the balance of augmentation and dispension may lead to appearance and disappearance of MA, i.e., a MA cycle occurs although egg cleavage does not always occur.

Effects of Glycol and pH on Polymerization of Brain Tubulin

The effects we have described of MA regulation in living cells cannot be interpreted without work on isolated tubulin since we have no way to judge whether the regulators act directly or indirectly on MA material. Unfortunately, it is not possible yet to polymerize microtubules from supernatants of clam or sea urchin eggs in the same manner as is possible in brain supernates. Weisenberg (18) has succeeded in polymerizing microtubules onto "organizing centers" obtained from eggs, but this seasonal material was not available at the time these experiments were done and would not be suitable to answer questions discussed below. Thus, there is a gap in the logic since we will be asking questions of brain microtubules and applying the answers to egg MAs. A partial bridge between these systems was constructed with results obtained last summer. We were interested in the interaction of brain tubulin with clam isolated MA to see if this heterologous material would polymerize in situ. As material we utilized a new method of isolating MAs developed by Smith (56) which yielded spindles whose microtubules were cold labile and which were stable at room temperature – this is in contrast to MAs isolated with organic solvents which are stable in the cold but not at room temperature. When such MAs are held at 0 to 4°C for 10 to 15 min, their birefringence is reduced to 1/3 to 1/4 of that of controls and almost all microtubules are lost when they are examined with the electron microscope (56). Incubation of these MA remnants with chick brain tubulin in polymerization mixtures caused a re-

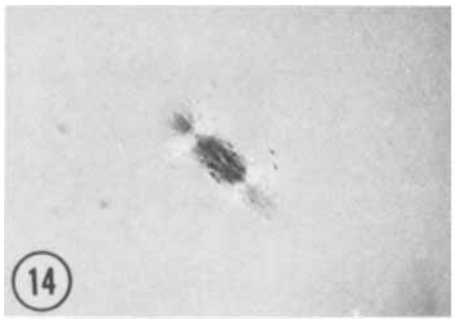


Fig. 14. Normal MA isolated from eggs of *Spisula* at metaphase of the first meiotic division. Isolation medium is modified from Weisenberg's polymerization medium (57) as worked out in detail by Smith and coworkers (54, 56) (× 500).

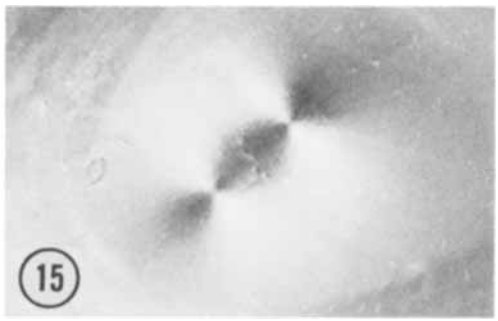


Fig. 15. MA reconstructed in vitro from a *Spisula* MA. MAs were isolated and depleted of their native microtubules by cold depolymerization. They were then incubated with chick brain tubulin under polymerization conditions. Chick tubulin incorporates into the MA remnant which grows slowly in length and width and may attain lengths up to three times the original (54). In this case a volume increase of 3.5 times has occurred (× 500).

appearance of the birefringence in situ and caused growth of the MAs so that they attained lengths up to three times normal (Figs. 14 and 15). Thus, brain tubulin can replace native clam tubulin as far as birefringence is concerned and can cause a further increase in growth reminiscent of in vivo augmentation. This suggests that studies of the effects of our regulatory agents on brain tubulin should be relevant to understanding their in vivo effects on the MA.

Brain tubulin polymerization. Tubulin was prepared from rabbit brains within 1 hr of sacrifice of the animal. The chilled brain was removed, rapidly cut into slices, and homogenized in a volume (in milliliters) of ice cold buffer equal to one and one-half times the weight of the brain in grams. Homogenization was accomplished with a Teflon pestle homogenizer driven at moderate speed for about 1 min. The homogenate was centrifuged for one-half hour at 50,000 × g at 2°C and the supernatant was then removed and polymerized at 37°C for 10 to 30 min. Microtubules were harvested at room temperature by centrifugation at 100,000 × g for 20 min, the pellet washed thoroughly with buffer, and then redissolved in the cold with homogenization. The aggregated material was removed by centrifugation at 100,000 × g for 20 min. A second cycle of polymerization was sometimes used in further purification of the material. No differences were seen in ultracentrifuge patterns of preparations polymerized once compared to those polymerized twice. The homogenization buffer consisted of 0.1 M PIPES buffer, 1 mM EGTA, and 1 mM $MgCl_2$. One mM GTP was added immediately prior to polymerization and, occasionally, to the homogenization buffer. Several other buffers were utilized, as will be detailed elsewhere. Our results are independent of buffer type. Other preparations were made using glycol as described by Shelanski et al. (58). In the presence of glycerol the yield of microtubules is greatly increased and, indeed, polymerization can take place in the absence of GTP. We shall discuss the influence of pH and glycerol on the subunits present in depolymerized microtubule preparations.

As discussed in detail by Sander and Kirschner (59) and by Kirschner and Williams (59a),

solutions of depolymerized microtubules contain a 6S and a 36S unit when examined in the optical ultracentrifuge as long as the microtubules were not polymerized in glycerol or, if they were, as long as glycerol is thoroughly removed prior to depolymerization. In our own work, we have found that preparations which have been polymerized without glycerol at pH 6.4–6.5 (Fig. 16) contain a 6S and a 30S peak. At pH 6.9 we obtain preparations in which (Fig. 16) the 6S and 30S peaks are present but in addition, a peak of about 8–18S occurs. If brain tubulin is polymerized at pH 6.9 in the presence of glycerol and the microtubules spun out and resuspended in cold buffer so that the final concentration of glycerol is about 0.5 M, the depolymerized tubulin rarely has any trace of the 30S component.

From these results so far, it would appear that a 30S subunit is not necessary for polymerization (all of the samples run in the ultracentrifuge were tested for their ability to form microtubules) but a component with a sedimentation coefficient of about 8–18S exists which can substitute. In an occasional preparation which will not polymerize, no fast peak (faster than 6S) has been seen, so that some fast sedimenting material appears to be necessary for polymerization to occur.

Similar effects of glycerol in eliciting a 8–18S sedimenting material occur at pH 6.4 as at pH 6.9 (Fig. 17). Tubulin was polymerized at pH 6.4 in the presence of glycerol, the microtubules were spun out, and the material depolymerized in buffer with about 0.5 M glycerol remaining. Three peaks are seen, 6S, an intermediate peak of about 8–18S, and a faster sedimenting peak of about 30S. Thus, glycerol favors the formation of an intermediate peak even at a pH in which this peak does not normally occur. Glycerol does not have to be present during polymerization for this peak to be seen. In Fig. 18 tubulin is examined which was polymerized at pH 6.4 in the absence of glycerol but to which glycerol was added prior to pelleting. The pellet was depolymerized in buffer (which still contained about 0.5 M glycerol). The ultracentrifuge pattern is similar to that in which glycerol is present throughout polymerization, Thus, glycerol favors the occurrence of a peak intermediate to the 6S and 30S peaks, as does a pH of 6.9 compared to one of 6.4. A number of other experiments in which tubulin is shifted from low to high pH, in which glycerol is added at various stages or removed by rapid dialysis in an Amicon filtration system, strongly suggest that the 30S peak and the 8–18S peak can be interconverted and can be present in the same preparation.

Electron microscope studies have failed to reveal a 450 Å ring structure such as reported by other investigators (17a, 59a, 59b, 59c) in any preparation which does not contain 30S material. Thus, preparations with only 8–18S material do not contain 450 Å rings although such preparations are competent to polymerize.

These results may be interpreted in terms of several of the observations reported in this issue. Weisenberg (59b) has demonstrated that during polymerization of microtubules, as occurs in the optical ultracentrifuge during warming, a peak of lower sedimentation velocity forms which may be a species of polymerization intermediate. In addition, Kirschner and Williams (59a) have reported that rings can form fibers in the presence of GTP and colchicine, the alkaloid preventing polymerization of fully formed microtubules. Thus, at least one pathway to polymerization of microtubules may involve the formation of some form of intermediate from 30S material and this material may possibly be stabilized, at least in the presence of colchicine. We suggest that glycols represent an alternate mode of reaching the same state; that is, they shift the 30S material to an intermediate form which is the more direct precursor of polymerized microtubules.

A further potential effect of glycols in vivo relates to their ability to protect microtubules from depolymerizing effects of calcium. We have found that disappearance of

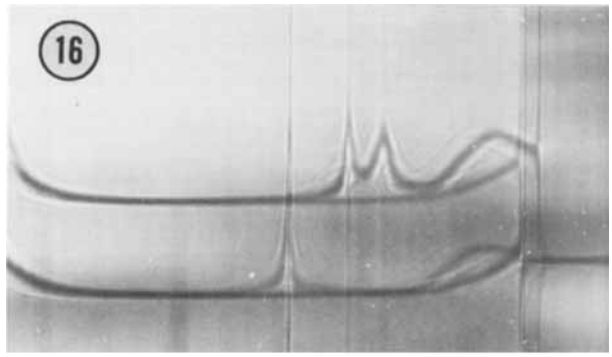
microtubules in 1 M glycerol requires approximately 15 mM of calcium compared to about 0.5 mM in the absence of glycerol. Microtubules in 0.1 M dimethylhexanediol required approximately 10 times as much calcium to cause depolymerization than in its absence. In addition, few rings were seen with the electron microscope in preparations depolymerized with calcium in the presence of glycols compared to those depolymerized in the absence of glycols.

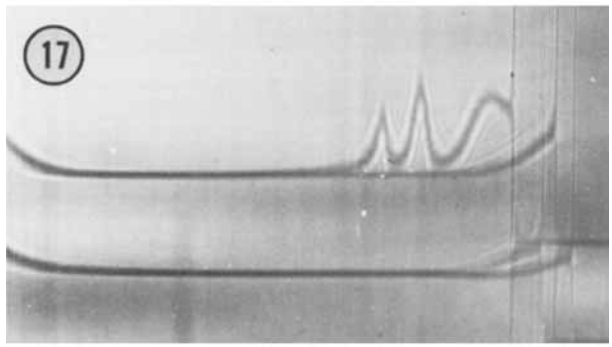
The effects of glycerol on microtubules appears to be threefold: it shifts the 30S ring structure to another slower sedimenting form which may be an intermediate in polymerization closer to the microtubules; it increases the level of calcium needed to depolymerize microtubules; it reduces the number of 450 Å rings forming during depolymerization (with either cold or calcium). We will attempt to interpret these results in terms of our in vivo observations on augmentation in the Discussion.

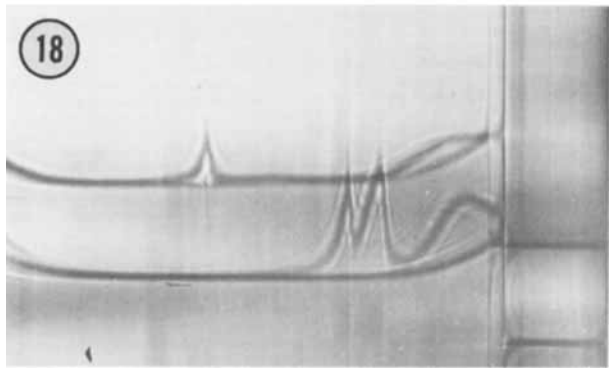
DISCUSSION

In the introduction to this article we briefly developed some of the evidence that the microtubules of the MA are in some form of equilibrium with a pool of subunits available for polymerization, that temperature determines the proportion of pool in polymerized form, and that assembly onto potential nucleating centers could be the basis for first order kinetics of in vivo assembly. While argument could be raised with any of these points, detailed observations on both augmentation and dispersion phenomena and especially the antagonisms between augmenting and dispersing agents find their simplest interpretation if polymerizable subunits can enter and leave the MA, i.e., if there is a process of cycling of subunits through the MA. Inoué and Sato's model (12) couples such a cycling process to the generation of motive force for chromosome movement by allowing a shift in the equilibrium toward disassembly in the chromosome-pole region during anaphase. However, the cycling process itself is not necessarily tied to any mechanism of chromosome movement nor is it incompatible with any model. The idea of a pool of polymerizable subunits does, however, require further discussion because of the possibility that several pools exist. We will now turn to observations of Stephens (16) for some insight into this problem.

Stephens utilized eggs of the sea urchin Strongylocentrotus drobachiensis which have the unusual property of being able to cleave at temperatures considerably below those necessary for most other cells to divide. In his experiments, eggs were grown at 1°C or at 8°C until metaphase of the first mitotic division and were then rapidly shifted to temperatures between 1° and 20°C where the retardation of the MA was measured after it had stabilized. For cells grown at 1°C and transferred at metaphase, a maximum birefringence of 1.81 nm was obtained at all temperatures between 12°C and 20°C, i.e., no increase in retardation occurred above 12°C. Similar experiments with cells grown at 8°C and then transferred also showed a maximum retardation at 12°C and above, but the maximum, 2.78 mM, was 50% greater than for cells grown at 1°C. Thus, the temperature at which the cells were grown appeared to determine the pool of subunits available for polymerization when the temperature was shifted at metaphase. If the shift in temperature took place prior to preprophase, however, then the MAs formed were characteristic of the temperature to which the cells were shifted, not of that at which the cells had been grown (60). These results suggested that some process could set the size of the available subunit pool and that the size was fixed as far as temperature effects were concerned at some time prior to prophase. D_2O was used to further specify the pool potentialities. In its presence the birefringence attained the maximum possible for cells grown at the







specified temperature, that is, 1° or 8°C. In other words, D_2O caused a mobilization of all those subunits which were available in the pool set by the temperature at which the cells were grown prior to shift in temperature at metaphase. Finally, D_2O applied in a different way [for details, see Stephens (16)] caused mobilization of a pool of tubulin not previously available to cells grown at 1°C. To summarize, S. drobachiensis eggs possess a total pool of tubulin which can be caused to enter the MA by growth of the eggs at a high enough temperature or by utilization of D_2O in a certain way if the eggs are grown at a lower temperature. Growth at 1°C mobilizes only part of the potentially available pool. It is clear from this work that some facilitation process occurs in the eggs which allows part of the potentially polymerizable subunit pool to become actualized and that manipulations with D_2O can change these relationships. The size of the pools at 1°C

Fig. 16. Brain tubulin was obtained as described by Weisenberg (57) with PIPES buffer instead of MES buffer (53). It was polymerized at pH 6.4 or at pH 6.9; microtubules were harvested at room temperature, resuspended in buffer of the proper pH, and depolymerized in the cold. The solution was then clarified by centrifugation at 75,000 × g × 30 min and examined at 4°C in a Beckman model E optical ultracentrifuge at 60,000 rpm at a concentration of about 12 mg/ml. At pH 6.4 we obtained two peaks, one at 6S and the sharp peak at 30S (corrected for temperature and concentration) (lower trace). The upper trace is from sample polymerized and depolymerized at pH 6.9. An intermediate peak of sedimentation coefficient 8–20S appears. All samples were tested for polymerization.

Fig. 17. Brain tubulin was obtained at pH 6.4 as described in Fig. 16. The material was polymerized after addition of glycerol to a final concentration of 4 M (58). Microtubules were harvested at room temperature and resuspended in pH 6.4 buffer. The pellet of microtubules polymerized in glycol still contained about 0.5 M glycerol. Microtubules were depolymerized in the cold and examined as described in Fig. 16. The upper trace has an intermediate peak sedimenting at about 20S.

Fig. 18. Brain tubulin was polymerized as described in Figs. 16 and 17. After polymerization, one-half the sample was made 4 M in glycerol and both samples were harvested at room temperature. Buffer at pH 6.4 (without glycerol) was added to *both* pellets and they were depolymerized at 0°C for 1 hr. After a clarification spin (see Fig. 16) the solutions were analyzed as in Figs. 16 and 17. The patterns observed in tubulin solutions at pH 6.4 depolymerized after treatment with glycerol (lower trace) resemble those in which microtubules polymerized in the presence of glycerol (Fig. 17). In both cases, about 0.5 M glycerol was present during depolymerization. The fast peak in the upper trace is probably the same as the 30S peak in samples polymerized without glycerol. In experiments in which glycerol is added at about 0.5 M to depolymerized tubulin solutions at 0°C at pH 6.4 and compared to those without glycerol, about the same decrease in sedimentation velocity as appears here is observed, but *no* intermediate peak is seen.

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and 8°C is not determined by protein synthesis; the requisite tubulin is already present before fertilization.

In Stephens' work a cell was utilized which apparently can mobilize all available tubulin simply by being grown at a suitably elevated temperature. For most other egg materials which have been studied this is not the case since the eggs usually die before a temperature is reached at which the MA retardation is comparable to that obtained in the presence of D_2O or glycols. Augmenting agents could work by shifting the equilibrium toward polymerization and thus utilizing more of a large pool, all of which is competent to polymerize. That more is involved is indicated by the experiment described in the section "In Vivo Interaction of Glycols with Colchicine, DNP, and MX." The observations on

application of glycols subsequent to the action of colchicine indicate that a pool of tubulin exists which is not available for colchicine binding but which becomes available after mobilization into the MA by glycols. The nature of this mobilization process is not clear, but we may speculate using some of the results obtained with purified brain tubulin. Sander and Kirschner (59) and Kirschner and Williams (59à) have reported that the 36S subunit binds little if any colchicine compared to the 6S unit. However, in the presence of GTP and colchicine the 36S unit appears to open and form a fibrous aggregate which is probably related to the 8–18S intermediate which we see at pH 6.9 or in the presence of glycerol. These observations suggest the following interpretation. The egg contains a pool of tubulin which may be in a form similar to that of the 36S ring, if it is not the ring itself. This material will not spontaneously polymerize in the egg environment and is not available for colchicine binding. A portion of this pool is mobilized by a temperature dependent process and becomes available for colchicine binding and for polymerization, but such polymerization does not occur spontaneously, only in relation to activated centers. This is indicated by our work in which augmentation occurs only in relation to asters or spindles and by Weisenberg's observations that egg tubulin can polymerize in homogenates, but only when activated centers are present (18). Glycols can activate the pool not mobilized by temperature and after activation, this tubulin can participate in MA formation. Once this material has participated in the MA it is cycled through that structure and becomes available for colchicine binding and, in the presence of the alkaloid, is prevented from entering the MA again.

The potential function of calcium in this system is to regulate the amount of tubulin in the polymerization competent pool which actually cycles through the MA. At high calcium levels more tubulin is inactivated (possibly in a reversible equilibrium reaction) and the MA shrinks. The action of methyl xanthines as discussed above is potentially at this level of the process. A model which incorporates these ideas, stresses the observation that augmentation requires activated centers for its occurrence, and stresses Weisenberg's incisive experiments which show that egg tubulin in egg homogenates only polymerizes in relation to centers is given in Fig. 19. In this model glycols act by mobilizing previously unavailable tubulin and by decreasing the interaction of calcium and tubulin, thus adding an additional pool of tubulin for MA augmentation. Methyl xanthines increase the concentration of free calcium which may also be the site of action of oxidative phosphorylation uncouplers, although the latter could also be involved in modification of the pH or availability of ATP and GTP. An additional possible site of action of D_2O and glycols may involve inhibition of release of calcium from cellular binding sites. The inhibition of contraction of striated muscle by D_2O was shown to involve just this mechanism by Kaminer and Kimura (61).

The MA offers the opportunity to study an important transient organelle in the cell on a number of different levels of organization. Microtubules can be polymerized in vitro from subunits and the mechanism of their assembly studied. While this has not yet been accomplished in systems other than brain it seems difficult to believe that this situation will continue. At a slightly higher level of organization tubulin from clam eggs can polymerize in relation to mitotic centers (18) so that the problem of organization of nucleating centers is available for study, if only in this limited but important system. Isolated MAs can now be studied in bulk although they have not been seen to function in vitro. This has, however, been accomplished in a partially lysed cell system in tissue culture (62) and it seems reasonable to believe that application of similar methods will succeed for isolated MAs.

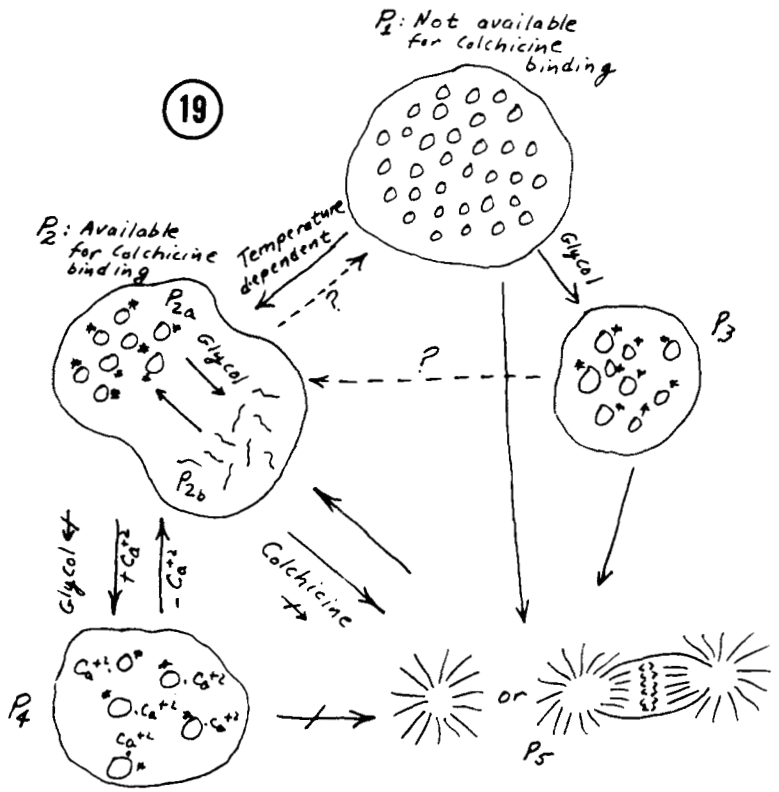

Fig. 19. A summary of many relations discussed in the text. P_1 is the pool of tubulin not available to colchicine binding. Some of this pool is made available for entry into the MA by temperature as indicated by the work of Stephens (60). Pool P_2 cycles through the MA but may exist as two subpools, P_{2a} and P_{2b}, the latter more directly a precursor of microtubules in the MA. Temperature may affect this equilibrium and glycols may favor P_{2b}. Glycols also shift P_1 into a pool P_3 (which may be the same as P_2 as indicated by the broken arrow) or may feed directly into the MA. Ca^{2+} is assumed to bind to a proportion of P_2, probably in an equilibrium reaction forming P_4. The complex is then incapable of polymerization. Glycol may shift this equilibrium toward P_{2b}. P_5 is the pool of polymerized microtubules in the MA (or aster). Spontaneous polymerization in the cell is assumed to be inhibited in the absence of activated centers.

At a higher level of organization one can study eggs in which a MA is formed but which only proceeds to metaphase of the first meiotic division and can remain quiescent in this stage for 8 to 10 hours. Such in vivo MAs can be modulated in size by various metabolic inhibitors or glycols (20, 37) and represent naturally arrested nondeveloping cells in which in vivo assembly and disassembly can be studied repetitively in the absence of protein synthesis. These eggs can then be started on the path to development by parthenogenetic activation or fertilization. Of course, many other cells are available for study of the mitotic process. We are, therefore, at the stage at which molecular biology and cell biology can cross-react in the study of the mitotic apparatus, a central structure in the life history of all eukaryotic cells.

ACKNOWLEDGMENT

This work has been supported by grants from the National Institutes of Health and the National Science Foundation. A considerable part of the observations were made at the Marine Biological Laboratory, Woods Hole, Massachusetts.

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