THE MITOTIC APPARATUS

Physical-Chemical Factors Controlling Stability

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ABSTRACT

Previous investigations have shown that the mitotic apparatus (MA) can be isolated from dividing sea urchin eggs in water buffered at pH 5.6 and that the addition of 1 M hexanediol to the solution raises the usable pH to 6.4. Long chain glycols appeared to be much more effective than related compounds in increasing the stability of the MA, and the aim of the investigations reported here was to determine the basis of this specificity. These experiments show that this impression of specificity is misleading and that under suitable experimental conditions a variety of compounds can be substituted for the glycols. A number of alcohols will duplicate the action of the glycols in stabilizing the MA at pH 6.4, but they must be used at a similar per cent concentration rather than at a similar molar concentration. Increases in the concentration of alcohol or glycol allow isolation at more alkaline pH values, and a pH–concentration relation for the stability of the MA, covering the range from pH 5.6–8, has been determined. These results indicate that the action of these compounds in stabilizing the mitotic apparatus is non-specific and is similar to their effects on the solubility of proteins. The isolation and stabilization of the mitotic apparatus can thus be viewed as a function of the solubility properties of its constituent proteins, opening a variety of new experimental approaches to this problem.

INTRODUCTION

More than ten years have passed since Mazia and Dan (13) first demonstrated that the intact mitotic apparatus could be isolated from dividing sea urchin eggs. In the intervening years, new methods of isolation have been developed and the structure and properties of the isolated MA investigated in some detail (4, 7, 8, 10, 12, 14–16, 19). The factors which control the stability of the MA are still poorly understood, however, as the development of isolation methods has been largely empirical and no general principles have emerged. Since some understanding of the stability properties of the MA *in vitro* seems a necessary preliminary to an attack on such problems *in vivo*, a more systematic study of the isolation process is clearly required. The object of such an investigation should be to define, in the most general terms possible, the conditions necessary for the isolation of the mitotic apparatus, thus allowing the formulation of testable hypotheses concerning the mechanism involved in the stabilization of the MA *in vitro*.

The original method of isolation developed by Mazia and Dan (13) was based on the extended treatment of metaphase eggs in a solution of 30 per cent ethanol at $-10°C$, after which the cytoplasm was dispersed, freeing the intact mitotic apparatus. This method was sensitive to both concentration and temperature. At lower alcohol concentrations the MA disintegrated, while at higher concentrations the cytoplasm of the cell coagulated. Similar

coagulation occurred at higher temperatures. The action of the alcohol was believed to be a "fixation" or stabilization which, under suitable conditions, preserved the MA without coagulating the remainder of the cell. It was suggested at the same time, however, that the alcohol might not act directly on the MA, but rather prevent the reactions which cause its disappearance. In more recent publications of the original authors (4, 14) the alcohol is considered to be acting directly on the MA, but no suggestion is made as to the mechanism of this action.

The resistance of the alcohol-isolated mitotic apparatus to solution and the absence of ATPase activity (although this has since been demonstrated by Miki (16)) indicated that the extended ethanol treatment was modifying the properties of the MA. To avoid these difficulties, Mazia *et al.* (14) developed a direct isolation method, based on the disulfide compound dithiodiglycol. Sulfhydryl compounds appear to be involved in some fashion in spindle structure as the SH compound mercaptoethanol has been found to block division, apparently by disorganizing the structure of the MA (11, 15). It was reasoned that dithiodiglycol, the oxidized form of mercaptoethanol, might act in reverse to stabilize the MA during isolation. The isolation procedure consisted in the transfer of metaphase eggs to a 0.15 M solution of dithiodiglycol, containing also sucrose and the chelating agent, ethylenediamine tetraacetic acid; after allowing time for the dithiodiglycol to penetrate, the eggs were broken mechanically. This method was successful in making possible the direct isolation of the MA and such MA were shown (12) to have ATPase activity, but the explanation advanced for the role of the dithiodiglycol in stabilizing the MA was not convincing.

The problem of direct isolation was later investigated by the author (7), and it was found that a number of non-sulfur-containing six-carbon glycols duplicated the effects of dithiodiglycol. A very useful isolation method based on hexanediol was developed, showing that disulfide compounds were not required for direct isolation. This method consists in the transfer of metaphase eggs to a 1 M solution of hexanediol, buffered at a pH in the range 6.2 to 6.4. The cells cytolyze osmotically in this medium and the intact mitotic apparatus can be released by very mild agitation. Further study showed that the pH of the solution was of fundamental importance in the stabilization of the mitotic apparatus, as it was found that isolation was possible in water without added hexanediol at a pH of 5.5 to 5.6. This was not a practical method of isolation however, as the cytoplasm coagulated in this pH range and made cell breakdown difficult. Hexanediol thus had the very useful effect of raising the pH at which the MA was stable to a point at which cytoplasmic coagulation did not interfere with isolation. The stability of the MA at low pH values could be attributed to protein solubility effects, but the specific requirement for glycol at higher pH values was unexplained.

The results of the present investigation show that the apparent specificity for a 1 M glycol solution is illusory, and that the mitotic apparatus can, in fact, be stabilized by a variety of penetrating nonelectrolytes. The results obtained suggest that these compounds act through a non-specific effect on the solubility properties of the constituent proteins of the MA.

MATERIALS AND METHODS

The eggs of the sea urchin, *Arbacia punctulata*, obtained at the Marine Biological Laboratory, Woods Hole, Massachusetts, were used as experimental material. The gametes were obtained by the application of 10 volts of alternating current across the test, using lead electrodes (6). The eggs were shed into 250 ml of sea water and washed in additional sea water before use. Sperm was diluted in the ratio of one drop of "dry" sperm to 25 ml of sea water, and several drops of the resulting mixture were added to each 10 ml of egg suspension. Fresh sperm was obtained and diluted for each experiment. Egg batches showing less than 95 per cent fertilizability were discarded.

The fertilization membrane was removed by treatment of the eggs with a 1 M solution of urea immediately after fertilization. The details of this method were given previously (7); the procedure used in the present experiments differed only in that 0.001 M KH_2PO_4, adjusted to pH 8 with KOH, was included in the urea solution to maintain the pH. The eggs were grown in calcium-free sea water at 19–21°C, washed two times in a 19–1 mixture of isotonic sodium and potassium chlorides (2), and then transferred to the isolation medium at the same temperature. The cells cytolyze rapidly, after which they are centrifuged down and the supernate, containing most of the pigment of the eggs, is discarded. Fresh isolation medium is added, and the tube swirled gently to disperse the cytolyzed cells and free the mitotic apparatus. The tube is then placed on ice and all subsequent operations carried out at or near 0°C.

Observations and photomicrographs were made with phase contrast optics.

RESULTS

Revised Glycol Procedure

Studies carried out since the publication of the glycol isolation procedure have resulted in the substitution of an isomer, 2 - methyl 2,4 - pentanediol (known commercially as hexylene glycol), for the 2,5 hexanediol originally used. This substitution was the result of investigations of the effect of several glycols on developing eggs. Aliquots of a batch of fertilized eggs were transferred to 0.1 M solutions of hexylene glycol, 2,5 hexanediol, and 1,5 pentanediol in sea water and observed at regular intervals for 48 hours. Hexylene glycol interfered least with development, causing only a slight retardation of division and allowing most of the eggs to develop to normal plutei. The other two glycols caused more abnormal development and some cell death during the period investigated. In another experiment, eggs and sperm were separately equilibrated with 0.1 M hexylene glycol in sea water and mixed; the sperm remained motile, all eggs formed fertilization membranes, and most developed to plutei. The fact that the cells tolerate this relatively high concentration of hexylene glycol during division provides additional evidence for the absence of any direct chemical effect on the MA.

In addition, the solubility properties of mitotic apparatuses stored in hexylene glycol are retained for a longer period than in hexanediol. Hexylene glycol also forms clear solutions at all concentrations used in these experiments, in contrast to the slightly opalescent solutions formed by hexanediol. In view of these advantages, all glycol experiments now utilize hexylene glycol.

The standard isolation medium contains hexylene glycol at a concentration of 1 M and is buffered with 0.01 M KH_2PO_4, adjusted to pH 6.4 with KOH. Fig. 1 shows the appearance of the mitotic apparatus after isolation in this medium. A delicate fibrous structure is visible in the spindle and aster and only a very few, or in many cases no, cytoplasmic granules are present at the periphery.

Experiments on Glycol Substitution

The isolation procedure as outlined above was quite successful, but the role of the glycol was unexplained. Since it is a relatively simple compound, it should be acting in a non-specific fashion, and one might expect that similar compounds could be substituted for it, but attempts to utilize compounds containing a higher proportion of hydroxyl groups, such as glucose, glycerol, and hexanetriol were unsuccessful (7). These compounds do not cause cytolysis, and mechanical breakage of the cells is necessary; stable mitotic apparatuses have not been found in the resulting suspensions. The failure of isolation in these compounds suggested that rapid penetration and lysis of the eggs is required for isolation.

No such simple explanation can be advanced to account for the failure of alcohols in isolation. A 1 M solution of ethanol, buffered at pH 6.4, penetrates the cells rapidly and causes lysis, yet produces only small yields of reduced and structureless MA. The poor performance of ethanol as a medium for direct isolation was particularly interesting, as this compound was used in the original isolation method (13). Further investigations with ethanol were undertaken in the hope that the relation between indirect and direct isolation methods might be clarified.

pH Limit on Cytoplasmic Dispersion

Since stable mitotic apparatuses can be isolated in water at pH 5.5 to 5.6, a reduction of the pH of the alcohol solution would be expected to increase the stability of the MA and improve the isolation. This effect is obtained, a pH of 6.0 to 6.1 producing somewhat larger and more structured MA. Further reduction in pH cannot be made, however, as such pH values are too low to allow satisfactory

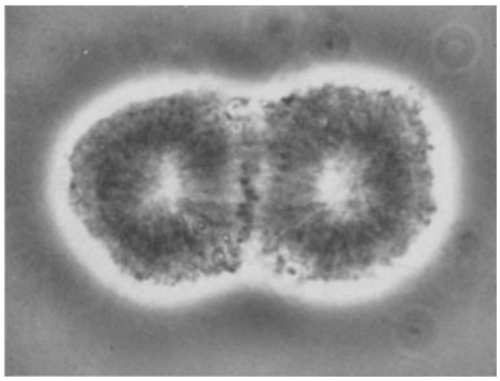

FIGURE 1 Mitotic apparatus isolated in 1 M (12.5 per cent) hexylene glycol, 0.01 M KH_2PO_4, pH 6.4. × 1000.

dispersion of the cytoplasm. At pH values below 6, the cytolyzed cells break up only upon the application of considerable mechanical stress and then merely fragment into clumps whose size increases with decreasing pH, the entire cell remaining intact below pH 5.4. At pH values from 6.0 to 6.2, the cells can be dispersed more easily, but the suspension remains coarse, consisting of aggregates of cytoplasmic granules. Complete dispersion of the cytoplasm occurs only at pH values of 6.4 and above. In this range, the cells are broken down by mild swirling of the solution and the resulting suspension is composed of granules which appear single in the light microscope. The low forces sufficient to break down the cells prevent damage to the mitotic apparatus and allow the MA to be easily separated from the suspension by differential centrifugation, as they are much larger than all other components present. Since the dispersion of the cytoplasm in this range depends only on the pH of the solution and is not influenced by the presence of non-electrolyte, only procedures which can be carried out at pH 6.4 or above are of interest here.

Use of Equal Per Cent Concentration

Investigations were then carried out to determine whether an ethanol-based direct isolation method was feasible at pH 6.4. Examination of the experimental data showed that the concentration of glycol used in the standard procedure, on a per cent basis, was approximately twice that of the alcohol. A 1 M hexylene glycol solution contains 12.5 per cent glycol by volume, while 1 M ethanol solution contains only 5.8 per cent alcohol. Solutions containing ethanol at a per cent concentration equal to the concentration of glycol in 1 M hexylene glycol solution were then investigated and found to duplicate the action of the standard glycol medium. A solution containing 11 to 12 per cent ethanol, buffered at pH 6.4, produces isolated mitotic apparatuses in the same yield and condition as 1 M hexylene glycol solution, as illustrated in Fig. 2. These MA also display the same solubility in solutions at high ionic strength as glycol-isolated MA. Thus, what had appeared to be a specific requirement for a 1 M concentration of long chain glycol is, in fact, the result of a fortuitous circumstance: the molecular weight of these compounds is such that a 1 M solution contains a per cent concentration of penetrating non-electrolyte suitable for isolation.

Extension to Other Compounds

Related penetrating compounds were investigated and found to be effective, providing additional evidence for the non-specificity of this action. Normal propanol and isopropanol acted similarly to ethanol at a concentration of 10 per cent. Acetone could be used at a concentration of 13 per cent, but the mitotic apparatuses which resulted were of poorer structure and greater insolubility than those isolated in other compounds. There is a marked difference in the action of the four butanol isomers. Secondary and tertiary butanol yield MA similar in structure and properties to those isolated in glycol, and these alcohols are effective at a concentration of 8 per cent, lower than the other compounds tested. Normal butanol and isobutanol could not be used at any concentration, only a few distorted and barely recognizable MA being present after lysis in the most successful experiments. The alcohols above butyl are not sufficiently soluble in water to be useful in isolation. Further evidence of the lack of specificity of this action is provided by the observation that the unrelated compound dimethylsulfoxide is also effective at a concentration of 12 per cent.

The shorter chain glycols were previously found (7) to be less effective in isolation than the six carbon compounds, as their use gives rise to reduced and less structured mitotic apparatuses. Isolation in ethylene glycol was reinvestigated and found not to be improved at higher concentrations, thus supporting the hypothesis that the slower penetration and delayed cytolysis which

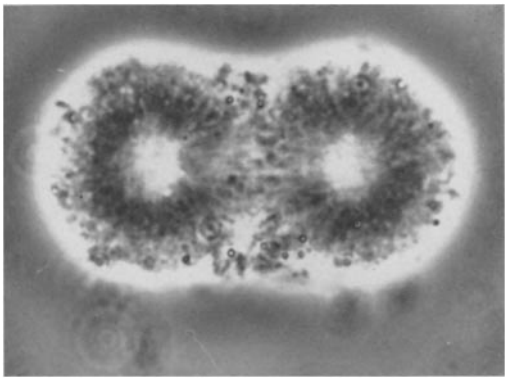

FIGURE 2 Mitotic apparatus isolated in 11 per cent ethanol, 0.01 M KH_2PO_4, pH 6.4. × 1000.

occur in solutions of these glycols render them unsuitable for isolation.

Concentration–pH Relations

With the specificity for 1 M glycol solutions eliminated, the only limitation on isolation remaining was that of pH. All direct isolation procedures require a pH of 6.4 or below, as higher pH values cause a reduction in the size and structure of the mitotic apparatus, which becomes unstable and disappears from lysates buffered above pH 7 (7). However, the studies on the alcohol-based isolation procedure suggest that this pH requirement may not be absolute. A 1 M (5.8 per cent) ethanol solution allows isolation only at pH 6.0–6.1, while an increase to 11 per cent raises the permissible pH to 6.4. By extrapolation, a further increase in concentration should allow isolation at higher pH values. Tests showed this relation to hold for both alcohol and hexylene glycol, suitable adjustment of concentration permitting isolation over a wide range of pH.

The Hexylene Glycol–pH Curve

The first concentration–pH relation investigated in detail was that for hexylene glycol, since this is the preferred non-electrolyte. At a given pH value, isolations were carried out in a graded series of glycol concentrations and the appearance of the resulting mitotic apparatuses compared. Their structure changes markedly with small changes in concentration, and an optimum concentration range can be defined for isolation at that pH. Repetition of this procedure at other pH values allows a concentration–pH relation for isolation to be determined.

Mitotic apparatuses isolated in glycol concentrations which are below the optimum value are reduced in size, particularly in the astral region, and lack visible structure. In the acid pH range, MA's isolated in slightly suboptimal concentrations are dense and granular in appearance and have well defined chromosomes (Fig. 3), while at more alkaline pH values suboptimal MA's appear transparent and fragile, with less visible chromosomes (Fig. 4). At concentrations further below the optimum range than those illustrated unstable MA's are produced, which soon disintegrate.

Glycol concentrations in excess of optimum cause the MA's to be surrounded with cytoplasmic granules. At values slightly above optimum, the granules appear in optical section as a narrow ring surrounding the MA's (Fig. 5), and at values further above the optimal range the coating of granules becomes so thick that it completely obscures the structure of the MA's (Fig. 6). This effect is identical throughout the pH range investigated. The amount of contamination varies with mitotic stage, anaphase MA's being more contaminated at a given concentration than metaphase figures. The conditions defined here are optimal for the isolation of metaphase MA's.

The shape and size of mitotic apparatuses isolated under optimal conditions remain constant

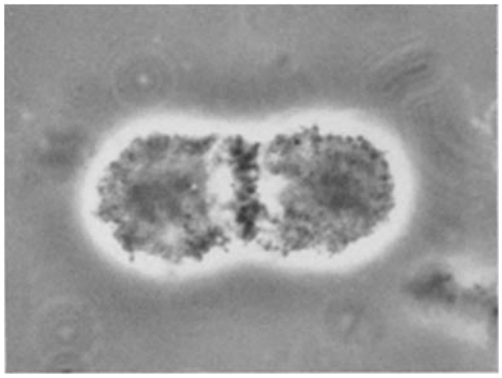

FIGURE 3 Mitotic apparatus isolated in 4 per cent hexylene glycol, 0.01 M KH_2PO_4, pH 6.0. × 1000.

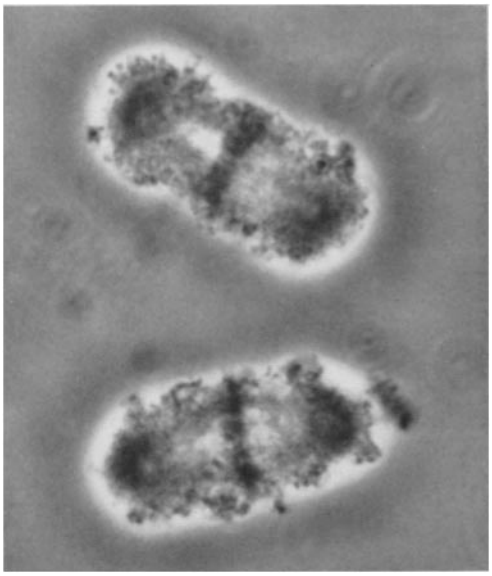

FIGURE 4 Mitotic apparatuses isolated in 16 per cent hexylene glycol, 0.01 M KH_2PO_4, pH 7.6. × 1000.

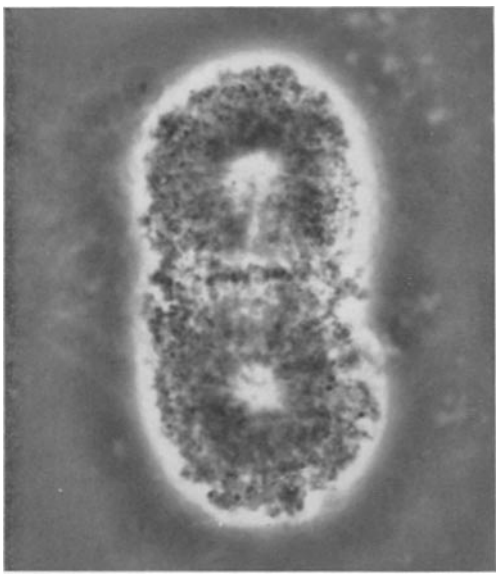

FIGURE 5 Mitotic apparatus isolated in 10 per cent hexylene glycol, 0.01 M KH_2PO_4, pH 6.0. × 1000.

throughout the pH range investigated, but the fibrous structure of the spindle and aster and the definition of the chromosomes are reduced at higher pH values. At the extreme value of the range investigated, pH 8, the MA is essentially structureless and the chromosomes invisible (Fig. 7).

The complete concentration–pH curve for hexylene glycol is given in Fig. 8. The optimum concentration range for isolation has a spread of approximately 2 per cent between its upper and lower limits and is indicated on the graph by shading. The curve begins at pH 5.5, the previously determined (7) point at which isolation can be accomplished in the absence of non-electrolyte. The hexylene glycol concentration required for stability rises linearly to approximately 16 per cent at pH 6.6 and remains at this value until a pH of 7.2 is reached, at which point it rises again to a new plateau of 19 per cent.

The concentration–pH curve for ethanol has also been determined and is essentially the same as that for glycol.

DISCUSSION

These experiments show that the stability of the mitotic apparatus *in vitro* is a function of the pH and per cent of organic solvent in the solution and that no specific chemical factors need be invoked. The action of these organic solvents in stabilizing the MA at higher pH values is similar to their effects on the solubility properties of proteins (3), if stabilization is considered to be a reduction in solubility. This result is in accord with studies of the chemical composition of the MA (10, 19), which show that protein is the major component present. This protein presumably forms the fibrous structure of the MA and changes in its solubility

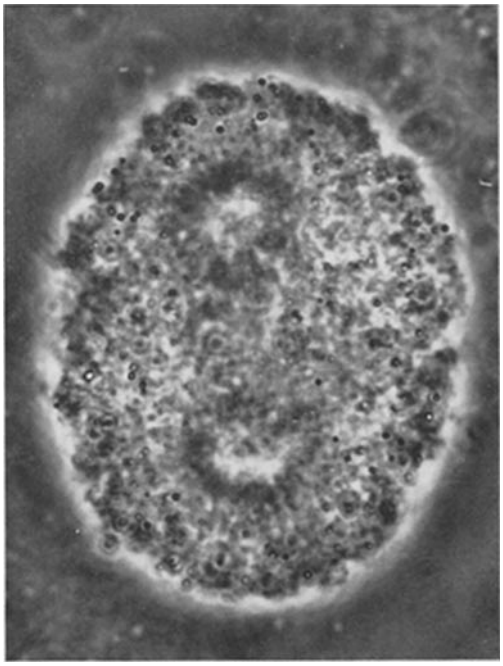

FIGURE 6 Mitotic apparatus isolated in 23 per cent hexylene glycol, 0.01 M KH_2PO_4, pH 8.0. × 1000.

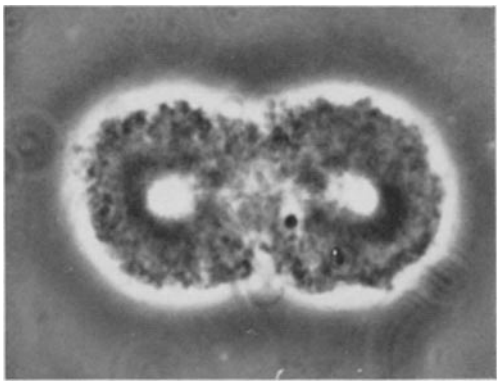

FIGURE 7 Mitotic apparatus isolated in 19 per cent hexylene glycol, 0.01 M KH_2PO_4, pH 8.0. × 1000.

caused by changes in the solvent medium would thus directly influence the structural integrity of the MA.

The results of these experiments provide evidence that the various isolation methods that have been developed have a common basis. Previous experiments of the author (7) demonstrated that long chain glycols would duplicate the effects of dithiodiglycol in isolation. The experiments reported here show that the alcohols act in the same manner as the glycols, leading to the conclusion that the original ethanol-based indirect isolation method is also based on protein solubility effects. The low temperature isolation method has a narrow con-

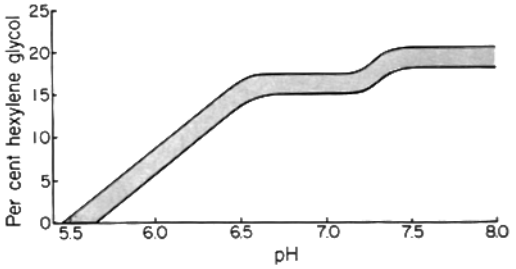

FIGURE 8 pH-concentration curve for isolation in hexylene glycol. Optimum conditions for isolation indicated by shading.

centration range for effectiveness, and values above and below this range produce effects similar to those observed here (4, 13). If one assumes that the pH of this unbuffered isolation medium is close to that of sea water after the addition of the eggs, the alcohol concentration used is somewhat higher than is required at the same pH in the experiments reported here, but it is difficult to estimate the effects of temperature on such a complex system.

The stability of the mitotic apparatus in water at pH 5.6 is believed to be due to the fact that this pH approaches the isoelectric point of its constituent proteins, thus decreasing their solubility. As the pH is raised, these proteins become more soluble, causing the breakdown of the mitotic apparatus and preventing the isolation of intact MA in water at more alkaline pH values. The original glycol isolation method was based on the observation that the addition of 1 M (12.5 per cent) hexanediol increased the stability of the MA sufficiently to allow isolation at pH values up to 6.4. The current experiments show that this action is nonspecific and that a variety of organic compounds, at a similar per cent concentration, will stabilize the MA at this pH. This effect is reasonable in terms of the properties of proteins, as the addition of organic solvents to the medium will reduce the solubility of many proteins. As the pH is raised to more alkaline values, the net charge on the proteins involved increases and higher concentrations of organic solvent are required to reduce their solubility and maintain the stability of the mitotic apparatus. The concentration–pH curve for isolation thus may be a function of the change in charge with pH and reflect the titration curve of the proteins involved. Concentrations of alcohol or glycol in excess of that required for the stabilization of the MA apparently cause additional cytoplasmic components to become insoluble and aggregate, resulting in the contamination of the MA.

Although the action of these organic solvents in increasing the stability of the mitotic apparatus is in accord with their effects on the solubility properties of proteins, the mechanism of these effects at the molecular level is difficult to determine unambiguously, even in much simpler systems. The addition of organic solvent has a number of effects on the properties of the solution which can influence the solubility properties of proteins. The addition of glycol or alcohol to water reduces the dielectric constant, which will increase the electrostatic free energy of the protein molecule and reduce its solubility (3). The solubility of serum albumin in a water-alcohol mixture, for example, has been found to depend on the dielectric constant of the solution (5). Although the change in dielectric constant brought about by the concentrations of organic solvent used in these experiments is rather small, it is of interest to compare the experimentally determined concentrations of organic solvents which are equivalent to 12 per cent ethanol in stabilizing the MA at pH 6.4 with the concentrations calculated from the literature (1) to produce a reduction in the dielectric constant equal to 12 per cent ethanol (calculated values in parentheses): *n*-propanol 10 per cent (10.0), isopropanol 10 per cent (9.6), tert-butanol 8 per cent (8.1), and acetone 13 per cent (12.6). These values are in rather good agreement to be fortuitous, but, of course, do not prove cause and effect.

The addition of alcohol or glycol to the medium will also influence the hydrophobic interactions between the non-polar groups of the protein molecule, which play an important role in controlling

the conformation of protein molecules in solution (9). The concentrations of organic solvent used in these experiments, of the order of 10 per cent, may be sufficient to influence such interactions, as a series of alcohols at a similar concentration has been found to have an effect on hydrophobic interactions in ribonuclease, reducing the transition temperature (17). Changes in such solvent-solute interactions caused by added organic solvent could also modify the solubility of proteins, but whether the effect of alcohols and glycols on the stability of the mitotic apparatus involves such interactions cannot be determined on the basis of the experimental evidence now available. However, the influence of non-aqueous solvents on the properties of protein molecules in solution is a field of active investigation (18), and the information gained from such studies should make possible the design of critical experiments which will allow a decision to be made among the various molecular interactions that may be controlling the stability of the MA *in vitro*.

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REFERENCES

1. AKERLÖF, G., *J. Am. Chem. Soc.*, 1932, **54,** 4125.
2. CHAMBERS, R., *Cold Spring Harbor Symp. Quant. Biol.*, 1940, **8,** 144.
3. COHN, E. J., and EDSALL, J. T., Proteins, Amino Acids and Peptides, New York, Reinhold Publishing Corp., 1943.
4. DAN, K., and NAKAJIMA, T., *Embryologia*, 1956, **3,** 187.
5. FRIGERIO, N. A., and HETTINGER, T. P., *Biochim. et Biophysica Acta*, 1962, **59,** 228.
6. HARVEY, E. B., *Biol. Bull.*, 1952, **103,** 284.
7. KANE, R. E., *J. Cell Biol.*, 1962, **12,** 47.
8. KANE, R. E., *J. Cell Biol.*, 1962, **15,** 279.
9. KAUZMANN, W., *Advances Protein Chem.*, 1959, **14,** 1.
10. MAZIA, D., *Symp. Soc. Exp. Biol.*, 1955, **9,** 335.
11. MAZIA, D., *Exp. Cell Research*, 1958, **14,** 486.
12. MAZIA, D., CHAFFEE, R. R., and IVERSON, R. M., *Proc. Nat. Acad. Sc.*, 1961, **47,** 788.
13. MAZIA, D., and DAN, K., *Proc. Nat. Acad. Sc.*, 1952, **38,** 826.
14. MAZIA, D., MITCHISON, J. M., MEDINA, H., and HARRIS, P., *J. Biophysic. and Biochem. Cytol.*, 1961, **10,** 467.
15. MAZIA, D., and ZIMMERMAN, A. M., *Exp. Cell Research*, 1958, **15,** 138.
16. MIKI, T., *Exp. Cell Research*, 1962, **29,** 92.
17. SCHRIER, E. E., AND SCHERAGA, H. A., *Biochim. et Biophysica Acta*, 1962, **64,** 406.
18. SINGER, S. J., *Advances Protein Chem.*, 1962, **17,** 1.
19. ZIMMERMAN, A. M., *Exp. Cell Research*, 1960, **20,** 529.