

SOME THEORETICAL ASPECTS OF OSMIUM TETROXIDE FIXATION WITH SPECIAL REFERENCE TO THE METAPHASE CHROMOSOMES OF CELL CULTURES

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ABSTRACT

Fixation of cell cultures with 1 per cent OsO_4 at constant pH and tonicity but variable cationic valence and dielectric constant causes profound changes in metaphase chromosomes. It is possible to make them disappear, flocculate, or show little change from the living cell in the phase contrast microscope. Conventional fixation for the electron microscope causes almost complete disappearance of metaphase chromosomes in phase contrast. Reasons for this behavior are discussed. It is postulated that intermolecular distances and consequently internal structure in chromosomes are governed by the same forces that govern these distances in colloidal sols.

INTRODUCTION

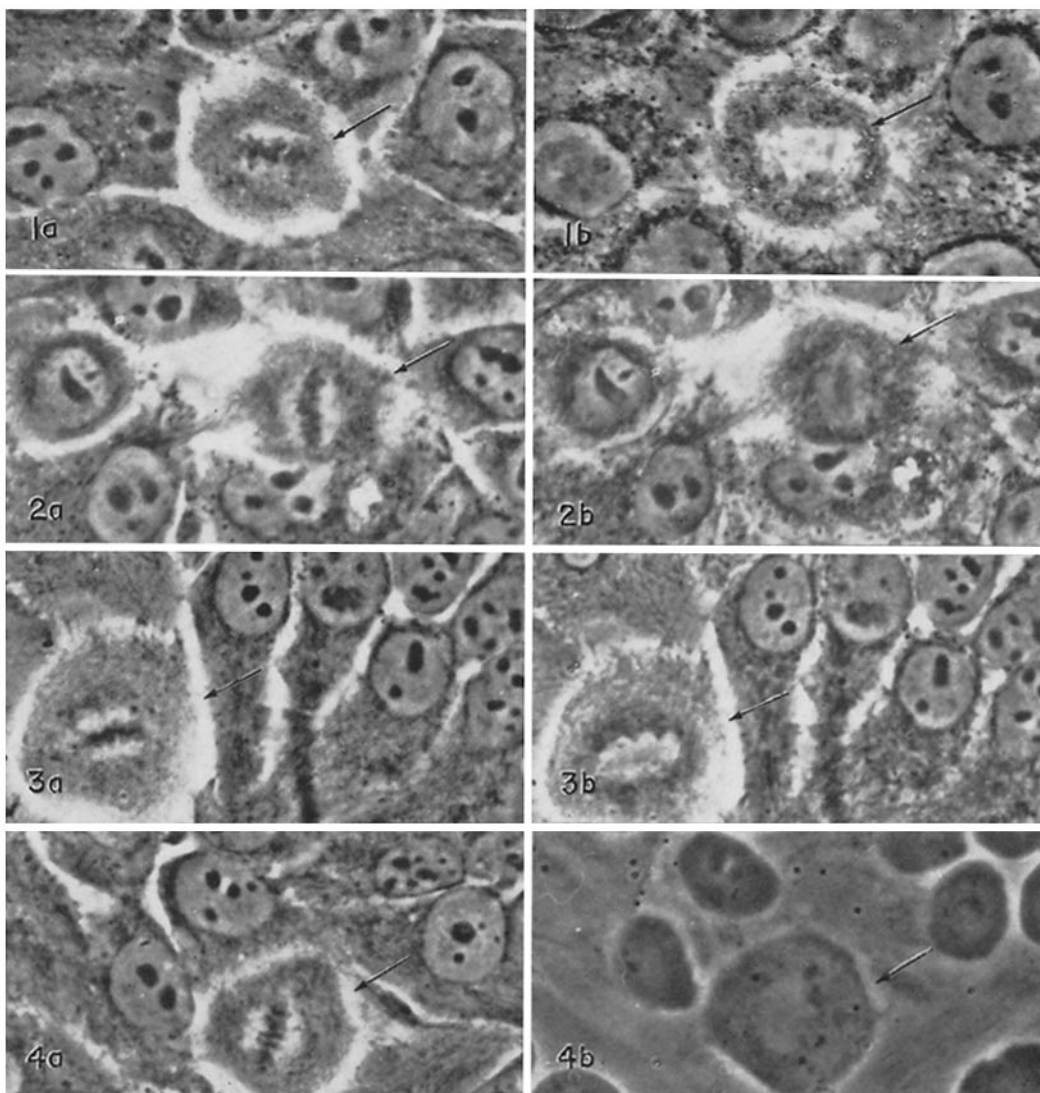
The difficulties of studying the chromosomes in the electron microscope have been pointed out many times (1-4). Chromosomes do not appear to be neatly delimited by membranes as are mitochondria, the Golgi apparatus, or the endoplasmic reticulum. This, combined with the two-dimensionality of thin sections, adds to the difficulty of recognizing significant structure at the electron microscope level. There are also the studies of Bahr (5) which demonstrated that nucleic acids do not react with OsO_4 in the test tube. However, the studies reported here strongly suggest that these factors are not the only ones responsible for the limited success obtained.

Since the metaphase chromosomes and the mitotic apparatus are colloidal systems, they must follow a pattern of behavior consistent with this fact. Much of the evidence relating to this subject has been reviewed by Anderson (6, 7). The present investigations support this view and indicate the importance of the various factors which influence colloidal suspensions in the fixation of metaphase chromosomes. It is demonstrated that varying

such factors as the dielectric constant or the divalent cation concentration of the fixative vehicle changes the character of the fixation in a fashion that is predictable from colloidal chemical theory.

METHODS AND MATERIALS

Since cell cultures were used in these studies, it was possible to observe cells both before and after fixation (8-10). A stock strain of HeLa cells was grown in milk dilution bottles with Parker's 199 supplemented with fetal calf serum as the growth medium. Monolayers were dissociated with 0.05 per cent Versene at pH 7.8. They then were washed with growth medium, centrifuged at 400 RPM (approx. 50 g) for 2 minutes, and resuspended in growth medium. Several drops of a crowded suspension (5×10^5 cells per cc) were placed in a perfusion chamber (see reference 10 for a description of the chamber). The chamber was placed in an atmosphere of 5 per cent CO_2 , 95 per cent air, and 100 per cent humidity at 37°C for 5 to 7 hours. At the end of this time many cells in metaphase could readily be found. After removal of the perfusion chamber from the



All figures in phase contrast. Cell in metaphase indicated by arrow. All pictures approx.

FIGURE 1

a, Living cells. *b*, Same cells fixed with OsO_4 in 0.001 M NaCl.

FIGURE 2

a, Living cells. *b*, Same cells fixed with OsO_4 in isotonic NaCl.

FIGURE 3

a, Living cells. *b*, Same cells fixed with OsO_4 in 0.3 M NaCl.

FIGURE 4

a, Living cells. *b*, Same cells fixed with OsO_4 in isotonic glycine.

incubator, specific cells for study were located under the phase contrast microscope and photographed; the test solution was then infused into the chamber and another photograph taken, usually at the end of 5 minutes. A large number of fixing solutions containing 1 per cent osmium tetroxide, but of variable tonicity, ionic types, and dielectric constant, were studied for their effects on cells in culture. All solutions were adjusted to pH 7. Special attention was paid to the metaphase chromosomes.¹ It should be mentioned that these methods and results cannot be applied to tissue blocks, unfortunately, because of the differential rates of penetration of fixative and vehicle in contrast to the condition of simultaneous penetration that exists in single cells.

RESULTS AND DISCUSSION

The results obtained were as follows:

1. Variations in the tonicity from 0.001 M NaCl to 0.3 M NaCl adjusted to pH 7 resulted in quite different qualities of fixation, but in all these experiments the image of the chromosomes of cells in metaphase almost completely disappeared in the phase contrast microscope. Figures 1 to 3 show the results of varying the tonicity. Using 1 per cent OsO₄ in a markedly hypotonic solution (0.001 M NaCl) with respect to the living cell causes a significant swelling of all cells, a blurring of the nucleoli, and increased homogeneity of the nucleoplasm, as well as what seems to be a dispersion of the chromosomes of cells in metaphase (Fig. 1).² OsO₄ in isotonic NaCl (0.15 M) adjusted to pH 7 resulted in apparent preservation of all visible structure in interphase cells, but the metaphase chromosomes have practically disappeared (Fig. 2). With 0.3 M NaCl, the results are surprisingly little different from those with the isotonic solution (Fig. 3);³ the

¹ It is important to stress that the results obtained apply *only to the metaphase chromosomes*. Cells in anaphase usually behave similarly, but the results are somewhat less striking depending on what stage of anaphase the cell is in. The later the stage, the less striking are the effects described below.

² The report of Strangeways and Canti (8) on the "almost perfect preservation" of cells fixed with OsO₄ in distilled water may have been due to their necessary reliance on camera lucida drawings and the unavailability of phase contrast.

³ It is interesting to note that this increase in tonicity does not cause cellular shrinkage. In fact, if spherical cells are used in these experiments, it is found that osmium tetroxide in isotonic solution causes swelling, and so do the hypertonic solutions, although less so.

same is true if the tonicity is decreased to 0.07 M. Changing the fixative vehicle to Tyrode's, Locke's, or Ringer's solution and performing the same series of experiments gave essentially the same results and therefore these are not discussed further.

2. Maintaining isotonicity and a pH of 7, but making the ratios of ionic constituents significantly different from those ordinarily found in physiologic solutions, had profound effects on the general character of the fixation and especially on the metaphase chromosomes. As already pointed out, OsO₄ in isotonic NaCl had little effect on interphase cells, but appeared to disperse metaphase chromosomes. OsO₄ in isotonic glycine, on the other hand, caused swelling of all cells, and in addition caused significant cellular destruction, as shown in Fig. 4. OsO₄ in isotonic NaCl to which MgCl₂ was added to a final concentration of 0.02 M resulted in a totally different picture, cells in interphase showing a nucleolar phase reversal and intranuclear flocculation (circles in Fig. 5 *b*) and in general a fixation which is inferior to that obtained with OsO₄ in NaCl solutions; however, it is noted that the chromosomes in the metaphase cell have not disappeared although they are changed in character, showing a phase reversal. A marked increase in the refractility of asters and spindle fibers also is noted frequently, although it is not evident in the figure shown. The effects obtained with glycine occurred with other α -amino acids and urea. The effects obtained with MgCl₂ were comparable to those with other magnesium salts. Calcium salts were less able to prevent chromosomal dispersion. The character of the fixation remained the same, however, there being intranuclear flocculation and nucleolar phase reversal.

3. Significant raising or lowering of the dielectric constant of the fixative vehicle from the value of isotonic NaCl caused marked changes in the fixation even if isotonicity and a pH of 7 were maintained. The first indication that the dielectric constant might be important in determining the character of the fixation came from the results shown in Fig. 4. Glycine, along with other α -amino acids, and urea are among the few biologi-

This is, however, not perceived in spread cells, which being anchored at many points may only swell in an upward direction (11). This observation is consistent with those of Bahr *et al.* (12) on ascites cells.

cal compounds that raise the dielectric constant of water—this fact being contingent on its very high dipole moment. The extensive cellular destruction occurring with OsO_4 in isotonic glycine was thought to be due possibly to this increase in the dielectric constant of the fixative

5 and 10 per cent alcohol in the fixative vehicle caused very slight change in the results obtained with NaCl ; however, addition of 20 per cent alcohol resulted in intranuclear flocculation, nucleolar phase reversal, and the prevention of chromosomal dispersal (Fig. 6). These findings

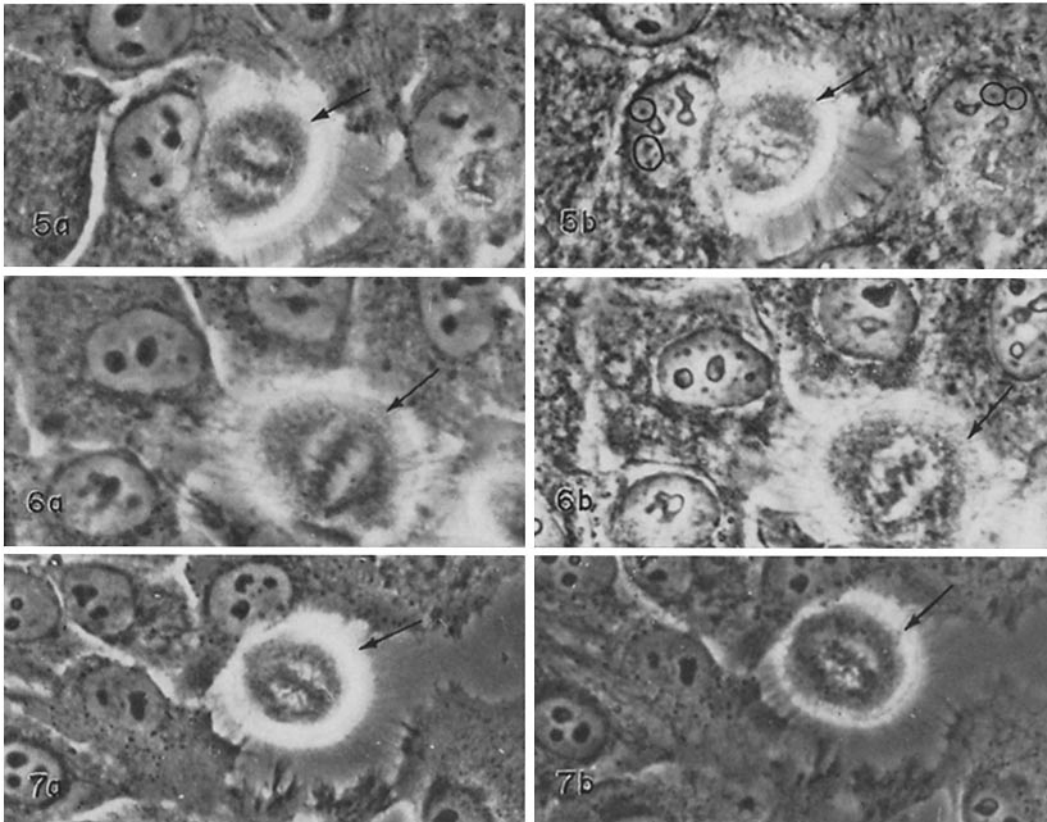


FIGURE 5

a, Living cells. *b*, Same cells fixed with OsO_4 in isotonic NaCl + 0.02 M MgCl_2 .

FIGURE 6

a, Living cells. *b*, Same cells fixed with OsO_4 in isotonic NaCl + 20 per cent ethyl alcohol.

FIGURE 7

a, Living cells. *b*, Same cells fixed with OsO_4 in 0.3 M NaCl + 19.5 per cent acetone.

vehicle. A series of experiments was therefore designed to test the effects of *lowering* the dielectric constant of the fixative vehicle by adding various volumes of alcohol in isotonic NaCl , with the expectation that flocculation would occur. The concentrations used ranged from 5 to 20 per cent of the final volume. The results were as expected.

are consistent with those of Gross *et al.* (13), who found that a fixative containing 30 per cent ethanol and 1 per cent OsO_4 tended to coarsen fine structure and " OsO_4 [alone] to disperse it." As is further discussed below, however, OsO_4 *per se* is probably not responsible for the dispersal of fine structure noted by these authors.

The phase reversal seen in Figs. 5 *b* and 6 *b*, although bearing some resemblance to the image seen with slight defocusing, is nevertheless real. Qualitatively similar results were obtained with other water-soluble organic solvents including methanol, acetone and Cellosolve (ethylene glycol monoethyl ether). The effects of these organic solvents on the tonicity of the fixative have been neglected in these studies. Because of the high rate of penetration of these substances into the cell, their osmotic effect is very small, in spite of their high concentration.

Manipulation of the variables discussed above led to the results shown in Fig. 7. The fixative solution used contained absolute acetone (19.5 per cent) and 1 per cent OsO₄ (80.5 per cent) made up in 0.3 M NaCl adjusted to pH 7 with NaHCO₃. The solution is hypertonic and contains no divalent cations. By addition of acetone the dielectric constant has been substantially decreased below that of pure water. For reasons that are not yet clear, experience with different cell strains has demonstrated that optimal concentrations of acetone must be empirically determined. These have ranged between 18 and 22 per cent. Not quite as good preservation was obtained by variation of the concentration of ethanol, methanol, etc. Since similar results were obtained with these other solvents which lowered the dielectric constant, a similarity of mechanisms is suggested; on the other hand, the inability to obtain exactly the *same* results suggests that unknown factors also play a role in the result.

It is important to emphasize here that the disappearance of any structure in the phase contrast microscope does not necessarily mean that the structure is no longer present. It may mean merely that the optical path length through it has become identical with that of its surroundings. In the case of chromosomes it is quite possible that they have not been completely dispersed during the fixation (Figs. 1 to 4), but rather that intramolecular reorientation such as protein unfolding has sufficiently changed the optical properties to bring about the changes illustrated. In any case there have been changes, and these changes may well be responsible for the difficulty encountered in visualizing metaphase chromosomes in the electron microscope.

Although determination of the precise physicochemical nature of the observations described here

awaits further study, some conclusions and preliminary explanations may be offered at this time.

First, tonicity and pH in themselves do not seem to be of sole importance in determining the quality of the fixation of metaphase chromosomes. Several isotonic solutions adjusted to pH 7 and 1 per cent OsO₄ concentration gave significantly different qualities of fixation.

Second, the type of ion (especially the valence of cations) and the dielectric constant of the fixative vehicle seem to play a critical role in chromosomal fixation.

Third, OsO₄ itself does not cause metaphase chromosomal destruction as has been suggested by Grassé (2) and others. The results illustrated here indicate that it is possible to obtain fixation of the chromosomes of the metaphase cell with OsO₄. The paucity of electron microscopic studies of the metaphase cell is due partly to its destruction by the fixative vehicle, not just to the OsO₄. However, studies to be reported at a later date show that significant changes may occur in metaphase chromosomes during dehydration, embedding, and polymerization in spite of their being "fixed." These difficulties have not yet been overcome.

The validity of applying the concepts of colloidal chemistry, in particular those of the electric double layer, to the results reported here can only be proved by further investigations. However, a simple explanation solely in terms of pH or tonicity is incomplete even though there is no question of the importance of these factors in general cytological (mainly extranuclear) preservation (14-16). It therefore seems worth while to briefly discuss the double layer because of its possible applicability to the above results.

The behavior of a hydrophobic colloidal suspension is contingent on the electric double layer which surrounds the colloidal particle if electrolyte is present (17). In this double layer the charges on the particle constitute one layer and the ions of opposite charge in the surrounding environment the second, "diffuse" layer. Colloidal particles in suspension are acted upon by at least two sets of forces: the coulombic repulsive forces of particles surrounded by like-charged double layers, and the van der Waals attractive forces which act over appreciable distances for particles of large size. The interplay between these two sets of forces determines the interparticulate distances (*i.e.*, whether a sol flocculates or remains stable).

It should be mentioned that although double layer theory has been quantified only for hydrophobic colloids (colloids whose interstices do not contain water), many biological (hydrophilic) colloids behave in a qualitatively similar fashion. Bungenberg de Jong (18) and Kruyt and Bungenberg de Jong (19) have amassed considerable experimental evidence illustrating the similarity in physical properties (although not always the stability with respect to flocculation) of hydrophobic and hydrophilic colloids.

Hydrophobic colloids are ordinarily treated in terms of relatively simple geometrical shapes such as spheres or plates; their charge is usually considered as being distributed uniformly over their surface and of one sign. While these conditions simplify things, the problem is still quite complex and only approximate theories have been evolved. Biocolloids (proteins in particular) in contrast cannot be considered to have any of these simplifying characteristics. They are intricately interwoven, and have no simple geometric shape and no uniform or even single type of charge. In addition, the charge as well as potential-determining ions are located in their interstices. Thus the problem is considerably more difficult. Nevertheless, certain variables such as the dielectric constant, the suspending medium, and the cationic valence which profoundly influence the behavior of the negatively charged hydrophobic sol have been shown to have qualitatively similar effects on the hydrophilic sol even though a quantitative theory cannot yet be presented.

In the theory of hydrophobic colloids as discussed by Verwey and Overbeek (17) the relationship between cationic valence, v , and the double-layer repulsive potential, ψ , is inverse in that as v increases, the double-layer repulsive potential between two particles decreases, and they can, therefore, more closely approach each other. The opposite is true of the dielectric constant.

At any instant the variables governing a colloidal sol may be considered to have a definite value especially if changes that are taking place do so over a matter of minutes. Thus a sudden change such as, for example, an increase in the dielectric constant will result in an increase in the repulsive potential between two particles, causing them to move farther apart if they are not restrained by more powerful forces. The converse is also true and is particularly significant since if

two macromolecules move close enough to one another, van der Waals attractive forces may bring them into contiguity. In a colloidal sol this is coincident with flocculation.

With reference to the cell, while it is impossible to change only the intracellular dielectric constant or divalent cation concentration while keeping everything else constant, useful information can be obtained by manipulating these variables in the extracellular environment, since extra- and intracellular milieux become confluent with regard to small ions when the cell is killed. It is suggested that OsO_4 kills the cell, but does not instantly fix it; therefore, there is an appreciable period of time between exposure of the intracellular to the extracellular environment and total macromolecular immobilization by OsO_4 . The ultimate quality of the fixation is probably determined during this brief prefixation period of interaction between the intracellular and extracellular environments. The problem thus becomes a colloidal chemical one. For various cytoplasmic structures such as mitochondria, the endoplasmic reticulum, and other membrane-bounded structures, the intermolecular bonds apparently are stronger than the forces tending to disrupt them during conventional fixation procedures. In the case of the metaphase chromosomes, however, the bonds are apparently more tenuous and the structures are dispersed. It is interesting in this regard to note the work of Mazia on the isolation of the mitotic apparatus of sea urchin eggs (20). He found that the exposure of these structures to the extracellular environment, untreated with any stabilizing agent, resulted in their immediate dispersal. On the other hand, Ca^{++} in low concentration and ethanol were found to stabilize them. As in the results reported here, this is what would be expected for colloidal systems. However, in Mazia's studies magnesium ion was ineffective in preventing dispersal, in contrast to our findings. The reason for this single discrepancy is not known.

In view of the above discussion, the results obtained may thus be explained as follows:

1. The fixing solutions which contain only monovalent cations are less effective than solutions containing divalent cations in suppressing the double layer potential. With reference to the metaphase chromosomes, this potential increases upon exposure to monovalent cations over what it is in the living state, causing these structures to disperse. It is interesting in this regard to point

out that several investigators have stressed the importance of calcium in chromosome condensation in the living cell. In fact, it has been demonstrated in high concentration in the immediate vicinity of condensed chromosomes (21, 22).

2. The presence of glycine or similar highly dipolar molecules in the fixative vehicle raises the dielectric constant. This increases the double layer potential to a point which through intermolecular repulsion disperses not only the chromosomes but other more stable structures as well. This occurs before the OsO₄ has a chance to fix. In contrast, alcohol and other organic solvents in relatively low concentrations have their effects by lowering the dielectric constant. This suppresses the diffuse double layer and consequently lowers the intermolecular repulsive potential in the same way as does an increase in the concentration of divalent cations. When this potential is lowered sufficiently, thermal activity brings the

particles close enough for the van der Waals forces to cause contiguity and visible flocculation.

This picture as presented is somewhat abbreviated since the detailed processes taking place are complicated. The fixation and the cellular interaction with the vehicle take place simultaneously but at different rates, and the end result is the product of the effects of these two processes and the rates at which they occur. However, the information obtained may provide some clues as to possible pathways of approach to the problem of chromosomal preservation for electron microscopy.

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