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INVITED PAPER

DIIMIDOESTERS: ROLE IN ELECTRON AND LIGHT MICROSCOPY

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Abstract—Diimidoesters are a class of bifunctional reagents which cross-link by reacting with α- and ε-amino groups of proteins. The structure and properties of these reagents as well as their reaction with proteins or cells are described. The importance of the type and concentration of the buffer and of the pH as well as the osmolarity and penetration of these fixatives are discussed. Possible artifacts produced during fixation and the use of diimidoesters in enzyme cytochemistry and immunocytochemistry are also reported. A comparison of the cross-linking and structures obtained by diimidoesters with those obtained by aldehydes is stated.

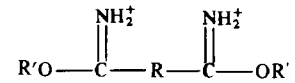
Index key words: Diimidoesters, electron microscopy, light microscopy.

INTRODUCTION

The diimidoesters are a class of bifunctional reagents ('fixatives'), i.e. reagents with two reactive groups per molecule capable of reacting with, and forming bridges between, the side chains of the amino acids in the proteins. They cross-link proteins by reacting solely with their α- and ε-amino groups, in the pH range 7–10 (Dutton *et al.*, 1966; Wold, 1972; Peters and Richards, 1977).

Diimidoesters have been used in electron and light microscopy to cross-link red blood cells (McLean and Singer, 1970), rat liver (Hassell and Hand, 1974; Hand and Hassell, 1976), collagen (Tzaphlidou and Chapman, 1984; Tzaphlidou 1986, 1987) and monkey kidney CV1 and COS1 cells (Matthopoulos and Tzaphlidou, 1987). They have also been used in general biochemical studies to cross-link bovine pancreatic ribonuclease A (Hartman and Wold, 1967), oligomeric proteins (Davies and Stark, 1970), ribosomal proteins (Bickle *et al.*, 1972; Sillers and Moore, 1981), erythrocyte membranes (Marinetti *et al.*, 1973; Ji, 1974; Mentzer *et al.*, 1978; Mentzer and Lubin, 1979; Roemer-Luethi *et al.*, 1979), sarcoplasmic reticulum (Louis and Shooter, 1972; Yuthavong *et al.*, 1975), mitochondrial proteins (Tinberg *et al.*, 1975, 1976; Rendon and Waksman, 1980; Anderson and Fisher, 1981; Chernyak *et al.*, 1981), peripheral neurons (Pulliam *et al.*, 1975), histones (Thomas and Kornberg, 1975; Bakayev *et al.*, 1981), chloroplast membranes (Henriques and Park, 1978), synaptosomal membrane proteins (Smith and Loh, 1978; Zisapel, 1982), viral polypeptides (Nagai *et al.*, 1978; Hordern *et al.*, 1979; Wetz and Habermehl, 1979), tropomyosin (Ohara *et al.*, 1980), hemoglobin (Pennathur-Das *et al.*, 1979, 1982, 1984a; Ferris and Smith, 1985), thyroglobulin (Jeso *et al.*, 1985), aminohexyl-FAD to proteins (Schroeder *et al.*, 1985) and yeast aldehyde dehydrogenase (Tamaki *et al.*, 1978). Also, these reagents have been useful in the study of sickling of red blood cells *in vitro* (Change *et al.*, 1983; Guis *et al.*, 1984; Pennathur-Das *et al.*, 1984b).

STRUCTURE AND PROPERTIES



The molecular formula of diimidoesters shows that they carry an amido group (NH_2^+) adjacent

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to each functional group and as a result leave the charge distribution of the protein unaltered by the reaction (Dutton *et al.*, 1966; Inman *et al.*, 1983). An interesting feature of these reagents is the fact that the size and geometry of the connecting structure R can be varied. This family of reagents may therefore be especially useful in mapping out the distances between different lysyl residues in the native conformation of a protein molecule (Wold, 1961). For example, dimethylsuberimidate (DMS) can cross-link proteins intermolecularly within a distance of 1.1 nm (Bickle *et al.*, 1972). As the chain length of diimidoesters becomes longer more intra-molecular cross-links can be achieved (Ji, 1979). This is indeed confirmed by the observations of Hassell and Hand (1974) and Tzaphlidou (1987).

Two classes of reaction products might form in the reaction of diimidoesters with proteins. In one class, both functional groups of a reagent molecule might react to yield amidine linkages and release of methanol (Tinberg *et al.*, 1975). Clearly, no change in net charge on the protein should occur with this class of reaction products. In the other class, one functional group of a reagent molecule might amidinate a lysyl residue, but the second might be hydrolyzed. Under ordinary conditions, the most likely hydrolysis product of a diimidoester is an ester, a less likely product is a nitrile (Dutton *et al.*, 1966). Diimidoesters are highly unstable in aqueous solutions so no unreacted, unhydrolyzed diimidoester groups could remain on the protein. By either of these combinations of monofunctional and hydrolytic reactions, the net charge on the protein molecule should not be affected. The relative extents of the two classes of reactions depend critically on the pH. The rate of amidine formation can therefore be controlled by an appropriate choice of pH (Ludwig and Hunter, 1967).

The relative concentration of diimidoesters and available ε-amino groups seems to be important. With a low concentration of diimidoesters, insufficient cross-links form to stabilize the proteins. With high concentrations an increasing number of the reagent molecules react monofunctionally, thus blocking available amino groups without producing cross-links. Studies of DMS-fixed rat liver by Hassell and Hand (1974) suggest a DMS concentration of 16–20 mg/ml for optimal cross-linking. Other studies on shrinkage temperature of collagen after fixation with DMS or dimethyladipimidate (DMA) by Tzaphlidou and Chapman (1984) and Tzaphlidou (1987), confirm the observations of Hassell and Hand (1974). Also, fixation of monkey kidney cells CV1 and COS1 with DMS or DMA gave the best appearance of cells, in light microscopy, with the same concentration (Matthopoulos and Tzaphlidou, 1987). The critical value for the diimidoesters concentration for optimal cross-linking shows that polymerization with diimidoesters does not occur. This is further supported by the unaltered negative staining pattern of collagen after DMS fixation (Tzaphlidou, 1986). The duration of fixation of liver blocks and collagen for electron microscopy is $2\frac{1}{2}$ h at room temperature (Hassell and Hand, 1974; Tzaphlidou and Chapman, 1984; Tzaphlidou, 1987). Much shorter durations are preferred for immunocytochemistry and cells for light microscopy (Matthopoulos and Tzaphlidou, 1987, 1988).

REACTION WITH PROTEINS

Hand and Hassell (1976), using paraffin sections of rat liver fixed with DMS at pH 9.5, found that they were weakly stained with the ninhydrin–Schiff procedure, indicating extensive reaction of NH_3^+ groups with the fixative. When the pH of the DMS was lowered, the ninhydrin–Schiff reaction increased in intensity, indicating that a greater number of free amino groups remain after fixation and thus less extensive cross-linking occurs as the pH lowers.

Tzaphlidou and Chapman (1984) and Tzaphlidou (1987) by selective deamination of collagen, replacing the ε-amino group of the lys and hyl side chains by a hydroxyl group, found that DMS and DMA cross-link collagen by reacting specifically with ε-amino groups (ignoring the minor contribution from the α-amino groups at the N-ends of the chains). That no reaction takes place between diimidoesters and arginyl residues was shown by the same authors by blocking arg with cyclohexanedione.

Reconstituted calf skin collagen incubated in dimethylsuberimidate solution and then stained positively with phosphotungstic acid and uranyl nitrate gives the fibril band pattern shown in Fig. 1(a). For comparison, Fig. 1(b) is the pattern from unfixed collagen. The two patterns resemble one another fairly closely, both exhibiting the same D-periodic distribution of 11 to 12 dark staining bands, identifiable with regions rich in charged residues (Chapman, 1974; Meek

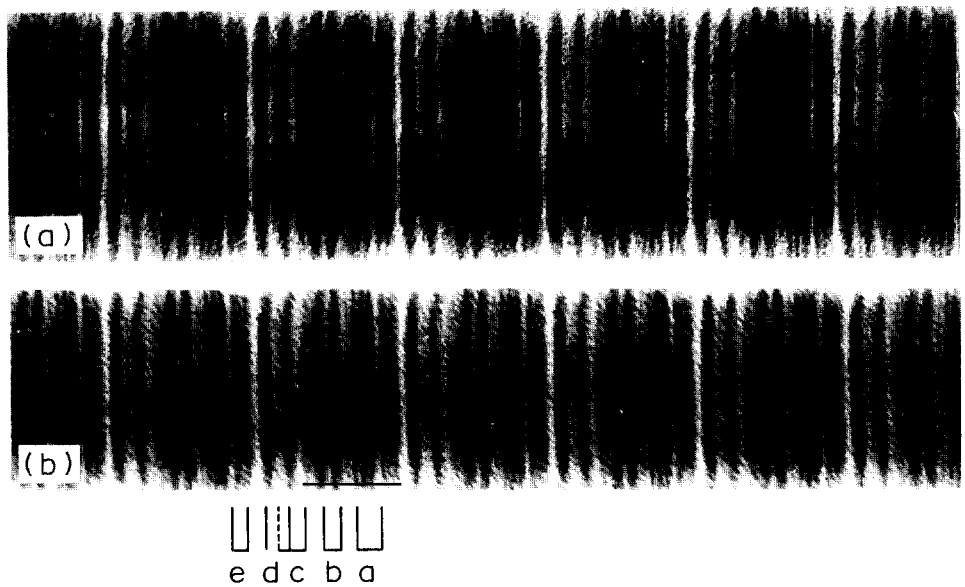

Fig. 1. Reconstituted collagen fibrils positively stained with phosphotungstic acid and uranyl nitrate with identification of bands: (a) fixed in dimethylsuberimidate, (b) unfixed (× 300,000).

et al., 1979). A high degree of contrast is preserved in all the bands in the fixed material. This also occurs in fixed collagen but stained with an anionic stain only, such as phosphotungstic acid (PTA) or ammonium tungstate (AT) (Tzaphlidou and Chapman, 1984) or with a cationic stain such as uranyl nitrate (Tzaphlidou, 1980). No changes in the positive staining behaviour are detectable and this must be due to the unaltered charge distribution along the collagen molecule after fixation. As noted earlier, this is because the amido group adjacent to each functional group on the DMS molecule leaves the net charge of the protein unaltered by the reaction. This was established by comparing the averaged staining pattern from DMS-treated fibrils with the pattern predicted from the amino acid sequence, using the correlation coefficient as a quantitative indication of the agreement between sequence-generated and experimental data (Chapman and Hardcastle, 1974; Tzaphlidou and Hardcastle, 1984). Using anionic stains (e.g. PTA or AT) known to react only with the positively charged side chains on the collagen (Tzaphlidou *et al.*, 1982), Tzaphlidou and Chapman (1984), after DMS fixation, found that similar correlations with unfixed material arose when experimental and positive-charge-derived histograms were compared. This indicates, that, as in unfixed collagen, all the positively charged residues in DMS-fixed collagen contribute to the PTA- or AT-staining pattern. This was also observed after fixation of collagen with DMA (Tzaphlidou, 1987). In Fig. 2(a) the positive charge distribution in a D-period, as predicted from the sequence data, has been smoothed by $n=9$ residues and is compared with averaged

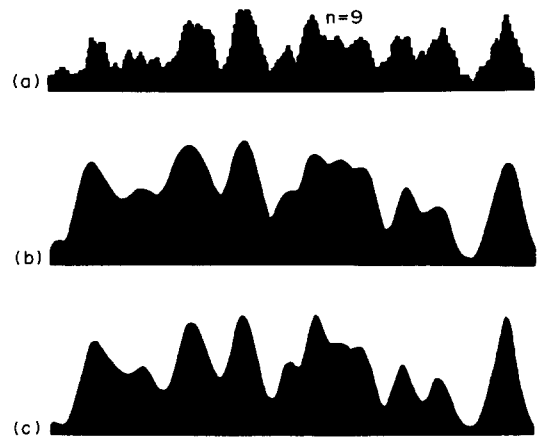

Fig. 2. The sequence-generated positive charge distribution, smoothed by $n=9$ residue spacings (a), compared with averaged densitometric traces from band patterns from dimethylsuberimidate fixed collagen fibrils. One D-period is shown in each figure, appropriately aligned. Data from at least 30 D-periods were averaged in each case. In (b) the collagen was positively stained with phosphotungstic acid; in (c) with ammonium tungstate.

densitometric traces from DMS-fixed fibrils, PTA- and AT-stained [Figs. 2(b) and 2(c)]. Visual inspection shows that there is reasonable agreement between sequence-generated and observed patterns. That after treatment with DMS no alterations occur in the positive staining pattern of collagen is also confirmed by a statistical comparison of averaged microdensitometric data from DMS-fixed and unfixed fibrils (Fig. 3). Each band in the fixed pattern can be matched in position to the corresponding band in

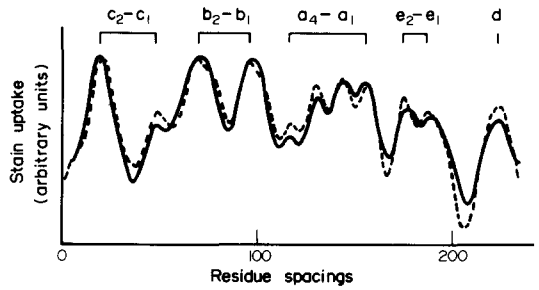

Fig. 3. The computer averaged microdensitometric trace from positively stained dimethylsuberimidate-treated collagen (broken line) compared with that from untreated collagen (continuous line).

the non-fixed fibril. A slight movement in the position of the c_2 band after DMS-treatment is the only shift detectable (about 0.5 nm towards the c_1 band). Similar results were obtained after DMA-treatment (Tzaphlidou, 1987). No changes are therefore detectable in electron-optical positive staining patterns after fixation of collagen with diimidoesters similar to those observed after glutaraldehyde fixation (Meek and Chapman, 1985).

Diimidoesters could also find an application in the preservation of biological structures prior to embedding and sectioning. Indeed, not only is the structure stabilized by cross-linking, but, in addition, staining is enhanced. Using thin sections of DMS-fixed collagen and stained with anionic stains, reveal staining patterns in which a high degree of contrast is preserved (Fig. 4). High correlations are obtained when densitometric traces from such patterns are compared with positive-charge-derived histograms (Tzaphlidou and Chapman, 1984).

This class of reagents does not produce the marked alterations in the negative staining pattern of collagen which result from glutaraldehyde fixation (Grant *et al.*, 1967; Bairati *et al.*, 1972). Figures 5(a) and (b) compare the patterns from unfixed and DMS-fixed fibrils, both negatively stained with lithium tungstate at pH 7.2. The two patterns are nearly identical, although the bands from DMS-fixed collagen tend to be more sharply defined than those from unfixed material, particularly at the C-terminal gap/overlap junction. The results from the quantitative comparison between negative staining patterns from DMS-fixed fibrils and chemical data show that the 'bulkiness' (average cross-sectional area or 'plumpness') of amino acid side chains is the dominant factor in determining the stain-excluding property of a DMS-fixed negatively stained collagen fibril as it is in unfixed collagen (Tzaphlidou, 1986). Some contribution of positive staining can also be demonstrated after DMS-fixation by partial correlation analysis.

REACTION WITH CELLS

The effect of DMS on DNA, in rat liver, was evaluated by the Feulgen reaction (Hand and Hassell, 1976). Fixation with DMS at pH 8.5 resulted in uniform staining of nuclei in cells

Fig. 4. A section of a reconstituted collagen fibril fixed in dimethylsuberimidate and positively stained with ammonium tungstate (× 300,000).

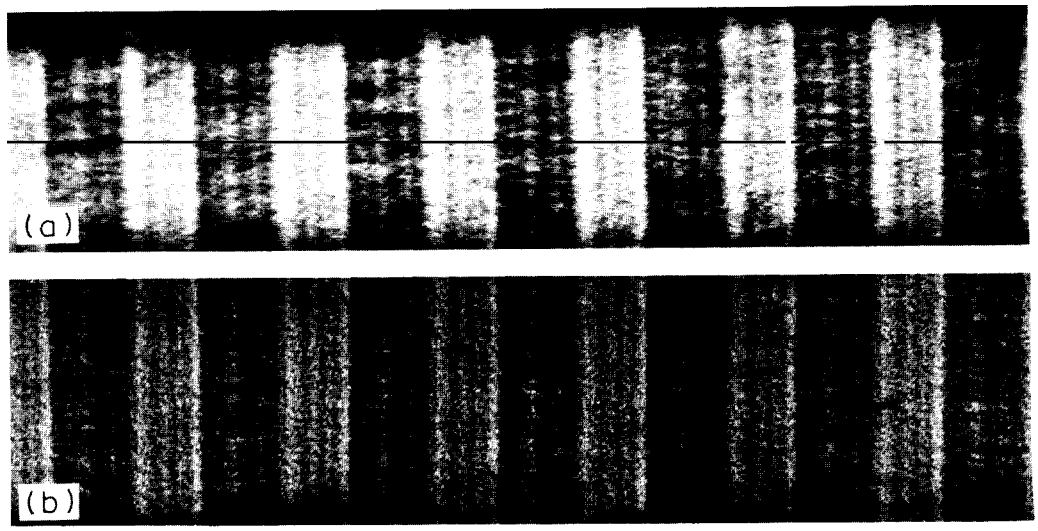

Fig. 5. Reconstituted collagen fibrils: (a) unfixed and negatively stained with lithium tungstate, (b) fixed in dimethylsuberimidate and similarly negatively stained (× 300,000).

located near the edge of the block, but hepatocyte nuclei in the centre of the block appeared vacuolated. At pH 9.5 uniformly stained nuclei throughout the block were obtained. However, in light microscopy, fixation with DMA or DMS at pH 8.0 of CV1 cells, grown on glass substrate, resulted in non-uniformly stained nuclei after hematoxylin/eosin staining (Matthopoulos and Tzaphlidou, 1987). A high degree of contrast was preserved in those cells fixed with DMA or DMS [see Fig. 8(b,c)].

EFFECT OF pH

Among all the factors that influence the interaction of diimidoesters with proteins, pH is the most important in obtaining the maximum binding with biological materials. An increase in pH generally results in an increase in the binding capacity of diimidoesters.

Hassell and Hand (1974) using rat liver and based on the assumption that intermolecular cross-linking would increase the amount of water-insoluble protein in the tissue block, found that the amount of protein that could be insolubilized increased as the pH raised from values nearer the physiologic range (pH 8.0) to pH 9.5.

Tzaphlidou and Chapman (1984) and Tzaphlidou (1987), with measurements of the shrinkage temperature of collagen after fixation with DMS or DMA, suggest that cross-linking by these reagents is more extensive at (or slightly above) pH 9.5 than at pH values closer to physiological. However, despite the necessity of high pH to maximize cross-linking, it cannot be assumed that all biological materials will tolerate such alkaline conditions. Although collagen (Tzaphlidou and Chapman, 1984; Tzaphlidou 1986, 1987) and rat liver (Hassell and Hand, 1974) appeared to be unaffected by the high pH, CV1 cells appeared to suffer structural changes. At higher pH values than 8.0, these cells tended to have blebs (Matthopoulos and Tzaphlidou, 1987).

BUFFERS

The degree of cross-linking is also influenced by the type of buffer. The same concentration of diimidoesters in different buffers may result in divergent cross-linking rates. According to Hassell and Hand (1974), DMS in Tris–HCl buffer is a more effective protein cross-linker than are glycine, borax, carbonate and ammediol buffers. The buffer system can cause marked variations in the appearance of the tissue, irrespective of the similar cross-linking values. The details of these effects are described elsewhere (Hassell and Hand, 1974). The same authors pointed out that the concentration of Tris–HCl appeared to affect the degree of cross-linking obtained with DMS.

These differences in cross-linking may be due to the differences in ionic and osmotic composition of the fixative solutions.

The concentration and type of buffer also play an important role in the fixation of cells in tissue culture. The concentration of Tris buffer that resulted in the best preservation of structure of DMA- or DMS-fixed CV1 cells, as judged by their appearance in the phase contrast microscope after hematoxylin/eosin staining, was found to be 0.25 M pH 8.0 (Matthopoulos and Tzaphlidou, 1987). At lower or higher Tris concentrations, the cells were retracted from their substrate and after a certain period, depending on Tris molarity, were detached.

It is worth noting that Dulbecco's phosphate buffer saline (PBS) gave a better cellular appearance than Earle's basal salt solution (BSS). Furthermore, in studies on the cytoskeleton, PBS gave better defined individual filaments in vimentin networks than Earle's BSS (Matthopoulos and Tzaphlidou, 1988).

OSMOLARITY

The osmolarity of diimidoester fixative solutions is important for satisfactory preservation of cellular structures for electron and light microscopy. The effective osmotic pressure of the fixative solution depends on the type and molarity of the buffer, the concentration of diimidoesters and the type of specimen in terms of its water content. However, it is not yet known if the contribution of diimidoesters to the effective osmotic pressure is more important than the contribution of the buffer.

Increased concentrations of diimidoesters (>15 mg/ml) generally result in increased retraction and a blebby appearance of cells (Matthopoulos and Tzaphlidou, 1987). The reason for this is that in the beginning of fixation, diimidoesters exert osmotic pressure as the water penetrates the cellular membrane more easily than them. This has as a result the movement of the water out of the cell, causing cell retraction and the presence of blebs. As the membrane becomes fixed it also becomes permeable to diimidoesters. At this stage of fixation diimidoesters will not exert any osmotic pressure. It is interesting that mitochondria treated with dimethylsuberimidate at concentrations greater than 5 mM (~ 1.4 mg/ml) did not respond osmotically when placed in deionized water (Tinberg *et al.*, 1975).

As reported by Hassell and Hand (1974) the osmolarity of dimethylsuberimidate in a 0.05 M Tris–HCl buffer is about 210 mosmols. According to the same authors, the osmolarity of dimethylsuberimidate is higher than that of glutaraldehyde.

PENETRATION

The rate of diimidoester penetration is influenced by the type of biological material, fixative concentration, pH, temperature and type of buffer vehicle.

Fixative penetration can be calculated by the expression $d = k\sqrt{t}$ (Medawar, 1941), where d = depth of fixative penetration in mm, t = fixation time in min and k = constant (coefficient of diffusibility of the fixative). Various model systems show different rates of diimidoester penetration. Dimethyladipimidate penetrates into 10% gelatin gel at a rate of 90 μm min^{-1} for the first 10 min and then drops off (Tzaphlidou and Matthopoulos, to be published). The penetration by this diimidoester into 10% gelatin gel that contains 0.5% bovine serum albumin is 150 μm min^{-1} in 10 min, declining to 18 μm min^{-1} in 2 h.

Dimethyladipimidate (DMA) penetrates faster at room temperature than in the cold. Increasing concentration of DMA from 1 to 20 mg/ml enhances its rate of penetration while concentrations greater than 20 mg/ml result in a decrease in penetration rate. Penetration is faster at a high pH. The rate of penetration is only slightly affected by a change in the molarity of the buffer (50–250 mM). Tris buffer seems to facilitate deeper penetration compared with that obtained with Dulbecco's phosphate buffer saline and Earle's basal salt solution (Tzaphlidou and Matthopoulos, to be published).

ARTIFACTS

As noted by Hayat (1986), during chemical fixation water movement contributes to swelling or shrinkage artifacts at the tissue, cell, organelle and macromolecular levels. During fixation with diimidoesters, osmotic effects as well as concentration and pH of the fixative solution may be responsible for artifacts.

In hepatocytes of liver blocks fixed with DMS and examined with electron microscopy, decreasing DMS concentration causes a progressive increase in the swelling of the Golgi saccules and

smooth endoplasmic reticulum (SER), vesiculation of the rough endoplasmic reticulum (RER), swelling and extraction of mitochondria and loss of nuclear structure (Hassell and Hand, 1974). Decreasing the pH of the fixative solution from 9.5 to 8.5 produces variable degrees of swelling of the Golgi, RER and SER and a tendency for the clumping of chromatin and apparent extraction of nuclear material. Studies with light microscopy of CV1 cells fixed with DMA or DMS show that a decrease of pH of the fixative solution from 9.5 to 8.0 results in a gradual reduction of the degree of cellular swelling. In short, structural preservation, i.e. absence of swelling and extraction of cytoplasm, no local distortions and detachment of cells from their substrate, are apparent as the pH values approach the physiological range (Matthopoulos and Tzaphlidou, 1987). As the fixative concentration decreases from 15 to 1 mg/ml cellular membranes appear dilated.

High resolution electron microscopy has shown no changes in the negative staining pattern of the DMS-fixed reconstituted collagen fibrils even at high pH, 9.5 (Tzaphlidou, 1986). Also the uptake of phosphotungstate and tungstate ions in positive staining was inhibited at *all* the positively charged residues, i.e. lysyl, arginyl and histidyl (Tzaphlidou and Chapman, 1984). The ability of collagen to take up the stain was not reduced.

ENZYMES

Studies with purified proteins have shown that enzymatic activity and immunologic properties are retained even after extensive reaction with certain imidoesters (for reviews see Hunter and Ludwig, 1972; Wold, 1972). In particular, the activity of glucose-6-phosphatase after fixation of liver with DMS appeared considerably greater than after glutaraldehyde fixation (Hand and Hassell, 1976). The activity of L-α-hydroxy acid oxidase of rat liver was also shown to be greater after DMS fixation than after glutaraldehyde fixation (Hand, 1975). Treatment of isolated mitochondrial inner membranes with DMS revealed a strong inhibition of electron transport and ATPase activity (Tinberg *et al.*, 1976). The same authors suggest that inhibition by DMS may be due, in part, to the molecular cross-linking of inner membrane components. Amidination of human liver aldehyde reductase with monofunctional *n*-alkane methylimidates increases the enzymic activity by 10–30%, whereas analogous bifunctional imidoesters cause a loss of activity of about 80% (Wermuth *et al.*, 1979). Both effects are prevented in the presence of the coenzyme NADPH or $NADP^+$ but not of the substrate 4-nitrobenzaldehyde.

USE IN IMMUNOCYTOCHEMISTRY

Several workers (McLean and Singer, 1970; Wofsy and Singer, 1963) suggest that little if any loss of antigenicity occurs after extensive amidination or cross-linking with certain diimidoesters. This is indeed an important property of these fixatives as the use of monoclonal antibodies requires the particular antigenic sites that are recognized to remain unaffected by the fixation.

Matthopoulos and Tzaphlidou (1988), by fixing monkey kidney CV1 cells with diimidoesters (DMA and DMS) and staining them with anti-vimentin monoclonal antibody, found that diimidoester-fixed cells appeared with more complexed vimentin type intermediate filament networks [Fig. 6(b,c)] than formaldehyde fixed ones [Fig. 6(a)]. In spite of this complexity, individual filaments can be distinguished even in the very complexed parts of the filamentous network.

Also, COS1 cells (SV40 transformed CV1 cells, Gluzman, 1981), fixed with DMA or DMS retained their nuclear/cytoplasmic SV40 large T antigen distribution (Matthopoulos and Tzaphlidou, 1987) as probed by the monoclonal antibody Pab 419 (Harlow *et al.*, 1981).

EFFECT AND COMPARISON OF GLUTARALDEHYDE OR FORMALDEHYDE

Aldehydes have successfully been used in electron and light microscopy to cross-link a variety of biological materials. Their use as fixatives began a long time ago (Sabatini *et al.*, 1963). As their usefulness in fine structural and cytochemical studies is great, it is interesting to compare the cross-linking and ultrastructure obtained by these fixatives with those obtained by diimidoesters.

Ultrastructurally, hepatocytes of DMS-fixed liver and glutaraldehyde-fixed liver are similar except that with the former fixative the Golgi saccules and smooth endoplasmic reticulum are dilated and mitochondrial matrices exhibit an

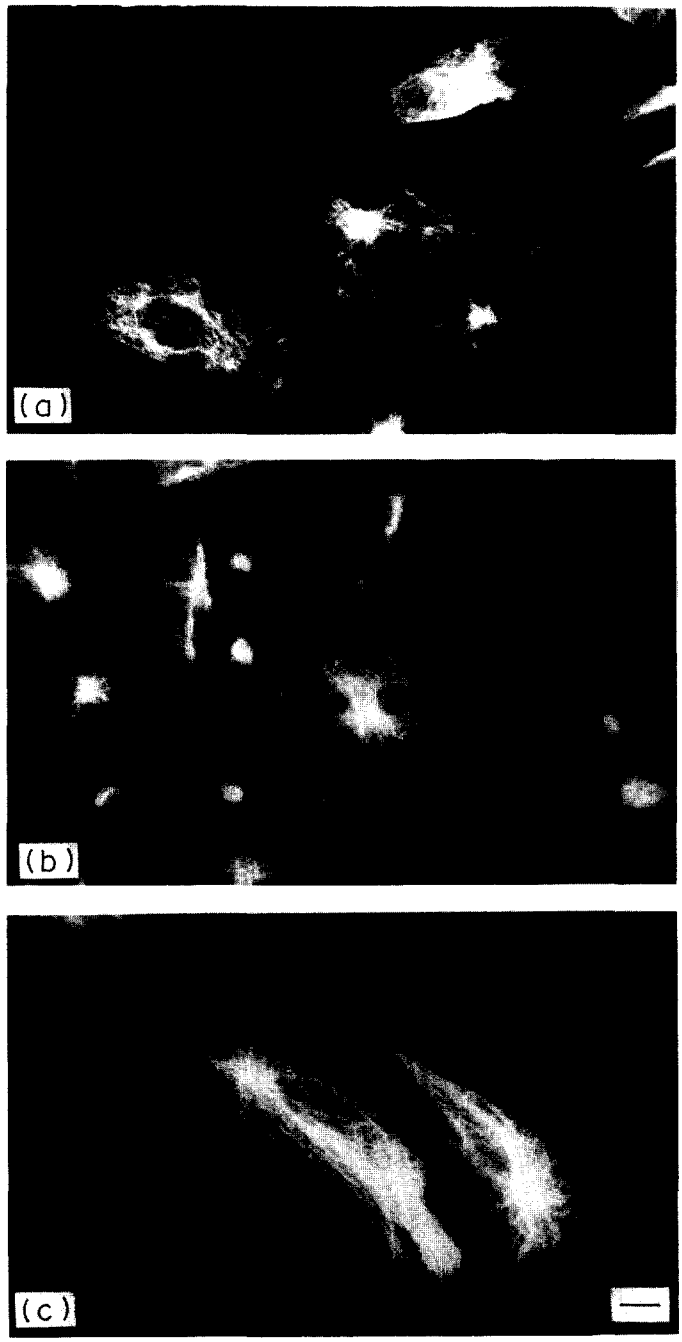

Fig. 6. Vimentin filament networks of CV1 cells fixed with: (a) formaldehyde, (b) dimethyladipimidate and (c) dimethylsuberimidate. Bar = 10 μm.

increased electron density (Hassell and Hand, 1974). The appearance of glutaraldehyde and DMS-fixed liver is more readily correlated with the degree of cross-linking than with the pH of the fixative solution *per se*. Also, it is reported by Hand (1975) that DMS maintains better structural preservation of rat hepatocytes than formaldehyde.

The fixative behavior of glutaraldehyde/DMS in combination was studied by Tzaphlidou (1983) using reconstituted fibrils of type I collagen. The same author pointed out that the structural preservation of collagen obtained using prefixation in glutaraldehyde followed by DMS fixation is less than that when using DMS and post-fixation in glutaraldehyde. Furthermore, the morphology and the distribution of electron density in the fixed collagen fibrils with glutaraldehyde differ from those in DMS-fixed fibrils (Fig. 7). Glutaraldehyde fixation of collagen is known to produce conformational changes (Meek and Chapman, 1985) while DMS fixation appears not to produce such changes (Tzaphlidou and Chapman, 1984; Tzaphlidou, 1986). Therefore, DMS may protect the collagen from those conformational changes that can be produced by glutaraldehyde when DMS treatment is followed by glutaraldehyde treatment.

CV1 cells fixed with diimidoesters, DMA or DMS, and stained with hematoxylin/eosin [Fig. 8(b,c)] show almost the same cytoplasmic appearance with cells fixed in formaldehyde [Fig. 8(a)] but the nuclear structure differs extensively. In DMA or DMS fixed cells the nucleus appears granular with thread-like structures over a transluscent background. The nucleolus, although more heavily stained, also shows a more granular appearance than when fixed

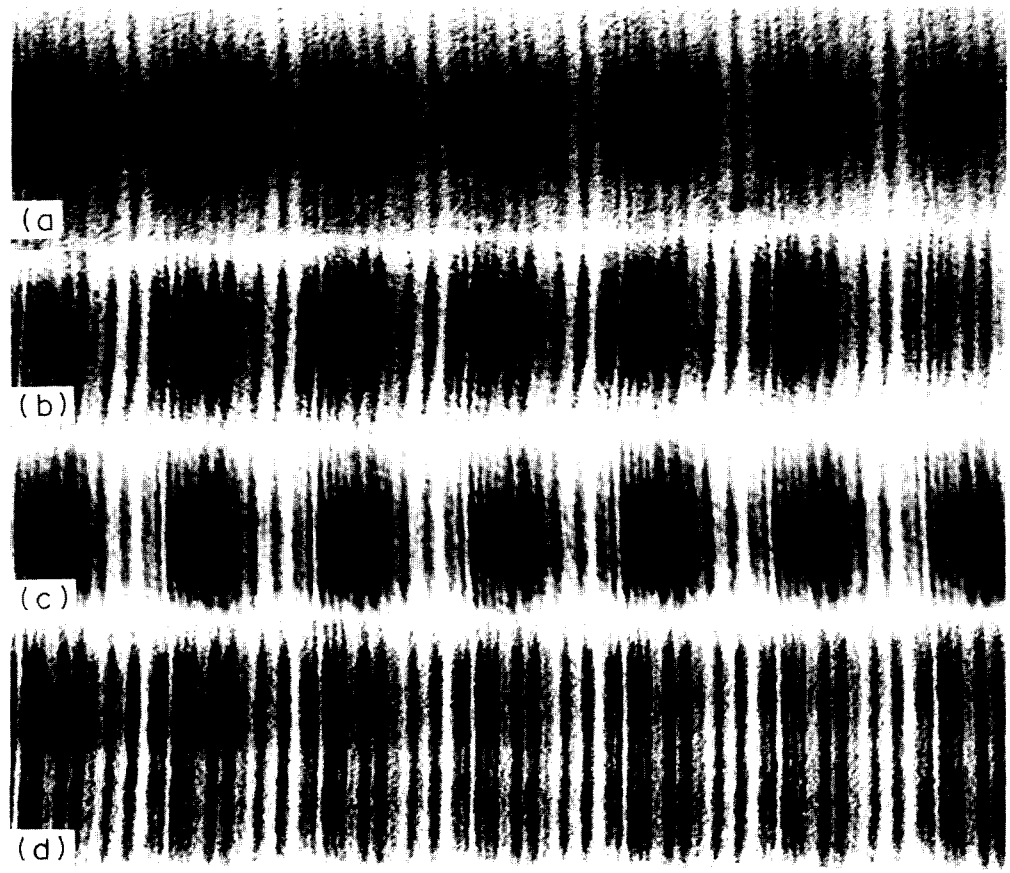

Fig. 7. Reconstituted collagen fibrils fixed with: (a) dimethylsuberimidate, (b) dimethylsuberimidate/glutaraldehyde, (c) glutaraldehyde/dimethylsuberimidate and (d) glutaraldehyde. All are positively stained with phosphotungstic acid and uranyl nitrate (× 300,000).

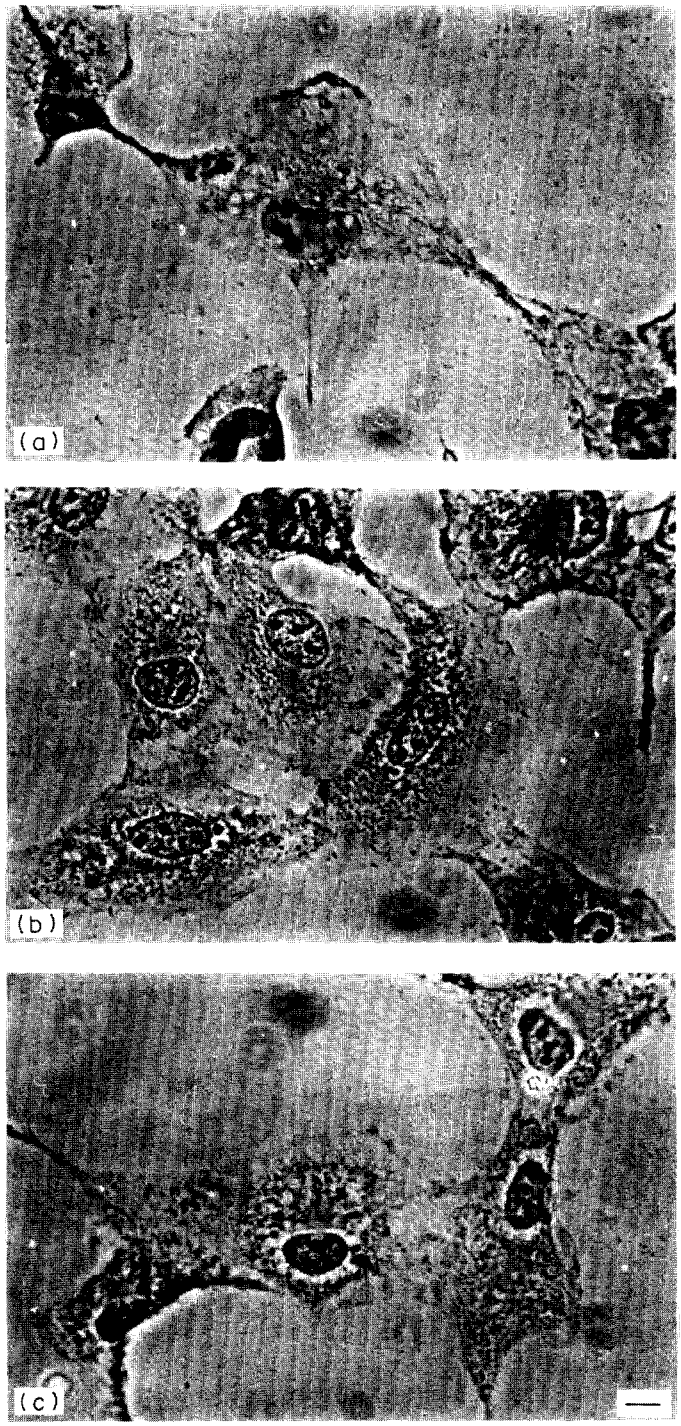

Fig. 8. Hematoxylin/eosin stained CV1 cells fixed with: (a) formaldehyde, (b) dimethyladipimidate and (c) dimethylsuberimidate. Bar = 10 μm.

with formaldehyde (Matthopoulos and Tzaphlidou, 1987). In addition, the diimidoester-fixed cells give better identifiable mitotic pictures than the formaldehyde-fixed ones.

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