ALTERATION OF THE CONFORMATION OF PROTEINS IN RED BLOOD CELL MEMBRANES AND IN SOLUTION BY FIXATIVES USED IN ELECTRON MICROSCOPY

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ABSTRACT

The effects of several commonly employed fixatives on the three-dimensional conformations of two soluble proteins and the protein of intact red blood cell membranes have been studied by means of circular dichroism measurements in the spectral region of the peptide absorption bands. The fixatives used produced significant and parallel conformational changes in all of the proteins, in the increasing order: glutaraldehyde; OsO_4; glutaraldehyde followed by OsO_4; and $KMnO_4$. The last two treatments obliterated most of the helical character of the proteins. The significance of these observations to the preparation of specimens for electron microscopy is discussed.

INTRODUCTION

The effect of chemical fixation on biological ultrastructure is a critical problem in electron microscopy. The major criterion used to determine whether the original ultrastructure had been preserved upon fixation is the reproducibility with which particular structural features are observed in specimens fixed and embedded in various ways. Recent examples of this approach are to be found in studies by Trump and Ericsson (1965) and Maunsbach (1966). While such evaluation is indispensable, it is clearly not sufficient. It is conceivable, for example, that several different fixation procedures may all produce the same structural alterations or artifacts. One would like to have independent and more direct information about the structural effects accompanying a particular fixation procedure. In this connection, a number of studies of the chemical reactions accompanying OsO_4 and $KMnO_4$ treatment of biologically important molecules have been published (see Bahr, 1954; Stoeckenius and Mahr, 1965; Korn, 1966, 1967; Hake, 1965); they have shown that chemical changes are indeed produced in such treatments.

The important role played by protein molecules in most ultrastructural elements of cells is well recognized. What is not so well appreciated is the role played by the three-dimensional folding (the conformation) of the individual protein chains in determining the structure of these elements. The specific noncovalent aggregation of protein and other molecules into an organized element must depend on the appropriate fitting together of their individual three-dimensional structures. Thus any treatment which leads to significant changes in the conformation of individual protein

chains (such as pH extremes, urea, or various chemical modifications) always results in the disorganization and disruption of the structure of the organized element containing these chains (see Reithel, 1963). Even if chemical cross-linkages are introduced between the individual protein molecules, as by certain fixation treatments, these linkages can not be expected to maintain the structure of the organized elements against the effects of protein denaturation any more than the naturally occurring disulfide bridges of individual protein molecules can protect them from the conformational changes produced by denaturing treatments.

From these considerations, it follows that a rational approach to the problem of fixation is the determination of the effect of different fixatives on the conformation of protein molecules. This information should be even more pertinent than direct chemical studies, because some kinds of chemical modification produced by fixation might have important ultrastructural effects and others might not. Fixation procedures which produce the least alteration in protein conformation, other things being equal, are clearly to be preferred for ultrastructural studies.

In recent years, direct investigation of the conformation of protein molecules has been greatly facilitated by the techniques of optical rotatory dispersion (ORD) and circular dichroism (CD) in the ultraviolet. In particular, CD in the spectral region from 190 to 240 mμ, in which a major peptide bond absorption band occurs, has become exceedingly useful in protein structural studies (see Holzwarth and Doty, 1965; Beychock, 1966). Different protein conformations are characterized by markedly different CD spectra; in addition, changes in the conformation of a particular protein can be detected and quantitated by changes in its CD spectrum. Furthermore, as has recently been shown (Lenard and Singer, 1966; Wallach and Zahler, 1966) characteristic ORD and CD spectra of the proteins in intact cell membranes and membrane fragments can be obtained.

In this paper, we report the effects of four commonly used fixation procedures on the CD spectra of two soluble proteins, bovine serum albumin (BSA) and sperm whale apomyoglobin (WAM), and of the protein of intact human red blood cell membranes. OsO_4, $KMnO_4$, glutaraldehyde, and glutaraldehyde followed by OsO_4 were the fixative reagents used. Significant changes in protein conformation were produced, and the bearing of these results on specimen preparation for electron microscopy is discussed.

MATERIALS AND METHODS

Human red blood cell membranes were prepared by the method of Dodge et al. (1963). BSA was obtained from Armour Pharmaceutical Co., Kankakee, Ill. Apomyoglobin was prepared from sperm whale myoglobin (Mann Research Labs Inc., New York) by the method of Teale (1959).

Fixation treatments were carried out by mixing together equal volumes of either the protein solution or membrane suspension in 0.008 M phosphate buffer, pH 7.7, and one of the following in 0.132 M phosphate buffer, pH 7.4; 4% OsO_4, 2% $KMnO_4$, or 6% glutaraldehyde. Treatments with OsO_4 and with $KMnO_4$ were carried out at 4° C for 30 min, while treatment with glutaraldehyde was performed at room temperature for 2 hr. The treated preparations were washed by dialysis against 0.008 M phosphate, pH 7.7. In the double-fixing experiments, the glutaraldehyde-fixed material was fixed with OsO_4 after it had been washed as described above. In one experiment the membranes were washed by repeated centrifugation and resuspension with very similar results.

The CD spectra are presented as the mean molar ellipticity, $[\theta]$, as a function of wavelength (see Beychock, 1966). For the determination of $[\theta]$, the protein concentration in the solution is required. Protein concentrations on all samples except those fixed with permanganate were determined from Kjeldahl nitrogen analyses. For the BSA solutions, the determination was made directly by assuming that the protein contained 15% nitrogen and had a mean amino acid residue weight of 114. The nitrogen content of the membranes was related to protein content by amino acid analysis on a Beckman Model 120B amino acid analyzer. 1 μmole of amino acid residue (mean residue weight 114) was found to correspond to 19 μg of nitrogen. The color produced by permanganate treatment interfered with the Kjeldahl determinations, so the concentrations of these samples were calculated from the concentration of stable acidic and neutral amino acids as determined by amino acid analysis.

CD measurements were made on a Durrum-Jasco UV-5 spectropolarimeter (Durrum Instrument Corp., Palo Alto, Calif.). All CD measurements reported, except those in 90% 2-chloroethanol, were obtained by using a cell of 0.5 mm path length and a 1 mm slit width at a concentration of 2.5–3.2 μmoles of amino acid residues per milliliter. The CD measurements in 90% 2-chloroethanol were obtained by diluting the aqueous samples with 9 vol of 2-chloro-

ethanol (Lenard and Singer, 1966) and by using a cell of 5.0 mm path length.

RESULTS

The CD spectra of untreated apomyoglobin (Breslow et al., 1965) and BSA both show two minima in the ultraviolet region, at 208 and 222 mμ (Figs. 1, 2). These two minima arise from, and uniquely characterize, the α-helical portions of the protein chain (see Beychock, 1966). The CD spectrum of unfixed human red cell membranes (Fig. 3) also shows the two minima characteristic of a helical protein. In this case, however, the minima are somewhat altered, both in position and in relative magnitude, from the spectrum observed with soluble helical proteins such as BSA and apomyoglobin. These findings have been reported and discussed in detail elsewhere (Lenard and Singer, 1966).

As seen in Figs. 1–3, treatment of all three protein preparations by each fixative resulted in significant alteration of the CD spectra. $KMnO_4$ treatment changed the spectra most drastically, completely obliterating the characteristic helical minima. Treatment with glutaraldehyde, OsO_4, or glutaraldehyde followed by OsO_4 decreased the depths of the CD minima. The decrease in helix content produced in each protein by each of these fixatives was estimated from the magnitude of the minimum at 222–224 mμ (Table I). It will be noted that for each protein the residual helical content varied with fixative in the same order: no fixative > glutaraldehyde > OsO_4 > glutaraldehyde plus OsO_4.

In addition, the CD spectra in 90% 2-chlorethanol of the untreated and variously treated red blood cell membranes were obtained. We have

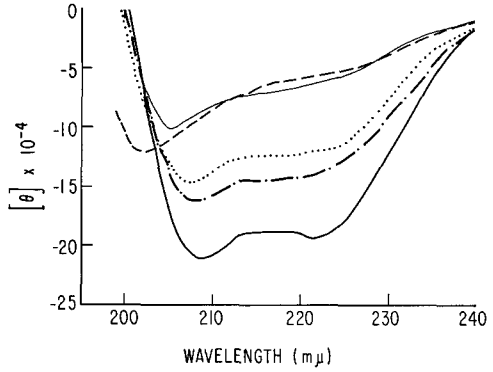

FIGURE 1 Circular dichroism spectra of bovine serum albumin in 0.008 M phosphate buffer, pH 7.7. Untreated, thick solid line; glutaraldehyde-treated dashed and dotted line; OsO_4-treated, dotted line; glutaraldehyde- plus OsO_4-treated, thin solid line; and $KMnO_4$- treated dashed line.

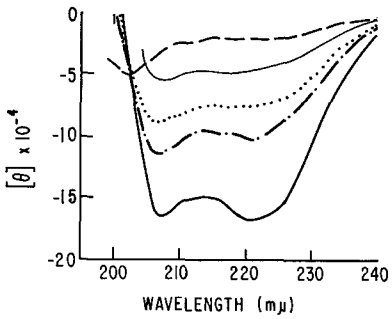

FIGURE 2 Circular dichroism spectra of whale apomyoglobin in 0.008 M phosphate buffer, pH 7.7. See legend of Fig. 1.

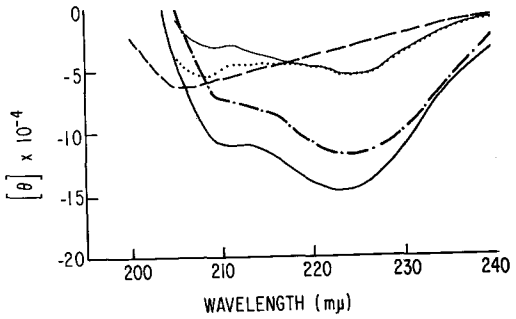

FIGURE 3 Circular dichroism spectra of red blood cell membranes in 0.008 M phosphate buffer, pH 7.7. See legend of Fig. 1.

TABLE I
Helical Loss on Fixation

Protein	Helix lost		
	Glutaraldehyde	OsO_4	Glutaraldehyde plus OsO_4
	%	%	%
Red blood cell membranes	22	63	70
BSA	26	39	70
WAM	29	55	72

shown earlier, from CD spectra (Lenard and Singer, 1966), that the membrane protein assumes a much more helical conformation in this solvent than it does in the intact membrane in aqueous suspension. A decrease in the helix-forming capacity of the membrane protein in 2-chloroethanol after fixation treatment is therefore another measure of chemical changes produced in the protein by the fixative. The results shown in Table II are consistent with those in Fig. 3 in that they indicate that glutaraldehyde does affect the structure of the membrane protein but considerably less so than does OsO_4 treatment. Prior treatment with glutaraldehyde does not protect against the effect of OsO_4.

DISCUSSION

The CD spectra shown are primarily determined by, and highly specific for, the helical portions of the protein chains in the solution. In particular, other optically absorbing species in these solutions (as, for example, the lipids in the red blood cell membranes[1]) or new chromophores introduced by the fixation treatments (as with $KMnO_4$ treatment) do not influence the CD spectra significantly in the spectral range of the peptide absorption band. The reductions in the magnitude of $[\theta]$ upon fixation treatments in all cases must therefore be due to a disruption of some helical portions of the protein molecules and their conversion to a more random conformation. (The random coil form of a polypeptide chain is characterized by a very small value of $[\theta]$ above 210 mμ; see Holzwarth and Doty, 1965).

TABLE II

$[\theta]$ of Membrane Protein In 90% 2-Chloroethanol Before and After Fixation

Fixative	$\theta_{222\ m\mu}$
None	−22000
Glutaraldehyde	−17400
OsO_4	−7300
Glutaraldehyde + OsO_4	−6400

The results indicate that the different fixative treatments caused similar relative changes in molecular conformation with all three protein systems that were examined. With each protein, glutaraldehyde treatment induced some significant structural change in aqueous solutions, but less than that resulting from OsO_4 treatment; glutaraldehyde followed by OsO_4 caused larger structural changes than either reagent alone; and $KMnO_4$ had the most drastic effect.

The magnitudes of the conformational changes particularly those induced by glutaraldehyde followed by OsO_4, or by $KMnO_4$, were as great as, or greater than, those which would be induced in these proteins by powerful denaturants such as 8 M urea. Also to be noted is the fact that the presence of the membrane protein in an organized structure in no way protected it from the effects of the fixatives.

The changes induced by OsO_4 and $KMnO_4$ were presumably due to chemical changes in the primary structure of the protein chains; this is suggested by the fact that after fixation the helicity attained by the membrane protein in 2-chloroethanol solution was reduced far below that of the unmodified protein in this solvent. These findings are consistent with the chemical studies of Hake (1965), who showed that both OsO_4 and $KMnO_4$ treatment of proteins liberated NH_3 and oxidized a number of amino acid side chains as well as disulfide bridges.[2]

It is gratifying that by ultrastructural criteria alone glutaraldehyde has come to be widely accepted as a most satisfactory fixative, since our studies indicate that among the commonly employed fixatives it causes the least protein conformational change. On the other hand, our results suggest that even this reagent causes some significant structural modification in the proteins studied. In this connection, the recent observation by De Jong et al. (1967) is of interest. They found, that by an unknown mechanism,

[1] It has been claimed (Urry et al., 1967) that β-lecithin contributes significantly to the CD and ORD spectra of red blood cell membranes in the spectral region of the peptide absorption band. This was shown, however, by Lenard and Singer (1966) not to be true.

[2] The choice of BSA and WAM as the two soluble proteins for this study was made in part because the former has a number of intrachain disulfide bridges and the latter has none. From the similar structural effects produced by a given fixative on the two proteins, one can conclude that the alterations are not solely attributable to the oxidation of disulfide bridges.

glutaraldehyde fixation activates a nuclear acid phosphatase enzymic activity, and they suggest that caution be exercised in the use of glutaraldehyde as a routine fixative in cytochemistry.

Furthermore, however, our results indicate that OsO_4 following glutaraldehyde treatment induces very marked conformational changes in the proteins examined. In the case of the red blood cell membrane, this double fixation treatment caused as much structural damage as that produced by $KMnO_4$. These results therefore suggest that this double fixative treatment (see Trump and Bulger, 1966) be viewed with some suspicion.

The extensive protein conformational changes produced by $KMnO_4$ are disturbing. This fixative has been used particularly, for example, to elicit reproducibly the "railroad track" unit membrane structure of a wide variety of biological membranes (Robertson, 1963). It is entirely possible, however, that this structure is markedly different from that of an unmodified membrane since the transformation results from the profound chemical and structural alterations induced in the membrane protein by this fixative.[3] For example, if the membrane protein is converted by reaction with $KMnO_4$ largely into a random coil form and if, in addition, some of its hydrophobic side chains are made more hydrophilic by oxidation, then it is possible that the preferred structure for the protein-lipid system would be one in which the protein lies flat and extended on the surface of a bimolecular lipid leaflet. In contrast, the lipoprotein structure in the native state might be an integrated one such as we have proposed (Lenard and Singer, 1966). One must seriously question, on these as well as other (Korn, 1966, 1967) grounds, the significance of micrographs of $KMnO_4$-fixed specimens.

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[3] One must also consider structural alterations that may be caused by the embedding procedure. In most polymer embedding, the monomers are nonaqueous liquids which most likely can induce conformational changes in protein molecules (see Singer, 1962). The change in the conformation of the protein of unfixed red blood cell membranes dissolved in 2-chloroethanol (Table II) is an example of this effect.