OXIDATION OF LIPIDS AND MEMBRANES II: Effects of Oxidants on Cerebral White Matter Homogenates

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Bovine cerebral white matter homogenates were oxidized by various oxidizing solutions of equal molarity and subsequently extracted with water. Most of the oxidants tested (K-dichromate, $FeCl_3$, H_2O_2, O_2, and chloroperbenzoic, ascorbic, performic, and periodic acids) rendered the various myelin constituents less extractable than the constituents of unoxidized control homogenates. $KMnO_4$, and to a lesser degree hemoglobin, rendered myelin constituents more extractable with water than those of the control homogenates. The findings indicate that most of the oxidants produced stabilization of the lamellar pattern, probably through cross-linking and polymerization. $KMnO_4$ and hemoglobin caused labilization and breakdown of the membranous structure. Proof that stabilization of membranes is caused by some oxidants and that fragmentation occurs by the action of $KMnO_4$ and hemoglobin was obtained by the light-scattering technique and by electron microscopy of the oxidized homogenates. Evidence obtained indicated that formation of hydrophobic end groups during oxidation favors polymerization, while prevalence of hydrophilic groups is associated with fragmentation.

INTRODUCTION

Oxidation is known to disrupt different cellular membranes. Tappel and Zalkin (1) have shown that oxidation can labilize mitochondrial membranes. Other studies have shown that lipid peroxidation may cause rupture of different cellular membranes, such as microsomes and lysosomes (2, 3). Mengel and Kann (4) have found that lipid peroxidation of erythrocyte membranes increased the fragility of these cells and caused hemolysis. A similar effect — increased permeability and subsequently labilization — has been observed in a study of oxidation of artificial lipid membranes by hydrogen peroxide (5).

It is known that oxidation, in addition to having a destructive effect on membranes, may sometimes result in the formation of stable polymers. In fact, oxidizing agents have been used for years in histology and in electron microscopy as stabilizers of lipids and membranes. Fixation with OsO_4, K-dichromate, or $KMnO_4$ may preserve membrane structure by polymerization of the constituents and formation of insoluble complexes. In vitro studies have also shown that oxidation of unsaturated lipids may cause stabilization of membranes and polymerization (6, 7). Furthermore, oxidation in vivo is known sometimes to result in polymerization rather than in fragmentation of lipid micelles with consequent formation of lipid pigment granules.

The present paper deals with the effects of various oxidants on biological membranes and mainly with attempts to elucidate conditions inducing peroxidative polymerization and those causing breakdown of membranes. White matter of brain which contains mainly myelin is known to consist of a concentrate of membranes. It has, therefore, been chosen as a substrate for the study of the effects of different oxidizing agents. As oxidants are known to affect various molecular groups differently, with resultant fragmentation or polymerization (8), the effects of a number of different oxidants on white matter have been studied.

White matter of brain has been previously used for studies of phase transitions in this laboratory. Evidence obtained indicated that myelin can be changed into o/w and w/o types of emulsions as a result of changes in the ionic milieu (9). These emulsions were expected to differ in their reaction to oxidants, as chaotropic agents were shown to increase the sensitivity of membranes to oxidation (10). In the present study increased extractability of lipids with water was considered an indication of decreased stability and fragmentation of membranes. Decreased extractability with water was considered a presumptive indication of polymerization. Further checks on these conclusions were made by examining the materials for autofluorescence, by solubility tests, by electron microscopy, and by light-scattering techniques.

MATERIALS AND METHODS

Brains of freshly slaughtered cows were obtained at the abattoir, and white matter was dissected out and homogenized in a Virtis homogenizer.

1. In one series of experiments 500 mg aliquots of white matter homogenate were suspended in 2.5 ml of either 1 M NaCl or M/2 $CaCl_2$ to which different oxidants were added, mostly in a final concentration of M/200. The oxidants used were: K-permanganate, K-dichromate, ferric chloride, chloroperbenzoic acid, ascorbic acid, hemoglobin, H_2O_2 1%, and O_2 (which was bubbled through the solutions). As a control $M/10^6$ of BHT was used. After 10—15 min the homogenates were dialyzed for 46 hr at 4°C against distilled water containing the same oxidant at the same concentration with 2 changes of the dialysis fluid. In other experiments the oxidant concentrations were varied. In all experiments the homogenates were exposed to the oxidants for about 48 hr. The contents of the dialyzed bags were centrifuged in a refrigerated centrifuge at 14,500 g for 10 min. Aliquots of the supernatant and of the sediment were taken and the supernatant was examined as described later. Aliquots (200 mg) of the sediments were then extracted 4 times with 1 ml of distilled water with centrifugal separation of the supernatants as in previous experiments (9). Three fractions were obtained: dialysis bag supernatant, supernatant obtained after extractions with water of the dialysis sediment, and the final sediment. All three fractions were examined for protein, phospholipid, cerebroside, and cholesterol as above (9). The amounts of constituents present in both supernatants were added and called the extractable fraction, while the amount of constituents present in the final sediment represented the nonextractable fraction of the samples. Each of these experiments was repeated 3 or 4 times and the average of the data is given in Figs. 1 and 2.

2. In other experiments the homogenates with the salts and some oxidants were

left at 4°C without dialysis and extractions. After 24 and 72 hr the tubes were examined for the presence of fluorescence when exhibited by UV light at 254 nm.

3. Some sediments obtained after dialysis and extractions with water were fixed with 3% buffered glutaraldehyde, postosmicated, dehydrated, and embedded in Epon. Thin sections were double stained with uranyl acetate and lead citrate and examined electron microscopically.

4. In one series of experiments the contents of the dialysis bags were centrifuged for 20 min at 14,500 g. The sediments were lyophilized and 100 mg aliquots were extracted with 20 ml of butanol. The insoluble residues were again dried and weighed, yielding the amount of treated homogenate which was insoluble in butanol. The results were checked against the weight of the residue obtained by drying an aliquot of 2 ml of the butanol.

On the advice of Prof. A. Katchalsky-Katzir of the Polymer Department, the Weizmann Institute of Science, Rehovoth, the butanol solutions were studied by the light-scattering technique in order to estimate the size and form of the solute micelles. The measurements and calculations were all kindly performed by Dr. Emil Reisler following the technique used in that laboratory (11).

5. In another series of experiments the amount of peroxides in the oxidation mixtures were determined by a thiobarbituric acid procedure (12). To 20 mg of homogenate 4 ml of 10% trichloroacetic acid were added. After centrifugation 4 ml of the supernatant were separated and 1.25 ml of 0.75% thiobarbituric acid were added. The tube was heated for 10 min in a boiling water bath and after cooling the color was read with a Klett photometer with a No. 52 filter.

RESULTS

As can be seen in Fig. 1 the various oxidants at M/200 concentration differ in their tendency to form complexes which resist extraction with water. Considering BHT as point of reference, most oxidants tested rendered myelin constituents less extractable with water and seemed, therefore, to enhance stabilization of structure. Only $KMnO_4$ seemed to render myelin constituents more extractable than normal, with H_2O_2 and hemoglobin having the least stabilizing activity. The order in which the various oxidants promoted stabilization did not seem to correspond to the order of oxidation potentials of these oxidants (6). The differences between emulsions containing NaCl and those containing $CaCl_2$ were not significant in most instances. Figure 2 shows the effect of variations in the concentration of oxidants. With the compounds tested smaller oxidant concentrations reduced extractability with water and presumably, therefore, induced more polymerization than the higher oxidant concentrations.

Examination of oxidized homogenates for fluorescence (using $KMnO_4$, $K_2Cr_2O_7$ $FeCl_3$, BHT, O_2, H_2O_2, and hemoglobin) revealed that the homogenates treated with $KMnO_4$ and hemoglobin did not exhibit any fluorescence, presumably because of quenching due to an inner filter effect (13). Dichromate oxidation produced the most intense fluorescence and ferric chloride followed, with the homogenate containing BHT being third in intensity. All the other oxidants produced less fluorescence than BHT. There were no obvious differences between the NaCl- and $CaCl_2$-treated homogenates.
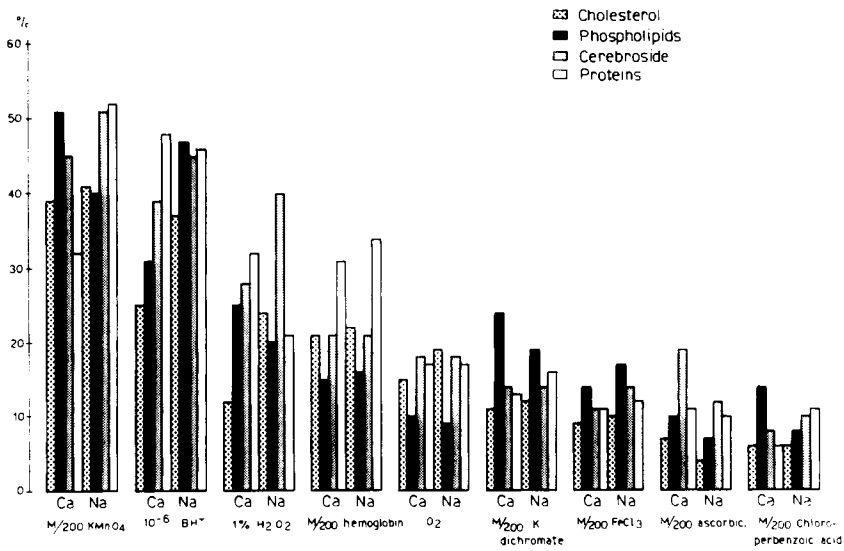
Fig. 1. Extractability of bovine white matter constituents by NaCl and CaCl$_2$ solutions after oxidation with different agents.
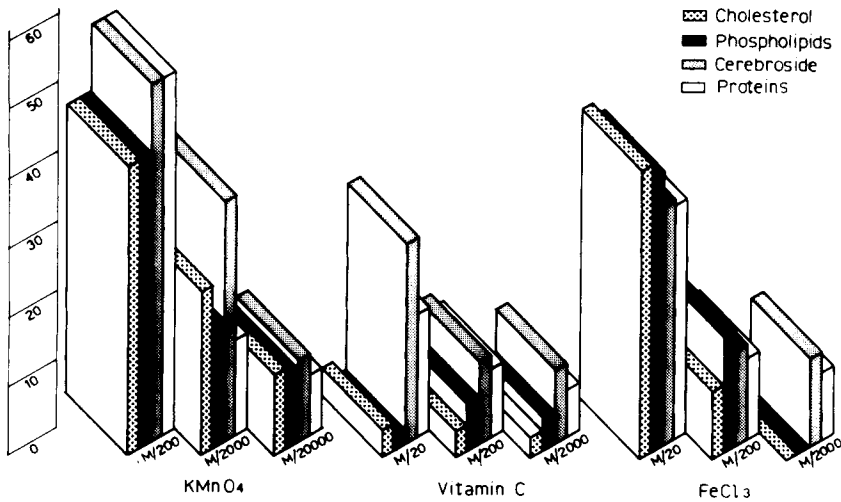

Fig. 2. Extractability of bovine white matter constituents by M/1 NaCl after oxidation with different concentrations of three oxidants. The scale on the left indicates the percentage of total constituents which was extracted.

Electron microscopic study of the sediments of homogenates prepared with BHT revealed membranous structures with typical myelin pattern — namely, alternating main dense and intraperiod lines with periodicity of about 130 Å (Fig. 3). A similar preservation of myelin structure was obtained in homogenates oxidized with FeCl$_3$, K-dichromate, chloroperbenzoate, and ascorbic acid.
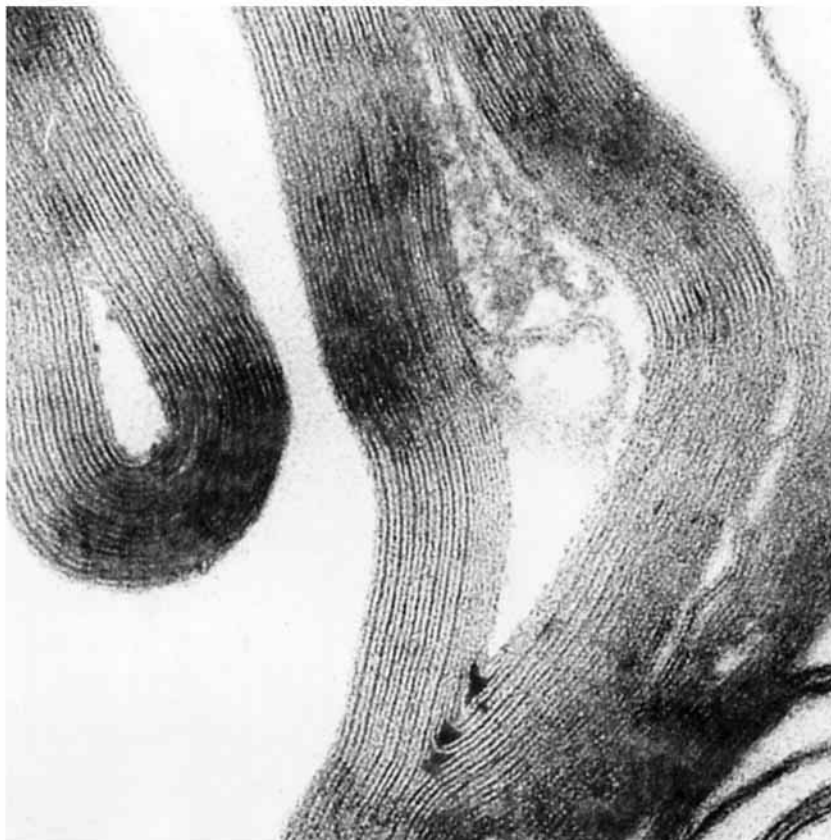
Fig. 3. Electron micrograph of cerebral white matter homogenate treated with BHT 10^{-6} M. The 130 Å lamellar periodicity typical of myelin with major dense and intraperiod lines is obvious (× 131,000)

In homogenates oxidized with K-permanganate most of the material lost its membranous structure and appeared either finely granular or in short and slender rods about 430 Å in length and about 40 Å in diameter. In a few places scanty fragments with myelinlike pattern were discerned. Also, these preserved fragments showed different degrees of distortion of the lamellae. In such areas transition between the orderly lamellar pattern and granular appearances could be seen (Figs. 4 and 5).

Oxidation of homogenates by hemoglobin resulted in preservation of lamellar structures in part of the material. It seemed that there was a smaller loss of membranous structure than in the homogenates treated by permanganate. In many places the regular architecture continued with a band made of granular material (Fig. 6). No rodlike structures were seen in these micrographs.

The presence of either NaCl or $CaCl_2$ in the various oxidizing solutions did not produce any remarkable differences in the electron microscopic appearances.

Extraction with butanol of the oxidized homogenate yielded completely different results from the extraction with water. As can be seen in Fig. 7 in the homogenates treated with NaCl 82% of the total dry weight was extracted after oxidation with $FeCl_3$, while

Fig. 4. Electron micrograph of cerebral white matter homogenate treated with $KMnO_4$ The lamellar pattern is replaced by granular material which is aggregated in some places in a distorted lamellar pattern. ($\times$ 36,000)

only 10% was extracted after permanganate oxidation. The differences in solubility of the constituents in butanol (Fig. 7) seem to follow an inverse pattern to that observed with water (Fig. 1). In most instances solubility in butanol was higher in homogenates treated with NaCl than in those oxidized in the presence of $CaCl_2$.

The light-scattering studies made on butanol solutions of oxidized homogenates were evaluated in terms of the conventional quantity of $\dfrac{K}{\triangle R \theta}$. ($\triangle R\theta$ is the reduced scattering intensity, corrected for solvent contribution, at angle θ with the incident beam, and K is equal to $\dfrac{2\pi^2 n^2}{\lambda^2 N_A}$, when n is the refractive index of the medium, λ is the wavelength in vacuo, and N_A is Avogadro's number). The values of $\dfrac{K}{\triangle R \theta}$ were plotted against $\sin^2 \dfrac{\theta}{2}$ and extrapolated to zero angle, yielding, after multiplication by the concentration of the sample, approximately $\dfrac{1}{\text{mol. weight}}$. In order to make these
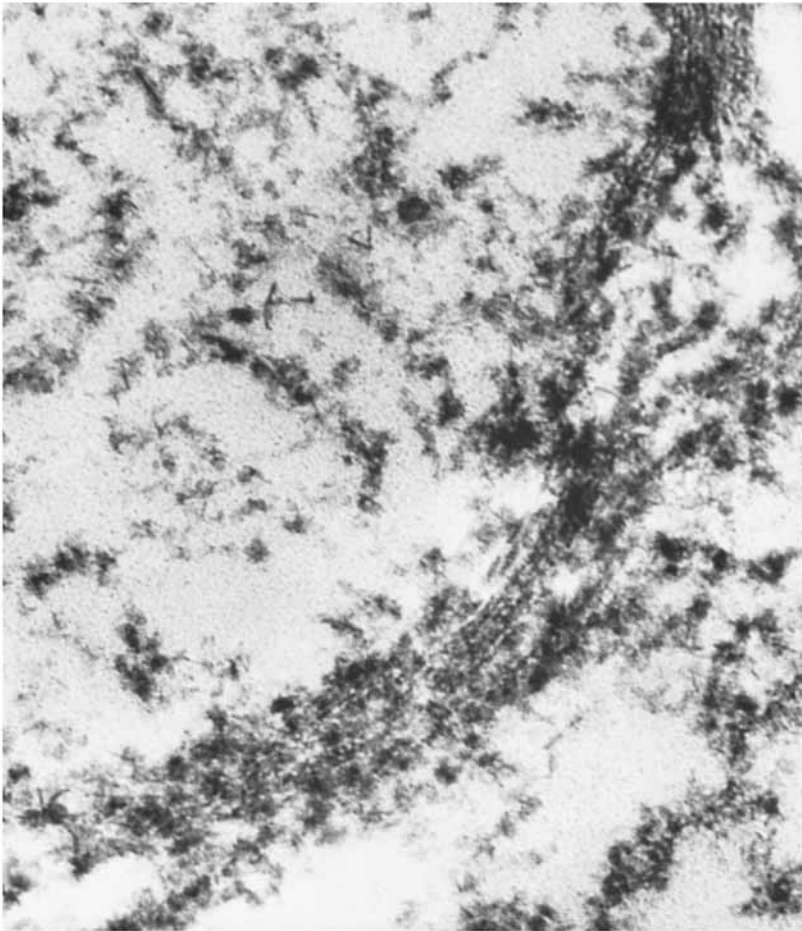
Fig. 5. High-power electron micrograph of same material as in Fig. 4. The lamellar pattern is replaced by granules and rods which are arranged in some places in parallel arrays. ($\times$ 120,000)

evaluations possible, it has been assumed that all the samples examined have the same refractive index increment. The obtained values of $\dfrac{1}{\text{mol. weight}}$ are presented in Fig. 8. It is obvious that this fraction, which represents approximately $\dfrac{1}{\text{average mol. weight}}$, is highest in the homogenate oxidized by K-permanganate and lowest in that oxidized by $FeCl_3$. The findings, therefore, again indicate that $KMnO_4$ breaks down myelin to small lipid-soluble particles, while the products of oxidation by $FeCl_3$ are the largest, or most polymerized.

The plots of light scattering also gave an indication of the degree of dissymetry of the oxidation products. The dissymetry was calculated by dividing the voltage at $\sin^2 \dfrac{\theta}{2} = 1$
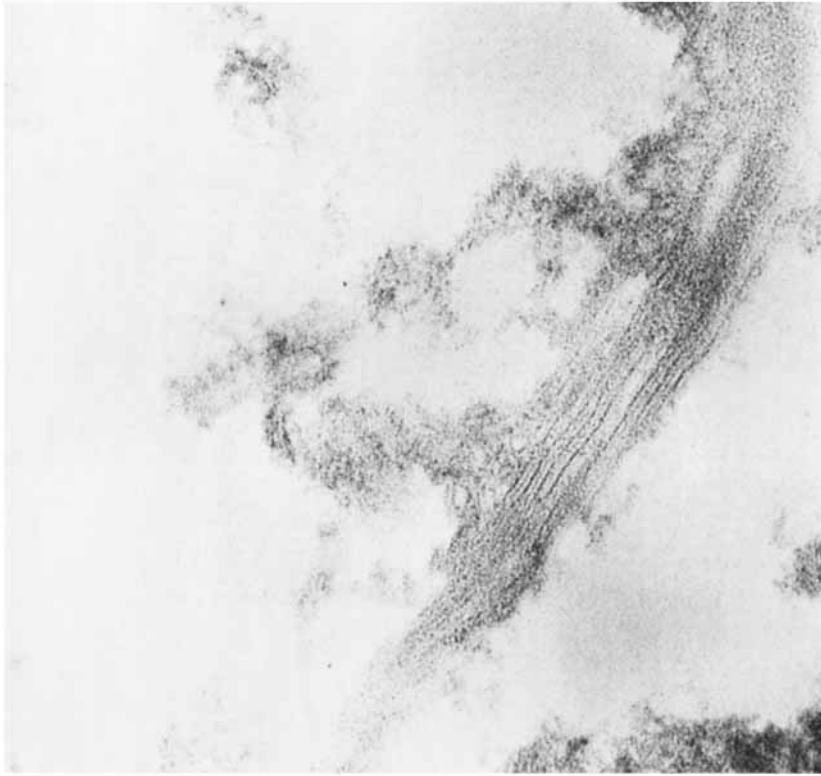
Fig. 6. Electron micrograph of white matter homogenate treated with hemoglobin. In some areas the lamellar pattern is preserved. In others it is replaced by a granular pattern. ($\times$ 120,000)

by the voltage at the $\sin^2$ value of zero and it roughly indicates the degree of departure of the micelle from spherical or compact conformation. The lowest degree of dissymetry (about 3 units) was obtained in the Na-BHT sample, followed by the samples oxidized by $FeCl_3$. The highest dissymetry was observed in the samples oxidized by ascorbic acid and by K-permanganate (10–14 units).

The changes with time in the concentration of peroxides in homogenates oxidized by different agents are shown in Fig. 9. The slight increase in peroxides observed in the homogenate treated by ascorbic acid (M/200) and H_2O_2 (1%) did not appreciably differ from the curve observed in the homogenate without any additions. With all the other oxidants, whenever the estimates were available for both 24 and 48 hr, the peroxide curve was biphasic. With $KMnO_4$ the amount of peroxide was higher in the homogenate oxidized with an M/200 than with the higher or the lower concentration. With $FeCl_3$ the higher oxidant concentration yielded higher lipid peroxide level.
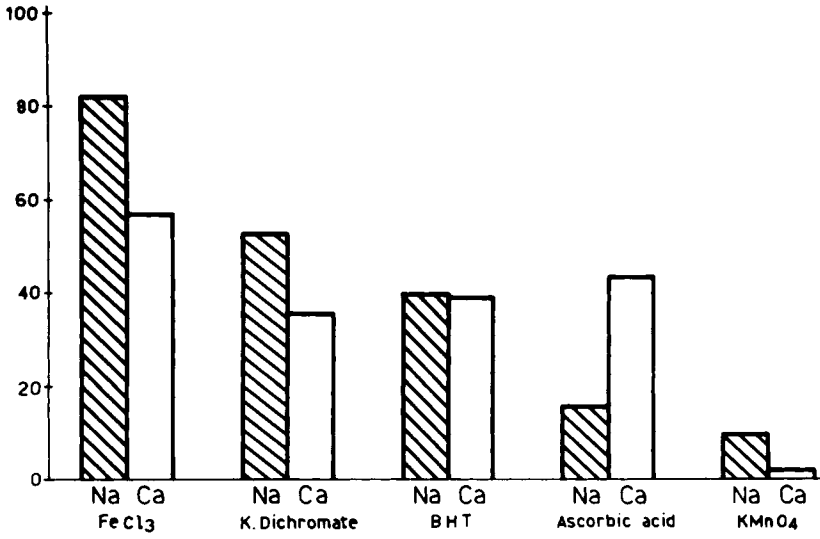
Fig. 7. Extractability by butanol of white matter constituents after oxidation with different agents in the presence of 1 M NaCl or M/2 CaCl$_2$. The scale on the left indicates the weight of the butanol-soluble substance as a percent fraction of the total dry mass.
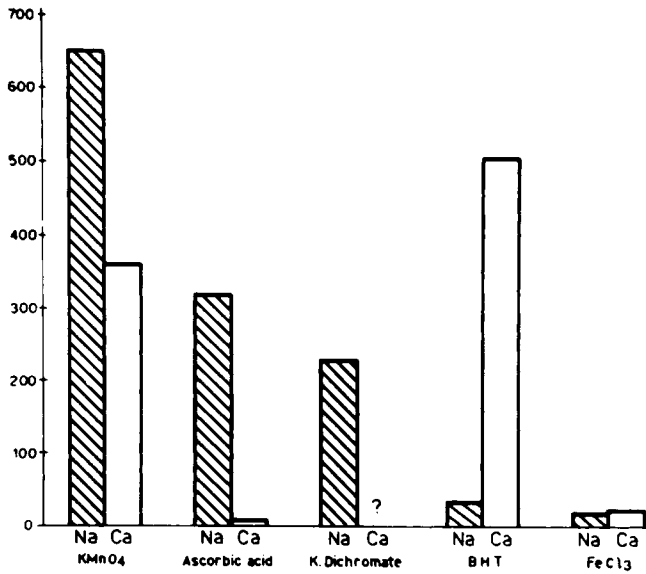

Fig. 8. Light-scattering values of butanol solutions of white matter homogenates treated with different oxidizing agents in the presence of 1 M NaCl or M/2 CaCl$_2$. The scale on the left represents $\dfrac{1}{\text{mol. weight}}$ of the dissolved material (see text).
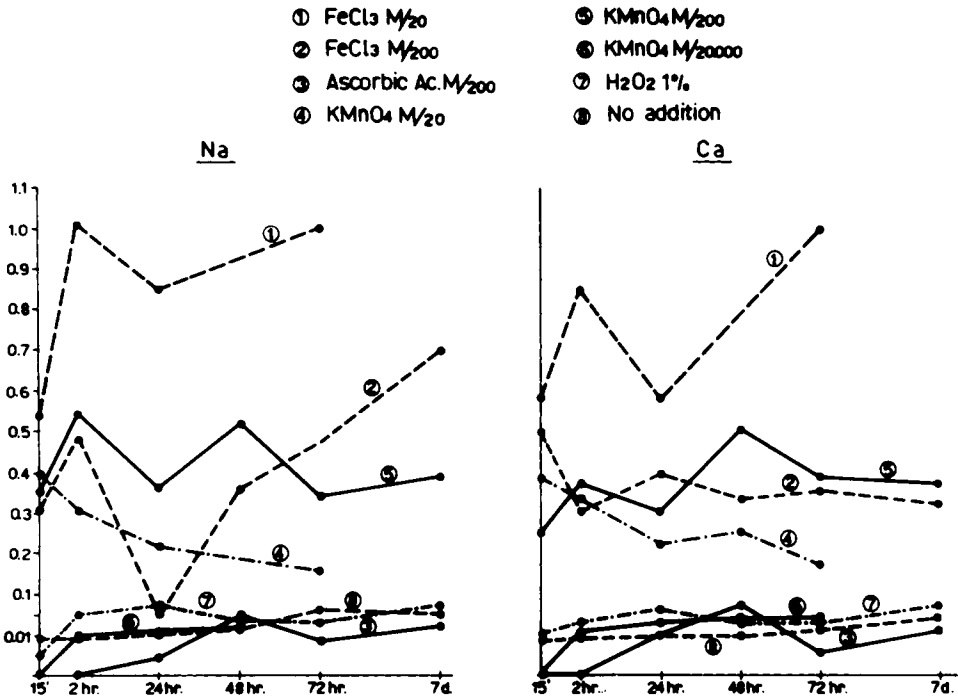
Fig. 9. Concentration of lipid peroxides as a function of time in white matter homogenates treated with different oxidizing agents in the presence of 1 M NaCl (left) or M/2 CaCl₂ (right). The scale on the left represents arbitrary photometric units.

DISCUSSION

The oxidants used in this study were shown to differ in their effects on white matter constituents. The extraction experiments (Fig. 1) showed that in the conditions used most oxidants inhibited extraction with water. Only $KMnO_4$ enhanced extractability with water, while H_2O_2 and hemoglobin had a relatively mild effect. In some instances (with $KMnO_4$ and with BHT) emulsions containing NaCl (presumably of the o/w type) contained more extractable moieties after oxidation than did emulsions containing $CaCl_2$ (presumed to be of the w/o type).

The meaning of the extractibility with water of oxidized homogenates is presumed to be as follows. The emulsions which can be extracted with water contain micelles with hydrophilic groups on the surface. The tendency of emulsions to resist aqueous extraction is probably due to formation of cross-links among molecules and to polymerization. Breakdown of the membranes, or of already formed polymers, to small units with polar groups on the surface will facilitate extraction with water. In view of these considerations it appears that K-permanganate and to some extent H_2O_2 and hemoglobin caused mainly breakdown of membranes, whereas the other oxidants aided polymerization. The electron microscopic findings confirmed these conclusions. The products of oxidation breakdown of myelin in the material treated by $KMnO_4$ in the electron micrographs were of two

types, granules and rods. After hemoglobin oxidation the breakdown products were entirely of the granule type. It is possible that the rodlike structures in the permanganate-oxidized homogenate represent precipitates of a manganese compound.

The findings presented in Fig. 2 indicate that lower oxidant concentrations are associated with increased polymerization and decreased breakdown. Also the light-scattering data confirm the conclusion that $KMnO_4$ is associated with breakdown of the membranes, while most other oxidants cause mainly cross-linking and polymerization.

As none of these effects was of the "all or none" type it appears that oxidative processes produce both stabilization and breakdown. The nature of the oxidizing agent, its concentration, and possibly other factors may enhance or inhibit the one or the other process. The two processes may follow each other, as was shown more than 30 years ago (14) in a study of acid permanganate oxidation of a monolayer of erucic acid. Oxidation caused at first rapid expansion of the film, then slower expansion, followed by contraction (increased packing), and finally dissolution of the film.

The observation that the amount of lipid peroxides in the oxidizing homogenates mostly follows a biphasic curve indicates that peroxide formation and peroxide disappearance are two concurrent but independent processes. It is known (15) that these two processes are independent steps of lipid peroxidation and that it is the breakdown of lipid peroxides which may result in the formation of polymers. The data represented in Fig. 9 together with those of Figs. 1, 2, and 8 do not show any correlation between the amount of lipid peroxides formed and the action of the oxidant in promoting polymerization.

Extraction of oxidized homogenates by butanol showed that permanganate produced more insoluble residue and ferric chloride more lipid-soluble moieties than all the other oxidants. As solubility in lipid solvents is mainly a function of the presence of hydrophobic groups and paucity of hydrophilic groups, it appears that permanganate oxidation results in the formation of numerous hydrophilic groups while ferric oxidation produces mainly hydrophobic groups. It is likely that the tendency to enhance polymerization is mostly associated with the formation of hydrophobic groups, while formation of hydrophilic groups on the surface of micelles favors breakdown and emulsification.

The finding that the fluorescence of homogenate did not run parallel to the polymerizing effect of the various oxidants indicates that rate of polymerization cannot be determined by the intensity of fluorescence.

The formation of polymers through cross-links between adjacent molecules (with or without inclusion of the oxidizing agent in the polymer) has a stabilizing effect on the structure of membranes, as shown in the electron microscopic observations. Whether such effects occurring in vivo would have a stabilizing effect on membranes remains an open question. It is possible that membrane stabilization, when occurring at one site of a membrane, might result in rupture, as the rigid part of a membrane will not be able to follow changes in adjoining sites. It is also possible, however, that mild stabilization might render a membrane more rigid and less adapted than a normal membrane to changes in the milieu.

Polymerization of membranes through oxidation of the lipoprotein moieties is known to be the cause of formation of lipid pigments known as chromolipids, lipofuscins, and so on. These pigments are known to be autofluorescent. The finding that $KMnO_4$, the most efficient oxidizing agent among those tested, did not produce polymers indicates that the balance between oxidants and antioxidants is not the only factor responsible for

the formation of lipid pigments. The balance which determines whether lipid pigments will form depends on the nature and concentration of polymerizing oxidants and of their antagonists, while nonpolymerizing oxidants might allow cells to get rid of the oxidized products.

ACKNOWLEDGMENTS

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