Journal of Supramolecular Structure 3:80–89 (1975)

OXIDATION OF LIPIDS AND MEMBRANES I: In Vitro Formation of Peroxidative Lipid Polymers

Moshe Wolman

Departments of Pathology and of Cell Biology and Histology, Tel-Aviv University Medical School, Chaim Sheba Medical Center, Tel-Hashomer, Israel

Fluorescent polymers were obtained by oxidizing partly emulsified linolenic acid with different oxidants. The speed of formation of polymers differed for the various oxidants, and the difference was not a simple function of the oxidation potential. The speed of polymerization also depended on the nature of the emulsion.

The presence of egg albumen in the emulsion enhanced polymer formation with all oxidants. When the oxidants used are arranged in the order of decreasing speed of polymer formation, the order is different in the presence of albumen from what it is in the absence of albumen.

With different oxidation catalysts most antioxidants and amino acids tested enhanced polymerization. In oxidation with ferric ions, with K-dichromate, and without added oxidants the only antioxidants which delayed polymerization were "inhibitors." "Retarders" enhanced polymerization. With $KMnO_4$ slight delay was caused by some retarders.

The findings indicate that not only oxidation catalysts, but also proteins, amino acids, and antioxidants enhance polymerization. The possibility is suggested that in animal cells lipid pigment formation might represent a mechanism for neutralizing free radicals.

INTRODUCTION

Autoxidation of unsaturated lipids is responsible for a number of biological processes. Food fats exposed to atmospheric oxygen become rancid (1). Extraneous lipids or lipid-containing biological materials introduced into the bodies of animals are transformed into pigmented polymers (2–4). Similar lipid-containing polymers are formed in various organs of the body during the usual wear and tear process, and as a rule their amount increases with age (5). In some pathological processes, such as avitaminosis E, Batten's disease, and chronic granulomatous disease of childhood (6–8), deposition of lipid pigments might be of major pathogenic importance. Autoxidation and formation of polymeric pigments in vivo are known to be enhanced by increased unsaturation and by conditions favoring oxidation and to be delayed by antioxidants and radical trapping agents. Similar phenomena have been repeatedly observed in autoxidation experiments in vitro (9).

It has been suggested that lipid autoxidation per se, with the local formation of free radicals, is a deleterious process (10). It is not known whether the formation of pigmented lipid polymer does or does not damage the cells. The present study deals with the conditions which affect the tendency of peroxidizing lipids to polymerize.

Technical assistance, J. Gorski

Preliminary experiments have indicated that oxidation of an unsaturated fatty acid (linolenic acid) emulsified with water or with an aqueous oxidant solution resulted in the formation of a sticky precipitate which could be spun down by centrifugation. This is in agreement with data from the literature which indicate that naturally occurring lipid pigments (chromolipid, lipofuscin, ceroid) are heavier than water (11). The formation of these precipitates and their fluorescence served, therefore, as criteria for studying lipid pigment formation under different conditions.

This paper reports on experiments dealing with the in vitro formation of polymers from linolenic acid acted upon by different oxidants and antioxidants in different types of emulsions, in the presence and absence of amino acids, albumen, and heparin.

MATERIALS AND METHODS

All experiments were performed with 0.2 ml of linolenic acid overlying 1.0 ml of an aqueous medium. The tubes were vigorously stirred with a Vortex Genie mixer (Scientif. Instr., Springfield, Mass.) at speed 5 and left at room temperature. The tubes were centrifuged 2–3 times weekly with a clinical centrifuge at a low speed for 3 min and the various layers examined. After noting the findings the tubes were again stirred.

In the first experiment the following oxidizing agents were used at a final concentration of M/400: K-dichromate, chloroperbenzoic acid, ferric chloride, and ascorbic acid. An additional control tube not containing any oxidant was also used. The aqueous phase also contained 0.8% Na taurocholate. For each oxidant 3 tubes were used: the first contained no further additions (to the linolenic acid, taurocholate, and oxidant); the second contained NaCl in a final concentration of M/1; the third contained $CaCl_2$ in a final concentration of M/2.

In the second and third experiments the same oxidants were used and no salts were added. In the second experiment the aqueous phase contained albumen of a fresh egg in a final concentration of 4% (i.e., egg white diluted 1:25), or about 0.5% protein. In the third experiment the aqueous phase contained heparin in a final concentration of about 2%.

In the fourth experiment 4 series of tubes including one series without oxidants and 3 with the following oxidants were tested: K-permanganate, K-dichromate, and $FeCl_3$, at M/400 concentration. Also in this experiment Na taurocholate in a final concentration of 0.8% was included. Each series consisted of 13 tubes; one contained no other agents, while the others contained different amino compounds and antioxidants at a final concentration of M/400. The list of the amines and antioxidants is given in Figs. 3–6.

In the fifth experiment, three series of tubes were set: the first series contained K-permanganate and the second $FeCl_3$, both at a final concentration of M/400; the third series was left without any oxidant. In each series two tubes contained vitamin E acetate (dissolved in the linolenic acid), another two contained tyrosine, a third couple contained centrophenoxine, and a fourth couple did not contain any antioxidants. The antioxidants were at a final M/400 concentration in the 1 ml of the aqueous phase. One tube of each couple contained NaCl at a final M/1 concentration, and the other contained $CaCl_2$ at M/2 concentration.

At the end of each experiment a sample of the sediment was deposited on a glass

plate and examined for yellow fluorescence when excited by a UV light of 336 nm. In some experiments sediments were smeared on slides and examined under UV light directly and after having been repeatedly extracted with ethanol-ether (1/1 by volume).

RESULTS

Figure 1 gives in a graphic form the results of the first experiment. Comparison of the tubes to which no ions were added indicates that permanganate and dichromate were the fastest producers of heavy polymeric precipitates, with ascorbic acid, chloroperbenzoic acid, ferric chloride, and water following in this sequence. Emulsions in water were more rapidly oxidized than those containing salts. With permanganate, chloroperbenzoate, and ascorbate the process was more rapid with Ca than with Na; with dichromate and ferric chloride both Na and Ca retarded polymerization to the same extent, whereas without added oxidants ("water") the process was more rapid with Na than with Ca.

Examination by UV light of smears of sediments obtained after 40 days showed marked differences in the intensity of fluorescence in the untreated smears and in smears extracted with ethanol-ether. The fluorescence intensity decreased in the following order: dichromate, chloroperbenzoate, permanganate, ferric chloride, ascorbate, water. With all oxidants fluorescence was most intense in the Ca-containing solution, less in the Na, and least in the solutions not containing salts. Figure 2 gives results of the second experiment, in which egg albumen was added to the aqueous medium. Comparison of the tubes to which no ions were added shows that ferric chloride was the fastest producer of precipitate, followed by chloroperbenzoic acid, permanganate, and water, and then by dichromate and ascorbic acid. The presence of Ca ions or either Ca or Na enhanced the oxidative polymerization activity by dichromate, by chloroperbenzoic acid, and possibly also by ferric chloride. Comparison of Fig. 2 with Fig. 1 shows that the presence of albumen enhanced the formation of polymeric precipitates.

Examination by UV light of smears of sediments obtained in the presence of albumen showed a more intense fluorescence than that observed in the first experiment, with insignificant differences between the different sediments.

The findings in the third experiment were almost identical with those of the first experiment both with regard to the time of appearance of a sediment and to the fluorescence indicating that heparin did not affect polymerization.

In the fourth experiment the effects of antioxidants and amino acids differed with the various oxidants. Figure 3 shows the results obtained with K-permanganate. Considering the last line (water, no antioxidant) as baseline, slight retardation of the onset of polymerization occurred with methylene blue and with the antioxidants glutathione and AET (2-aminoethylisothiouronium bromide hydrobromide). All other antioxidants and amino compounds had either a marked or slight enhancing effect on polymer formation or practically no effect.

Figure 4 shows the results obtained with ferric chloride. On comparing the time of appearance of polymers in the test tubes containing various antioxidants and in the test tubes containing no antioxidants (bottom line in the figure), it is obvious that some of the agents tested had a marked retarding effect, while others had hardly any effect at all. The three compounds having a marked retarding effect on polymerization were:

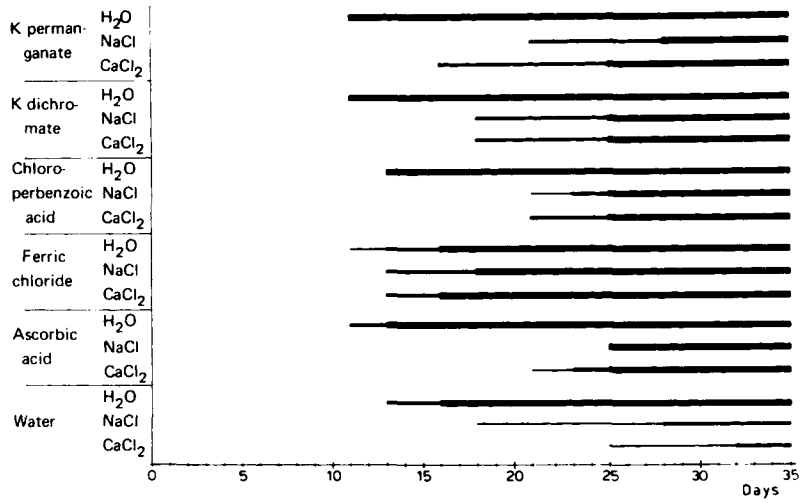

Fig. 1. Thickness of lines indicates the amount of sediment. Thin line – slight sediment; medium line – about half the lipid was present in the sediment; thick line – all or most of the lipid was in the sediment.

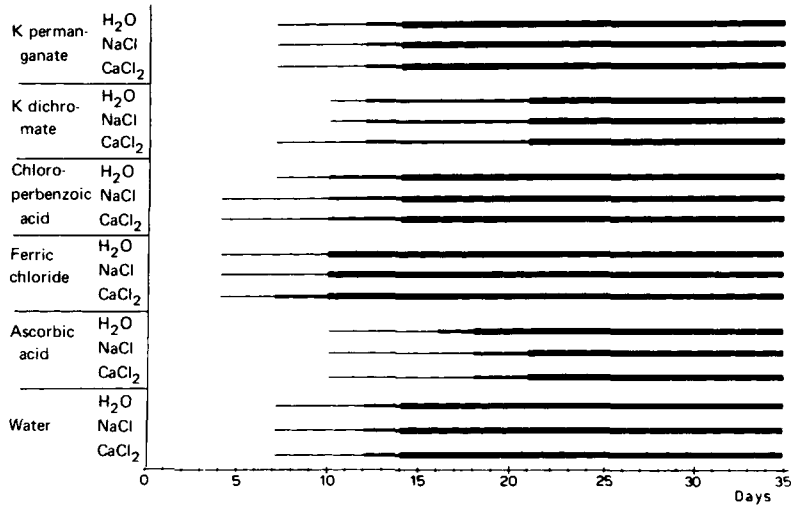

Fig. 2. Thickness of lines indicates the amount of sediment. Thin line – slight sediment; medium line – about half the lipid was present in the sediment; thick line – all or most of lipid was in the sediment.

p-aminodiphenylamine, hydroquinone, and BHT (butylated hydroxytoluene).

Figure 5 shows the results when the oxidant was K-dichromate, which are remarkably similar to those obtained with $FeCl_3$. The same three antioxidants (p-aminodiphenylamine, hydroquinone, and BHT) caused retarded polymerization. All other antioxidants and amino acids did not appreciably affect the polymerization.

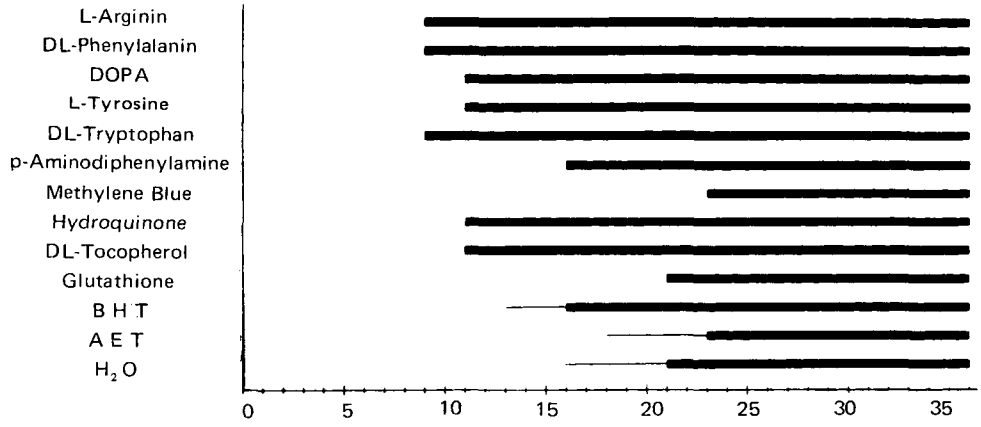

Fig. 3. Thickness of lines indicates the amount of sediment. Thin line – slight sediment; medium line – about half the lipid was present in the sediment; thick line – all or most of the lipid was in the sediment.

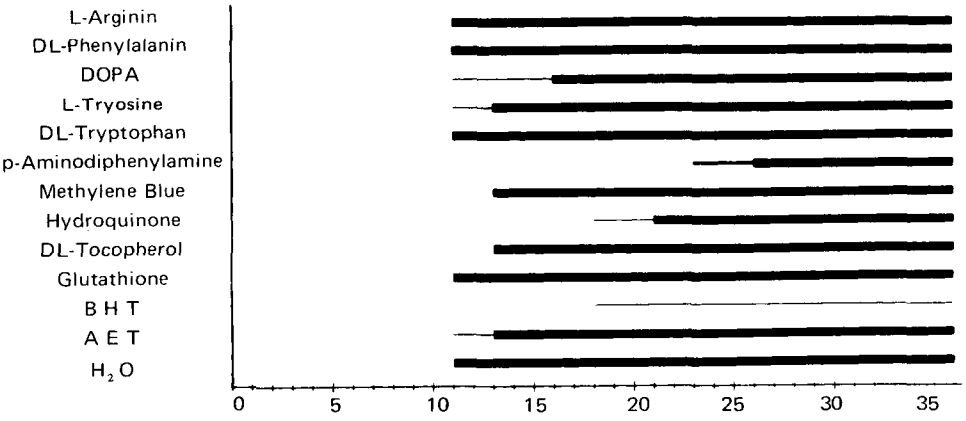

Fig. 4. Thickness of lines indicates the amount of sediment Thin line – slight sediment; medium line – about half the lipid was present in the sediment; thick line – all or most of the lipid was in the sediment.

Figure 6 represents uncatalyzed autoxidation, as no oxidants were added. Slight polymerization retarding activity was exhibited by vitamin E (tocopherol) and possibly also by DOPA (3, 4 dihydroxy phenylalanine) and tyrosine. Effective retardation of polymerization was again given by the same three antioxidants as with ferric chloride and K-dichromate.

It should be noted that these experiments were performed in various seasons with different room temperature ranges. Since in experiments done in the summer polymerization time was half that observed in the cold season (for example, the uppermost line in Fig. 1 in comparison to the top line of Fig. 3), each experiment has been considered

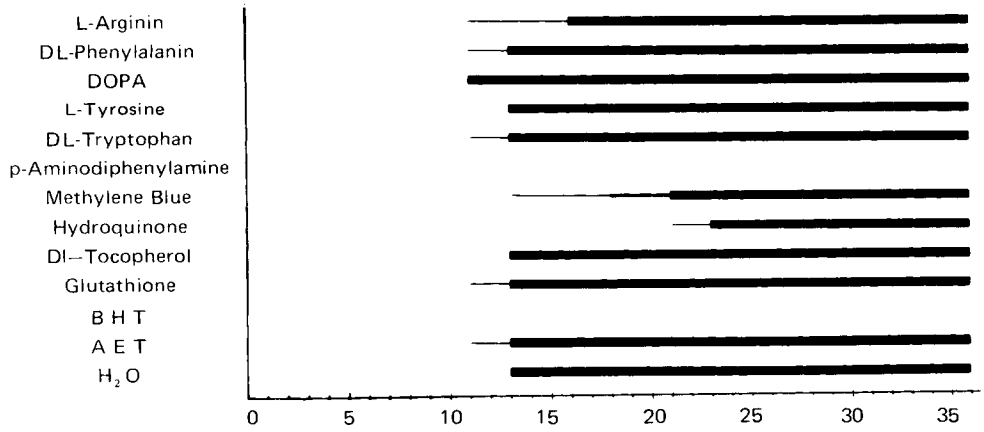

Fig. 5. Thickness of lines indicates the amount of sediment. Thin line – slight sediment; medium line – about half the lipid was present in the sediment; thick line – all or most of the lipid was in the sediment.

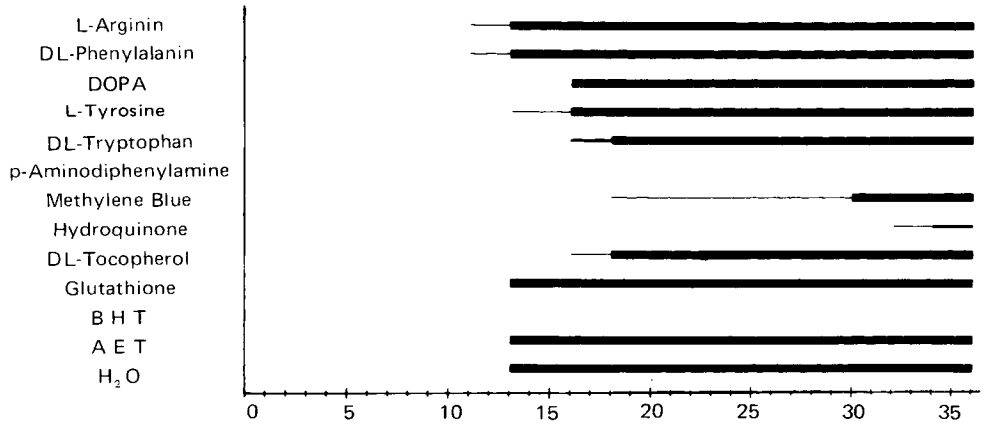

Fig. 6. Thickness of lines indicates the amount of sediment. Thin line – slight sediment; medium line – about half the lipid was present in the sediment; thick line – all or most of the lipid was in the sediment.

separately. Figures 1 and 2, which were repeated concurrently and were therefore compared to each other, constitute the only exception to this rule.

Examination of fluorescence of the polymers obtained at the end of this experiment showed that the most intense yellow fluorescence was obtained in all four series (three oxidants and one no oxidant series) in the presence of p-aminodiphenylamine, tryptophane, methylene blue, and DOPA. Least fluorescence was obtained with BHT and without any antioxidants.

In the fifth experiment some of the points tested in the fourth experiment were examined again. If we consider the tubes containing NaCl only, Fig. 7 confirms the following observations made in the preceding experiment: with $KMnO_4$, vitamin E had practically no effect, while tyrosine and centrophenoxine enhanced polymerization. With $FeCl_3$ all of the three compounds tested enhanced polymerization.

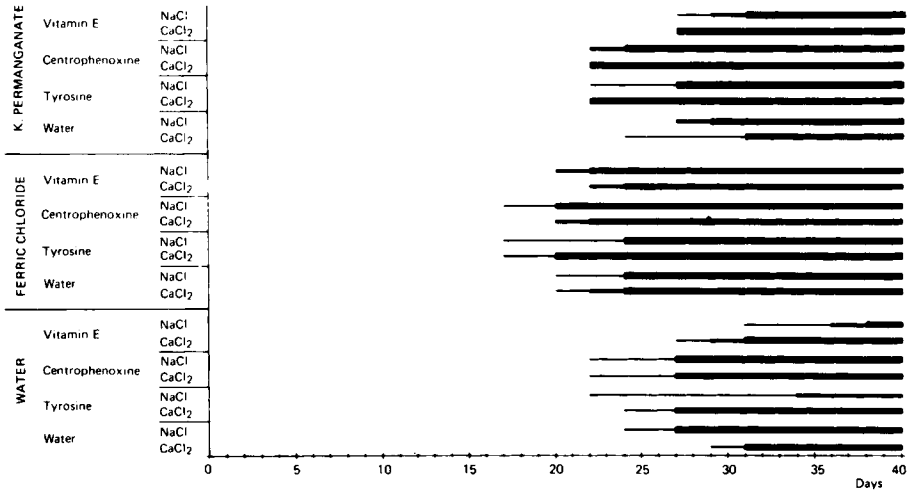

Fig. 7. Thickness of lines indicates the amount of sediment. Thin line – slight sediment; medium line – about half the lipid was present in the sediment; thick line – all or most of the lipid was in the sediment.

In uncatalyzed autoxidation (in the presence of NaCl), however, polymerization was obviously slowed down by vitamin E and to a lesser extent by tyrosine.

It can be seen in Fig. 7 that the effects of oxidants and antioxidants depend also upon the salts. In the uncatalyzed autoxidation and in the absence of antioxidants, $CaCl_2$ retarded polymerization, whereas in the presence of vitamin E as antioxidant, $CaCl_2$ enhanced the process.

It may be noted that in many tubes the sediment started rising to the surface some days after its formation. As this phenomenon might have been due to any of a number of causes, it has not been included in the tables and the discussion.

DISCUSSION

The aim of the presently described experiments was to find out under which conditions does the autoxidation of lipids produce lipid polymers and what are the conditions favoring disaggregation and breakdown of the lipid molecules. It has been found that different oxidants differ in their polymerizing action on the same lipid. An attempt was made to roughly calculate the oxidation potential of the different solutions, and the order in which the oxidants are listed in Fig. 1 represents an approximate descending order of potential. Although the calculations were inaccurate, as the nature of the reactions is not well known, it appears that the rate of polymer formation is not proportional to the oxidation potential. This is in agreement with known facts, as oxidants vary in their tendency to form complexes with lipids, and some oxidants (OsO_4, $K_2Cr_2O_7$) are used in cytology to fix and render tissue lipids insoluble by cross-linking them. Therefore, it may be concluded that the nature, rather than the potential, of the oxidation catalyst plays an important role in determining whether polymerization is the major pathway of the oxidative process.

Proteins are known to enhance the rate of formation of oxidative lipid polymers (12, 13). It has been shown that in these polymers the protein structure is changed (14). The present findings indicate that although egg albumen always increases the rate of polymer formation, the promoting effect differs with various oxidants. Thus, the copolymerization of oxidizing lipids with protein differs in its nature from the oxidative polymerization of lipids alone, indicating that protein is an integral part of the polymer rather than an inclusion compound. Furthermore, the fact that the order of decreasing speed in which the various oxidants induced polymerization was different with albumen from the order of induction times without albumen indicates that the rate of polymerization does not primarily depend on the oxidation potential.

Heparin, which in the present experiments was chosen to represent hexosamine-containing compounds, did not have any appreciable effect on oxidative polymerization of lipids.

The rate of oxidation of unsaturated lipids is known to depend on the nature of the lipid emulsion. Monomolecular lipid films autoxidize more rapidly than bulkier lipid masses (15, p. 41). In the present experiments emulsions of the "oil in water" (O/W) and "water in oil" (W/O) types were prepared by adding NaCl and $CaCl_2$, respectively (16, 17), in order to determine which type of emulsion is more rapidly oxidized. In the O/W type of emulsion, water is the continuous phase and with water-soluble oxidants the lipid droplets are exposed to freely moving oxidant molecules. In the W/O emulsion, oil represents the continuous phase and water-soluble oxidant molecules are enclosed in tiny aqueous bags. Examination of Fig. 1 indicates that oxidative polymerization is more rapid in the aqueous (ion-poor) medium than in the presence of ions.

This is not quite unequivocal as the specific weights of the aqueous solutions were not the same, and the stage at which precipitation occurred differed for the various salts and oxidants. With most oxidants the addition of $CaCl_2$ caused more rapid formation of polymers than the addition of NaCl, which might be related to the effect of Ca ions in increasing the lipophilia and thereby facilitating the diffusion of oxidants into the lipophilic hydrocarbon layer. The differences between the effects of Na and Ca ions on the various oxidants might be related to the different lipophilia of the oxidants and their derivatives.

As can be seen in Fig. 7, autoxidation of linolenic acid in the presence of atmospheric oxygen present in the aqueous phase and without the addition of any oxidation catalysts or antioxidants resulted in a more rapid polymerization in the presence of NaCl than $CaCl_2$.

Autoxidation of polyunsaturated lipids is a three-stage process (15). In the first stage a hydroperoxide is formed, probably through loss of one hydrogen from the carbon atom situated between the two double bonds, with formation of free radicals. This stage, chain initiation, is often associated with a long induction period in which free radicals are formed, and it is in this stage that inhibitor-type antioxidants act by preventing peroxidation and free radical formation. In the second stage, chain propagation, the reaction is autocatalytic, with formation of new chain-initiating radicals and possibly also of a growing polymer. The third stage, the chain-growth–stopping reaction, might be due to a number of causes. Two peroxide radicals might combine, or a radical might become attached to another moiety, thus ending the autocatalytic process of free radical formation. Free radical acceptors act by terminating the chain propagation (18).

On theoretical grounds the nature of the reaction products (whether large polymer masses are formed or not) depends in part on whether chain initiation has occurred and on the rate of the chain-stopping reaction, which might end chain growth at different stages. No polymer formation occurs when the oxidative attack breaks down molecules into smaller nonreactive units or if the chain-stopping reaction is faster than the initiation.

The findings summarized in Fig. 3 show that with permanganate oxidation many oxidizable amines and other antioxidants enhance polymer formation. The most active were phenylalanine, arginine, and tryptophane, but tyrosine, DOPA, hydroquinone, and tocopherol also exhibited polymerization-enhancing activity.

Thus, amino acids which are antioxidants may often promote polymerization by reacting with the free radicals. It is probable that the complex is formed by the formation of bonds between the amino acids and the oxidizing lipid radicals. The reaction of lipid peroxides with proteins might depend on a similar mechanism.

Although Venolia and Tappel (13) have shown that amine-aldehyde condensation is not the major mechanism responsible for the formation of brown lipid-protein polymers, other interactions and bonds obviously play a major role in polymerization. It appears that a protein which probably is an antioxidant [as most of its constituent amino acids are (19)] might enhance formation of lipid-protein polymers through formation of lipid-protein bonds.

The findings summarized in Figs. 4, 5, 6, and 7 are similar with respect to the effect of antioxidants and amines on polymerization. They probably represent the course of events in lipid autoxidation as it usually occurs in the animal body (with only trace amounts of oxidation catalysts or in the presence of excess iron). Only three antioxidants among those tested were effective in delaying polymerization (p-aminodiphenylamine, hydroquinone, and BHT). It is interesting to note that these compounds belong to the antioxidants termed inhibitors, which prolong the induction period of autoxidation, in contrast to retarders, such as amino acids, which lower the rate of autoxidation by donating electrons and by chelating catalytic traces of metals (20). The data indicate that while all types of antioxidants have an effect in delaying autoxidation, autoxidative polymer formation in the conditions of the present experiments seems to be delayed only by inhibitors. This observation is probably generally valid, as retarders are free radical trappers, which combine with free radicals, while inhibitors act mainly in delaying hydroperoxide breakdown and formation of free radicals. As has been noted in Fig. 3, and as can be seen also in Figs. 4, 5, 6, and 7, retarders (for example the amino acids tested) do not delay polymerization and often promote it for the reasons explained above (with $KMnO_4$ oxidation some retarders do, however, somewhat delay the process). It is obvious, therefore, that autoxidation and polymerization do not necessarily proceed pari passu, and factors enhancing the one may inhibit the other.

The conclusions regarding inhibitors and retarders are only statistically valid. Polymerization and breakdown probably occur side by side in all instances, and the question is mainly which process predominates under the various conditions.

Coleman et al. (21) have found that the products of autoxidation of methyl oleate could be divided into two fractions. The one, non-complex forming, was rich in peroxides and hydroxyls. The other, complex forming, was poor in these groups but rich in oxirane and carbonyl groups.

The observation (22) that those antioxidants which prolong the induction period of unsaturated lipid peroxidation up to 7–12 times cause formation of complexes of higher molecular weights than the complexes formed in the absence of antioxidants shows that the inhibitor-type antioxidants also participate in cross-linking reaction. Pokorny et al. (23) have shown that the products of oxidation of tocopherols also react with proteins to form brown and insoluble complexes.

The present studies might be of relevance for the understanding of the formation of lipid pigments in the animal body. The naturally occurring chromolipids (also called lipofuscin, ceroid, and wear and tear pigment) exhibit histochemical characteristics similar to those of artificially prepared lipid and lipid-protein polymers (24, 25). It is possible that lipid pigment formation in animal cells represents a defensive reaction on the part of the cell – neutralization of free radicals by trapping agents with consequent formation of mixed polymers.

REFERENCES

1. Lundberg, W. O., in "Autoxidation and Antioxidants," W. O. Lundberg (Ed.), vol. 2. Interscience, New York, p. 451 (1962).
2. Hirsch, E. F., Arch. Path. (Chicago) 31:516–527 (1941).
3. Gedigk, P., and Pioch, W., Virchows Arch. Path. Anat. 339:100–135 (1965).
4. Hartroft, W. S., Science 113:673–674 (1951).
5. Wolman, M., in "Handbuch der Histochemie," W. Graumann and K. Neumann (Eds.), vol. 5/2, p. 96 (1964).
6. Dam, H., and Mason, K. E., Fed. Proc. 4:153 (1945).
7. Zeman, W., and Hoffman, J., J. Neurol. Neurosurg. Psychiat. 25:352–362 (1962).
8. Crocker, A. C., in "Pigments in Pathology," M. Wolman (Ed.), Academic Press, New York, p. 304 (1969).
9. Sims, R. P. A., and Hoffman, W. H., in "Autoxidation and Antioxidants," W. O. Lundberg (Ed.), vol. 2, Interscience, New York, pp. 629–682 (1962).
10. Tappel, A. L., Fed. Proc. 24:73–78 (1965).
11. Björkerud, S., Adv. Geront. Res. 1:257–288 (1964).
12. Casselman, W. G. B., J. Exp. Med. 94:549–562 (1951).
13. Venolia, A. W., and Tappel, A. L., J. Amer. Oil Chem. Soc. 35:135–138 (1958).
14. Andrews, F., Björksten, J., Trenk, F. B., Henick, A. S., and Koch, R. B., J. Amer. Oil Chem. Soc. 42:779–781 (1965).
15. Swern, D., in "Autoxidation and Antioxidants," W. O. Lundberg (Ed.), vol. 1. Interscience, New York, pp. 1–54 (1961).
16. Clowes, G. H. A., J. Physic. Chem. 20:407–451 (1916).
17. Wolman, M., and Wiener, H., Biochim. Biophys. Acta (Amsterdam) 102:269–279 (1965).
18. Uri, N., in "Autoxidation and Antioxidants," W. O. Lundberg (Ed.), vol. 1. Intersceinces, New York, pp. 133–169 (1961).
19. Tappel, A. L., Arch. Biochem. Biophys. 54:266–280 (1955).
20. Marcuse, R., and Fredriksson, P. O., J. Amer. Oil Chem. Soc. 46:262–268 (1968).
21. Coleman, J. E., Knight, H. B., and Swern, D., J. Amer. Oil Chem. Soc. 32:135–137 (1955).
22. Fukuzumi, K., and Ikeda, N., J. Amer. Oil Chem. Soc. 47:369–370 (1970).
23. Pokorný, J., Luân, Nguyên-Thiên, and Janicek, G., Z. Lebensm. Unters.-Forsch. 152:65–70 (1973).
24. Ciaccio, C., Biochem. Z. 69:313–333 (1915).
25. Wolman, M., and Shoshan, S., Histochemie 2:69–75 (1960).