Cross-Linking of Collagen in the Presence of Oxidizing Lipid

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ABSTRACT

Gelatin films containing unsaturated lipid have been exposed to ultraviolet and visible irradiation. No sign of paramagnetism could be detected in the films, although the gelatin was undergoing cross-linking reactions. The addition of nitroxyl-forming radical scavengers decreased the rate of cross-linking, as did addition of ascorbic acid to the reacting mixture. Nitroxyls could not be detected in the gels, however. The conclusion is drawn that the main reaction in the cross-linking reaction of collagen is a condensation of amino groups and extrinsic or intrinsic carbonyl groups. The extrinsic aldehydes are formed in the autoxidation of unsaturated lipid.

An object of the present study is to increase our knowledge of the causative mechanisms of the fully demonstrated premature aging of human skin which has been excessively exposed to solar radiation.

On one hand, it has been postulated by Harman (1) and others that free radicals are primary causative factors in the aging process and that these are formed in the process of lipid oxidation (2,3).

On the other hand, doubt has been raised regarding the necessity for free radicals in this reaction. The free radicals observed by Roubal (3) would seem expectable from the enzyme reactions connected with the material used.

The reaction between oxidizing fat and protein was known in the hectograph industry prior to 1915 (4). Fahrion (5,6) postulated that the reactive groups involved in the cross-linkage reaction or "tannage" with oxidizing lipids are peroxide groups formed by the action of oxygen in air on the unsaturated fatty acids of the oil (7).

The formation of acrolein and other pungent compounds in the tanning process has been known to generations of chamois tanners (8). Procter (9), suggested that acrolein was the principal tanning agent. Indeed, acrolein is an effective cross-linkage agent. Salway (10) showed that acrolein is formed by the oxidation of free unsaturated fatty acids from linseed oil. Balfe, in his review (11), stated that acrolein is the major tanning agent in oil tanning. On this basis, Kuntzel and Nungesser (12) reinvestigated the matter. Cod liver oil, exposed to air oxidation in a Mackay tester, gave acrolein yields of 0.7-1.5% of the oil being oxidized under conditions corresponding to oil tanning. When hide powder was added to the oxidizing oil, no free acrolein could be found and the hide powder became cross-linked.

Farmer (13), has shown that fatty hydroperoxides alone can effect a cross-linkage in proteins. On the other hand, the cross-linkage of proteins in proteinaceous products containing unsaturated lipids has been ascribed to effects of free radicals by Desai and Tappel (14).

Okamura and Shirai (15) studied oil tanning by incubating preoxidized cod oil in hide powders. They conclude that oil tanning follows a complex course including the combination of peroxides of cod oil with hide. No electron spin resonance (ESR) investigation was undertaken, however.

In view of the fact that both aldehydes and peroxides can induce cross-linkage and damaging reactions in proteins, it was of interest whether free radicals can be observed with ESR spectroscopy while a demonstrable accelerated protein cross-linkage by oxidizing unsaturated lipid is in progress.

EXPERIMENTAL PROCEDURES

Gelatin films containing 0, 0.17, 1, 5, and 10% of lipid, respectively, were prepared (16). As the gelatin component, pigskin gelatin (275 Bloom) was used. The lipid used was a commercially available corn oil containing 86% (w/v) unsaturated fat and free from added antioxidants. The antioxidant ascorbic acid (Pharmaceutical grade F) was dissolved in a small volume of water prior to addition. The ascorbic acid content of the gel was 0.00074%. The radical scavenger tert.-nitrosobutane was prepared as described (17), and the radical scavenger 2,4,6-tri-*tert.*-butyl nitrosobenzene was prepared by the procedure outlined (18). The scavenger was ground in a mortar together with the dry gelatin before the swelling procedure. The final concentration of the scavenger was 0.001 and 0.1%, respectively. The gels were stored before and after irradiation as previously described (16).

The gels were irradiated in a Rayonet Photochemical chamber reactor model RRR-100. The

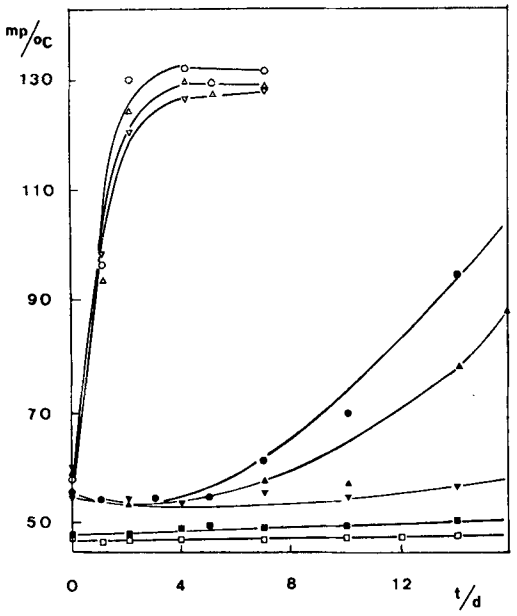

FIG. 1. Crosslinking of lipid containing collagen. Solid figures refer to samples irradiated 5 hr at 360 nm; empty figures refer to samples irradiated 60 min at 253 nm □, ■ no oil added, ▽, ▼ 1 % oil added, △, ▲ 5 % oil added, and ○, ● 10 % oil added.

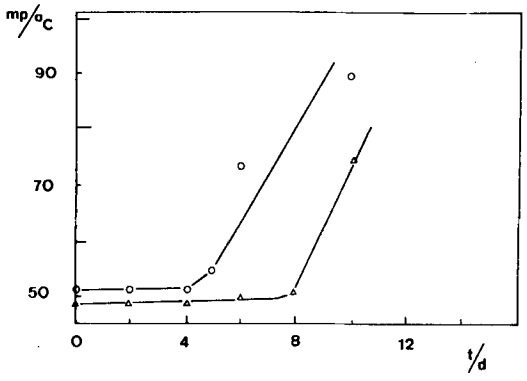

FIG. 2. Crosslinking of lipid containing collagen in presence of 7.4 x 10^{-4} % ascorbic acid. Irradiation 60 min at 253 nm., Δ 5 % oil added, ○ 10 % oil added.

gelatin films were cooled during the irradiation. The films were exposed to both daylight (360 nm) and UV-light (263 nm) in different experiments. The experimental values represent the average of at least five measurements.

The cross-linking of the collagen was followed by measuring melting points from time to time. The melting points were determined in mineral oil as has been described by Bjorksten and Collbring (16).

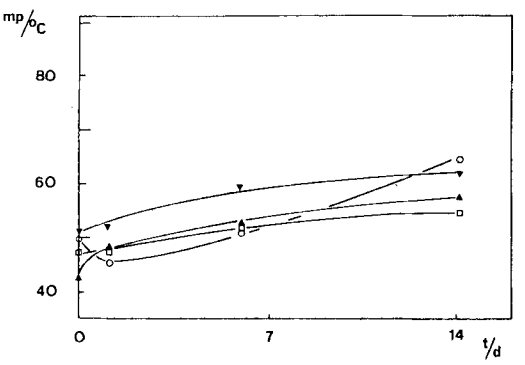

FIG. 3. Crosslinking of lipid containing collagen in presence of 7.4 x 10^{-4} % 2,4,6-tri-*tert.*-butylnitroso-benzene and 5 % oil (▼), 0.1 % 2,4,6-tri-*tert.*-butyl-nitroso-benzene and 5 % oil (▲), 0.1 % 2,4,6-tri-*tert.*-butyl-benzene (○), and no added compounds (□).

ESR measurements were performed on a Varian X-band E-3 spectrometer at temperatures ranging from +100 – -150 C with variation of all the measuring parameters. The gels were checked for paramagnetism before and after irradiation, and at time intervals equal to time between melting point measurements.

RESULTS AND DISCUSSION

The change in melting points was taken as a measure of the rate of cross-linking in the lipid-containing gels (16). The results of these determinations are collected in Figures 1, 2, and 3.

As judged by the change in melting point, there is no mechanistic difference between the reactions initiated at 263 nm and 360 nm, respectively. This finding could be explained as follows: The photochemically active chromophore (in the lipid or in the collagen) has its absorption maximum in the ultraviolet region, with some absorption probability also in the visible region, due to tailing. The difference is seen in the velocity of the aging reaction of the gels (Fig. 1). After 60 min irradiation at 263 nm, the rise in melting point is from about 50 C to about 100 C in one day, whereas 5 hr irradiation at 360 nm hardly affects the melting point in one day. By using the short wavelength light for the irradiation of the gels, we hoped to reach a higher and measurable concentration of free radicals in the gels. This was not found, however, and every effort to detect an ESR signal in the system of lipid-containing gelatin was unsuccessful.

Experiments have been reported (16) in which the effect of an added antioxidant on the melting point of the gel was tested. It was shown that tocopherol efficiently retarded the

melting point raising reaction. Accordingly, the effect of ascorbic acid was tested. The results are collected in Figure 2. The concentration of ascorbic acid was kept sufficiently low, $< 10^{-3}\%$, not to affect the pH of the gels. The addition of ascorbic acid quite evidently slows down the melting point raising reaction, after 60 min UV irradiation the melting point remains unaltered for 5 days.

The gels containing lipid and ascorbic acid were tested for paramagnetism at intervals equal to intervals between melting point determinations. No ESR signal could be detected.

Radical scavengers forming relatively stable paramagnetic products in systems where radicals are present have been developed. Among these nitroxyl-forming nitroso compounds or nitrones are commonly in use (22). The effect of the scavengers *tert.*-nitrosobutane (2-methyl-2-nitrosopropane) (17) and 2,4,6-tri-*tert.*-butylnitrosobenzene (18) on the reactions in the lipid-containing gels was tested.

In a preliminary experiment *tert.*-nitrosobutane was chosen as a scavenger. The choice was due to the high solubility of *tert.*-nitrosobutane in hydrophilic systems. The scavenger was carefully mixed in the gels after the irradiation to avoid its known photochemical degradation (19). The signal detected from the gels after this treatment was, however, shown to be due to di-*tert.*-butylnitroxyl.

Recently the use of the photochemically stable scavenger 2,4,6-tri-*tert.*-butylnitrosobenzene was reported (18) (note, however, ref. 20). The effect of this scavenger on the melting point raising reactions in the gels was similar to that of the antioxidants, tocopherol and ascorbic acid, but lasted over a longer period. The melting points of the gels remained unchanged within experimental error for 14 days. No sign of paramagnetism could be detected. The results of the measurements are collected in Figure 3.

The molecular reactions in the cross-linkage of collagen containing unsaturated lipid remain somewhat doubtful. Probably radical and condensation reactions take place in complicated sequences. It is possible that the irradiation, or an initially formed alkoxy- or alkylperoxy radical causes hydrogen abstraction in the peptide chain thereby causing intramolecular or intermolecular cross-linking between peptide chains. Reactions of these types are reported in the literature (21,22).

On the other hand, it has been pointed out that the normal sequence of events in the intramolecular cross-linkage of collagen appears to be: collagen molecules, which contain carbonyl groups, self-assemble into fibers which then become cross-linked because of the reactions that occur between the carbonyl groups and amino groups of adjoining amino acids. When aldehydes react with proteins, Schiff bases are commonly formed. Extrinsic, as well as intrinsic, aldehydes may cause this reaction (23). We, therefore, conclude that even if a free radical reaction might occur in the cross-linking reaction, the condensation between carbonyl compounds and amino groups is far more important in the cross-linking of autoxidizing lipid containing collagen. The effect of ascorbic acid or tocopherol to the lipid containing collagen as well as the radical scavengers *tert.*-nitrosobutane and 2,4,6-tri-*tert.*-butylnitrosobenzene could be the inhibition of the autoxidation of the lipid.

REFERENCES

1. Harman, D., J. Gerontol. 11:293 (1956).
2. Roubal, W.T., and A.L. Tappel, Arch. Biochem. Biophys. 113:5 (1966).
3. Roubal, W.T., J. Am. Oil Chem. Soc. 47:141 (1970).
4. Isaacs, A., U.S.Patent,151,951 (1915).
5. Fahrion, W.Z., Z. Angew. Chem. 4:172, 446, 691 (1891).
6. Fahrion, W.Z., Z. Angew, Chem. 16:665 (1903).
7. Knox, J.M., Tex. J. Med. 56:653 (1960).
8. Gustavson, K.H., in "The Chemistry of Tanning Processes," Academic Press, New York, 1956, pp. 295-307.
9. Procter, H.R., "Principles of Leather Manufacture," Spon, London, 1904.
10. Salway, A.H., J. Chem. Soc. 109:138 (1916).
11. Balfe, M.P., "Progress in Leather Science," Vol. 3 Brit. Leather Mfg. Res. Assoc., 1948, p. 495.
12. Küntzel, A., and T. Nungesser, Das Leder 2:233 (1951).
13. Farmer, E.H., Trans. Faraday Soc. 38:348 (1942).
14. Desai, I.D., and A.L. Tappel, J. Lipid Res. 4:204 (1963).
15. Okamura, H., and K. Shirai, J. Am. Leather Chem. Assoc. 69:322 (1974).
16. Bjorksten, J., and T. Collbring, Proc. Toilet Goods Assoc., Sci. Sect. 42:1 (1964).
17. Holman, R.J., and M.J. Perkins, J. Chem. Soc. C 2195 (1970).
18. Okazaki, R., T. Josogni, E. Idaware, M. Hashimoto, and N. Imamoto, Bull. Soc. Chem. Jpn. 42:3611 (1969).
19. Maassen, J.A., H. Mitterhausen, and Th.J. Boer, Tetrahedron Lett. 29:3213 (1971).
20. Barclay, L.R.C., and I.T. McMaster, Tetrahedron Lett. 4901 (1937).
21. Roubal, W.T., and A.L. Tappel, Arch. Biochem. Biophys. 113:150 (1966).
22. Davies, A.G., in "Organic Peroxides," Butterworths, London, 1961, p. 161.
23. Tanzer, M.L., Science 180:561 (1973).

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