Identification of some lipid peroxides by thin-layer chromatography

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SUMMARY Solvent systems are described which permit class separations of various peroxidized lipids by thin-layer chromatography. This procedure has proved useful in biological studies and for testing the deterioration of lipids.

Proof is given that the least polar peroxides of methyl esters, glycerides, and fatty acids are monoperoxides; it is then assumed that the least polar peroxides in the other tested groups (cholesterol, cholesterol esters, and phospholipids) are also monoperoxides. The more polar peroxides probably represent highly peroxidized and polymeric forms.

KEY WORDS thin-layer chromatography · lipid peroxides · monoperoxides · pancreatic lipase · tissue peroxides · vitamin E deficiency

UNSATURATED LIPIDS READILY form peroxides in the presence of oxygen and ultraviolet light or of metallic ions and biological catalysts that induce the production of free radicals. As autoxidation proceeds, polymerization and decomposition of peroxides take place; these processes lead to complex mixtures of products. When autoxidation conditions are mild, many of these compounds carry peroxide groups. For studies of lipid peroxides in biological systems we required a qualitative method which would enable us to show the presence of peroxides in complex lipid mixtures, to classify them, and to determine whether simple peroxides or higher peroxidation products were present. This was achieved by TLC on Silica Gel G plates with various solvent systems commonly applied in separations of unoxidized lipids; the KI-starch reaction served as the most sensitive indicator of peroxides.[1] Complete chemical characterization of lipid peroxides has not been attempted, but the method is useful as a test for the presence of peroxidized products in lipid samples.

Abbreviations: TLC, thin-layer chromatography; GLC, gas–liquid chromatography; EE, diethyl ether; PE, petroleum ether; TO, triolein; CO, cholesteryl oleate; $C_{18:1}$ME, methyl oleate; $C_{18:2}$ME, methyl linoleate; $C_{18:3}$ME, methyl linolenate.

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[1] Most of the lipid peroxides described in this report are presumed to carry hydroperoxide groups. However, since this was not rigorously proved, the more general term *peroxides* is used in this text.

METHODS

Materials and Equipment

TLC equipment was obtained from C. Desaga, Heidelberg, Germany, and E. Merck, Darmstadt, Germany.[2]

Reference substances and compounds for autoxidation included methyl oleate ($C_{18:1}$ME), methyl linoleate[3] ($C_{18:2}$ME), methyl linolenate ($C_{18:3}$ME), and methyl ricinoleate;[4] triolein[3] (TO), diolein, mono-olein; and triglycerides containing one or two oleic acid residues per molecule in specific positions,[5] namely SOS, SSO, SOO, and OSO, where S = stearic acid and O = oleic acid. Furthermore, we used commercial samples of corn and linseed oils, cholesterol, cholesteryl oleate (CO), cholesteryl stearate, soybean and rat liver lecithins, and phosphatidyl ethanolamine of human red cells.

Autoxidation

Autoxidations were carried out in ampules in a continuous stream of oxygen at room temperature under ultraviolet light. Solid compounds such as cholesterol were spread out in a thin layer on the wall of test tubes. Oxidation times (hours to days) varied inversely with the degree of unsaturation of the lipid. Most of the samples had

[2] Distributed by C. A. Brinkmann Instruments, Inc., Great Neck, N. Y.

[3] Hormel Institute, Austin, Minn.

[4] Applied Science Laboratories, Inc., State College, Pa.

[5] Courtesy of Dr. Fred Mattson, Procter & Gamble Company, Cincinnati, Ohio.

peroxide values (microequivalents per gram) up to 1000, as determined by a previously described amperometric method (1), and in some cases even more highly peroxidized products were analyzed. Certain peroxidations were performed with biological catalysts such as hemoglobin, lipoxidase, or peroxidase,[6] using emulsified $C_{18:2}ME$ and soybean lecithin as substrate; reactions were carried out in phosphate buffer (pH 7.4) over oxygen at room temperature in the dark for 24 hr. Peroxidase was not effective for the peroxidation of soybean lecithin.

Thin-Layer Chromatography

Chromatoplates (20 × 20 cm) were layered with Silica Gel G or H (0.25–0.40 mm) according to Stahl (2, 3) and Mangold (4, 5) and developed by the usual ascending technique for 10–15 cm.

Solvent systems useful in separating unoxidized lipids were applicable in studies of lipid peroxides. Various mixtures of ethyl ether (EE) in petroleum ether (bp 30–60°) (PE) were effective in chromatographing the nonpolar lipid classes (15, 30, 45, and 65% EE–PE). The latter two mixtures can be equilibrated with 2 ml of concentrated NH_4OH per 100 ml of solvent mixture for analyses of certain fatty acid peroxides and for reduction of tailing of higher peroxides. Although the possibility of reactions of NH_4OH with keto groups should be considered, the R_F value of palmityl aldehyde remained essentially unaffected in solvent systems containing NH_4OH. EE–acetic acid (HOAc) 98:2 separated peroxides of neutral lipids from those of phospholipids and permitted the detection of monoglyceride peroxides. Chloroform–methanol–water 75:25:4.2 (C–M–H_2O) was used for the separation of phospholipid peroxides.

Indicator Sprays

Most of the lipid peroxides described in this report are presumed to carry hydroperoxide groups, and these groups are specifically detected by spraying plates with 5% (w/v) potassium iodide solution, and 1 min later with 1–5% (w/v) starch in 1% acetic acid. Peroxides became visible as blue-brown spots within a few minutes. To exclude the creation of artifacts by atmospheric oxygen, the plates were sprayed immediately after development and then kept under nitrogen, but in most cases the second precaution was unnecessary. Since the plates sprayed with starch–iodide darken slowly in the presence of atmospheric oxygen, it is advisable to outline the peroxide spots immediately. Under the mild acidic conditions used (6, 7) KI–starch spray was found to react most rapidly with hydroperoxides and per-acids, and only slowly and incompletely with dialkyl peroxides. The sensitivity of the reaction in complex lipid mixtures was 1–3 μeq peroxides per g of lipid, which is comparable to the sensitivity of the micro-amperometric method (1) used in parallel with these studies.

Useful nonspecific indicators include iodine vapor, 50% sulfuric acid with heating at 150–200°, or 0.04% bromothymol blue in 0.01 N NaOH. For detection of sterol peroxides, plates were sprayed with 50% sulfuric acid and heated at 100°. Most of these peroxides stained blue, a few more violet or gray, while unoxidized sterols developed as violet spots. Glycolipids (cerebrosides) developed a similar range of colors with sulfuric acid, after chromatography in C–M–H_2O.

Bodansky's acid-molybdate reaction (8) was used to identify phospholipid peroxides. A stock solution of 60% stannous chloride was made up in 10 N HCl, from which the dilute working reagent was prepared by adding 0.5 ml of the stock solution to 200 ml of water. The acidic molybdate reagent was made by mixing one volume of freshly prepared 7.5% ammonium molybdate with one volume of 10 N sulfuric acid, while cooling. Plates were sprayed with acidic molybdate reagent and heated for about 2 min at 100°, then sprayed with dilute stannous chloride solution. Substances containing phosphorus appeared immediately as intense blue spots, while later certain nonspecific paler blue spots sometimes developed. It was found advisable to check the reagents before using, by mixing equal volumes of acidic stannous chloride and acidic molybdate solutions in a test tube: the mixture should remain colorless or nearly so.

Ancillary Reactions

Lipolysis of lipid peroxides was carried out according to Desnuelle, Naudet, and Rouzier (9) with pancreatic lipase, B grade,[7] at 37° in phosphate buffer (pH 7.4) containing 0.1 M sodium taurocholate.

Methylations were carried out with diazomethane prepared from *N*-methyl-*N*-nitroso-*N'*-nitroguanidine[8] and 2 N NaOH, according to McKay (10). GLC was performed as reported by Farquhar, Insull, Rosen, Stoffel, and Ahrens (11). Solutes were recovered from preparative TLC plates by the technique of Goldrick and Hirsch (12).

RESULTS

Development of chromatoplates in two pilot systems (45% EE–PE and C–M–H_2O) rapidly demonstrated the polarities and approximate amounts of lipid peroxides present in a mixture, and indicated whether to seek more definitive information through use of other systems. With highly polymerized viscous samples it was advisable to separate the mixture into two fractions on the basis of

[6] Worthington Biochemical Corporation, Freehold, N. J.

[7] Biochemical Research Corporation, Los Angeles 63, Calif.

[8] Aldrich Chemical Company, Inc., Milwaukee 12, Wis.

solubility in PE or 5% EE–PE. Separate analysis of the more and less soluble fractions resulted in better separations of the less polar peroxides and less tailing.

Figure 1 illustrates schematically the chromatographic behavior of lipid peroxides in various solvent systems. It is apparent that monoperoxides in each lipid class can be identified readily by use of appropriate eluents and reference compounds. Some insight into the polarities of 0.50, free cholesterol 0.26. Figure 2 illustrates the comparative polarities of various lipid peroxides developed in 45% EE–PE (NH_3), and Fig. 3 shows the same, after development in 30% EE–PE and C–M–H_2O.

Cholesterol and Cholesterol Esters

Peroxides of cholesterol esters were separable in the nonpolar systems, 15–65% EE–PE. After autoxidation of

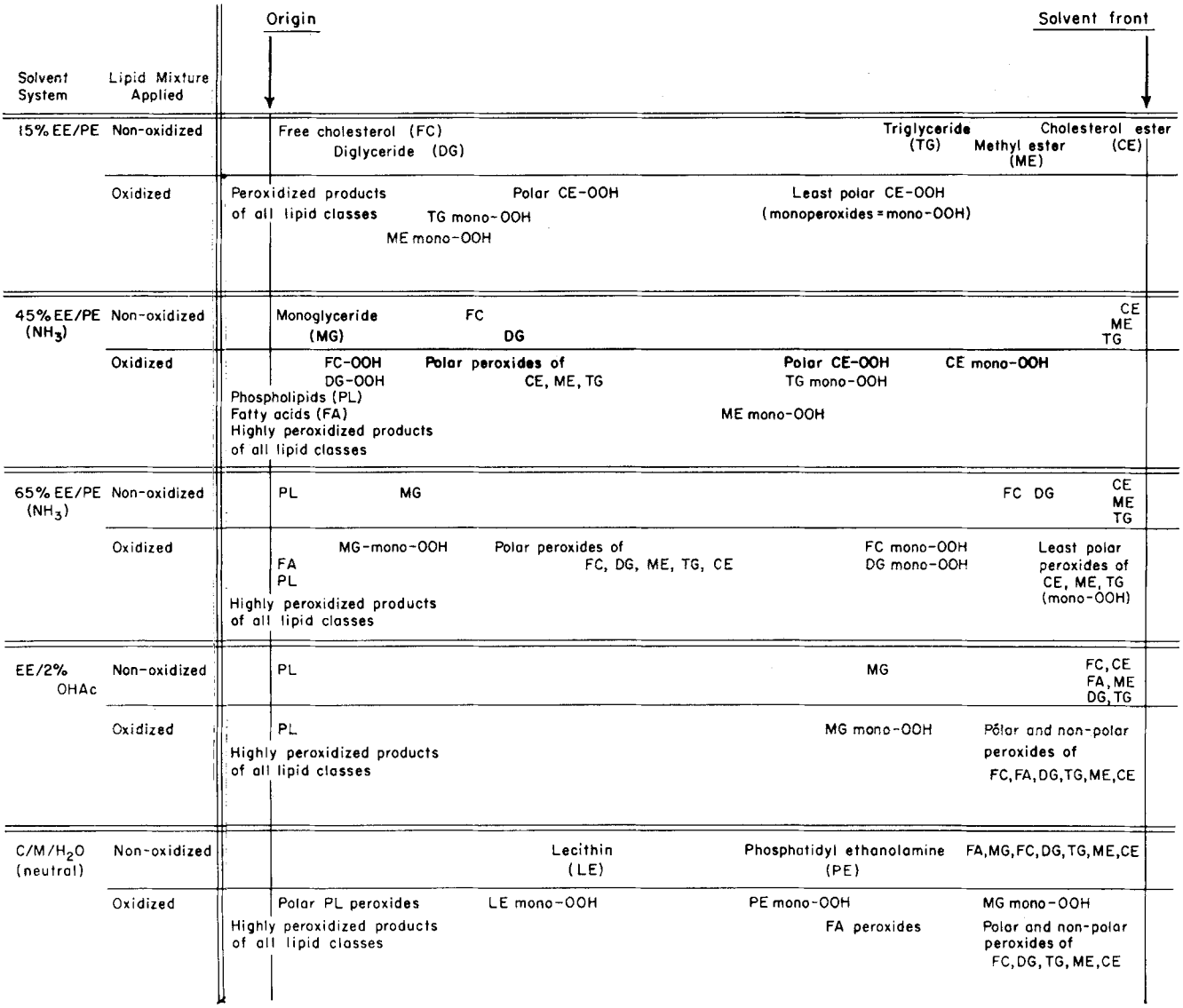

FIG. 1. Schematic presentation of approximate R_F values of unoxidized and oxidized lipids in five developing systems.

peroxy, hydroxy, and epoxy groups can be obtained by inspection of the following series of R_F values (listed in order of increasing polarity) in 30% EE–PE: cholesteryl oleate (CO) 1.00, $C_{18:1}$ME 0.97, triolein (TO) 0.95, least polar CO peroxides 0.85, epoxy-$C_{18:1}$ME 0.80, TO monoperoxide 0.72, more polar CO peroxides 0.68, $C_{18:1}$ME monoperoxide 0.65, hydroxy-$C_{18\ 1}$ME

cholesteryl oleate (CO), chromatoplates developed in 30% EE–PE showed as many as 13 KI–starch positive spots. Inasmuch as similar analysis of autoxidized oleic acid shows a smaller number of peroxides, we concluded that autoxidation of CO caused oxidation in the ring as well as in the fatty acid chain. As further evidence for oxidative changes in the ring, cholesteryl stearate autoxi-

dized under the same conditions was found to have a TLC pattern similar to that of autoxidized CO, although the products were fewer in number and smaller in concentration. Autoxidation products of free cholesterol were best resolved in 65% EE–PE, with most products (even after extended autoxidation) moving between free cholesterol and monoglyceride. These products turned blue at 100° after spraying with sulfuric acid.

Fatty Acid Methyl Esters

In the systems 30–65% EE–PE various fatty acid methyl ester peroxides of $C_{18:1}$ME were resolved, as were those of $C_{18:2}$ME and $C_{18:3}$ME (Figs. 2, 3), whereas in EE–acetic acid and C–M–H_2O these products migrated with or close to the solvent front. Highly polymerized products remained at the origin in all systems.

The structure of the major component running in 30% EE–PE between esterified cholesterol and free cholesterol was elucidated with the aid of preparative TLC: the recovered solute proved to have a peroxide value which was 87% of the theoretical value of methyl oleate monoperoxide. The compound could be brominated, proving that the double bond was still present, and could be reduced with KI in aqueous acetic acid with formation of a reduction product which had the same R_F value by TLC as methyl ricinoleate. GLC analysis of the reduction products on a polyester stationary phase column showed a group of three peaks in the region of methyl ricinoleate. These peaks may represent the hydroxyesters corresponding to the four known $C_{18:1}$ME hydroperoxide isomers (13).

Time studies (by TLC) of the autoxidation of $C_{18:2}$ME and $C_{18:3}$ME showed the appearance first of a single spot with the same R_F value as $C_{18:1}$ monoperoxide (Fig. 2). In the case of $C_{18:2}$ME this spot presumably contained the two known isomers (14–16) of methyl linoleate monohydroperoxide, which would not be expected to differ greatly in polarity. Later autoxidation products moved between monoperoxide and monoglyceride; they were formed much more readily with $C_{18:2}$ME and $C_{18:3}$ME than with $C_{18:1}$ME. These products may be dimers and trimers containing peroxide groups, compounds related to the secondary oxidation products studied by Dulog, Selz, and Kern (17).

Glycerides

Peroxides of triglycerides and fatty acid methyl esters showed rather similar chromatographic properties (Figs. 2, 3). Best resolutions of glyceride peroxides were obtained in 30–65% EE–PE whereas in EE–acetic acid and in C–M–H_2O the less oxidized products of triglycerides and diglycerides moved nearly with the solvent front and the most highly polymerized fraction remained at the origin. In EE–acetic acid, monoglyceride peroxides (Fig. 1) were readily distinguished from tri- and diglyceride peroxides.

Studies by TLC of the autoxidation of triglycerides carrying oleic acid residues in various combinations of α- and β-positions were carried out in order to show whether monoperoxides were produced in the early stages. TO, SOS, and SSO were autoxidized, and the major (least polar) spots, all having the same R_F, were isolated by preparative TLC (25% EE–PE). The isolated solutes were then subjected to the action of pancreatic lipase for short periods in order to release the fatty acid in the α-positions, with production of free fatty acid and diglyceride moieties. With TO and SSO the only peroxides obtained were in the free fatty acid fraction, while in the case of SOS, diglyceride peroxides and (to a lesser extent) monoglyceride peroxides and traces of free fatty acid peroxides were produced. Furthermore, the fatty acid peroxides were extracted and reesterified with diazomethane. The R_F value of the reesterified peroxides was identical with that of $C_{18:1}$ME monoperoxide. This proves that the triglyceride peroxides were monoperoxides.

To obtain information concerning the behavior on TLC of di- and triperoxides of triglycerides, triglycerides containing two and three oleic acid radicals were autoxidized. Chromatograms of autoxidized SOO or OSO showed two major peroxidation products. The less polar monoperoxide spot predominated; the more polar product had an R_F value similar to that of unoxidized diglyceride. These studies indicated an equivalence in polarity between one glycerol hydroxy group and two peroxide groups. Extensive autoxidation of SOO, OSO, triolein, and natural triglyceride mixtures (corn oil and linseed oil) produced monoperoxides, but also complex mixtures of oxidation products of higher polarity (which chromatographed between unoxidized diglyceride and monoglyceride).

Fatty Acids

Peroxides of 18:1, 18:2, and 18:3 fatty acids remained at or near the origin when nonpolar solvent systems were used (Figs. 2, 3) and migrated with or close to the solvent front in EE–acetic acid. Highly polymerized products of fatty acids remained at the origin in all systems. In C–M–H_2O, peroxides of fatty acids overlapped with phospholipid peroxides.

To ascertain the presence of fatty acid peroxides in a complex lipid mixture, chromatograms were run both in acidic and in neutral or alkaline systems; fatty acid peroxides were held at or near the origin in an alkaline system. Moreover, they could then be isolated by equilibrating a chloroform solution of the lipid mixture with aqueous carbonate; the alkaline extract containing the fatty acid peroxides was acidified and extracted with

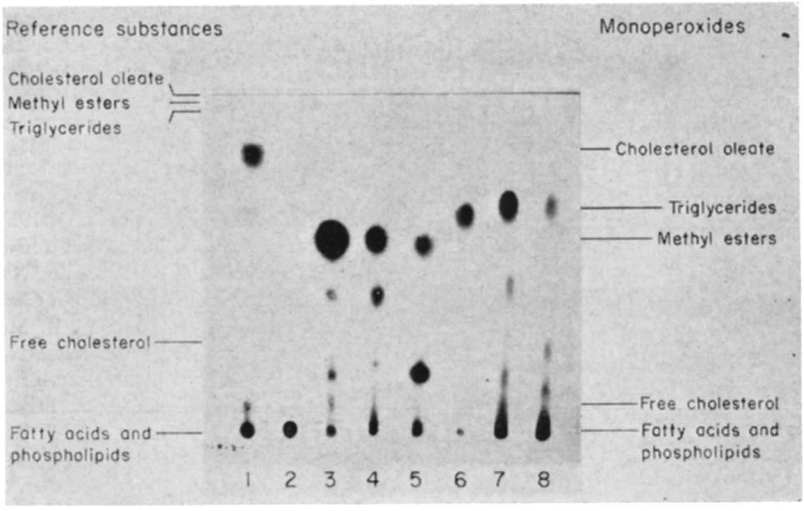

FIG. 2. TLC of various lipids mildly peroxidized by UV-autoxidation. System: 45% EE–PE (NH_3); peroxides indicated by KI–starch reaction.

1, cholesteryl oleate and free cholesterol. *2*, fatty acids and phospholipids. *3*, $C_{18:1}$ methyl esters (ME). *4*, $C_{18:2}$ME. *5*, $C_{18:3}$ME. *6*, triolein (moderately oxidized). *7*, corn oil. *8*, linseed oil (heavily oxidized).

ethyl ether. (Extractions must be performed quickly to avoid decomposition and further oxidation of peroxides.) Solutes in the ethereal extract were esterified with diazomethane, and the chromatographic behavior of the methyl ester peroxides was compared with that of reference materials.

Phospholipids

Resolution of phospholipid peroxides was achieved only in C–M–H_2O (Fig. 3). In this system highly polymerized compounds of all lipid classes remained at the origin, and the neutral lipid peroxides migrated with or close to the solvent front. Fatty acid peroxides interfered particularly with peroxides of phosphatidyl ethanolamine. If both were present in a mixture, it was helpful to develop the plates firstly in EE–acetic acid and then (after drying in N_2) in C–M–H_2O, or alternatively to make a single run in C–M–H_2O acidified with 2% of acetic acid. With both procedures the fatty acid peroxides migrated well ahead of the phospholipid peroxides.

After only 5 hr of UV-autoxidation, highly purified red cell phosphatidyl ethanolamine (PE) showed three closely connected peroxide spots which partly overlapped unoxidized PE (Fig. 3, III), while smaller amounts of other peroxides tailed from the origin. (All PE peroxides were phosphorus- and ninhydrin-positive.) After 24 hours' autoxidation no material reacting to the KI–starch indicator remained; the oxidation products appeared mainly as a large phosphorus-positive fraction tailing from the origin. Since PE peroxides are quickly converted into more polar, peroxide-free polymers, the absence of materials reacting with KI–starch may be misleading when analyses are made of

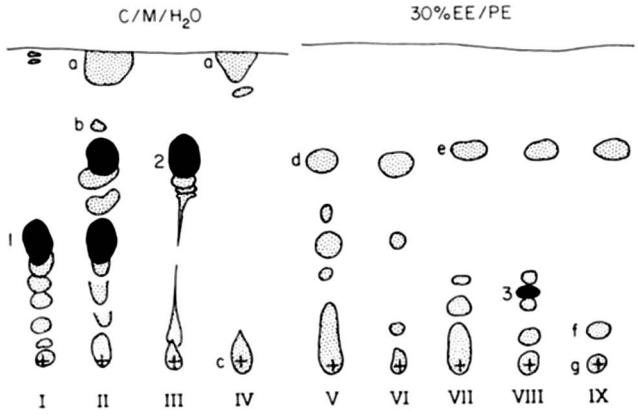

FIG. 3. TLC patterns of peroxides in polar and nonpolar systems (C–M–H_2O = chloroform–methanol–water 75:25:4.2; 30% EE–PE = ethyl ether–petroleum ether 3:7). Indicator: KI–starch. Peroxides = stippled areas; unoxidized reference substances = solid.

I, autoxidized soybean lecithin (LE) (UV-catalyzed). *II*, mixed muscle lipids of vitamin E-deficient animals fed corn oil methyl esters (ME). *III*, autoxidized phosphatidyl ethanolamine (PE). *IV*, neutral lipid peroxides. *V*, $C_{18:2}$ME peroxides (UV-catalyzed). *VI*, $C_{18:2}$ME peroxides (lipoxidase or peroxidase catalyzed). *VII*, adipose tissue lipid peroxides of vitamin E-deficient rats on linseed oil ME. *VIII*, adipose tissue lipid peroxides of vitamin E-deficient rats on corn oil ME. *IX*, lipolytic products of triglyceride monoperoxides (TG mono-OOH) with the OOH-group in β-position. *1*, lecithin; *2*, phosphatidyl ethanolamine; *3*, free cholesterol.

(*a*) neutral lipid peroxides; (*b*) phospholipid peroxide (?); (*c*) highly polymerized peroxides of all lipid classes; (*d*) $C_{18:2}$ME mono-OOH; (*e*) TG mono-OOH, (*f*) diglyceride mono-OOH; (*g*) fatty acid and monoglyceride peroxides.

highly unsaturated compounds such as PE. Phosphatidyl serine peroxides appeared in the region of lecithin and also near or at the origin. Soybean lecithin, after 5 hours' UV-autoxidation, showed a series of six peroxide spots, all containing phosphate groups (Fig. 3, I).

Biological Aspects

Qualitative analyses of lipid peroxides in biological systems will be presented elsewhere.[9] Under normal dietary conditions lipid peroxides were found to be absent from extracts of liver, brain, testis, muscle tissue, adipose tissue, serum, and red cells of rats. In vitamin E deficiency, lipid peroxides accumulate in several tissues, particularly in the adipose tissue; in the tissue extracts peroxides derived from triglycerides and phospholipids were found, but none from other lipid classes.

The TLC patterns of lipid peroxides produced by mild UV-autoxidation were comparable with those caused by lipoxidase and by heme catalysis, and with those found in tissue extracts of vitamin E-deficient rats on highly unsaturated fat diets. Under all conditions monoperoxides were found in significant amounts. However, in biological studies the peroxides of intermediate polarity were less complex and were present in lower concentrations, and highly polymerized material (at the origin in C–M–H_2O) was almost absent.

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