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Note

Permeation chromatography of fluorescent products from tissues and peroxidized lipids

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Peroxidation of phosphatidylethanolamine (PE), mitochondria, microsomes, and unsaturated fatty acids in the presence of amines gives rise to fluorescent products[1–3]. Since fluorescence spectroscopy is a powerful technique capable of detecting nanogram levels of substances, it is a very attractive method for following lipid peroxidation *in vivo* where other methods have been found limiting. One problem with this technique is the presence in tissues of interfering fluorophores such as vitamin A. Previously, this problem was decreased by irradiating samples with UV light[4]; however, this method can give variable results, as shown by Csallany and Ayaz[5]. These workers used Sephadex LH-20 to separate chromatographically the fluorescent (350 nm excitation, 435 nm emission) products of rat and mouse tissues from interfering compounds. The fluorescent products were found in the void volume of the Sephadex LH-20 eluate. In the present study, silylated glass beads were employed for permeation chromatography of fluorescent lipid peroxidation products and tissue extracts in order to determine more accurately the molecular size of fluorescent products and to eliminate the swelling problems of Sephadex LH-20 as solvents are changed.

MATERIALS AND METHODS

Soybean and bovine brain PE were purchased from Avanti Biochemicals (Birmingham, Ala., U.S.A.). Egg PE and hexamethyldisilazane were purchased from Sigma (St. Louis, Mo., U.S.A.). Burdick and Jackson Labs. (Muskegon, Mich., U.S.A.) was the source of glass distilled chloroform and methanol. Lot No. 360 120–200 mesh CPG00120B glass beads with 118 Å mean pore diameter were purchased from Electro Nucleonics (Fairfield, N.J., U.S.A.). These beads had a surface area of 186 m^2/g, a pore volume of 0.71 cm^3/g, and a pore distance $\pm 7.8\%$. Vitamin A was purchased from Nutritional Biochemical Corp. (Cleveland, Ohio, U.S.A.). Polystyrene molecular weight standards were obtained from Waters Assoc. (Milford, Mass., U.S.A.). All other chemicals used were reagent grade. The rats used were randomly bred albino male retired breeders (12–15 months) from Holtzman (Madison, Wisc., U.S.A.).

Glass beads (100 cm^3) were silylated by refluxing with 100 ml of hexamethyldisilazane and 100 ml of hexane for 8 h. The beads were dried and exposed to N,O-bis(trimethylsilyl)acetamide overnight before they were washed with chloroform.

Samples were chromatographed on a 100 × 0.6 cm I.D. glass column of silylated glass beads at 30°. Solvents were delivered to the column under nitrogen at 10–50 p.s.i. as needed to give a flow-rate of *ca.* 1 ml/min, and samples were introduced on-line by injecting each in 5–100 μl of the eluting solvent. Fraction size was 1.2–1.5 ml. The column was standardized with 233,000, 35,000, 17,500, 15,000, and 3600 dalton polystyrene polymers using chloroform as the eluting solvent. The elution volume of 233,000 dalton polystyrene was 13 ml (void) and benzene was 23 ml (contained volume). The standards were detected by absorbance at 260 nm.

In order to minimize the problems of peroxidation of tissue lipids during the preparation of naturally occurring fluorescent products, the rat tissues were rapidly removed from the animal, placed in 20 times their volume of extracting solvent, homogenized with a PTFE–glass homogenizer, and water (one-fifth of total volume) was added to make two phases. The chloroform phase was treated as described below.

Microsomes were obtained from rat liver homogenized in 1.15% potassium chloride (w/v) and 10 m*M* phosphate buffer, pH 7.0 (1 g liver per 5 ml of buffer) by centrifugation of the homogenate for 30 min at 27,000 *g* followed by centrifugation of the supernatant at 95,000 *g* for 45 min. The microsomes were washed with 80 m*M* phosphate buffer, pH 7.0, and suspended in the same buffer. Peroxidation of the microsomes was carried out in an oxygen atmosphere at 37° for 4 h (7.8 mg lipid/ml) after the addition of ferric chloride and ascorbate to make the solution 1 m*M* in each. Soybean or bovine PE was peroxidized[1] for 24 h prior to extraction. Egg PE was peroxidized in an air atmosphere and in chloroform–methanol (2:1) for 24 h.

Lipids from all preparations were extracted by the addition of 20 volumes of chloroform–methanol (2:1) to the reaction mixtures, followed by the addition of four volumes of water. For the peroxidized PE mixtures, 10 m*M* hydrochloric acid was substituted for water. The fluorescence of the chloroform phase was measured and then the sample was concentrated by rotary evaporation and/or under a stream of nitrogen. The chloroform–methanol mixture contained 0.05% 2,6-di-*tert.*-butyl-*p*-cresol and was usually deaerated. Fluorescence was measured with an Aminco-Bowman spectrophotofluorometer (American Instrument Co.).[1] Instrument parameters were set so that one unit of fluorescence represented 220 pg quinine sulfate/ml 0.05 *M* sulfuric acid. Quinine is a convenient standard for the fluorescent pigments under study. The column fractions were assayed for phosphate in order to determine where phospholipids eluted[6].

RESULTS AND DISCUSSION

Peroxidized bovine brain PE was chromatographed on the silylated glass bead column with chloroform–methanol (1:9) as the eluting solvent (Fig. 1). The fluorescent peroxidation products eluted immediately after the void volume and had fluorescence excitation and emission maxima of 350 nm and 430 nm, respectively. Chromatography of peroxidized egg or soybean PE or peroxidized rat liver microsomes gave the same chromatographic pattern. When the eluting solvent was changed to chloroform–methanol–acetic acid (66:33:1), the fluorescent products eluted in the contained volume (Fig. 1), indicating that they were smaller than 3500 daltons as compared to polystyrene standards. When chloroform–methanol–acetic acid (10:90:1) or chloroform–methanol (2:1) were used as solvents, the fluorescence eluted at a position

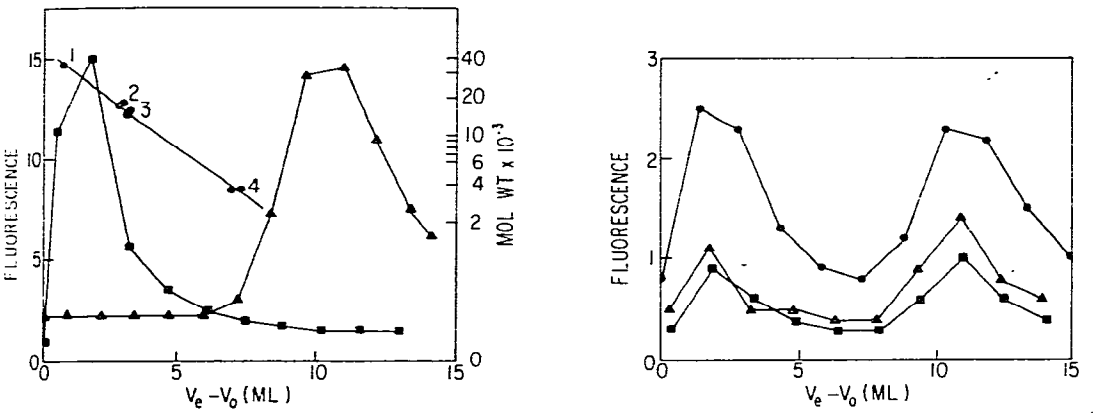

Fig. 1. Permeation chromatography of peroxidized bovine brain PE in chloroform–methanol (1:9) (■), and in chloroform–methanol–acetic acid (66:33:1) (▲). The column was standardized with 35,000 (1), 17,500 (2), 15,000 (3) and 3600 (4) dalton polystyrene standards (●) as described in Materials and methods.

Fig. 2. Permeation chromatography of fluorescent products of rat testes (■), kidney (▲), and heart (●) lipids. Sample sizes of 100 μl were applied (100 mg/ml). The eluting solvent was chloroform–methanol (1:9). Excitation was set at 350 nm and emission at 430 nm.

between the two peaks shown in Fig. 1, and the elution profile was very broad, indicating that there was no single sized fluorescent moiety. These elution profiles indicate that the fluorescent products were predominantly large micelles of one size in chloroform–methanol (1:9) and were single molecules or micelles smaller than 3500 daltons in chloroform–methanol–acetic acid (66:33:1). The fluorescent moieties appear to be acidic since protonation with acetic acid is needed to break up the micellar interactions. Fluorescence depolarization studies in glycerol of similar products isolated from a crude preparation of commercial cephalin indicated a molecular size of 1 - 10^3 daltons. These fluorescent products also occurred as micelles in water and chromatographed as large particles on Sephadex G-100. Chloroform–methanol (1:9) was used by Csallany and Ayaz[5] to separate fluorescent products extracted from rat and mouse tissues on Sephadex LH-20. These workers found characteristic ceroid fluorescence in the void volume, indicating that the fluorescent material was probably a micelle. The micellar nature of the fluorescent peroxidized PE products might also explain the observations that lipofuscin from human brain fractionated in the range of 5,000 to 10,000 daltons and contained fatty acids[7].

The lipids from heart, kidneys and testes of male retired breeder rats were chromatographed on the same silylated glass bead column (Fig. 2). Blood, muscle, adipose and brain lipids were also examined. Fluorescent products were found in amounts that varied from tissue to tissue and in the same tissue from rat to rat. The least amount of fluorescence was found in adipose and brain lipids. The lower levels of fluorescence shown in Fig. 2 were at the limit of detectability. The early eluting fluorescent products eluted in about the same volume and had similar fluorescence spectra as did fluorescent products from peroxidized PE (excitation maximum, 350 nm; emission maximum 430 nm). Little or no fluorescence was found in the early eluting fractions when extracts from young rats (5–7 months) were chromatographed.

The fluorescent products eluting in the contained volume of all samples had fluorescence spectra distinctly dissimilar to those of the large molecular sized fractions. Non-peroxidized PE and vitamin A also eluted in the total column volume. This indicates that the early eluting fluorescent moieties should have been partially free of other lipids and interfering fluorescent moieties, but only partially free since they were micelles and could have contained other lipids. Upon re-chromatography the early eluting fluorescent fractions from the heart lipids eluted as a single peak with a similar elution volume as obtained in the first chromatography. When the fluorescent fractions from heart lipids which eluted in the contained volume were re-chromatographed, two peaks of fluorescence eluted, one in the contained volume and the other in the same volume as the early eluting fractions. These early eluting fractions had the same fluorescence excitation and emission maxima as the early eluting fractions in the original chromatograph. This phenomenon makes quantitation impractical, since all of the fluorescent moieties of interest do not always co-chromatograph. Another problem encountered that affected quantitation was that at the concentrations needed to detect fluorescence, all of the lipids would not dissolve in the solvent mixture used.

Heart lipids chromatographed in chloroform–methanol–acetic acid (66:33:1) gave one peak of fluorescence in the contained column volume fractions and had the same fluorescence excitation and emission maxima as peroxidized PE in the same solvent system. This indicates that the fluorescent moieties were similar, if not the same.

These results show that the fluorescent products with excitation and emission maxima at 350 nm and 430 nm, respectively, from peroxidized soybean and bovine brain PE, peroxidized microsomes, and tissues of old rats behave similarly when chromatographed on silylated glass beads with two different solvent systems. Considering the fluorescence spectra and the acidic nature of the peroxidation products and tissue extracts examined in this study, it appears that they were similar to, if not the same as the ceroid described by Siakotos and Armstrong[8].

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