Autoxidation as a cause of altered lipid distribution in extracts from human red cells

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ABSTRACT A characteristic alteration in the distribution of human red cell phospholipids represents an artifact due to autoxidation of the lipid extract. This alteration is manifested on silicic acid chromatography by a decrease mainly in the phosphatidyl ethanolamine and phosphatidyl serine fractions (probably because of their abundance of highly unsaturated fatty acids) and an increase in the phospholipid recovered with the more polar fractions, sphingomyelin and lysolecithin. No evidence was found for "lysocephalin" formation or plasmalogen breakdown in dry lipid extracts after autoxidation by exposure to air at room temperature for 24–35 hr. On thin-layer chromatography, however, the ninhydrin-positive streaking in the autoxidized samples may be erroneously attributed to the presence of "lyso" derivatives. When the alterations in lipid distribution described above are found, the possibility of this artifact should be considered.

KEY WORDS autoxidation · lipids · human red cells · lipid artifact · phospholipids · lysophosphatides · fatty acids · antioxidants · spurious peaks · gas–liquid chromatography

In studies of human red cell lipids by chromatographic methods, several investigators have noted a characteristic alteration of phospholipid distribution manifested by a decrease in the "cephalin" fraction and an increase in more polar fractions (2–5). This alteration has been attributed to the formation of "lyso" derivatives resulting either from use of heparin (2) or from storage of dry lipid extracts (3). Preliminary observations in this laboratory, however, suggested that lyso derivatives were not responsible. In addition, the infrequent appearance of this alteration when heparin was used routinely suggested that heparin alone was not responsible. Nevertheless, the occasional appearance of the phospholipid alterations in only one of a number of lipid extracts from the same sample of red cells indicated that these changes were artifactual. Because of the importance of this artifact to any study of red cell lipids, the present investigation was carried out to characterize these alterations and to define the basis for their formation.

MATERIALS AND METHODS

Blood was collected from normal subjects with heparin, EDTA, or ACD Solution (USP Formula A) as the anticoagulant. Red cells were isolated by centrifugation and washed three times with isotonic saline; the buffy coat was discarded. Lipids were extracted with methanol–chloroform 1:2 (v/v) as previously described (6), or with methanol–chloroform 1:1 (v/v) as described by Reed, Swisher, Marinetti, and Eden (7); all extracts were washed with 0.05 M KCl (8) and centrifuged, and the lower phase was collected, dried under reduced pressure, and stored in darkness either dry in an evacuated flask at −25°C or as a chloroform solution at −25°C.

Phospholipid distribution was determined by silicic acid column chromatography with solutions of chloroform, methanol, and water as eluents as previously described (9) or by TLC on Silica Gel H (Brinkmann Instruments Inc., Westbury, N.Y.),[1] 0.5 mm in thick-

Abbreviations: TLC, thin-layer chromatography; BHT, 2,6-di-*tert*-butyl 4-methyl phenol; GLC, gas–liquid chromatography.

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[1] Contaminants in the Silica Gel H that gave high blank values in the phosphorus determination were removed by a washing procedure kindly suggested by Dr. Frank Parker prior to his publication of the method (10). Subsequently, another grade of material, Silica Gel H-HR (Brinkmann Instruments Inc., Westbury, N.Y.), was found to be sufficiently free from phosphorus contamination to make washing unnecessary.

ness, with the developing solvent chloroform–methanol–glacial acetic acid–water 25:15:4:2 (v/v) described by Skipski, Peterson, and Barclay (11). The chromatogram was made visible with iodine vapor, ninhydrin spray, or 18 N H_2SO_4 spray. For quantification, the chromatogram was lightly stained with iodine vapor and the spots were outlined; each spot was scraped into a long-stemmed funnel that was plugged near the bottom with glass wool and the phospholipid was eluted with approximately 2 ml of HCl–methanol 1:19 (v/v). Elution of phospholipid by this method was shown to be complete unless the phospholipid was autoxidized (see below). Phosphorus was determined by the method of Bartlett, modified by digestion with perchloric acid according to Marinetti (12).

Fatty acid esters were determined as the hydroxamate (13), and plasmalogens as the *p*-nitrophenylhydrazine derivative or by specific iodination of the vinyl ether as described by Camejo, Rapport, and Morrill (14). Autoxidation products were determined by the thiobarbituric acid reaction in water-saturated butanol with absorbancy measurement at 535 mμ (15), and by measurement of ultraviolet absorbancy (16, 17) at 234 and 268 mμ. Methyl esters of the fatty acids were prepared by the method of Stoffel, Chu, and Ahrens (18). GLC was performed with a Perkin Elmer instrument equipped with a flame ionization detector; a 6 ft column of butanediol succinate polyester on Chromosorb W was employed at 190°C with nitrogen at a flow rate of 37 ml/min at the outlet. The major fatty acid methyl ester peaks were identified by comparison with the data of Farquhar (4) and Ways and Hanahan (19).

DL-α-Tocopherol with an activity of 1.1 IU/mg was obtained from Nutritional Biochemicals Corp., Cleveland, Ohio; 2-thiobarbituric acid from Eastman Organic Chemicals, Rochester, N.Y.; heparin sodium, USP, and EDTA ("Certified Reagent") from Fisher Scientific Co., Fair Lawn, N.J., and 2,6-di-*tert*-butyl 4-methyl phenol (BHT) (Ionol, CP) as a gift from Shell Chemical Co., New York, N.Y. Chloroform was redistilled in an all glass system and methanol was added as a preservative, 1:50 (v/v). Other reagents were "analytical" grade.

RESULTS

After silicic acid column chromatography, the characteristic alteration of phospholipid distribution appeared as a decrease in the "cephalin" fraction, which consisted mainly of phosphatidyl ethanolamine and phosphatidyl serine, with an increase in the more polar fractions, sphingomyelin and lysolecithin (Table 1). The percentage of cholesterol also appeared to have decreased, but data in that regard were limited to those from only four altered samples, for which the controls were duplicate extracts from the same sample of cells. That this lipid alteration after silicic acid column chromatography occurred either to an obvious degree or not at all suggests an all-or-none phenomenon (see Fig. 3, p. 393). Of 70 consecutive red cell lipid samples analyzed, the alteration appeared spontaneously in 13. Results on the 9 of the 13 with the most complete data are shown in Table 1. Although the alteration was obvious in these samples, considerable variation can be seen from the standard deviations.

TABLE 1 DISTRIBUTION OF ALTERED LIPIDS OF HUMAN RED CELLS ON SILICIC ACID COLUMN CHROMATOGRAPHY

	Lipid Sample	
	Controls (13)*	Altered (9)
	mmoles/liter packed cells	
Cholesterol†	3.70	3.34
Phospholipids		
Total lipid P	4.50 ± 0.36	4.52 ± 0.41‡
	per cent	
"Cephalin" fraction§	42.4 ± 1.0	30.5 ± 2.7
Lecithin "	32.7 ± 2.0	32.7 ± 3.8
Sphingomyelin "	23.1 ± 1.9	28.0 ± 3.4
Lysolecithin "	1.8 ± 0.2	8.7 ± 2.4
Recovery from columns	97 ± 3	96 ± 4

* Data on 13 normal subjects reported previously (5).
† Determined on paired extracts from four subjects in which one of the duplicates became altered and the other unaltered duplicate served as a control.
‡ Determined on only four samples.
§ Includes phosphatidyl ethanolamine and phosphatidyl serine.

The similar values found in the altered and control series for total lipid phosphorus extracted from the cells and for recovery of lipid phosphorus from the columns indicated that part of the "cephalin" fraction was modified and eluted with the more polar fractions, rather than lost from the red cells or from the lipid extracts. When chromatographed on silicic acid-impregnated filter paper or on thin-layer plates, samples of the altered lipid gave a ninhydrin-positive streak from the origin to phosphatidyl ethanolamine, a finding that supported the concept of modification rather than loss of the "cephalins."

Several factors in the processing of red cells were evaluated for a possible relationship to the production of these phospholipid alterations. Heparin was used as the anticoagulant in vitro in amounts up to 6 mg/ml of blood and the heparin-containing blood was allowed to stand at room temperature for 1 hr before the red cells were washed. No abnormality in phospholipid distribution was found in 23 consecutive samples that were analyzed immediately after completion of the extraction procedure. This finding indicated that heparin *alone* was not responsible. The possibility of exposure to acidic conditions

TABLE 2 RELATIONSHIP OF THE DURATION OF STORAGE OF DRY HUMAN RED CELL LIPID EXTRACTS TO THE DEVELOPMENT OF PHOSPHOLIPID ALTERATIONS*

Storage Time	Number of Samples	Number Altered	Per Cent Altered
days			
0–2	28	1	4
3–20	42	12	29

* Stored in evacuated flasks at −25°C in darkness.

TABLE 3 ANALYSIS OF RED CELL LIPID EXTRACTS THAT DEVELOPED SPONTANEOUS ALTERATIONS DURING STORAGE*

Determination	Lipid Sample	
	Control	Altered†
	OD for mM P	
Thiobarbituric acid test	0.42	1.74
Fatty acid distribution in total phospholipids		
	weight per cent	
16:0	19.1	19.1
18:0	16.3	15.4
18:1	15.9	14.9
18:2	9.9	4.0
20:4	15.1	1.1
24:0	3.0	3.0
22:4 + 22:6 ?‡	7.5	1.9
Sum of 7 minor peaks	13.2	8.1

* Average values from two normal subjects. All samples were stored dry in vacuo at −25°C in darkness.

† Fatty acid percentages in the altered sample were recalculated to make the percentage of palmitic acid equal to that of the control sample.

‡ Sum of two peaks with retention times of 5.06 and 6.93 relative to methyl stearate on butanediol succinate polyester.

was considered, but hemolysis in a hypotonic acetate buffer at pH 4.6 caused no evident phospholipid alteration. Nor were discernible phospholipid alterations, caused by mechanical stress and oxygen exposure, obtained by shaking washed red cells in saline with glass beads while bubbling through 100% oxygen for 15 min before extraction. Methemoglobin production and Heinz body formation by acetylphenylhydrazine were not accompanied by phospholipid abnormalities that could be detected on column chromatography. Addition of the detergent "Tide" (Procter & Gamble), used to wash the glassware, at a concentration of 0.7 mg/ml of a suspension of red cells in saline (hematocrit 50) and incubation for 1 hr at room temperature caused no apparent alterations. Since none of these manipulations of red cells appeared to be responsible for the observed phospholipid alterations, attention was directed to the lipid extracts.

A relationship to the duration of storage of the lipid extract was evident from the data in Table 2, which shows the occurrence of the lipid alterations in 13 of the 70 consecutive extracts that had been stored dry in evacuated flasks at −25°C in darkness for various periods of time before analysis. The finding of lipid alterations after storage in the same extracts that were previously shown to be normal confirmed the relationship to storage.

Table 3 shows analyses on two altered lipid extracts for which ideal controls were available, namely, duplicate extracts from the same blood samples which had been stored, apparently, in the same manner but which developed no alterations. The thiobarbituric acid test for oxidation products was four times higher in the altered lipid extracts, which suggests autoxidation as the basis for these changes. In support of the postulate of autoxidation were the values for fatty acid distribution. Losses of unsaturated fatty acids, particularly the highly unsaturated ones, were evident in the altered samples. The ninhydrin-positive streaking on TLC and the increased thiobarbituric acid reaction seen with the altered samples could be reproduced by exposure of dry lipid extracts to air at room temperature and prevented by addition of antioxidants (see below). These findings indicated that the alterations resulted from autoxidation.

To study further the changes accompanying autoxidation, we analyzed lipid extracts that had been exposed to air (Table 4). A convenient method for determination of autoxidation in a lipid sample was the measurement of UV absorbancy. The UV spectrum of a sample of red cell phospholipid that was dried and exposed to air for 35 hr compared to that of an unexposed sample is shown in Fig. 1. These spectra resemble closely those reported by Lea (16) for autoxidized and native phosphatidyl ethanolamine derived from hen's egg. Absorbancy at about 234 and 268 mμ has been correlated with the appearance of conjugated dienes and conjugated trienes, respectively, as a consequence of autoxidation (17). An increase in both UV absorbancy and the thiobarbituric acid reaction was observed in the air-exposed samples (Table 4) and was incompletely prevented under these conditions by the presence of α-tocopherol.

TLC showed streaking of ninhydrin-positive material from the origin to phosphatidyl ethanolamine in the air-exposed samples (see also Fig. 3). Quantification of the phospholipid distribution by TLC showed changes analogous to those found on column chromatography. The phosphatidyl ethanolamine, phosphatidyl serine, and lecithin fractions were decreased, while phosphorus recovered in the region of sphingomyelin, lysolecithin, and the origin was increased. The low phosphorus recovery of the autoxidized samples suggested tighter binding of the lipids to the Silica Gel H as a result of autoxidation.

We performed the following studies to determine whether the trailing of autoxidized phospholipid on

chromatography was explained entirely by the formation of more polar autoxidation products or represented the concomitant formation of lyso derivatives. The fatty acid ester-to-phosphorus molar ratio in autoxidized lipid was consistently equal to the control values (Table 4). The other possible basis of lyso derivative formation was also evaluated, namely, cleavage of the vinyl ether bond of plasmalogens, found abundantly in the "cephalin" fraction (4, 19). Oxidation products, however, interfered with attempts at measurement of plasmalogen by two established methods. Falsely low values resulted with the iodimetric method, presumably because of oxidation of iodide to iodine by the lipid peroxides. Such oxidation of iodide by lipid peroxides, but in acidic organic solvent systems, is the basis of many methods for peroxide and hydroperoxide determination (17). Falsely high values resulted with the *p*-nitrophenylhydrazine method, a finding consistent with the appearance of α,β-unsaturated carbonyl groups among the autoxidation products (20). Lyso derivatives of red cell phospholipids prepared by mild acid hydrolysis gave rise to *discrete* ninhydrin-positive spots with mobilities of lysophosphatidyl ethanolamine and lysophosphatidyl serine, rather than the streaking observed in the autoxidized samples. Furthermore, lysophosphatidyl ethanolamine added to a normal red cell lipid extract was recovered with the lecithin fraction on silicic acid column chromatography rather than with the sphingomyelin and lysolecithin fractions as observed with spontaneously autoxidized "cephalin." The release of long-chain aldehydes as a result of autoxidation, moreover, could not be demonstrated on thin-layer silicic acid chromatography using petroleum ether–diethyl ether 9:1 (v/v) as solvent and the Schiff aldehyde stain (21). These aldehydes were released, however, when either 3 N HCl or 0.005 M $HgCl_2$ was added to the autoxidized lipid spot before chromatography. The native lipid behaved in the same manner. These results indicated that lyso compounds, derived either from the cleavage of ester or vinyl ether bonds, were not formed as a result of autoxidation under these conditions.

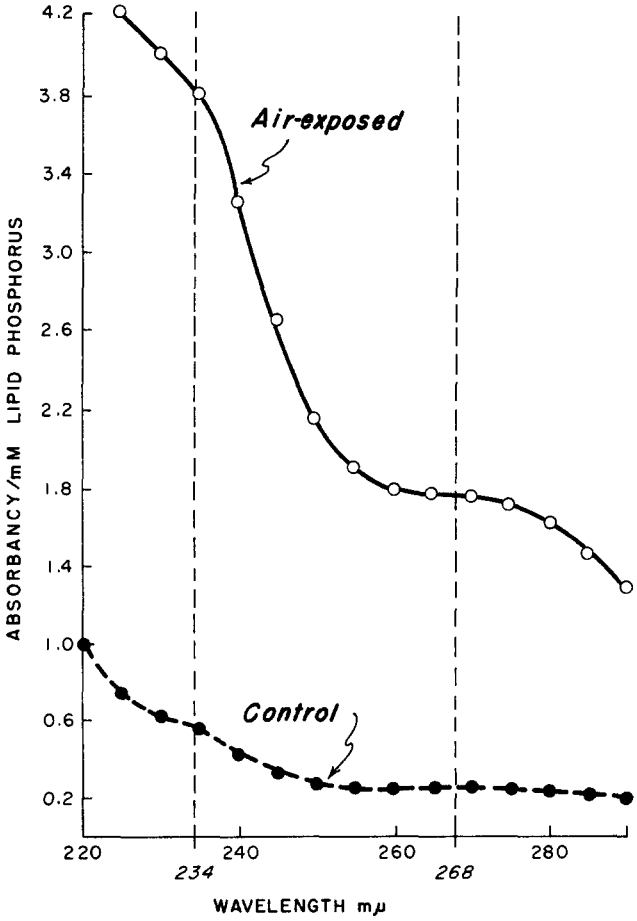

FIG. 1. Effect of air exposure on UV absorbancy in methanol of human red cell phospholipids. The control sample was stored at −20°C in chloroform, while the air-exposed sample was an aliquot of the control, dried and exposed to room air for 40 hr. Spectra were traced on a Cary recording spectrophotometer and absolute values for absorbancy determined on a Beckman DU spectrophotometer. Absorbancy measurements at 234 and 268 mμ were used as one criterion for autoxidation.

The protection of red cell lipids by antioxidants was demonstrated. Fig. 2 shows the effect of α-tocopherol on prevention of autoxidation as measured by absorbancy at 268 mμ; 2 μg of α-tocopherol per mg of lipid protected under these conditions. Fig. 3 illustrates protection of phospholipids by a synthetic antioxidant, 2,6-di-*tert*-butyl 4-methyl phenol (BHT). Under these conditions, BHT at a concentration of 0.7 μg/mg of phospolipid protected. In the presence of the lower amounts of BHT, samples showed the same ninhydrin-positive streaking that was characteristic of the spontaneous phospholipid alterations. The sudden transition from discrete spots to marked streaking seen in Fig. 3 suggested an all-or-none phenomenon consistent with the chain-reaction nature of autoxidation. Ultraviolet absorbancy was correlated with the degree of streaking.

DISCUSSION

These studies provide evidence for autoxidation as the basis for the spontaneous alteration of phospholipid distribution observed in lipid extracts of human red cells. This evidence includes an increase in absorbancy at 535 mμ in the thiobarbituric acid test for autoxidation products and a decrease in the relative amount of polyunsaturated fatty acids in the altered samples, as well as reproduction of these alterations on exposure of dry lipid extracts to air and their prevention by addition of antioxidants before exposure. Autoxidation was found predominantly in the phosphatidyl ethanolamine and phosphatidyl serine fractions, possibly because of their high

TABLE 4 ANALYSIS OF AIR-EXPOSED PHOSPHOLIPID EXTRACTS OF HUMAN RED CELLS*

Determination	Control	Exposed to Air†	
		α-Tocopherol Added‡	No α-Tocopherol Added
Ultraviolet absorbancy (OD for mmolar P)			
234 mμ	0.60	1.66§	4.09
268 mμ	0.21	0.68§	1.71
Thiobarbituric acid test (OD for mmolar P)	0.09	0.39§	1.19
Fatty acid ester to phosphorus molar ratio	1.32	1.31	1.37
Plasmalogen to phosphorus molar ratio			
p-Nitrophenylhydrazine method	0.12	0.38	0.44
Iodimetric method	0.13	‖	‖
Thin-layer chromatography			
Appearance of PE and PS areas¶ with ninhydrin spray	discrete spots	faint streaking	gross streaking
Phospholipid distribution		*per cent*	
Phosphatidyl ethanolamine	29.0	23.9	8.5
Phosphatidyl serine**	13.6	13.9	9.3
Lecithin	29.1	30.8	24.6
Sphingomyelin	24.5	26.4	31.4
Lysolecithin and origin	0.6	0.8	12.7
Recovery of applied phosphorus	96.8	95.8	86.5

* Data averaged from duplicate determinations on blood from two normal subjects. Phospholipids were isolated by silicic acid column chromatography before exposure to air.

† Exposed 35 hr at 25°C in light.

‡ 3.7 μg of α-tocopherol per mg of phospholipid added to an aliquot from one subject and 20 μg/mg added to an aliquot from the other subject afforded approximately equal degrees of protection.

§ Corrected for the absorbancy of α-tocopherol.

‖ Values were either low or negative in the air-exposed samples, presumably because of the oxidation of iodide to iodine by the lipid peroxides (see text).

¶ PE, phosphatidyl ethanolamine, PS, phosphatidyl serine.

** Phosphatidyl inositol made up about 1% of the total lipid P and was included with the phosphatidyl serine fractions.

content of polyunsaturated fatty acids (2, 4, 19). Evidence was also found by TLC for lesser amounts of autoxidation of the lecithin fraction, which contains smaller amounts of polyunsaturated fatty acids (2, 4, 19). The involvement of lecithin was probably masked on column chromatography by the trailing of autoxidized "cephalin" into the lecithin fraction. Cholesterol also appeared to be affected, but the data in that regard are limited. The susceptibility of cholesterol to autoxidation is a well-known phenomenon and has been reviewed recently (22). The apparent increase in sphingomyelin and lysolecithin on silicic acid chromatography was evidently due to recovery of the more polar oxidation products of the "cephalin" and lecithin with those fractions.

A vast literature has accumulated on peroxidation of biological lipids (23). Lea (16) studied in detail the autoxidation of the individual phospholipids of hen's egg and noted the particular sensitivity of phosphatidyl ethanolamine. Subsequent studies (4, 24–33) have called attention to the occurrence and manifestations of autoxidation of individual phospholipids from various sources. Similar autoxidative changes have been observed in this laboratory in phospholipids of beef brain and of subcellular fractions of rat liver, and can probably occur in any lipid extract containing unsaturated fatty acid or cholesterol.

Various hemoglobin derivatives have been shown to catalyze the autoxidation of animal tissue lipids and purified fatty acids (24, 34–36). Since methanol–chloroform extracts of red cells are contaminated with hemoglobin derivatives, these extracts may be particularly vulnerable to rapid autoxidation. These derivatives, such as hematin and protoporphyrin (37, 38), have an affinity for lipids and are difficult to remove from a lipid extract. To prevent inclusion of heme derivatives in the lipid extract, Rose and Oklander (39) proposed substitution of isopropanol for methanol in the extracting mixture.

Alteration of the "cephalin" fraction of human red cell lipid extracts was reported by Hanahan, Watts, and Pappajohn (2) and attributed to the formation of lyso derivatives resulting from the use of heparin. As a consequence, many investigators have considered heparin-anticoagulated blood an unreliable source for native phospholipids. Since, in the present studies, use of heparin did not result in alterations of phospholipid distribution, alternative hypotheses were investigated. De Gier and van Deenen (3) noted a similar alteration of the "cephalin" fraction, which they attributed to lyso compound formation resulting from storage of red cell "ghost"

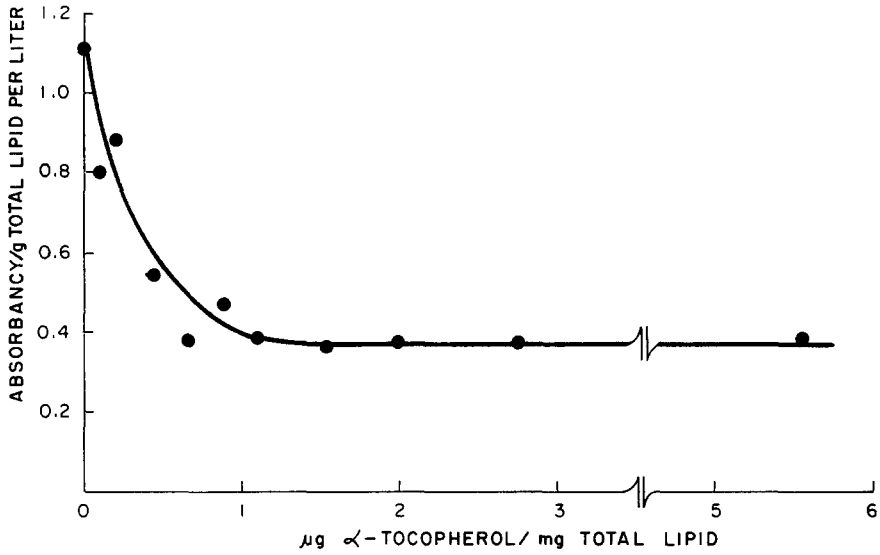

FIG. 2. Inhibition of autoxidation in a human red cell lipid extract by α-tocopherol, as measured by UV absorbancy. Aliquots of a human red cell total lipid extract with α-tocopherol added in increasing amounts were dried, exposed to room air for 24 hr, and dissolved in methanol for absorbancy measurements at 268 mμ. Absorbancy before exposure to air was 0.310 OD units for 1 g of total lipid per liter. These data were corrected for the absorbancy of α-tocopherol.

lipid extracts over phosphorus pentoxide in darkness for ten days. The "cephalin" fraction decreased from 31.5 ± 1% to 11.0 ± 1%, while the sphingomyelin fraction, which included lysophosphatides, increased from 33 ± 3% to 51 ± 1%. They concluded that most of the phosphatidal ethanolamine, or a chromatographically similar compound, was converted to its lyso derivative through cleavage of the labile vinyl ether bond. That conclusion was supported by chromatographic evidence, which was reported to show the ninhydrin-positive breakdown product to migrate similarly to a synthetic lysophosphatidyl ethanolamine. In the present studies the "cephalin" alteration was not associated with lyso compound formation. The trailing autoxidized phospholipids on silicic acid chromatography, either on paper (12, 24, 29, 31), column (27, 28, 32), or thin layers (31, 32), however, may be mistaken for lysophosphatides. Rouser, O'Brien, and Heller (27) and O'Brien, Fillerup, and Mead (31) emphasized the similar migration of phosphatidyl ethanolamine and phosphatidyl serine that were exposed to air compared to that of the corresponding lyso derivatives. Moreover, a spuriously low plasmalogen content obtained by iodimetric methods, caused presumably by oxidation of iodide by lipid peroxides, may be misinterpreted as evidence for cleavage of the labile vinyl ether bond. Therefore, an alternative explanation to hydrolysis as the basis for the "cephalin" alterations described by Hanahan et al. (2) and by de Gier and van Deenen (3) is autoxidation in the lipid extracts.

These autoxidative changes may lead to erroneous interpretation of analytical data. One example may be the reported decrease in phosphatidyl ethanolamine with a corresponding increase in lysophosphatidyl ethanolamine in the red cells from patients with hereditary spherocytosis (40), a finding refuted by others (5, 41, 42). These abnormal findings may be explained, in part, by hydrolysis of plasmalogens during chromatography on silicic acid-impregnated filter paper in a solvent system that contained acetic acid at *room temperature* (24). Fleischer and Rouser (43), however, have indicated that simulated lyso compounds are formed from the breakdown of oxidized phospholipid in this system. The concomitant contribution of autoxidation may be considered in view of the low values for arachidonic acid found in the spherocytic red cells and the controls, 6.4 and 7.9%, respectively. The latter values for the weight percent of arachidonic acid in total human red cell fatty acid methyl ester are considerably lower than recent estimates from other laboratories: 11.5% recalculated from Farquhar (4); 16.1% recalculated from Ways and Hanahan (19); and 19.5% according to de Gier, van Deenen, Verloop, and van Gastel (44). A decrease in arachidonic acid may be a sensitive index of autoxidation, because its high degree of unsaturation makes it particularly susceptible. Farquhar (4) took precautions to prevent artifactual development of hydrolytic and autoxidative changes and found no evidence for "lysocephalin" compounds in human red cell lipid. Moreover, he noted that the omission of these precautionary meas-

ures resulted in losses of plasmalogen, appearance of lyso derivatives, and alteration of the fatty acid distribution due to autoxidation of polyenoic components.

Another example of red cell phospholipid analysis suggesting autoxidation is the work of Szeinberg, Zaidman, and Clejan (45), who reported abnormalities in phospholipid distribution in cells deficient in glucose-6-phosphate dehydrogenase, with further alteration on exposure of these cells to acetylphenylhydrazine or nitrofurantoin. The low values found for phosphatidyl ethanolamine and phosphatidyl serine and high values for lecithin and sphingomyelin both in control and test samples

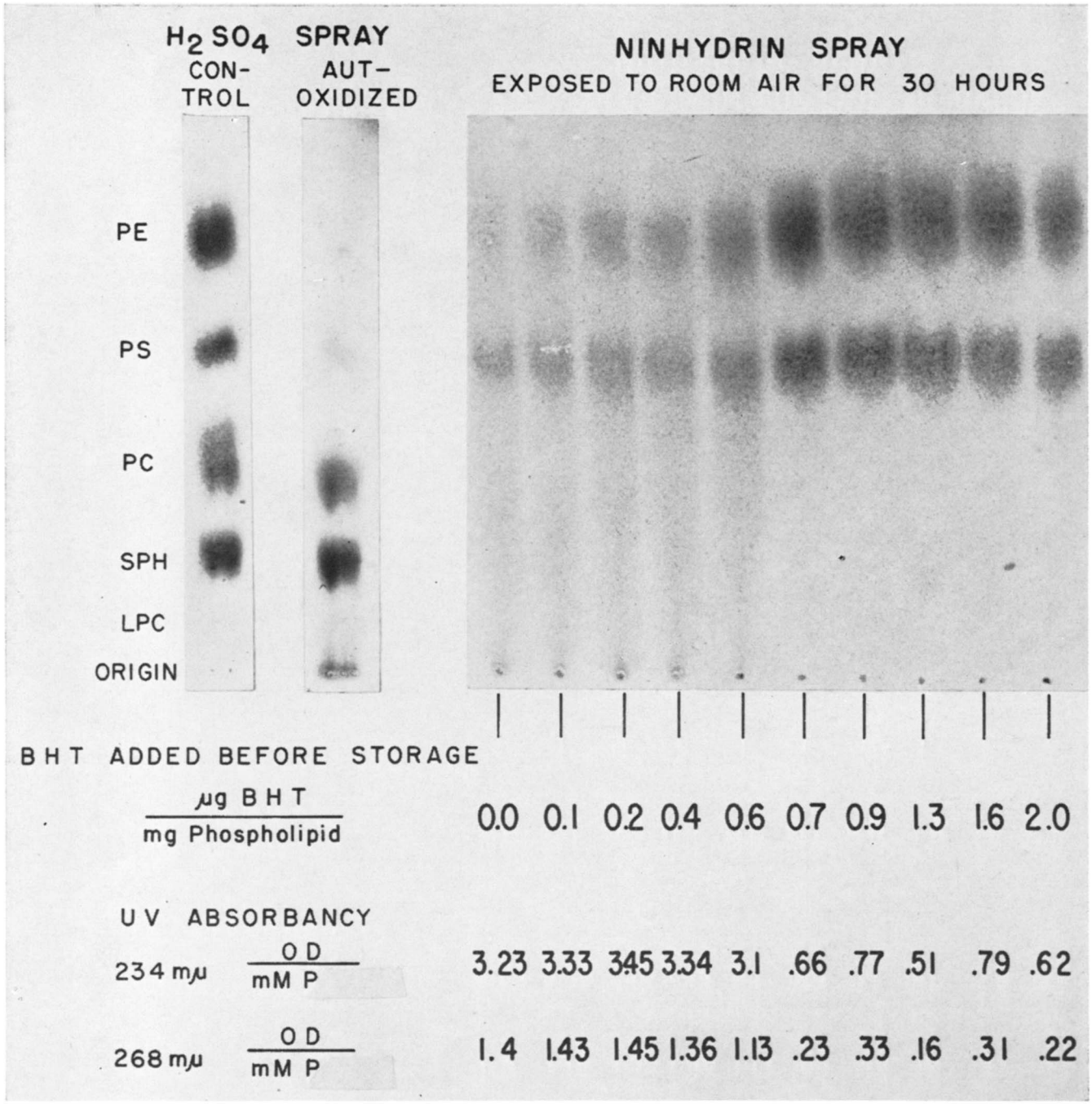

FIG. 3. Inhibition of autoxidation of human red cell phospholipid by BHT, as demonstrated by TLC and UV absorbancy. To aliquots of a sample of human red cell total phospholipid isolated by silicic acid column chromatography, BHT was added in increasing amounts. The aliquots were then dried, exposed to room air for 30 hr, and dissolved in methanol. TLC was performed, as described in the text, with 8.9 μg of lipid phosphorus per lane. Typical lanes from another plate prepared from the same phospholipid were sprayed with 50% aqueous H_2SO_4, and are shown on the left of the photograph to illustrate migration of the phospholipid fractions that do not stain with ninhydrin. PE, phosphatidyl ethanolamine; PS, phosphatidyl serine; PC, lecithin; SPH, sphingomyelin; LPC, lysolecithin.

suggest that autoxidation was involved. Since the acidic chromatography method of Marinetti (24) was used in this study, "lyso" compound formation may also have occurred.

For protection of unsaturated fatty acids from autoxidation, Mattson and Volpenhein (46) suggested the addition of any one of a number of antioxidants to the solvents during synthesis and subsequent storage of glycerides. Wren and Szczepanowska (32) prevented autoxidation during thin-layer and column chromatography by addition of BHT to the solvents. In the present investigation both α-tocopherol and BHT were shown to be effective. Additional precautions recommended for prevention of lipid autoxidation include use of an inert atmosphere such as nitrogen or carbon dioxide, low temperature, oxygen-free solvents, avoidance of chloroform as the solvent for storage, exclusion of metallic catalysts, and protection from ultraviolet light (4, 12, 24, 27–29, 32).

Autoxidation, therefore, appears to be an important artifact to be considered in analyses of lipids from any source even when measures are taken to prevent its formation. Because of the difficulty of distinguishing the artifacts caused by autoxidation from true lipid abnormalities in the tissue under study, it seems desirable to include measurements for autoxidation with the data on any exhaustive analysis of tissue lipid, particularly when evidence for "lyso" derivatives of the glycerophosphatides or unusual fatty acids such as hydroxy derivatives is found.

ADDENDUM

Subsequent studies have shown that BHT may interfere with certain assay procedures. On TLC with hexane–diethyl ether–acetic acid 80:20:1 (v/v) as solvent, BHT migrates just behind cholesterol ester but with considerable overlap. BHT, furthermore, interferes with the determination of cholesterol in the ferric chloride method (47) by producing a brown product that absorbs at 560 mμ. On GLC with 8% EGSS-X on 100–120 mesh Gas-Chrom P (Applied Science Laboratories, Inc., State College, Pa.) at 165°C with nitrogen flow rate of 50 ml/min, BHT had a retention time relative to methyl palmitate of 0.51 (retention time of methyl palmitate, 9.4 min), similar to that of methyl myristate (0.53). When taken through a boron–trifluoride methylation procedure (48), BHT under the above conditions gave rise to an additional peak with a retention time relative to methyl palmitate of 1.25, similar to that of methyl palmitoleate (1.23). In addition, BHT absorbs in the UV region (49) and may interfere with UV spectrophotometric methods, such as that used for measurement of autoxidation products.

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