Metabolism of 1-^{14}C-Methyl Linoleate Hydroperoxide in the Rabbit

G.M. FINDLAY, H.H. DRAPER and **J.G. BERGAN,**
Department of Food Science, University of Illinois at Urbana-Champaign, Illinois 61801

ABSTRACT

The metabolism of 1-^{14}C-methyl linoleate hydroperoxide (1-^{14}C-MLHP) by the rabbit was investigated. Administration of 1.1-1.9 mg of 1-^{14}C-MLHP by ear vein injection proved lethal to four of the nine experimental animals. After 2 hr the lungs and liver contained 3.3% and 7.2%, respectively, of the dose. This radioactivity was found to be associated primarily with intact 1-^{14}C-MLHP. The triglycerides from these tissues also contained ^{14}C-trienoic and ^{14}C-dienoic fatty acids. Of the dose, 68% was recovered as $^{14}CO_2$ in 2 hr compared to 39% after 1-^{14}C-methyl linoleate injection. The triglycerides from kidney adipose tissue contained a small amount of ^{14}C-hydroxy fatty acid, providing confirmation of previous evidence for the presence of a fatty acid hydroperoxide reductase in animal tissues.

INTRODUCTION

Whether fatty acid peroxides are formed in animal tissues in vivo has been a controversial question for some years (1-6). Substances which presumably are derived from peroxides or their degradation products have been detected in tissue extracts (7-10), but it is difficult to prove their existence in the live organism. It seems apparent that only minute concentrations of peroxides, if any, occur in vivo, and in the absence of information on their turnover rate, it is impossible to draw conclusions as to the amounts formed. Although the evidence is largely circumstantial, present indications favor the view that small amounts of lipid peroxides are formed in vivo and that vitamin E, serving as a natural antioxidant, is an important factor in their metabolism. Infusion of small quantities of methyl linoleate hydroperoxide has been reported to induce incipient symptoms of vitamin E deficiency, including encephalomalacia in chicks (11) and creatinuria and hemolysis in rabbits (12). Lipid peroxides also have been implicated in aging, CCl_4 hepatotoxicity and ionizing radiation damage.

Studies on the toxicity of lipoperoxides indicate that only trace quantities in the tissues are tolerated. Fatty acid hydroperoxides are highly toxic when administered intraperitoneally (13,14). When administered orally they are considerably less toxic, and failure to

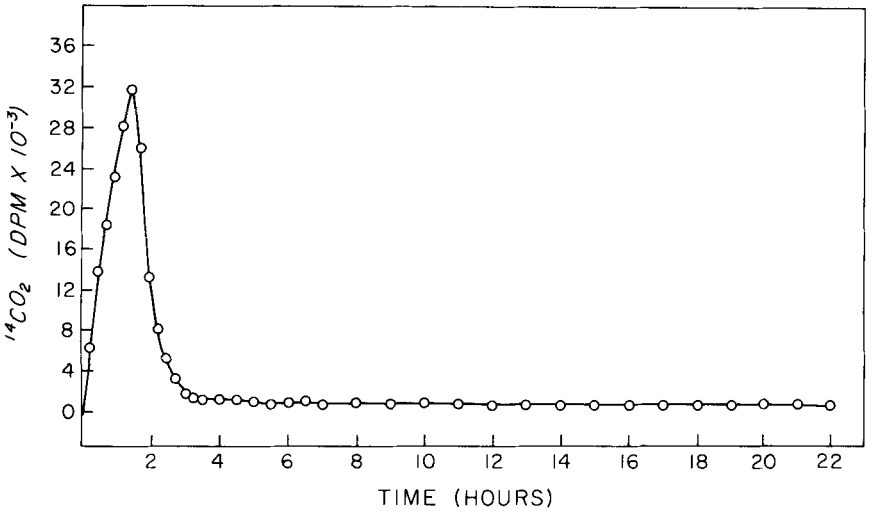

FIG. 1. $^{14}CO_2$ expiration during 22 hr following 1-^{14}C-MLHP injection.

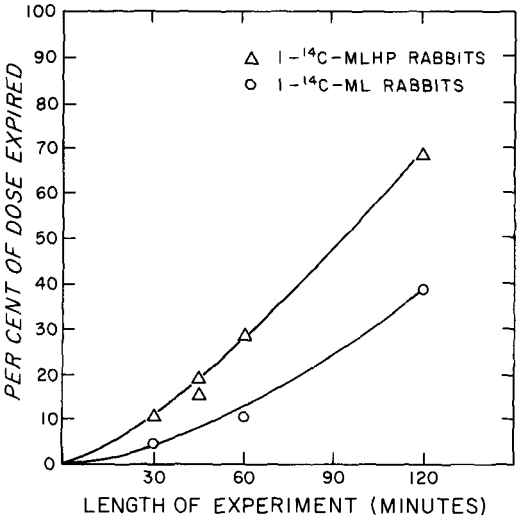

FIG. 2. Per cent of injected dose expired as $^{14}CO_2$. Each point represents the total $^{14}CO_2$ expired by a single animal.

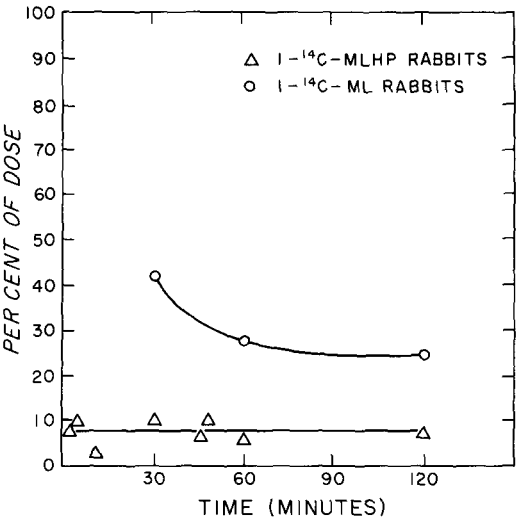

FIG. 3. Levels of radioactivity in liver following 1-^{14}C-MLHP or 1-^{14}C-ML administration.

find them in the tissues subsequently (15) indicates that their oral toxicity is due either to damage to the gut wall or to a systemic effect of decomposition products formed in the intestine. Methyl linoleate hydroperoxide is apparently not converted to linoleic acid in the tissues (16).

The present study was designed to investigate the metabolism of intravenously administered 1-^{14}C-methyl linoleate hydroperoxide in the rabbit.

EXPERIMENTAL PROCEDURES

Skelly Solve solvents were distilled twice before use; other reagents and solvents were of analytical grade. 1-^{14}C-Methyl linoleate (1-^{14}C-ML) with a specific activity of 9.3 mc/mM was obtained from Tracerlab (Waltham, Mass.). 1-^{14}C-Methyl linoleate hydroperoxide (1-^{14}C-MLHP) was prepared by controlled oxidation of 1-^{14}C-ML according to a modification of the method of Banks et al. (17). The oxygenation tube (2.6 x 30 cm) was loaded with 25 ml of Skelly Solve C (bp 88-100 C), 83 μc of 1-^{14}C-ML and 52.4 mg of methyl linoleate. Crude 1-^{14}C-MLHP obtained by stripping the solution with 87% ethanol was purified by the method of Kokatnur et al. (18).

The purity of the hydroperoxide was established using unlabeled ML. A peroxide number of 6022-6145 meq/kg was obtained by the Wheeler (19) method as modified by Kokatnur et al. (18). The theoretical value for pure MLHP is 6125 (18-20). A single spot (Rf = 0.52) was obtained by thin layer chromatography (TLC) on Silica Gel G using a 1% methanolic benzene solvent system. IR analysis revealed a hydroperoxy group (2.9 μ) and *trans,trans* conjugation (10.15μ). UV spectroscopy yielded a peak at 233 mμ for conjugated double bonds which gave a calculated molar extinction coefficient of 28,455. Banks et al. (17) have reported a value of 29,000 and Kokatnur et al. (18) found 27,873. The peroxide obtained by this procedure has been characterized as a mixture of 9-hydroperoxy-10,12-*trans,trans*-octadecadienoate and 13-hydroperoxy-9,11-*trans,trans*-octadecadienoate (17).

1-^{14}C-MLHP, 1.1-1.9 mg, was dissolved in 0.5 ml of ethanol and emulsified with 0.5 ml of physiological saline containing 2 mg/ml Tween 80. This material was injected slowly, over a period of 1.5-2 min, into the marginal ear vein of nine New Zealand White rabbits. The rabbits, weighing 1.7-2.3 kg, either died or were killed at 2, 4, 11, 30, 45, 47, 60, 120 min or 22 hr after injection. Three additional rabbits were injected with a like amount of 1-^{14}C-ML as a reference compound and were killed after 30, 60 or 120 min. Each animal was deprived of food for 16 hr prior to injection and was placed in a metabolism unit immediately after injection. $^{14}CO_2$ was monitored at 5 min intervals by passing the expired gases through 2 ml of Hyamine for 30 seconds. The $^{14}CO_2$ samples were counted in a Packard Tri-Carb liquid scintillation spectrometer, model 3003, after adding 15 ml of 0.3% 2,5-diphenyloxazole (PPO) in toluene.

TABLE I

Distribution of Radioactivity in the Tissues and Excreta of the 1-^{14}C-MLHP and 1-^{14}C-ML Injected Rabbits (% of Dose)

^{14}C-Compound administered	MLHP	MLHP	MLHP	ML	ML
Time after dose, hr	1	2	22	1	2
Tissues					
Liver	6.1	7.2	3.3	28.2	26.2
Lung	0.9	3.3	<0.1	8.0	0.6
Heart	<0.1	0.6	<0.1	---	---
Kidney	<0.1	1.0	0.4	---	---
Kidney adipose	<0.1	0.3	0.4	---	---
Brain	<0.1	0.2	0.1	---	---
Blood[a]	2.8	3.0	1.1	6.5	11.7
Excreta					
Urine	0	1.6	3.8	---	---
Feces	0	0	0.2	---	---
$^{14}CO_2$	29.0	68.0	78.0	10.0	39.1

[a]Calculated on basis of 6.1% body weight.

At the termination of the experiment various tissues were removed for analysis. Radioactivity in the blood serum was determined using Cab-O-Sil and 15 ml of 0.3% PPO in toluene. The excised tissues and red blood cells were ground with Na_2SO_4 in a mortar and extracted with $CHCl_3/CH_3OH$ (2:1) for 12 hr on a mechanical shaker. The extract was washed with 20 vol of water (21) and the residue was fractionated using silicic acid column chromatography (22). Trimethyl silyl (TMS) derivatives were formed using Tri·SIL/BSA (Pierce Chemical Company) (23). Separation of the silyl mono- and diglycerides and starting compounds was carried out on silica gel using a Skelly Solve B (bp 60-80 C)-diethyl ether-acetic acid (65:35:0.25) solvent system.

The lipid fractions were saponified with 1 N ethanolic KOH for 16 hr at room temperature (24). Fatty acids were decarboxylated with NaN_3 and H_2SO_4 (25). An ethereal solution of diazomethane was used to methylate the free fatty acids. Methyl esters of normal fatty acids were separated from the esters of polar acids using the 1% methanolic benzene TLC system. This system also was used for the separation of the methyl esters of polar acids and their silyl derivatives. Fractionation of normal fatty acid methyl esters according to their degree of saturation was accomplished by TLC on Silica Gel G impregnated with 5% $AgNO_3$ using a Skelly Solve F (bp 30-60 C)-diethyl ether (85:15) system (26).

Pure ricinoleic acid (12-hydroxy-9-*cis*-octadecaenoic acid) and dimorphecolic acid (9-hydroxy-10,12-*trans,trans*-octadecadienoic acid) were used as standard hydroxy fatty acids. MLHP was reduced to the corresponding hydroxy fatty acid using KBH_4 (27).

TABLE II

Distribution of Radioactivity in Lipid Fractions Isolated by Silicic Acid Column Chromatography (% of total DPM)

Tissue	Lung			Liver			
^{14}C-Compound administered	ML		MLHP	ML		MLHP	
Time after dose, hr	1	2	2	1	2	1	2
Cholesterol esters	1.1	0.2	0	1.1	1.8	0.2	0.2
Triglycerides	15.0	14.0	0.5	24.0	41.1	10.5	24.7
Free fatty acids	11.0	4.1	10.5	11.2	10.0	13.1	6.4
Cholesterol							
Diglycerides	6.4	6.5	37.4	6.1	8.8	25.1	27.6
Monoglycerides	4.4	5.4	34.8	10.2	3.3	40.7	32.1
Phospholipids	62.0	68.0	16.8	47.3	34.6	9.9	9.0

RESULTS AND DISCUSSION

$^{14}CO_2$

Figure 1 illustrates the rate of $^{14}CO_2$ expiration following intravenous injection of 1-^{14}C-MLHP. Peak expiration occurred at 85 min, 68% of the injected radioactivity being expired by the end of the second hr and 78% by the end of 22 hr. By comparison, after 1-^{14}C-ML injection only 39% of the dose was expired as $^{14}CO_2$ during the initial 2 hr period and peak expiration occurred 50 min after administration of the dose.

Figure 2 gives a comparison of the total $^{14}CO_2$ expired after administration of either 1-^{14}C-MLHP or 1-^{14}C-ML. Obviously 1-^{14}C-MLHP is much more rapidly oxidized to $^{14}CO_2$.

Toxicity of 1-^{14}C-MLHP

Four of the nine rabbits injected with 1-^{14}C-MLHP (3.5-6.0 μM) developed torpidity and died at 2, 4, 11 and 47 min, respectively, following administration of the hydroperoxide. No such reaction was observed in the animals injected with 1-^{14}C-ML. On an equivalent body weight basis the amount of MLHP administered was 0.83% and .17% of the LD_{50} dose reported for mice (13) and rats (14), respectively, following intraperitoneal injection. Differences in routes of administration, species, chemical form and purity of compounds administered are factors which may contribute to this wide range in toxicity values. It also was observed that those animals to which the dose proved fatal all contained high concentrations of radioactivity in the lungs. This observation, in addition to the nature of the symptoms induced, suggests that an impairment of the respiratory function may have been the cause of death. Lung tissue is known to be sensitive to small concentrations of gaseous oxidants and the lungs represented the first capillary bed encountered by the administered dose in these experiments.

Distribution of Radioactivity in the Tissue

Table I shows the distribution of radioactivity in the tissues and excreta at various times after 1-^{14}C-MLHP or 1-^{14}C-ML administration. The only tissues except blood which contained a significant amount of activity were the liver and lungs. Figure 3 illustrates the relatively constant level of radioactivity found in the liver during the initial 2 hr after 1-^{14}C-MLHP administration and the greater activity found following 1-^{14}C-ML injection.

Identification of ^{14}C-Compounds in Liver and Lung

Table II gives the distribution of radioactivity among the lipid classes of lung and liver 1 and 2 hr after 1-^{14}C-ML and 1-^{14}C-MLHP injection. As expected, the major concentrations of activity following 1-^{14}C-ML administration were in the TG and PL fractions. Saponification of these fractions, TLC of the fatty acid methyl esters on $AgNO_3$-impregnated Silica Gel G and subsequent decarboxylation of the fractionated acids indicated that 90% of this radioactivity was present in 1-^{14}C-linoleic acid.

In contrast, following 1-^{14}C-MLHP injection the radioactivity in both lung and liver lipids was located predominately in the DG and MG fractions which were eluted with 25% and 100% diethyl ether in Skelly Solve B, respectively. However, the ^{14}C compounds in the DG and MG fractions did not form silyl derivatives, indicating that the radioactivity in these two fractions was not present in esters of glycerol but in compounds with similar polarity. The ^{14}C compounds in the DG and MG fractions from the animals injected with 1-^{14}C-ML could be easily silylated.

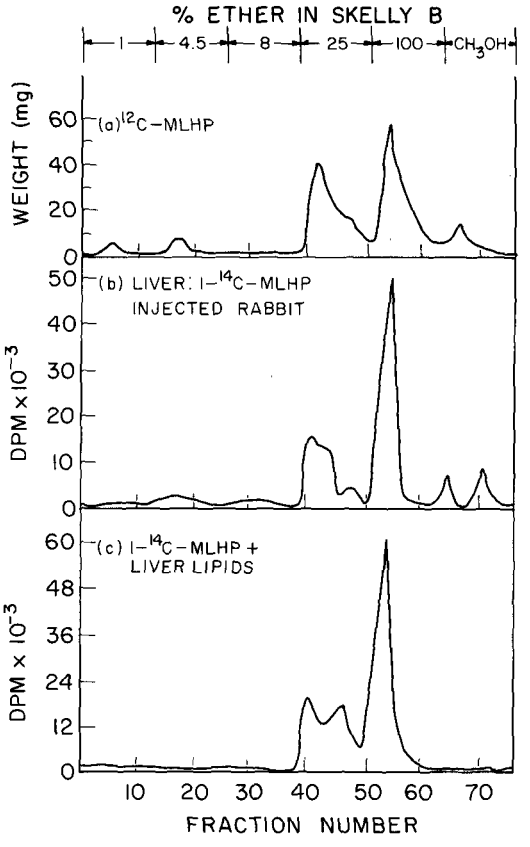

FIG. 4. Comparative silicic acid column elution patterns.

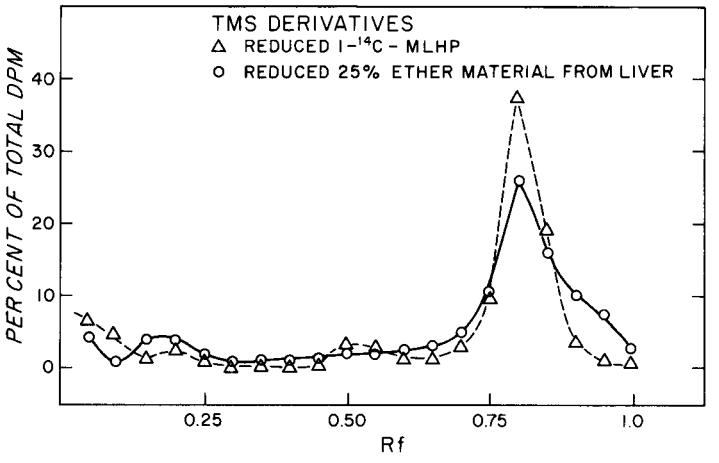

FIG. 5. TLC of pure 1-^{14}C-MLHP and the 25% ether fraction from the liver of a 1-^{14}C-MLHP-injected rabbit after KBH_4 reduction and silylation. The developing solvent was 1% methanolic benzene.

Standardization of the silicic acid column with radioactive and nonradioactive MLHP revealed that intact hydroperoxide was eluted with 25% diethyl ether in Skelly Solve B and that 100% diethyl ether eluted degradation products of the peroxide which formed on the column (Fig. 4). After applying a 230 mg sample of ^{12}C-MLHP (peroxide number 6000) to the column it was observed that the material eluted with the 25% diethyl ether comprised 42% of the sample weight, had a peroxide number of 5400 and an IR spectrum indistinguishable from that of the original peroxide. The 100% diethyl ether eluate represented 36% of the original mass and had a peroxide number of 2800 (Fig. 4a). Comparison of the IR spectrum of this fraction with that of the original sample revealed numerous discrepancies. When 1-^{14}C-MLHP was combined with liver lipids and chromatographed on a silicic acid column, the radioactivity was recovered in the same two solvents in similar proportions (Fig. 4c). The 25% diethyl ether eluate contained 41% of the radioactivity and the 100% diethyl ether fraction contained 49%. Comparison of these two elution patterns with that of the liver lipid after 1-^{14}C-MLHP injection (Fig. 4b) showed that all three elution profiles were similar and suggested that the ^{14}C materials eluted with the DG and MG from the injected animals were unhydrolyzed 1-^{14}C-MLHP and products of its degradation on the column.

Exposure of 1-^{14}C-MLHP and the 25% diethyl ether fraction from the liver of a 1-^{14}C-MLHP-injected rabbit to Tri•SIL/BSA and TLC of the products showed that neither 1-^{14}C-MLHP nor the isolate from liver formed a silyl derivative. After reduction of the two samples both formed a silyl derivative (Fig. 5), confirming the chromatographic evidence that they were identical. Decarboxylation of the 25% and 100% diethyl ether fractions showed that the materials present were entirely carboxyl-labeled. Consequently it was concluded that a large portion of the radioactivity remaining in the liver and lung lipids 2 hr after 1-^{14}C-MLHP injection was still present in the form of unhydrolyzed hydroperoxide. These results support the results of earlier studies (28) in which intact peroxides were observed in rat liver 48 hr after intravenous administration of ethyl linoleate hydroperoxide. Evidently these hydroperoxy fatty acid esters can survive in biological systems longer than generally presumed.

After saponification of the TG fraction from lung lipids the fatty acid methyl esters were chromatographed using the 1% methanolic benzene system and then $AgNO_3$-impregnated Silica Gel G. It was observed that following 1-^{14}C-MLHP injection this fraction contained labeled trienoic fatty acids. Although only 0.5% of the lung radioactivity was present in the TG 2 hr after injection, 75% of this activity was found in the trienoic and another 22% was present in the dienoic acids. In addition, 19% and 6% of the radioactivity in the liver TG was associated with the dienoic and trienoic acids, respectively. It is noteworthy in this connection that in an associated study on the metabolism of the hydroxy acid obtained by reduction of 1-^{14}C-MLHP, labeled dienoic and trienoic acids were detected in liver triglycerides (29).

Saponification and TLC of the fatty acid methyl esters of kidney adipose tissue (1% methanolic benzene) revealed that 24% of the activity in this tissue was associated with polar fatty acids. Silylation of the methyl esters indicated that a hydroxy fatty acid comprised a portion of this fraction. Unfortunately, the low levels of radioactivity present, coupled with a comparatively large tissue weight, made further analysis impossible. A subsequent paper in this series indicates that hydroxy and trienoic acids are formed from 1-^{14}C-MLHP during absorption (30).

The results of these experiments show that intravenous MLHP is rapidly oxidized to CO_2 in rabbit tissues and that the peroxide is not incorporated into tissue lipids. No evidence was obtained for the presence of free peroxide. However, substantial amounts of MLHP were found in the lungs and liver 2 hr after administration. This finding indicates that the limiting factor in MLHP oxidation was the hydrolysis of the ester, and suggests that fatty acid peroxides formed in vivo might be even more rapidly oxidized. The detection of a hydroxy fatty acid in the kidney TG supports the proposals of O'Brien and Little (31) and Christophersen (32) that a reductase is involved in peroxide metabolism.

ACKNOWLEDGMENT

This research was carried out with the aid of Training Grant GM-00653 and Research Grant NB-04201 from the U.S. Public Health Service.

REFERENCES

1. Tappel, A.L., in "Symposium on Foods: Lipids and Their Oxidation," Edited by H.W. Schultz, E.A. Day and R.O. Sinnhuber, The Avi Publishing Co. Inc., Westport, Connecticut, 1962, p. 122-138.
2. Tappel, A.L., Fed. Proc. 24:73-84 (1965).
3. Witting, L.A., Ibid. 24:912-916 (1965).
4. Bunyan, J., J. Green, E.A. Murrell, A.T. Diplock and M.A. Cawthorne, Brit. J. Nut. 22:97-110 (1968).
5. Yoshikawa, M., and S. Hirai, J. Gerontol. 22:162-165 (1967).
6. Witting, L.A., and M.K. Horwitt, Lipids 2:89-96 (1967).
7. Hendley, D.D., A.S. Mildvan, M.C. Reporter and B.L. Strehler, J. Gerontol. 18:250-259 (1963).
8. Schornagel, H.E., J. Pathol. Bacteriol. 72:267-272 (1956).
9. Glavind, J., S. Hartman, J. Clemmesen, K.E. Jensen and H. Dam, Acta Pathol. Microbiol. Scand. 30:1-6 (1952).
10. Tappel, A.L., Nutr. Today 2:2-7 (1967).
11. Nishida, T., H. Tsuchiyama, M. Inone and F.A. Kummerow, Proc. Soc. Exp. Biol. Med. 105:308-312 (1960).
12. Kokatnur, M.G., J.G. Bergan and H.H. Draper, Ibid. 123:254-258 (1966).
13. Horgan, V.J., J.S.L. Philpot, B.W. Porter and D.B. Roodyn, Biochem. J. 67:551-558 (1957).
14. Olcott, H.S., and A. Dolev, Proc. Soc. Expt. Biol. Med. 114:820-822 (1963).
15. Andrews, J.S., W.H. Wendell, J.F. Mead and R.A. Stein, J. Nutr. 70:199-210 (1960).
16. Freeman, I.P., and P.J. O'Brien, Biochem. J. 102:9p (1967).
17. Banks, A., S. Fazakerley, J.N. Keay and J.G.M. Smith, J. Sci. Food Agr. 12:724-728 (1961).
18. Kokatnur, M.G., J.G. Bergan and H.H. Draper, Anal. Biochem. 12:325-331 (1965).
19. Wheeler, J., Official and Tentative Methods of the AOCS, 1964, Cd-8-53.
20. Frankel, E.N., in "Symposium on Foods: Lipids and Their Oxidation," Edited by H.W. Schultz, E.A. Day and R.O. Sinnhuber, The Avi Publishing Co. Inc., Westport, Conneticut, 1962, p. 51-78.
21. Folch, J., I. Ascoli, M. Lees, J.A. Meath and F.N. LeBaron, J. Biol. Chem. 191:833-841 (1951).
22. Hirsch, J., and E.H. Ahrens, J. Biol. Chem. 233:311-320 (1958).
23. Sweely, C.C., R. Bentley, M. Makita and W.W. Wells, J. Amer. Chem. Soc. 85:2495 (1963).
24. Chisholm, M.J., and C.Y. Hopkins, JAOCS 43:390-392 (1966).
25. Phares, E.F., Arch. Biochem. Biophys. 33:173-178 (1951).
26. Morris, L.J., Chem. Ind. (London) 1962:1238.
27. Mattic, M., and D.A. Sutton, Chem. Ind. 1953:666.
28. Glavind, J., E. Sondergaard and H. Dam, Acta Pharmacol. Toxicol. 18:267-277 (1961).
29. Reber, R.J., and H.H. Draper, Lipids 5:983-987 (1970).
30. Bergan, J.G., and H.H. Draper, Lipids 5:976-982 (1970).
31. O'Brien, P.J., and C. Little, Can. J. Biochem. 47:493-499 (1969).
32. Christophersen, B.O., Biochim. Biophys. Acta 176:463-470 (1969).

[Received August 24, 1970]