Calcium ions cannot replace chloride ions in the activation of pancreatic amylase at *p*H 7.2. The data given in Table IV show no activity for pancreatic amylase in the absence of chloride ions even when 0.02 *M* calcium ions were present in the hydrolyzates at *p*H 7.2. The marked influence of slight changes in the *p*H of its hydrolyzates upon the action of pancreatic amylase is emphasized by the lower activity of the amylase in the otherwise comparable but unbuffered or poorly buffered hydrolyzates used for the study of the influence of calcium ions. Phosphate ions[5a,b] and acetate ions[5a] had been found previously to have no influence on the activity of pancreatic amylase.

Protection or Activation of Pancreatic Amylase

Distinction between Protection and Activation of Pancreatic Amylase.—The data given in Tables III and IV show that there is a fundamental difference between the influence exerted by chloride ions in activating pancreatic amylase and the influence exerted by calcium ions and by chloride ions in protecting the amylase from inactivation on standing in dilute aqueous solution. It appears probable that chloride ions, and to a less extent certain other anions,[5b] are necessary for the effective union of the amylase protein and its substrate. Attempts to clarify the fundamental differences between the activation of pancreatic amylase by anions such as chloride and the protection of the amylase by ions such as chloride ions and calcium ions are being continued.

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[CONTRIBUTION FROM THE EASTERN REGIONAL RESEARCH LABORATORY[1]]

Reactions of Fatty Materials with Oxygen. XIV.[2] Polarographic and Infrared Spectrophotometric Investigation of Peroxides from Autoxidized Methyl Oleate

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Methyl oleate has been autoxidized from 35 to 120° in the presence or absence of ultraviolet radiation. Polarographic and iodometric analysis of the autoxidation mixtures, and peroxide concentrates obtained from them by urea complex precipitation of unoxidized methyl oleate, has shown that, although the bulk of the peroxides formed are hydroperoxides, a significant proportion is not. Evidence is presented which indicates that the non-hydroperoxide portion probably consists of cyclic peroxides. Furthermore, the hydroperoxides have the *trans* configuration, predominantly. It is extremely unlikely that methyl oleate hydroperoxide (*cis* configuration) has ever been isolated since the free-radical nature of the autoxidation reaction precludes its formation to any appreciable extent.

Three major points bearing on the autoxidation of methyl oleate are reported and discussed in this paper. First, although it will be shown that hydroperoxides are the major early products of autoxidation of methyl oleate, a significant proportion (as much as 28%) of the total peroxides is not. Second, evidence will be presented which leads to the conclusion that the non-hydroperoxide portion of the total peroxides isolated from autoxidized methyl oleate is probably cyclic, at least in part. Third, the hydroperoxides from autoxidized methyl oleate have the *trans* configuration predominantly and are preferably referred to as methyl *trans*-octadecenoate hydroperoxides. A consequence of this last statement is that, in view of the isolation procedures presently available, methyl oleate (*cis*) hydroperoxide has undoubtedly never been isolated. Furthermore, the present investigation suggests that it will be extremely difficult to isolate a pure *cis* hydroperoxide from autoxidized methyl oleate, or from any other long-chain *cis* olefinic compound.

All the conclusions drawn in this paper refer to autoxidized methyl oleate in which the peroxide content has not yet reached its peak. After the peak in peroxide content has been reached and the peroxide values are decreasing, the extent of oxidation is so great and the mixture so complicated that there is serious question regarding the reliability of the various analytical methods.

Non-hydroperoxide Content of Autoxidized Methyl Oleate.—Polarographic analysis is a convenient and accurate way to determine hydroperoxides in the presence of other peroxide types.[3] Study of the polarographic properties of methyl oleate autoxidized to different peroxide contents and peroxide concentrates obtained from them clearly demonstrates (Table I) that hydroperoxides are not the sole peroxide substances formed, although they predominate, and that the non-hydroperoxide portion may amount to as much as 28% of the total peroxides (no. 9, Table I). In every case studied so far, in which the peroxide concentrates were not further fractionated by solvent partition methods, the values obtained for total peroxides by chemical (iodometric) analysis[4] exceed those obtained for hydroperoxides by polarographic analysis.[3] Results representative of over fifty autoxidations and concentrations of peroxides are listed in Table I.

It appeared to make little or no difference in the results obtained whether methyl oleate was oxidized in the dark or in the presence of ultraviolet radiation, between 35 and 120°, or in the presence or absence of metal deactivators. A benzoyl peroxide-

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[2] Presented in part at the Meeting-in-Miniature of the Philadelphia Section of the American Chemical Society, January 29, 1953, and at the meeting of the American Chemical Society held in Los Angeles, California, March 15-20, 1953. The preceding paper in this series is *J. Polymer Sci.*, in press.

[3] C. O. Willits, C. Ricciuti, H. B. Knight and D. Swern, *Anal. Chem.*, **24**, 785 (1952).

[4] D. H. Wheeler, *Oil and Soap*, **9**, 89 (1932); W. O. Lundberg and J. R. Chipault, THIS JOURNAL, **69**, 833 (1947).

Table I

Comparison of Total Peroxide with Hydroperoxide Content in Autoxidized Methyl Oleate and Peroxide Concentrates

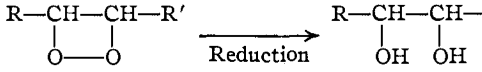
	No.	Total peroxide content, %	Hydroperoxide content, %
A.M.O.[a]	1	18.5[d]	16.6
P.C.[b]	1	88.3	71.0
A.M.O.	2	35.3[e]	32.3
P.C.	2	72.1	60.5
P.C.	3	79.7[f]	74.6
P.C.	4	81.8[g]	68.2
P.C.	5	83.9[g]	64.8
P.C.	6	86.8[h]	77.3
A.M.O.	7[c]	8.8	8.6
A.M.O.	8	17.8	14.9
A.M.O.	9	28.6	20.5
A.M.O.	10	3.8[i]	..
P.C.	10	81.9	67.8

[a] A.M.O. = autoxidized methyl oleate. [b] P.C. = peroxide concentrate. [c] Numbers 7, 8, 9 are samples withdrawn at 4, 6 and 12 hours from a methyl oleate autoxidation at 100° in the presence of ultraviolet. [d] Autoxidized at 80° in the dark. [e] Autoxidized at 80° in the dark in the presence or absence of metal deactivators (0.02–0.1%), such as phytic acid, ascorbic acid, phosphoric acid or citric acid. [f] Autoxidized to 17% peroxide content in the dark at 35–40° in the presence of 1.0 mole per cent. of benzoyl peroxide, followed by concentration. [g] Autoxidized to 13–15% peroxide content at 35–40° in the presence of ultraviolet radiation, followed by concentration. [h] Autoxidized to 14% peroxide content at 100° in the presence of ultraviolet radiation followed by concentration. [i] Autoxidized at 35–40° in the presence of ultraviolet radiation.

initiated experiment conducted in the dark at 35° also gave similar results. That the differences between total peroxide and hydroperoxide are real and that the newly-developed polarographic method is reliable was shown by the fact that on a purified hydroperoxide isolated from autoxidized methyl oleate by a combination of the urea complex technique and countercurrent distribution, both the chemical analysis and polarographic analysis gave the same value. Furthermore, as Table I shows, even at initial levels of autoxidation of as little as 3.8% peroxide, hydroperoxides are not the sole products of oxidation (Table I, peroxide concentrate no. 10). It should be emphasized that at low peroxide contents the difference between total peroxide and hydroperoxide must necessarily be small. In the peroxide concentrates, however, the difference can be clearly observed. Repetition of the urea complex separation technique on peroxide concentrates, in an attempt to obtain even higher peroxide contents, resulted in only slight increases in peroxide content, no loss of the total peroxide was observed, and the difference between total peroxide and hydroperoxide remained unchanged. These experiments showed that the isolation technique was not preferentially destroying one peroxide type and giving a spurious difference between the two peroxide analyses.

In view of the methods of fractionation employed by earlier workers,[5,6] it is evident that a peroxide consisting solely of hydroperoxide has not yet been isolated from autoxidized methyl oleate.

Cyclic Peroxide Content of Autoxidized Methyl Oleate.—Until a cyclic peroxide is actually isolated from autoxidized methyl oleate or until model cyclic peroxides are prepared synthetically, and their physical and chemical properties determined, evidence for the presence of cyclic peroxides in autoxidized methyl oleate must remain circumstantial. The major evidence we have obtained for the presence of cyclic peroxides in autoxidized methyl oleate is the fact that reduced peroxide concentrates show an α-glycol content which, in several instances, exactly equals the difference between total peroxide and hydroperoxide originally observed in the peroxide concentrate before reduction. Reduction of the cyclic peroxide (I) should yield an α-glycol (II).



$$\underset{\text{O---O}}{\text{R—CH—CH—R}'} \xrightarrow{\text{Reduction}} \underset{\text{OH OH}}{\text{R—CH—CH—R}'}$$

I II

Unfortunately, the agreement between the α-glycol content of the reduced peroxide and the difference between total peroxide and hydroperoxide has not been as good as we should have liked in all cases.

An additional piece of circumstantial evidence for the presence of cyclic peroxides is the fact that the oxirane content of some of the peroxide concentrates also agrees with the difference between total peroxide and hydroperoxide. Cyclic peroxides (I or Staudinger's moloxide)[7] contain a labile ring which should be attacked by the hydrochloric acid–ether reagent employed for determining oxirane oxygen.[8] However, α,β-unsaturated ketone structures known to be present in autoxidized unsaturated fatty esters in small amounts are also attacked by this reagent and may render this analytical technique questionable when applied to autoxidized substances.

***trans*-Content of Peroxide Concentrates Obtained from Autoxidized Methyl Oleate.**—In a previous paper[9] infrared spectrophotometric data were presented which led to the conclusion that during the earliest stages of autoxidation of methyl oleate at 35° and up to a peroxide content of about 22%, 90% of the peroxides produced were *trans*. At the relatively low levels of autoxidation studied in that investigation, however, it was not possible to state with certainty whether any small observed differences between peroxide and *trans* content were real, or whether an observed agreement was not, in fact, masking a significant difference. Just as was the case in taking full advantage of the polarographic method of analysis,[3] the picture was considerably clarified by investigating peroxide concentrates as well as autoxidation mixtures.

The results obtained in determining the *trans* content of several autoxidation mixtures and peroxide concentrates by the infrared spectrophoto-

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[6] C. E. Swift, F. G. Dollear and R. T. O'Connor, *Oil and Soap*, **23**, 355 (1946).

[7] H. Staudinger, *Ber.*, **58**, 1075 (1925).

[8] D. Swern, T. W. Findley, G. N. Billen and J. T. Scanlan, *Anal. Chem.*, **19**, 414 (1947).

[9] H. B. Knight, C. R. Eddy and D. Swern, *J. Am. Oil Chemists' Soc.*, **28**, 188 (1951).

TABLE II

COMPARISON OF TOTAL PEROXIDE, HYDROPEROXIDE AND *trans* CONTENT IN AUTOXIDIZED METHYL OLEATE AND PEROXIDE CONCENTRATES

Total peroxide content (iodometric), %	Hydroperoxide content (polarographic), %	*trans*-Content, %
14.2[a,b]	..	13
16.8[c,i]	15.1	12
18.5[d,e]	16.6	13
69.4[b]	61.2	68
73.5[f]	66.5	59
77.0[g]	72.9	59
78.2[g]	71.3	62
78.9[h]	68.4	53
79.7[i]	74.6	60
81.8[b]	68.2	65
88.3[e]	71.0	62

[a] Autoxidized at 35–40° in the presence of ultraviolet radiation. [b] These peroxides are an autoxidized methyl oleate and peroxide concentrates obtained from it on two separate occasions. [c] Autoxidized at 35–40° in the dark in the presence of 1.0 mole per cent. of benzoyl peroxide. [d] Autoxidized at 80° in the dark. [e] These two peroxides are an autoxidized methyl oleate and its corresponding peroxide concentrate. [f] Autoxidized at 80° to 37% peroxide content in the presence of ultraviolet radiation and ascorbic acid, followed by concentration. [g] Autoxidized at 80° to 33% peroxide content in the dark followed by concentration. [h] Autoxidized at 80° to 37% peroxide content in the dark in the presence of phosphoric acid, followed by concentration. [i] These two peroxides are an autoxidized methyl oleate and its corresponding peroxide concentrate.

metric method[10] are shown in Table II. It is difficult to assess the absolute accuracy of the analysis for *trans* components, since the reference *trans* standard employed is methyl *trans*-9-octadecenoate (elaidate) and not a synthetically prepared pure *trans* α-methylenic hydroperoxide. However, it seems quite clear from Table II that most, if not all, of the hydroperoxides formed during autoxidation of methyl *cis*-9-octadecenoate (oleate) have the *trans* configuration, thus substantiating our earlier conclusions.[9] It made little or no difference whether methyl oleate was autoxidized in the dark or in the presence of ultraviolet radiation between 35 and 120°.

It is safe to conclude that methyl oleate hydroperoxide described by Farmer and Sutton,[5] Swift, Dollear and O'Connor[6] and others is undoubtedly a mixture of *trans*-hydroperoxides mainly. In view of the free-radical nature of the autoxidation reaction, it is also unlikely that the double bond is in the original 9-position[9,11,12] in most of the molecules.

Occasionally the *trans* content exceeded the hydroperoxide content. This situation may be caused by the presence of non-peroxidic *trans* materials which could form as a result of the facile free-radical transformations discussed in our previous paper[9] or it may be a reflection of the use of non-ideal reference *trans* compounds.

Experimental

Starting Material.—Methyl oleate (composition, 97–99% methyl oleate, 1–2% methyl stearate and/or palmitate, 0.2% methyl esters of polyunsaturated acids) was prepared from olive oil.[13]

Autoxidation Procedure.—Methyl oleate in quartz flasks was autoxidized with pure oxygen in the presence of ultraviolet radiation, as described previously[9,14] or in Pyrex flasks in the dark. Reaction temperatures ranged from 35 to 120°.

Preparation of Peroxide Concentrates.—Unoxidized methyl oleate was separated from the autoxidation reaction mixture by precipitation as urea complex.[15] The peroxide concentrate was isolated from the methanol filtrate in the usual way.

Reduction of Peroxides.—Peroxides were reduced with sodium bisulfite,[16] with potassium iodide[4] (in glacial acetic acid–chloroform solution followed by titration of the liberated iodine with sodium thiosulfate and isolation of the reduction product by ether extraction), or with hydrogen in acetic acid solution in the presence of palladium on carbon as catalyst.

Analytical Procedures.—Peroxides were determined iodometrically[4] as well as polarographically.[3] α-Glycol content of reduced peroxides was determined by reaction with periodic acid.[17] *trans*-Content of autoxidation mixtures and peroxide concentrates was determined by infrared analysis,[9,10] using a Beckman 1R-3 infrared spectrophotometer.

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