The Autoxidative Contraction of Monolayers of Linoleic Acid at a Gas-Water Interface[1]

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Abstract

A monolayer of linoleic acid spread at an air-water interface was found to undergo an immediate oxygen-dependent contraction when compressed at a constant high film pressure. This is in contrast to the expansion of monolayers of other unsaturated acids at lower film pressures as reported by other workers. The process shows quasi-first-order kinetics, and there is little evidence for an induction period, in contrast to bulk-phase autoxidation.

Initial rate of contraction is a function of pH between 6 and 9. This and other data suggest that contraction is due to the titration of the packed adjacent carboxyl groups in the monolayer and to the solution of short-chain soaps resulting from the oxidative scission of linoleic acid.

Although complete anaerobiasis prevented film contraction, neither *dl*-alpha-tocopherol deposited in the film, nor hydroquinone in the substrate solution were inhibitory alone. Butylated hydroxyanisole (BHA) present in an aerobic gas phase produced inhibition of contraction of linoleic acid films spread on completely deaerated substrates. Inhibition was released upon removal of the BHA vapor, the restored rate in air approaching that before the BHA treatment. A method of specific testing of antioxidants is thus provided, which will differentiate between those presented in the vapor, the lipid, or the solution phases.

Introduction

AUTOXIDATION OF UNSATURATED lipids, the molecules of which have been partially immobilized as in concentrated or freeze-dried foods, or paint films, is very much a phenomenon of surfaces. These may be air-lipid, water-lipid, or protein-lipid interfaces, to mention a few. Monolayers of surface-active lipids spread at gas-water interfaces offer a simple model for such studies, provided a method to measure the extent of oxidation is available. In this paper, we have reported results of autoxidation of linoleic acid monolayers spread on water solutions, measured by the rate of contraction of the monolayer due to solution of the soluble products of oxidation.

In the forty years of intensive study of monolayers, there has been seemingly no specific study of the autoxidation of linoleic acid monolayers spread on water substrates. The pioneering work of Rideal and co-workers during the 1930's on rate of oxidation of monolayers of unsaturated fatty acids dealt either with a) potassium permanganate oxidations of mono-unsaturated acids (1,2) or b) autoxidation of monolayers of the maleic anhydride adduct of beta-elaeostearin (3). This adduct has the interrupted diene structure characteristic of linoleic acid, but incorporated in a polycyclic structure which makes the chemical analogy much less secure. Their detailed kinetic study was concerned only with expansion of the monolayer, although dissolution after $KMnO_4$ oxidation was mentioned. Trice (4) has studied the effect of autoxidation on contact angles and the derived wettability of linoleic acid monolayers taken up from a water substrate onto glass or copper surfaces using the Blodgett technique. He concluded that oxidation in dry air produces a coherent, non-wettable monolayer, presumably by secondary oxidation; whereas, in humid air, the known wettability of pure linoleic acid layers on glass surfaces is unchanged. Bangham (5) in a study of vitamin A-lecithin-cholesterol monolayers under aerobic conditions made brief mention of certain suggestive film contraction phenomena without elaboration. Merker and Daubert (6) reported without detailed data that aging of l-monolinolenin in air gave some expansion of the monolayer.

Because of the known quasi-crystalline order and simplicity of the monolayer structure as revealed by x-ray studies after transfer to multilayers (7), it seemed probable that insight into the initial stages of autoxidation of unsaturated lipids might follow from detailed studies of such monolayers and their relations with pro- and antioxidants. In the course of preparing built-up multilayers of linoleic acid by the Langmuir-Blodgett technique from a monolayer film on a substrate at pH 7, a pronounced oxygen-dependent contraction of the film under castor oil piston pressure on water was noted. The contraction showed quasi-first-order kinetics, without evidence of any induction period. Conditions could be altered so that the contraction, which was also pH-dependent, would result in dissolution of the entire film, or alternatively, in a static final condition with a substantial area of the floating film intact. The contraction was studied as a function of conjugated diene content of the applied linoleic acid, oxygen content of the gas phase, pH of the substrate, and added pro- or antioxidant in the monolayer, substrate or gas phase.

Experimental

Linoleic acid monolayers were spread on approximately one liter of substrate solution contained in a Teflon coated Cenco Hydrophil surface balance trough (14 × 64 × 2 cm), which was frequently recoated and buffed. The monolayer was kept under a constant piston pressure of approximately 16 dynes per cm by use of a close-fitting polyethylene barrier float (6 × 14 cm) on the opposite side of which a drop of pure castor oil was applied with a fine glass rod, cleaned in acid and redistilled water (Fig. 1). Under the conditions of the experiments, visible lenses of the castor oil indicate that the collapse pressure is being maintained, the oil being very resistant to oxidation. Linoleic acid was applied from a micropipette as approximately 0.04 ml of a 1 mg/ml solution in redistilled, deoxygenated low boiling (30–60C) petroleum ether, dropped upon the substrate solution sur-

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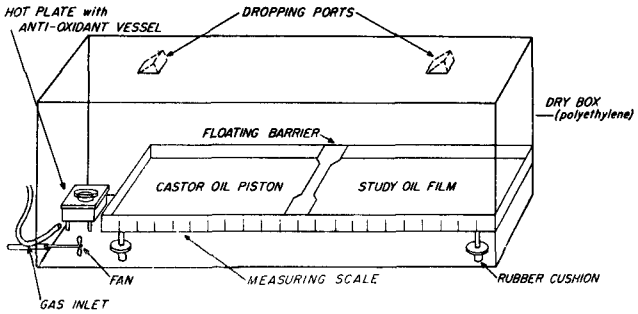

FIG. 1. Monolayer contraction apparatus.

face. After approximately a one-minute equilibration period for solvent evaporation, the position of the barrier edge was marked and subsequent measurements were made at timed intervals by sighting without parallax onto a scale outside the trough. The rectilinear position of the float was usually dependably maintained during movement, and its freedom of motion was maintained by the barely visible vibrations transmitted through the rubber cushioned feet from laboratory apparatus. It was found that with a sufficiently small amount of the castor oil "piston" little leakage of oil lenses around the ends of the barrier occurred, as checked with talc. However, on the few occasions when this occurred, as evidenced by lenses on the linoleic acid side of the barrier, a drop in contraction rate of the film was noted. In general, the steadiness and reproducibility of contraction rate was evidence that negligible sporadic leakage was occurring.

The substrate solution was made up from laboratory distilled water which had been deionized by an Illco-way Ion-exchange column (Illinois Water Treatment Co., Rockford, Ill.) until its conductivity was below 1.2 μmho/cm at 25C. The temperature of the substrate solution was monitored during the experiments, although an isothermal cabinet was not found necessary. Temperature was 25 $\pm$ 2C in all experiments unless otherwise stated. Chemicals used in preparation of the buffer and metal content of the substrate solutions were reagent grade. The *dl*-alpha-tocopherol was procured from Nutritional Biochemicals Corp. Thin-layer chromatography showed a single spot.

Linoleic acid used in preparation of the monolayers was from three sources: General Biochemicals (95% pure with respect to diene conjugation based on UV absorption at 233 mμ, as determined in our laboratory); Hormel Institute (99% pure); and Hormel Institute linoleic acid rechromatographed on silicic acid by partition chromatography with 2% methanol in benzene to separate hydroperoxide and secondary oxidation products. The product of the latter chromatography in our hands was not noticeably more free from conjugated diene than the original Hormel material, and, therefore, the former was used in many of our experiments. Diene conjugation, which was found correlated with monolayer contraction, was used as an index of purity for the experimental purposes. The nitrogen used was dried and purified by Medical-Technical Gases, Inc., Medford, Mass.

Fig. 1 shows the experimental set-up used to test the effects of volatile antioxidants. The experimental trough and substrate solution, together with a hot plate 5 cm in diameter and a small fan were inserted within a dry-box through which pure dry nitrogen could be circulated. Linoleic acid was spread on one side of the barrier and a castor oil piston placed on the other side. The substrate was potassium phosphate buffer at pH 7. Under these conditions barrier movement occurred in air but no barrier movement occurred in nitrogen, even with the hot plate and fan on. At the end of a suitable control period, a 5-cm open Petri dish containing about 1 g of BHA (butylated hydroxyanisole) as a thin solid layer was placed on the hot plate. The temperature of the hot plate was maintained between 70 and 80C, since the melting range of the mixture of BHA isomers used is 48–55C, and it was desired to produce in the chamber the saturated vapor pressure of the antioxidant at its melting point.

Results and Discussion

Film Contraction

In contrast to the film expansion found in analogous studies of other workers referenced above, oxidation of a monolayer of *linoleic acid* compressed at a constant high film pressure of 16 dynes/cm on water was found to result in almost immediate initiation of a film contraction process (Fig. 2) showing quasi-first-order kinetics in the initial stages, with virtually no evidence of induction period. Both the area and the rate of change of area decreased with time, the plot of log Ao/(Ao-X) versus time being virtually linear in the early stages. With linoleic acid of high initial diene content at higher pH (8–10) at normal (20%) partial pressures of oxygen, the contraction of the film was virtually complete, the floating barrier migrating nearly to the end of the trough in 2 hr. With relatively diene-free linoleic acid, at pH 2 on a 0.01 N H_2SO_4 substrate solution, and after treatment with a pure oxygen atmosphere, the film contracted to a static area [Ao/(Ao-X) about 4] which, however, retained some fluidity as tested by talc and gentle currents of air.

Kinetics

As noted above, the process showed quasi-first-order kinetics in the initial stages (Fig. 4). A plot of the area fraction Ao/(Ao-X) on a log scale versus time was uniformly used to present the data, k being derived from the first 20 min of run.

$$\ln Ao/(Ao\text{-}X) = kt$$

where Ao is initial area and X is area of film lost by time t.

The semilog plots were never completely linear, however, but could be made nearly so, providing a

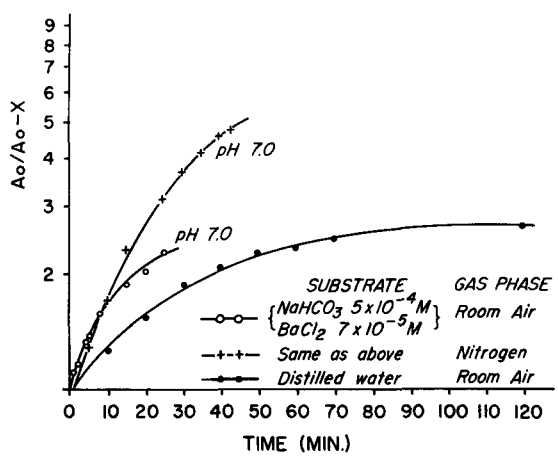

FIG. 2. Film contraction of barium linoleate and linoleic acid monolayers on aerobic substrate.

constant $A\infty$ (area of film at infinite time) were substracted from Ao to represent the fraction of molecules reacting to form insoluble products which thus reduced the possible extent of contraction. Such a corrected plot is shown in Fig. 4. The constant may be computed by equating the substituted rate expression for two successive equal periods of time as follows:

$$\ln (A_{01}-A\infty)/(A_{01}-X_1-A\infty) = \ln (A_{02}-A\infty)/(A_{02}-X_2-A\infty) = kt$$

where A_{01} and A_{02} are initial areas at two different times, and X_1 and X_2 are areas of film consumed in two successive but equal time periods. The proportion $Ao/A\infty$ is an, as yet, undefined function of partial pressure of oxygen and time of previous exposure to oxygen under conditions of slow contraction (low pH and freshly purified linoleic acid).

Effect of Anaerobiasis

Total anaerobiasis using a dry nitrogen atmosphere and a substrate solution of deionized, distilled water deaerated by boiling and dry nitrogen sparging, nearly completely inhibited the film contraction. Restoration of an air atmosphere produced virtually immediate contraction, and its replacement by nitrogen immediate cessation (Fig. 3). The evidence would suggest that the processes following the initial reaction with oxygen are very rapid and do not continue long after oxygen is removed. The almost immediate resumption of the process after restoration of oxygen with no induction period lag is also noteworthy.

In contrast to the results of other workers, however, complete anaerobiasis was found necessary at these relatively high film pressures. The use of nondeaerated water and a dry nitrogen blanket permitted contraction at rates comparable to those without nitrogen (Fig. 2). Hydroquinone present in the substrate solution at 0.2% (w/v) did not inhibit significantly, providing the atmosphere was air (Fig. 4) but did so if the atmosphere were N_2 (Fig. 3). This is in direct contrast to the results of other workers (1,3) at lower film pressures (2–8 dynes/cm) who found that hydroquinone (0.1%) in the substrate completely inhibited film oxidation. Their films, however, were in the vapor-expanded state, and the oxidation center was presumably free to seek the solution instead of being confined above it.

It will be noted from Fig. 3 that the rate of the resumed reaction is unaffected by an intervening period under nitrogen, confirming the fact that the reactions have been brought to a halt under anaerobic conditions.

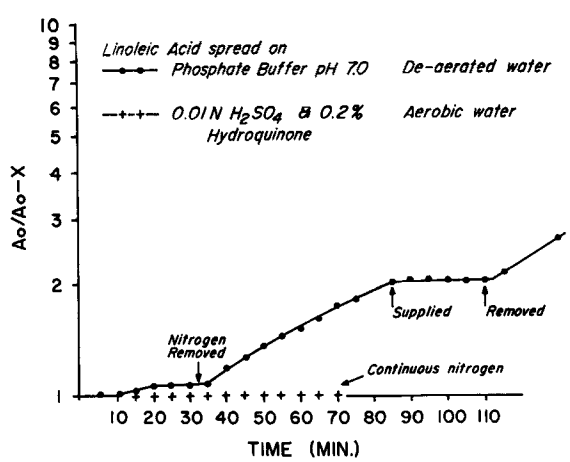

FIG. 3. Effect of complete anaerobiasis on film contraction.

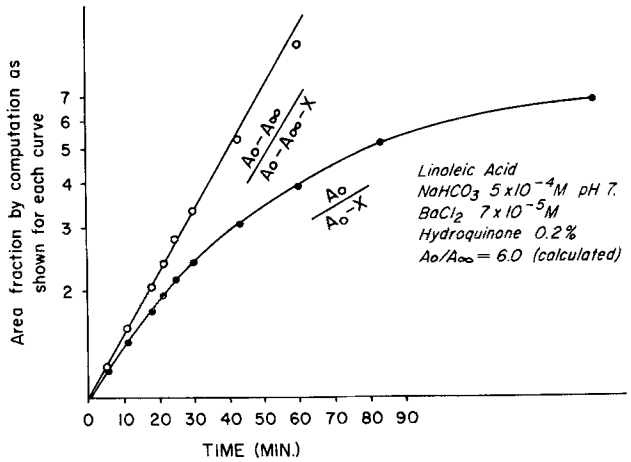

FIG. 4. Kinetic analysis of film contraction in room air.

Effect of pH and Titration

Fig. 5 shows that, for linoleic acid of a given diene content, the rate of monolayer contraction increases markedly with pH of the substrate solution above pH 5. At pH 9, half life of the film is less than 5 min. Fig. 6 shows a plot of rate versus pH. This bears a strong resemblance to a somewhat expanded titration curve with the half-maximum rate point falling at about pH 7.8. The curve resembles that obtained by Sobotka et al. (8) using stearic acid and radioactive calcium to determine per cent neutralization in floating monolayers and built-up multilayers. His 50% neutralization point occurred at 6.7, however.

The curves for pH 2 were obtained on 0.01 N H_2SO_4, for pH 5–8 on potassium phosphate buffer, and for pH 9–10 on sodium borate buffer. Although in some systems, phosphates have been considered as antioxidants, or synergists, and borates are sometimes used as complexing agents, the regularity of the results suggests that the added anions and cations had little to do with the effect. The pH dependency suggests that contraction may be due to the solution of short chain carboxylic acid derivatives resulting from the oxidative scission of linoleic acid, which are much more water soluble as their salts. The curves

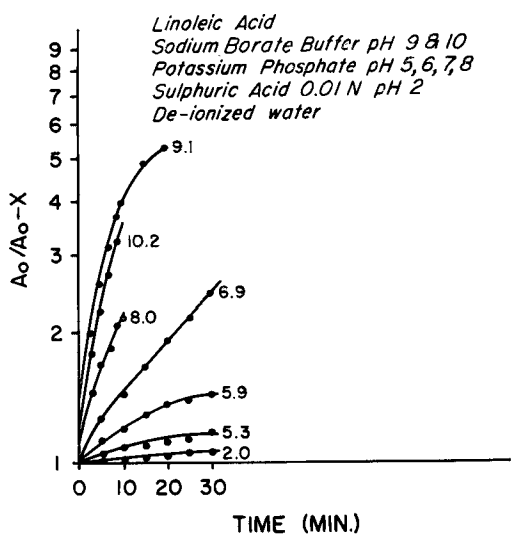

FIG. 5. Effect of pH of substrate solution on film contraction.

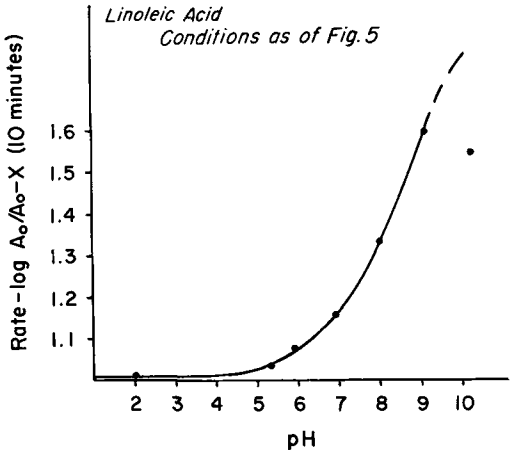

FIG. 6. Effect of pH of substrate solution on film contraction rate.

for pH 7 of Fig. 2 and Fig. 5 would suggest that the counterion has little effect, since in the former it was Ba^{++} and Na^{+} and in the latter, K^{+}. There is little difference in the rate at pH 7.

It was of interest to determine whether the reactions were proceeding as rapidly at low pH as at high, the difference in film contraction rates possibly being due to the solution of the short chain acids at high pH. Accordingly, films were spread on sulfuric acid substrate at pH 2 and exposed for varying periods of time, after which the substrate solution was carefully titrated by subsurface injection of 1 N NaOH, pH changes being monitored by electrodes submerged below the surface on the castor oil side of the barrier, and mixing accomplished by a magnetic stirrer in the substrate solution.

It was found (Fig. 7) that upon relatively rapid titration, the film underwent sudden collapse to a relatively static area, A∞, which was a larger proportion of Ao depending on the length of time the film was held at the slow contraction condition of low pH. It would appear from these data that at widely varying pH's the film is undergoing rapid oxidation at a somewhat similar rate from the moment of spreading and that the sudden contraction on titration is due to sudden solution of short chain acids. This conclusion is strengthened by the fact that the initial rapid contraction rate is similar for widely varying periods of preexposure to the slow contraction condition, but that the subsequent course of contraction to A∞ is very different. This must have been caused by further reaction of the oxidation centers when confined to the film for longer periods before titration. Were the films reacting at much lower rates on low pH substrates, one would expect their subsequent course of contraction when exposed by titration to high pH substrate to be substantially the same. Inspection of Fig. 7 shows that this is not the case.

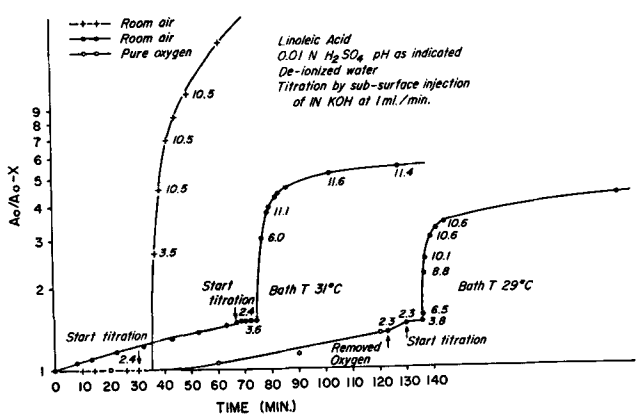

FIG. 7. Effect of substrate titration on film contraction.

In addition, the initial contraction rate upon sudden titration exceeds any obtained on any constant pH substrate tested, which would not be the case were the oxidation commencing at the time when the film is transferred to the higher pH.

It could be argued that only hydroperoxides are being formed on either high or low pH substrates, that the hydrophilic oxidation center subsequently dips into the substrate, but decomposes much faster at more alkaline pH. Apart from the fact that such molecular rearrangement of position would cause expansion of the monolayer, which was not observed, the argument is rejected by the data of Fig. 4 which show that hydroquinone in the substrate at pH 7 will not prevent film contraction without an anaerobic atmosphere whereas it is certain that it would prevent hydroperoxide decomposition to carboxylic acid residues if the peroxides were in contact with the substrate solution.

Effect of Location of Antioxidants in Substrate, Film or Gas Phase

One of the major objectives of these experiments was the study of the effect of antioxidants of both natural and synthetic types in a sterically ordered system of known components, steric distribution and ratio of components. For this purpose, monolayers were prepared from a petroleum ether solution containing known molecular ratios of linoleic acid and tocopherol, ranging from 19/1 to 1.8/1. Tocopherol prepared from hydrolysis of *dl*-alpha-tocopherol acetate was tested, as was *dl*-alpha-tocopherol procured as the oil. Under none of the experimental conditions used, including both aerobic and anaerobic atmospheres, was tocopherol shown to have any appreciable antioxidant action, and indeed, there appeared to be a slight prooxidant action (Fig. 8 and 9).

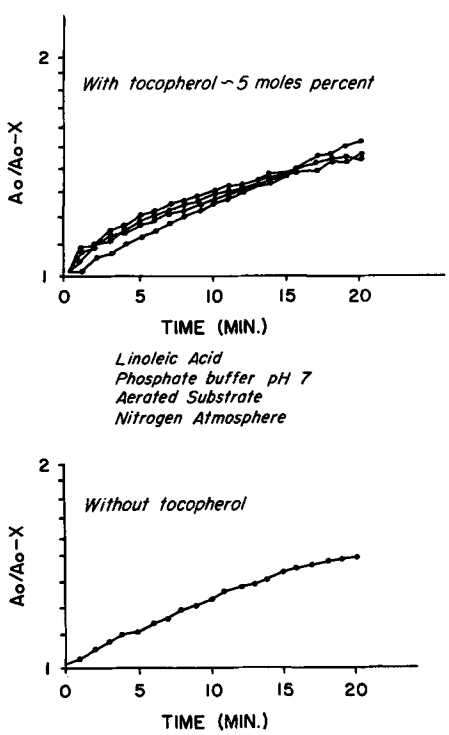

FIG. 8. Effect of tocopherol with nitrogen atmosphere.

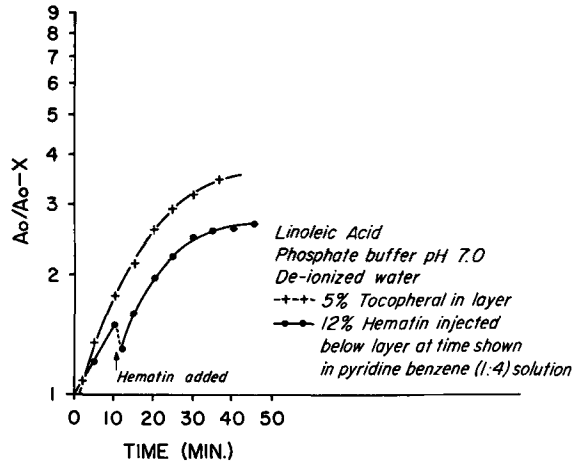

FIG. 9. Effect of tocopherol and hematin on film contraction with aerobic atmosphere.

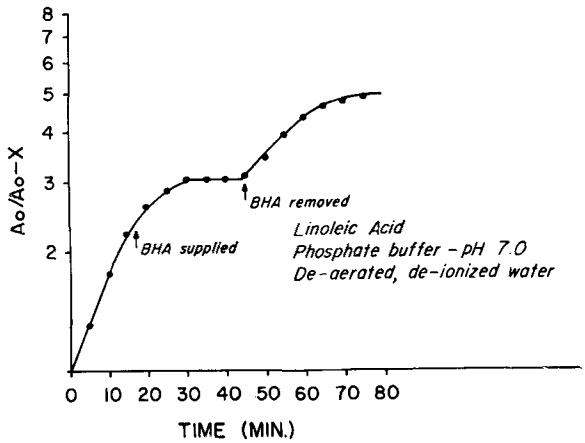

FIG. 10. Effect of BHA vapor in gas phase on film contraction under aerobic conditions.

It was noted above that hydroquinone in the substrate solution at 0.2% (w/v) did not inhibit the contraction, provided the trough was exposed to air. This is a contrast to the results of other workers at lower film pressures and is probably caused by the restriction on molecular movement at the higher pressure so that the double bond is kept away from the solution. In oxidations catalyzed by potassium permanganate in the substrate solution, the oxidation of oleic acid is drastically reduced at film pressures comparable to those of our experiments (1,2) and that of brassidic acid to virtually zero at only 8 dynes/cm, whereas at 2 dynes/cm oxidation proceeds rapidly on a potassium permanganate substrate for either acid. Similarly, Clowes (9), found complete inhibition of the photooxidation of polynuclear carcinogens like 3,4-benzpyrene when they were included as a 1:2 molecular complex with cholestanol or with lecithin-cholestanol in monolayer at an air-water interface. The hydrocarbon was, however, quickly photooxidized when ejected into the substrate solution containing dissolved oxygen, at higher film pressures.

It appears, then, that the steric restriction on molecular movement prevents contact of the double bonds with the substrate solution at high film pressures. In antioxidants like tocopherol the antioxidant moiety (the phenol ring) is held by its high surface energy close to the water surface, whereas hydroquinone is apparently confined to the substrate solution. Therefore, these antioxidants appear powerless to prevent reaction of oxygen from the gas or substrate phase with the 12–13 double bond. Therefore, it seemed wise to test the effect of moderately volatile antioxidants like the synthetic antioxidant BHA (butylated hydroxyanisole) introduced into the gas phase.

The introduction of the vapor from melted BHA at a vapor pressure corresponding to that at its melting point, to an oxidizing system having a steady film contraction rate in air stops the contraction within 5 min (Fig. 10). When BHA is removed, together with the dry box (since condensed BHA remains on the interior surfaces) the rate of film contraction is restored to its former level after fewer than 5 min. If, on the other hand, the vapor from melted BHA is added to a system where there is little to no contraction under a pure nitrogen atmosphere, very little change in the barrier position is noted. Where both the BHA and the nitrogen atmosphere are removed, normal film contraction and barrier movement commence within 30–40 min.

These data suggest that the BHA (at approximately 1 mm Hg partial pressure) acts to prevent film contraction by preventing oxidation, and not merely by its mechanical condensation on or penetration into the film, some of which must probably occur in both castor oil and linoleic acid films. Condensation or penetration could conceivably occur in a compensatory fashion on both sides of the barrier. However, oxidation and film contraction would not be expected to be gradually resumed at an *unchanged rate* after an interval (during which the BHA presumably sublimes away) if there had been substantial oxidation proceeding during the exposure to BHA.

Thus, whether the antioxidant effect of BHA in the gas phase is due to a blanketing by a condensed BHA film, or to the normal free radical trapping mechanism, the evidence suggests that it is a real effect on oxidation and not merely a compensatory plugging of holes left in a monolayer by oxidized and dissolved linoleic acid molecules.

The testing of antioxidants by the film contraction method would seem to discriminate between the action of the antioxidant when added to the substrate solution, the lipid or gas phase. Since each of these situations is encountered in actual reactions of oxygen with lipid, the method would seem to have promise in studying mode of action on a sterically specific basis.

Effect of Prooxidants

In an experiment to test prooxidant effects, hematin chloride in pyridine-benzene solution (1:4) was injected under a linoleic acid layer so as to produce a molecular ratio of linoleic acid to hematin of 8:1 after complete penetration of the hematin. That the hematin penetrated the layer was indicated by the expansion after equilibration and evaporation of solvent to a 16% larger area. The resultant oxidation proceeded at a nearly unchanged initial rate (Fig. 9), but $A\infty/Ao$, the static fraction of the area, was very large.

It would seem probable from results of these experiments that monolayers or the outermost layer of bulk liquid phases of linoleic acid, or its esters or pure triglycerides, commence being oxidized immediately, at initial rates involving half-lives of less than 3 min. Thus the precise surface pressure area studies of linoleic, linolenic, and arachidonic acids spread on

pH 2 substrates containing 0.12% hydroquinone, performed by Schneider et al. (10) would seem to be subject to considerable experimental error at film pressures of 16 dynes/cm and above.

The findings of the immediate inception of oxidation without evidence of induction period and of rates producing half lives of less than 3 min are similar to those of Gee and Rideal (3) using the maleic anhydride adduct of beta-elaeostearin. However, their results were based upon a compound whose oxidation resulted in film expansion, whereas in these results at high film pressures, contraction was the rule.

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