who furnished the complement. They likewise corroborated our results in finding specific antibodies in the specimen of complement examined. Measures have been taken by the commercial concern to enforce a previous order to destroy all used animals and further to test all lots of frozen and dried complement for the presence of typhus antibodies.

It is obvious from the above that serious errors in complement fixation can be obtained if complement from guinea pigs of unknown origin is used. It is therefore advisable when complement is obtained from unknown origin to test it for the presence of specific antibodies before use.

The neutralization tests were performed by Captain Byron L. Bennett, Sn.C., and Captain Howard L. Hamilton, Sn.C.; the psittacosis and lymphocytic choriomeningitis virus complement fixation tests were performed by First Lt. Joel Warren, Sn.C., of the Division of Virus and Rickettsial Diseases, Army Medical School.

## 14445

## Lipoxidase, a Dehydrogenase.

JOHN P. HUMMEL AND H. A. MATTILL.

*From the Biochemical Laboratory, State University of Iowa, Iowa City.*

The action of lipoxidase on unsaturated fats has recently been shown[1] to be of a complex nature; coupled with the oxidation of the fat is a loss of associated carotene which first directed attention to the existence of this enzyme. It appears to catalyze autoxidative rancidity, which has long been known to bleach carotene and hasten the oxidative destruction of fat-soluble vitamins.[2] Since autoxidation can be delayed by phenolic inhibitors and by tocopherol, application of these antioxidants offered a possible means of further studying the manner of action of lipoxidase, whether it is merely a catalyst or whether it modifies the course of autoxidation.

The substrate was the ethyl esters of lard fatty acids,[3] 75 mg properly emulsified in 75 cc of a solution comprising 60 cc water, 9 cc of 95% alcohol, 3 cc of acetone, and 3 cc of 0.1 M phosphate buffer at pH 6.5.

Oxygen absorption was measured at 38° in a macro-modification of the Warburg respirometer; when equilibrium conditions were attained, 3 cc of enzyme solution* were added by upsetting a small container within the flask. Iodimetric determinations of peroxide oxygen[4] were made on parallel samples under identical conditions.

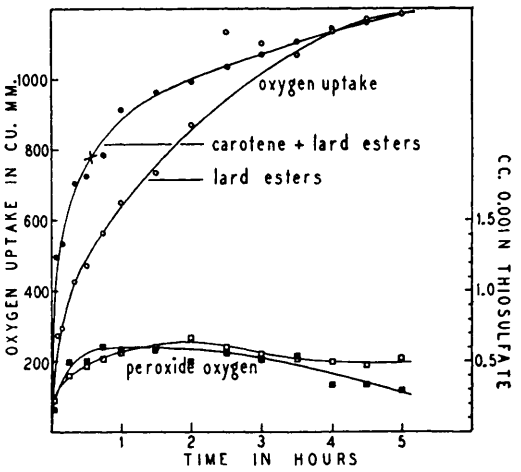


FIG. 1.

Oxygen uptake of esters with ● and without ○ carotene; peroxide oxygen with ■ and without □ carotene. X marks decolorization of carotene.

As seen in Fig. 1, the typical enzymatic

[1] Balls, A. K., Axelrod, B., and Kies, M. W., *J. Biol. Chem.*, 1943, **149**, 491.

[2] Mattill, H. A., *J. Am. Med. Assn.*, 1927, **89**, 1505.

[3] Olcott, H. S., and Mattill, H. A., *J. Am. Chem. Soc.*, 1936, **58**, 2204.

* This crude extract was prepared by suspending 5 g of powdered soybean protein (Glidden flakes, courtesy of Dr. P. L. Julian) in 100 cc of 2% sodium chloride solution. After standing for 30 minutes at room temperature the solution was centrifuged.

[4] French, R. B., Olcott, H. S., and Mattill, H. A., *Ind. Eng. Chem.*, 1935, **27**, 724.

oxidation of lard esters proceeded immediately on a hyperbolic course without a preceding induction period. During this time, there was only a small transient rise in peroxide oxygen.

When carotene was present,† there was a marked increase in the initial oxygen uptake, and this pro-oxidant effect persisted even after the carotene was decolorized. The time of decolorization varied with the amount of carotene present, as short as 1½ minutes in a concentration of 0.1% of the esters, and 90 minutes for 1% concentration. The reported failure of carotene to increase the rate of oxygen absorption[5,6] may be related to the amount of activator present.[1]

Since the simultaneous oxidation of fat is essential to the oxidation of carotene, the simplest explanation of the pro-oxidant effect of carotene is that the active fat peroxides bring about the autocatalytic formation of carotene peroxides which then also initiate new reaction chains, thus favoring the progress of the reaction. The persistence of the pro-oxidant effect of small amounts of carotene subsequent to decolorization might then be due to the additional reaction chains produced during the initial peroxidation.

The peroxide oxygen formed in the early stages of the reaction was small compared to that appearing in normal autoxidation, much too small to account for the oxygen absorbed. The ferrothiocyanate method of Sumner,[7] in our hands, failed to make any distinction between active and inactive peroxides.

In the course of ordinary autoxidation, when peroxides begin to disappear, secondary products make their appearance, thus suggesting that peroxide oxygen is in part used for this purpose. Since peroxide oxygen does not accumulate in the enzyme catalyzed reaction, this oxygen is apparently more promptly utilized; hence the effect of the enzyme on the peroxide oxygen became a matter of interest.

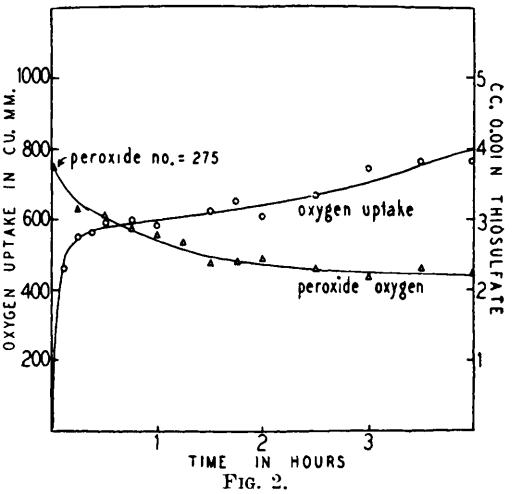


FIG. 2.

Oxygen uptake ○ and peroxide oxygen △ of highly rancid esters.

When rancid esters with a very high peroxide number were used as substrate (Fig. 2), the enzyme produced a marked fall in peroxide oxygen accompanied by a rapid absorption of oxygen only during the first 15 minutes; thereafter, the oxygen uptake was negligible. The only feasible destination of this oxygen was the formation of secondary products. This was confirmed by the presence of carbonyl groups[8] as shown by Schiff's reagent. Thus the enzyme not only accelerates the formation of peroxides, but facilitates their utilization in the creation of secondary products.

Antioxidants when added to the usual autoxidizing fat prolong the induction period. When hydroquinone was added to the enzyme-substrate emulsoid, it did not produce an induction period (Fig. 3); the oxygen uptake rose unimpeded to a level (A) at which it was interrupted until a later time (B) when it rose again to the normal ultimate value. This interruption of the oxygen uptake was noted only with relatively low concentrations of enzyme and was proportional to the amount of hydroquinone added. With higher concentrations of hydroquinone, the initial oxygen uptake proceeded more slowly and to a lower level, at which it remained for the rest of the experimental period.

† 50 mg crystalline carotene (10% α, 90% β) in 200 cc acetone. Desired amounts of this solution were added to the esters before their emulsification in water.

5 Süllmann, H., *Helv. chim. Acta*, 1941, **24**, 1360.

6 Sumner, R. J., *J. Biol. Chem.*, 1942, **146**, 215.

7 Sumner, R. J., *Ind. Eng. Chem., Anal. Ed.*, 1943, **15**, 14.

8 Süllmann, H., *Helv. chim. Acta*, 1942, **25**, 521.

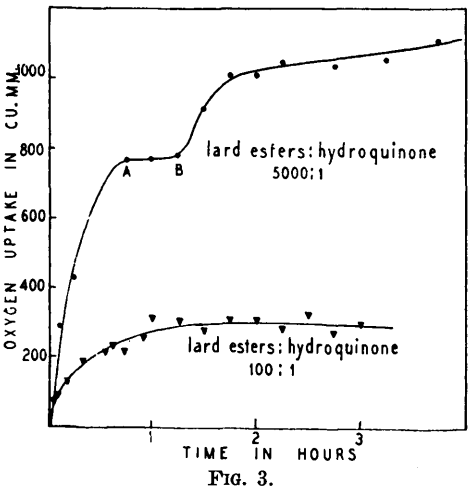


FIG. 3.

Effect of large ▲ and small ● amounts of antioxidant on oxygen uptake.

The initial oxygen uptake in the presence of small amounts of hydroquinone may imply that the enzyme directly attacks certain bonds in the fat molecule without involving the autocatalytic transfer of energy, such that hydroquinone cannot interrupt this initial process. As the reaction proceeds, part of the oxygen uptake may go to the further oxidation of the initially formed peroxides, and part to the formation of further reaction chains inaugurated by these peroxides; hydroquinone may then break these reaction chains.

For information as to the nature of the enzyme, the effects of some common enzyme inhibitors were tried, as well as the anaerobic reduction of a dye. The enzyme has been reported to be relatively uninhibited by cyanide, strongly inhibited by sodium azide and hydrogen sulfide.[5] Using regular Warburg respirometers, we found that sodium sulfide, iodoacetic acid, sodium pyrophosphate, and carbon monoxide markedly inhibited the action of the enzyme.

Two kinds of inhibitors must be taken into account, the true enzyme inhibitors, and the fat antioxidants. The above respiratory inhibitors probably have no antioxygenic action but with some compounds the distinction may not be clear. Of two surface active substances, sodium taurocholate had a very strong inhibitory action, whereas saponin had none.

Most of the respiratory inhibitors used above interfere with the action of the so-called aerobic dehydrogenases. Accordingly, the enzyme was tested (Thunberg tubes) for its ability to reduce a redox dye anaerobically; 2,6-dichlorophenol indophenol was chosen because of its high redox potential and its negligible antioxidant activity. Although some slow decolorization occurred in the absence of substrate, decolorization was very rapid in its presence, thus satisfying one of the most important criteria of an aerobic dehydrogenase. Among the properties which characterize aerobic dehydrogenases are the following: they activate the hydrogen of metabolites; they reduce certain dyes, and act in the absence of oxygen when such dyes are present; they catalyze the direct reaction between metabolites and oxygen, and produce peroxide in the presence of oxygen; they may or may not be inhibited by cyanide, and require neither coenzyme nor cytochrome systems.

The peroxide in this reaction is presumably a fatty acid peroxide rather than hydrogen peroxide which is usually encountered with aerobic dehydrogenases, but the fundamental characteristics of lipoxidase are those of an aerobic dehydrogenase rather than of an oxidase. Oxidases are inhibited by cyanide; this enzyme is not. Oxidases do not produce peroxides and do not decolorize dyes anaerobically. This enzyme does both. Little loss of activity follows dialysis.[5]

The evidence for the dehydrogenase nature of the enzyme may admittedly be criticized because the enzyme preparation was a crude extract; perhaps this contained a mixture of enzymes. If one of them is a dehydrogenase, it could activate the hydrogen of unsaturated fats, possibly by the creation of new double bonds. The answer to this question must await the further purification of the enzyme.

*Summary.* Lipoxidase appears to catalyze both the primary and secondary phases of fat autoxidation; in the primary phase carotene behaved as a pro-oxidant. Distinction must be made between inhibitors of enzyme action and the accepted inhibitors of the autoxidation of fats. A study of the effects of the former suggests that the enzyme is rather a dehydrogenase unless it is of multiple nature.