SCIENTIFIC SECTION

OXIDATION AND ANTIOXIDANTS.*

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One of the principal requisites of life, an indispensable, elemental necessity, is oxygen. We live by oxygen's favor only to spend our time and effort in an attempt to abate or prevent the damage done by this same element. Our food spoils and our property wastes away, in many instances due to the continual reaction which we know as oxidation. The first antioxidants were no doubt completely physical in their prevention of oxidation, such as pickling solutions, liquids or resins which protected some substance from contact with air. Our forefathers knew that their implements must be oil treated, lacquered or painted to preserve them, and we find that lacquer was used in China as a protective for buildings in the second century B. C., and a definite record of lacquer manufacture in Japan dates about A. D. 587. The lacquer used was an oxidizable substance extracted from a tree, *Rhus vernicifera*, and first oxidized to a yellow substance and then to a black.

An antioxidant is a substance which inhibits, prevents or stops the oxidation of some other substance or substances. This action is thought to occur in several ways.

By Inhibition.—Where the oxidation reaction is prevented by a substance which naturally repels the absorption of oxygen or takes up the oxygen which would combine with the substance being protected.

By Catalytic or Anticatalytic Action.—In which the antioxidant is regarded as a negative catalyst or a catalyst operating against change in the substance to which it is added. This catalytic action operates to cause a reaction to take some form other than the normal reaction with the principal substance involved.

By Chain Reactions.—In which the oxidizing reaction is viewed as one that starts and while going on gives off energy which in turn starts another reaction with the same results. The antioxidants operate to break up these subsequent reactions and stop this recurring chain.

These three conceptions while given here with titles to set them apart are not by any means entirely or distinctly different. There is some variation in viewpoint but each of them involves more or less a very kindred action. There are other theories which bear a connecting link between those just mentioned but are not sufficiently distinctive to continue to enumerate them.

Moureu and Dufraisse (1) have done quite extensive work on the study of auto-oxidation with the viewpoint that every reaction is a catalytic one, and there seems to be no legitimate reason to doubt that all auto-oxygenic or antioxygenic actions can be viewed from this standpoint. Whatever action takes place and by whatever means, it is easily resolved into one of catalysis. That every antioxidant is one that can be oxidized is seemingly very safe ground, especially when one

* Scientific Section, A. PH. A., Madison meeting, 1933.

considers that oxygen is oxidizable to ozone and acts as an inhibitor in the reaction between hydrogen and chlorine.

About twenty or twenty-five years ago considerable interest was aroused among chemists over oxydase, an enzymatic ferment, which was held as the principal causatory agent of oxidation in all products in which it was existent. Keegan (2) relates the observations of Lindet in 1893 regarding the oxidation of apple juice. It was found that the juice from boiled apples did not discolor in the air while the juice from fresh apples, cut and washed thoroughly with boiling water, did discolor. A conclusion was advanced that perhaps the vegetable tannin precipitated the oxydases. In the light of what we now know it seems more probable, however, that in boiling, a chemical reaction was responsible for some change in a natural constituent either destroying a pro-oxidant or forming an antioxidant. Such a conclusion has been reached by Overholser and Cruess (3) in their study of the browning of apples. They concluded that a peroxydase and an organic peroxide were responsible, and definitely detected the peroxide in fresh apple juice and could find no evidence of it in the boiled juice.

Lubimenko (4) observed, or detected, the formation of an antioxydase as a natural process during the ripening of the tomato.

It is well known that the heating or boiling of oils increases their susceptibility to oxidation. Especially is this practiced in the preparation of linseed oil, and in addition to boiling, traces of metals or metallic compounds have been added. Phokin (5) classified the activity of fifteen metals according to their activity as follows: *Group 1.*—Cobalt, manganese, chromium, nickel, iron, platinum, palladium. *Group 2.*—Lead, calcium, barium. *Group. 3*—Bismuth, mercury, uranium, cobalt, zinc. Observation of the increasing weight of a small sample is said to indicate the progress of the reaction.

Powick (6), in his study of the rancidity of fats, has given detailed consideration to the substances found in rancid fats with the idea of determining the agent responsible for the odor as well as seeking the agent responsible for the reaction in the Kreis test. Heptylic aldehyde is credited with the odor, and by spectroscopic methods the product of the reaction between acrolein and hydrogen peroxide has been found identical with that responsible for the color developed in the Kreis test, and identified as epihydrin aldehyde.

The Kreis test has had more or less discussion as to its reliability, and it may be said in regard to the work done in connection with this paper that the Kreis test was not depended upon except in a rough way. Powick found it unreliable in some cases and similar results have been obtained by many others. The Kreis test is performed by taking 5 cc. of the oil or fat to be examined, adding 5 cc. of concentrated hydrochloric acid, shaking thoroughly and then adding 5 cc. of 0.1% solution of phloroglucin in ether. A red coloration in the acid layer indicates rancidity. Holm and Greenbank (7) feel that the Kreis test is outranked by no other and are certain of its dependability. They do, however, state that the test does not indicate the degree of rancidity. Jones (8) regards the Kreis test as the best specific indicator of rancidity. But it is generally agreed that the degree of color is not proportionate to the rancidity, and that is the difficulty that has been encountered in the following experiments. The color developed is a very light pink in many cases and an indefinite pinkish brown in others, making it confusing in many instances

Richardson (9) reports that the test is not recommended for adoption by the American Oil Chemists Society. The most satisfactory results have been obtained in these experiments with a type of mercury manometer and a test in which a potassium iodide solution was used. Hyman and Wagner (10) used an alcoholic solution of potassium iodide and titrated the liberated iodine, after three minutes' standing in the dark, with 0.01 normal sodium thiosulphate, starch indicator. These authors state that an attempted manometric method gave erratic results and for this reason they abandoned it in favor of the chemical test.

Greenbank and Holm (11) concluded that methylene blue gave a good indication of the resistance offered by a fat to oxidation. We have found in this laboratory that the reduction of methylene blue in fats is very unreliable as to the powers possessed by an added antioxidant and if reliance were placed on this method, monoethanolamine would be quickly eliminated as a desirable antioxidant, however we are using it with great satisfaction in an aqueous solution of an organic mercurial.

In many instances refined oils and fats become rancid much more readily than the crude product and it has apparently been correctly assumed that refining removes some non-sterol portion which is a natural antioxidant.

TESTS USED IN DETECTION AND DETERMINATION OF OXIDATION.

The Methylene Blue Test.—Twenty cc. of fat are mixed with 1 or 2 cc. of a 0.025% solution of methylene blue in absolute alcohol. The use of alcohol other than absolute will introduce the factor of water in the experiment.

The Guaiac-Hemoglobin Test.—To a 10-Gm. sample add 4 or 5 drops of a one or two per cent solution of hemoglobin, 10 drops tincture guaiac and 10 cc. of distilled water. Allow to stand a few minutes when a blue color will be produced if oxygen has been absorbed.

The Kreis Test.—Has been given above.

Alcoholic Potassium Iodide Test.—To 5 Gm. potassium iodide add 10 cc. distilled water and ethyl alcohol 95% to make 100 cc. To a 5-cc. sample of the oil or melted fat add 5 cc. of this alcoholic solution and shake thoroughly, allow to stand about fifteen minutes and the alcoholic layer will appear as the supernatant liquid. It is essential that the samples for comparison be previously measured out and the addition of the potassium iodide solution be then made as near simultaneously as possible. Each sample is shaken the same length of time. The amount of color furnished by the liberated iodine will serve as an index to the amount of oxygen absorbed and the samples can be arranged accordingly.

Work in this laboratory was begun on cod liver oil, this being a very good example of a medicinal oil upon which feeding tests can be readily made, and also representative of a drying oil which will show resinous deposits or films when oxidized. From the latter standpoint work done upon linseed oil is applicable and considerable work has been done upon linseed oil and antioxidants. Mattill (12) has reported results with numerous chemicals both on lard and cod liver oil.

The first experiments undertaken were in connection with the antioxygenic activity of hydroxy-methyl-anethol, $CH_3.OH.CH_3CH.CH.C_6H_4O.CH_3$. Anethol is very rapidly oxidized even upon exposure to light, and since it is held more or less generally that the hydroxy compounds are a desirable class as antioxygens it appeared that this chemical might have satisfactory qualities.

Three 25-Gm. samples of cod liver oil were taken, plain oil, one containing 0.1% hydroxy-methyl-anethol (which for brevity will be designated as H. M. A.) and oil containing 0.2% H. M. A. Oxygen was slowly bubbled through the samples

and they were exposed to the light of a 100-watt lamp brought directly upon them. These samples were in a cabinet and were kept at a temperature of 35° C. The samples remained in this cabinet for a 24-hour period during which time 46-80 cc. of oxygen passed through each sample. Determinations were made for free fatty acids in an attempt to determine exactly the course of the reaction. The results follow:

Control	F. F. acids as % oleic	0.4022
Sample plain oil	F. F. acids as % oleic	0.4006
Oil with 0.1% H. M. A.	F. F. acids as % oleic	0.4089
Oil with 0.2% H. M. A.	F. F. acids as % oleic	0.4256

The results were too close together in the first three samples to make any conclusion whatever. The sample containing 0.2% H. M. A. did show that the induction of rancidity was definitely started.

Taking a new sample of cod liver oil, four 150-Gm. samples were taken, placed in a cabinet at 35° C. and exposed to the light of a 100-watt lamp for 27 hours. During this time oxygen was slowly bubbled through each sample until a total of 1600 cc. had been brought into contact with each sample of oil. Free fatty acid determinations were made (Col. 1) and the samples were set aside at room temperature in the dark, at the end of six months the samples were checked for free fatty acids (Col. 2).

The sample of plain oil was showing a film of oxidized oil on the surface, with a varnish-like deposit accumulating on the sides of the bottle at the surface. The control sample was in the same condition with approximately thirty per cent less oxidized deposit. The samples containing H. M. A. were free from any film or resinous deposit. The sample containing water was also free from any oxidized film. Both the control and the plain oil sample had a decidedly rancid odor while the other samples were comparatively free from this odor, the H. M. A. samples being best.

All of the samples were then kept in the dark, at room temperature, and at the end of two years, acidity determinations were again made (Col. 3).

Experiment "A."		1.	2.	3.
Control	F. F. acids as % oleic	0.379	0.6508	5.506
Sample plain oil	F. F. acids as % oleic	0.418	0.8606	6.809
Oil with 0.1% H. M. A.	F. F. acids as % oleic	0.427	0.6313	1.083
Oil with 0.2% H. M. A.	F. F. acids as % oleic	0.456	0.6603	2.085
Oil with 1.0% water	F. F. acids as % oleic	0.407	0.5751	1.040

The natural color was retained best by the sample to which water was added and was nearly as good in the sample containing 0.1% H. M. A. The sample containing 0.2% H. M. A. was slightly darkened, and the control and the plain oil sample were brown in color. The rancid odor was much greater in the control and plain oil sample.

Simultaneously with starting "A," 150-Gm. samples of the same oil were exposed to heat only, the temperature being 50° to 52° C. The control sample being the same as above.

Experiment "B."		End 1 Week.	End 1 Month.	End 6 Months.
Sample plain oil	F. F. acids as % oleic	0.4234	0.5051	1.448
Oil with 0.1% H. M. A.	F. F. acids as % oleic	0.4385	0.5320	1.131
Oil with 0.2% H. M. A.	F. F. acids as % oleic	0.4365	0.5170	1.286
Oil with 1.0% water	F. F. acids as % oleic	0.4079	0.5603	1.486

It will be observed, in the determinations made at the end of twenty-seven hours and at the end of one week, that the induction of rancidity is more rapid with the addition of H. M. A. and that the addition of water delayed this induction period. The determinations at the end of one month, on the heated samples, show the effect of the added water and the lesser acidity of the sample containing 0.2% H. M. A. The results are also shown for the end of a six months' period. The sample of plain oil showed a thick resinous film on the surface and resinous deposits on the sides of the bottle. The hydroxy-methyl-anethol samples were free from any film or deposit and the rancid odor was only about 25 per cent as great. The sample containing water was somewhat cloudy and had a pronounced rancid odor, but had only a slight deposit or resinous film on the sides of the bottle.

The apparent lengthening of the induction period, by the addition of water to cod liver oil, led to the washing of a sample of cod liver oil with three portions of distilled water. The volume of water used for each washing was the same as the oil volume. The oil was collected and filtered and samples were set aside for observation. At the end of eight months the following samples were observed:

Washed cod liver oil exposed to oxygen: Retained natural color, showed very thin film, and the odor was only approximately 50 per cent as rancid as the control. There was a slight deposit on the sides of the bottle.

Washed oil + 5 per cent added water: No film formed, a very slight deposit in bottle, 50 per cent, approximately, of rancid odor and a heavy white deposit in the bottom of the bottle.

Control: Dark yellow color, with a thin oxidized film, a strongly rancid odor and a small deposit on the sides of the bottle. Duplicate samples were held at 50° C. The control and washed-oil samples showed heavy gummy deposits, resinous in appearance. The washed oil + 5 per cent water showed no gummy deposit but did have a heavy film on the surface.

There is apparently some substance present in cod liver oil which can be removed or inactivated with water, thereby prolonging the induction period of oxidation. Perhaps in the sample washed with water, this substance reappears after oxidation begins and it may be that water present in the oil absorbs or hydrolizes this substance, thereby further preventing the oxidation.

COMPOUNDS FAVORED AS ANTIOXIDANTS.

The work done in all fields, for the prevention of oxidation has led to the establishment in general of favored types of compounds as antioxidants. Following are several of these more favored types: Phenolic compounds, hydroxy compounds, aldehydes, quinones, sterols, amines and alcohols.

Ammonium compounds, sugars, and in many isolated instances some other compounds have unexpectedly shown inhibitor action. Oxygen itself, acts as an inhibitor in the reaction between hydrogen and chlorine.

COMPOUNDS USED IN THIS EXPERIMENT.

A number of organic compounds were picked at random for determination of their antioxidant properties. Principally these compounds come under the classes named as favorable antioxidants. Flint-glass bottles of about one pint capacity were taken; in each bottle were placed 100 Gm. cod liver oil containing 0.5 per cent of the chemical, the bottles were filled with oxygen and stoppered tightly and connected to a mercury gage to measure the amount of oxygen absorbed. These bottles were set in a cabinet and a temperature of 58° to 60° C. was maintained. Two 100-watt lamps provided the heat, as well as light for whatever photochemical effect might be produced. The experiment ran for a forty-hour period and the following results show the absorbed oxygen, no regard being given to color developed.

Hydroxylamine Hydrochloride, $NH_2OH.HCl$	0.00 cc. O_2 absorbed in 40 hours
Alpha Naphthol $C_{10}H_7.OH$	0.50 cc. O_2 absorbed in 40 hours
p-Phenylenediamine, $NH_2C_6H_4NH_2$	3.90 cc. O_2 absorbed in 40 hours
p-Dimethylaminobenzaldehyde, $(CH_3)_2NC_6H_4CHO$	4.55 cc. O_2 absorbed in 40 hours
Thymoquinone, $C_6H_2O_2$ [1–4] CH_3 [2] C_3H_7 [5]	5.00 cc. O_2 absorbed in 40 hours
Blank Cod Liver Oil	5.05 cc. O_2 absorbed in 40 hours
Triphenylamine, $(C_6H_5)_3N$	5.15 cc. O_2 absorbed in 40 hours
p-Hydroxybenzaldehyde, C_6H_4OCHO	5.35 cc. O_2 absorbed in 40 hours

The *p*-Hydroxybenzaldehyde was in this case the least effective, oxidizing to such an extent in twenty-four hours that it was removed. This rapid indication of oxidation cannot be definitely regarded as disqualifying a chemical, for there are two views to be taken of the induction period. When an induction period is delayed or prolonged it may mean that the inhibitor used is one which will be protective by permanently preventing oxidation or it may be temporarily preventative and at the end of this temporary period oxidation may proceed with increased rapidity. A second view to take of the induction period is that when it is extremely short, it may be an indication that the chemical has a great affinity for oxygen and is taking up the oxygen for the protective benefit of the substance to which it has been added. From either viewpoint the possible value of the chemical cannot be definitely decided. However, those chemicals which show a prolonged induction period are usually of value.

A second experiment, made in the manner of the one just outlined, using a different sample of cod liver oil gave the following results:

Alpha Naphthol, $C_{10}H_7.OH$	0.00 cc. O_2 absorbed in 40 hours
Blank (plain cod liver oil)	3.95 cc. O_2 absorbed in 40 hours
Benzophenone, $(C_6H_5)_2CO$	5.00 cc. O_2 absorbed in 40 hours
8-Hydroxyquinoline, $(CH:CH)C_6H_3.OH.N.CH$	5.05 cc. O_2 absorbed in 40 hours
Ergosterol, $C_{27}H_{42}O.H_2O$	5.10 cc. O_2 absorbed in 40 hours
Phenyl-alpha-naphthylamine, $C_6H_5.C_{10}H_6.NH_2$	5.10 cc. O_2 absorbed in 40 hours

Duplicate cod liver oil samples of 100 Gm. each, with 0.5 per cent of chemical added, were placed in an incubator at 50° C. At the end of one month the samples appeared as follows: No resinous film had formed in samples containing: *p*-Phenylene diamine; *p*-Dimethylaminobenzaldehyde; Hydroxy-methyl-anethol; Alpha-naphthol; Thymoquinone; Phenyl-alpha-naphthylamine; Beta-naphthol; Thymol.

A slight resinous deposit formed in samples containing: 8-Hydroxy-quinoline; Triphenylamine; Benzophenone.

A noticeable deposit of resinous character formed, on the sides of the bottles at the surface, in samples containing: *p*-Hydroxy-benzaldehyde; Menthol; Ergosterol; Hydroxylamine hydrochloride; Plain cod liver oil.

By the same method samples of olive oil were tested using:

Menthol, $C_{10}H_{19}OH$	0.00 cc. O_2 absorbed in 40 hours
Alpha-naphthol, $C_{10}H_7OH$	0.00 cc. O_2 absorbed in 40 hours
Blank	3.80 cc. O_2 absorbed in 40 hours

Further tests were made with olive oil, measuring the absorbed oxygen in the same manner and using 100-Gm. samples of oil containing 0.5 per cent of chemical. In this test the chemicals used were hydroxy compounds and organic acids.

Tartaric acid, $C_2H_2(OH)_2COOH_2$	0.00 cc. O_2 absorbed in 49 hours
Anisic acid, $C_6H_4.OCH_3.COOH$ [1:4]	1.70 cc. O_2 absorbed in 40 hours
Hydroquinone, $C_6H_4(OH)_2$ [1:4]	3.30 cc. O_2 absorbed in 49 hours
Pyrocatechin, $C_6H_4(OH)_2$ [1:2]	4.70 cc. O_2 absorbed in 49 hours
Benzoic acid, C_6H_5COOH	4.90 cc. O_2 absorbed in 49 hours
Resorcin, $C_6H_4(OH)_2$ [1:3]	5.00 cc. O_2 absorbed in 40 hours
Camphoric acid, $C_8H_{14}(COOH)_2$	5.10 cc. O_2 absorbed in 49 hours
Salicylic acid, $C_6H_4(OH)COOH$ [1:2]	5.30 cc. O_2 absorbed in 40 hours
Blank (plain olive oil)	5.30 cc. O_2 absorbed in 49 hours

It will be noticed that three of these samples (salicylic acid, resorcin and anisic acid) ran only 40 hours, they were removed at that time because they were the only samples which were showing absorption of oxygen. The induction period on all other samples occurred after the fortieth hour.

As a check on the oxygen absorption, 5 cc. of each sample were shaken with 5 cc. of alcoholic potassium iodide solution. As soon as the mixture separated into two layers the tubes were arranged in order according to liberated iodine shown. The order was:

1. Salicylic acid
2. Resorcin
3. Anisic acid
4. Camphoric acid
5. Benzoic acid
6. Blank

(3–4–5–6)

7. Pyrocatechin
8. Hydroquinone
9. Tartaric acid
10. Unexposed olive oil

Numbers 3 to 6, inclusive, were so nearly equal in color that it was not possible to determine the order of arrangement. It should be noted that this test gave very consistent results with the measured oxygen absorption, the greatest difference by measurement being 0.4 cc. between the benzoic acid sample and the blank. Anisic acid would without dispute hold position No. 3 when its induction period of forty hours is considered. Blank tests run by adding these chemicals to the alcoholic potassium iodide did not change the positions. It is also seen that while three dihydroxy benzenes were used, no consistent results were obtained in regard to the positions of the hydroxyl groups. Hydroquinone, with the OH groups in the 1:4 positions, is the best antioxidant and if wide separation of the OH groups is an advantage, resorcin should be better than pyrocatechin, such, however, is not the case. Blank tests should always be made with the alcoholic potassium iodide to determine whether or not the chemical under examination liberates iodine. In a

further experiment phloroglucin, $C_6H_3(OH)_3 + 2H_2O$, pyrogallol $C_6H_3(OH)_3$ [1:2:3] and tartaric acid were found very effective in the order named.

Some of these chemicals used in these experiments were not soluble in a proportion of 0.5 per cent, but the results are given regardless, for the insolubility in this proportion would merely indicate that a smaller amount would be necessary.

Rogers and Voorhees (14) in their work on gasoline have used a few of these chemicals, in addition to many others, and with favorable results.

Three compounds typical of as many chemical groups were taken for feeding tests upon white rats, and Vitamin A assays. These three compounds beta-naphthol, thymoquinone and hydroxy-methyl-anethol preserved the appearance of the cod liver oil as it was naturally, and prevented the formation of any resinous film. There was no apparent difference in the Vitamin A activity as compared with a control sample, indicating that there was no deleterious effect of the compounds on the vitamin activity. The compounds did not, however, aid in retaining the vitamin activity of the oil to give it any advantages over the control sample of the same age. Feeding tests upon a more extensive scale and with several of the other compounds used as antioxidants might show some that preserved the vitamin activity in addition to the physical characteristics of the oil.

CONCLUSIONS.

It can be stated definitely, that there is no absolute means of pre-determining the value of an antioxidant in oils and fats merely from the chemical structure of the compound.

Apparently the naphthols, quinones and hydroxy compounds are three of the most dependable types.

The "trial and error" method must be used to determine two things—*first*, the possible value of the antioxidant and *second*, the amount necessary to give the maximum activity.

While this paper involves only the use of antioxidants in oils, work done of a similar nature in aqueous solutions bring conclusions that parallel those just given.

Thanks are due to Charles R. Eckler for his work on the vitamin activity of the cod liver oil, and to W. J. Rice for criticisms.

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