A STUDY OF ANTIOXIDANTS

WITH RESPECT TO VITAMIN A IN FISH OILS

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INTRODUCTION

Vitamin A is among the most labile of the vitamins; it was this property which enabled McCollum to differentiate it from vitamin D in cod liver oil. This lability of vitamin A is intimately associated with the instability of the oily vehicles, such as cod, halibut, and tuna liver oils, in which it is usually found. Oils, in themselves, are notoriously susceptible to rancidity, and this is particularly true of fish liver oils.

The production of oxidative rancidity in oils is due to atmospheric oxidation, which is possible because of the presence in the oils of unsaturated fatty acids, such as oleic, linoleic, and linolenic, with one, two, and three unsaturated linkages respectively. The relative rates of oxidation of these three fatty acids have been found to be in the ratios of 1, 12, and 100 (39). This accounts to some extent for the different rates of oxidation of various oils, although other factors, such as the presence of antioxidants, are of greater importance.

Under the influence of atmospheric oxygen oils containing vitamin A undergo autoxidation. This

catalytic oxidation of the oils usually does not begin at once, even under severe conditions, but is preceded by a period, of different lengths in different oils, during which practically no reaction takes place. Following this there is a period of rapid oxidation, during which the rate of oxidation gradually increases to a maximum, then falls off. Plotting this reaction rate against time gives rise to an S shaped curve, which is characteristic of autoxidation reactions.

This period of little change at the beginning of the autoxidation of oils is called the induction period. It is prolonged by the presence in the oils of antioxidants, and shortened by oxidation catalysts. The duration or length of the induction period of an oil is a measure of its keeping quality; when the induction period ends rancid odors and flavors develop and labile constituents of the oil, such as vitamin A, begin to break down. It has been assumed that the peroxides, which are formed as primary oxidation products in oils, are the auto-catalysts of this reaction (70). Greenbank and

Holm, however, conclude that acids and other products formed in the oxidation process are responsible for the increase in rate of reaction with time (29).

In the present study a standardized method has been developed for determining the induction periods of oils containing vitamin A. This method has been employed in evaluating the stability of various types of natural vitamin A oils; and also in studying the conditions affecting the stability of vitamin A in these oils, such as the additions of various inhibitors of oxidation, or antioxidants. It has been used further to determine the nature of natural antioxidants occurring in fish oils, and as a guide in attempting the isolation of these natural antioxidants.

REVIEW OF THE LITERATURE

Mechanism of Autoxidation

Theories of Autoxidation. There are two theories explaining the induction period of oils and fats. The older explanation (19) is that some product of the oxidation reaction, probably peroxide,

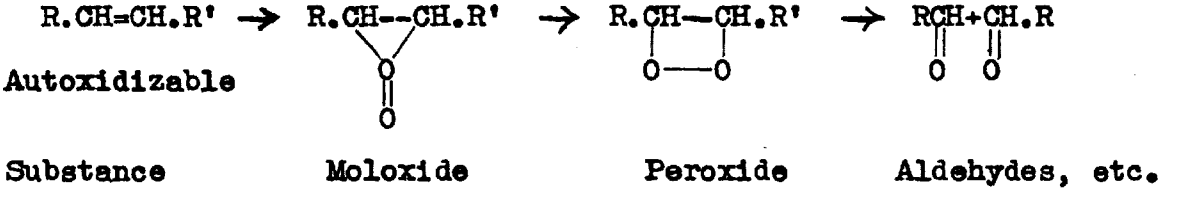
functions as a positive catalyst to promote the reaction. This substance is considered to accumulate in the fat until a certain critical concentration is reached, when a rapid oxidation is brought about. Early evidence for this explanation was the fact, reported by Fridericia (18), that adding rancid lard to fresh cod liver oil soon destroyed the vitamin A in the latter. He suggested that the effect might be due to organic peroxides. Rosenheim and Webster also reported (65) that if an oil high in peroxides was mixed with fresh cod liver oil, the growth promoting effect on the fat as well as the chromogen responsible for the antimony trichloride color test was destroyed.

Recently, however, it has been found that the induction periods of oils and other substances become progressively shorter with increased purification of the fat or oil. Thus Mattill and Crawford found that purifying corn oil increased its susceptibility to autoxidize (48). Also the induction period of glycerides resynthesized from the distilled acids of a natural oil has been found to be much less than that of the original oil (27). Hamilton and Olcott (24) found that purified methyl

oleate and oleic acid had practically no period of induction, beyond that required for the diffusion of oxygen. This evidence supports the view then that in natural oils the period of induction is due to the presence of inhibitors, and not to the slowness with which peroxides are initially formed.

Products Formed During Autoxidation of Oils.

There has been much speculation concerning the products formed during the autoxidation of the unsaturated fatty acid components of fats and oils. Staudinger (69) believes the steps in autoxidation are as follows:



Autoxidizable Substance — Moloxide — Peroxide — Aldehydes, etc.

The moloxides are believed to be extremely unstable and highly reactive. Powick (61) suggests that peroxides oxidize the fatty acid chain with the removal of two H atoms to form a new double bond, thereby being themselves converted to oxides. This formation of peroxides, followed by splitting of the new double bonds accounts for the series of saturated aldehydes and acids found in oxidized fats. Tschirch

(73) considers the peroxide formed to be decomposed by water to form an oxide, hydrogen peroxide and ozone. The ozone then reacts with other unsaturated molecules to form ozonides. These ozonides, in the presence of water, decompose into aldehydes and acids. It has been proposed by Browne (5) that a molecule of oxygen reacts with a double bond to produce a fatty oxide, and a liberation of an atom of active oxygen. This active oxygen attacks the glycerides in one of several ways--the resulting oxide isomerizes to a ketone, which breaks down on hydrolysis into substances of lower molecular weight. All these theories agree in requiring the primary formation of peroxides or oxides which either break down directly into aldehydes and acids of lower molecular weight, or isomerize, or react with water to form hydroxy and keto compounds. It is known that the double bond ruptures since azelaic and nonoic acids have been isolated from rancid oils.

Some time after the above theories were advanced concerning the products formed during autoxidation of unsaturated fatty acids, Hamilton and Olcott (21) published data which clarified the reactions to a great extent. They followed the

course of oxidation of highly purified oleic acid, methyl oleate and oleyl alcohol with special apparatus and methods which permitted the simultaneous measurement of the oxygen absorbed and its distribution among the transitory and final products of oxidation. These included water, carbon dioxide, and carboxyl, hydroxyl, peroxide and aldehydic compounds. Recently Deatherage and Mattill (10) after having refined Hamilton's and Olcott's apparatus made a further study of the oxidation products of several highly purified unsaturated fatty acids and related compounds. They found that oleic acid, oleyl alcohol, methyl oleate, butyl oleate and cis-9-octadecene all appear to be oxidized in a similar manner, yielding the same types of products--among others, peroxides, peracids, aldehydes, substituted ethylene oxides, acids, alcohols, combinations of these, and water. They concluded that after the addition of oxygen at the double bond to form peroxides, these peroxides may cleave to give aldehydes, they may either react with another double bond to give two moles of ethylene oxide, or they may aid in the further oxidation of the carbon chain. The aldehydes formed may also autoxidize to give peracids and acids. They

could not detect the presence of carbon monoxide and carbon dioxide. This work therefore does not support the theory of the mechanism of autoxidation of fats proposed by Powick (61), which necessitated the production of these two compounds during the process.

Factors Affecting Rancidity

Atmospheric Oxygen. Oxygen, either free or combined, is necessary for the autoxidation reaction. This is the basis for protecting fats from becoming rancid by enclosing them in completely filled containers. However, the amount of oxygen necessary to carry an oil to the end of its induction period is rather small. Therefore unless the space in the container is practically entirely filled by the oil, little advantage will result.

Peroxide Oxygen. Oxygen in loosely combined, or peroxide, form may play an important part in the development of rancidity in oils. As noted above, Fridericia (18) found that admixture with rancid lard slowly destroyed vitamin A in cod liver oil and suggested that the effect might be due to organic peroxides in the lard. Powick

(62) found that the inferiority of rancid lard as a component of a ration for rats was not due to its actual toxicity but to its ability to destroy the vitamin A in the rations with which it was mixed. This destruction appeared gradually over several days and was presumably due to the oxidation of vitamin A by the organic peroxides of the rancid lard. When the vitamin A was fed separately from the lard, the rats receiving the rancid lard seemed to require more vitamin A than did others receiving a corresponding ration containing sweet lard. The latter observation agrees with that of Lease, Steenbock, et al. (44), that rancid fats fed coincidently or shortly after ingestion of vitamin A reduce the storage of the latter in the liver. The assumption is that the peroxides of the rancid fat destroy the vitamin A after ingestion.

It has also been reported by Rosenheim and Webster (65) that when an oil high in peroxides is mixed with a fresh oil it gradually destroys the growth promoting effect as well as the chromogen responsible for the color test with antimony trichloride. Wokes and Willimot (80) studied the rate of destruction of vitamin A in cod liver oil aerated at various temperatures. They suggested that the

destruction of vitamin A under these conditions was due to the formation of volatile organic peroxides. Smith (68) found that vitamin A which had been dissolved in oil containing a large amount of peroxides was rapidly destroyed, even though sealed in glass tubes under nitrogen. Whipple (79) reported a drop in vitamin A potency of cod liver oils as the peroxide value increased. The same relationship was found by Lowen et al. (46), with the vitamin A in salmon and halibut oils.

Temperature. The rate of autoxidation of fats and oils is influenced by temperature, as are other chemical reactions. In fact, most accelerated tests for stability of fats are based on the increased rate of oxidation which occurs with a corresponding rise in temperature. Freyer (17) found that the rate of production of peroxides during the aeration of oils between 100° and 115° C was approximately doubled for each rise in temperature of 10° C. Lea (40) found that cod liver oil oxidizing at temperatures between -9 and 27° C, under the influence of weak illumination, oxidized 1.8 times as fast after a 10° increase in temperature. This accelerating effect of temperature is lessened

in the presence of active metal catalysts (64).

Light. The effect of light on the rancidity of fats and oils has been more or less confused with temperature and oxygen effects, with which it is often inter-related. It was formerly claimed (76) that light alone, in the absence of oxygen, was capable of producing rancidity. However, it has since been shown that this apparent production of rancidity by light alone was really due to traces of peroxides in the supposedly fresh fat. When great care is taken that the fats are peroxide-free and stored in nitrogen, they do not become rancid when exposed to light (36). A distinctive characteristic of rancidity or oxidation initiated by exposure to light is that once the process has begun it continues even if the samples are transferred to darkness (40). This is considered to be due to a stimulation of peroxide formation by the light, which later continues to catalyze the reaction, even in the dark.

Most of the recent data indicate that light simply acts as an accelerator of autoxidation of fats. This is due both to the intensity of illumination and to the wave length of the activating light. Lea (40) found that direct sunlight had the greatest effect, diffused daylight had a lesser effect, and

even a 100 watt lamp at a distance of 6 feet in an otherwise dark room had a pronounced accelerating action. Greenbank and Holm (21) studied the relative oxidation-stimulating effects on cottonseed oil of light of different wave lengths. Wave bands about 500 Å wide were studied by means of filter combinations, and the amount of energy passed through each filter was controlled by a thermocouple and galvanometer. The degree of rancidity was expressed as the amount of oxygen absorbed per 100 ml. of oil. The greatest effect was in the orange yellow band, being greater than in the orange red or the blue bands. These results are in opposition to those of Coe and LeClerc (8), who found that the greatest protection against rancidity caused by light was afforded by a dark green filter which transmitted only long wavelengths. Davies (9) also found that light of shorter wavelengths, especially in the region of the ultra-violet, was most effective in promoting rancidity. However, the depth of color of the protecting film was more important than its actual tint. Morgan (51) studied the protective effect against rancidity of fats of various cellophane films. He found that the efficiency

of a particular film in protecting against rancidity was in proportion to its absorption of ultra-violet and blue rays, rather than its color. He concluded that the effect of light of short wavelength increased rapidly with decreasing wavelengths.

Metal Catalysts. The role of metal catalysts in hastening oxygenation of oils has been known for a long time. This is the basis for the use of driers in paint. These are largely the soaps of cobalt, which have been found to be the most active of the metallic soaps in stimulating oxidation of oils (12). Copper is one of the most frequent offenders in causing rancidity in food fats, especially butter (31). A common method of evaluating the effect of metals on the oxidation of fats has been to immerse strips of the metals into samples of the fat and determine the rancidity at intervals. The results are compared with those for fat stored under similar conditions without metal. Using this method of examination and following the reaction by odor and the Kreis test, Emery and Henley (13) found that copper was the most active of the metals studied in promoting

rancidity in corn and cottonseed oils. Tin and aluminum were the least active, while lead, iron and zinc were intermediate.

These metal contaminants in oils can be removed by precipitation with thioglycolic acid, which has the property of forming complexes with metals (45). A similar mechanism has been suggested as being involved in the action of certain antioxidants. For instance it is claimed (74) that aliphatic diamines probably exert their stabilizing action by forming extremely stable complexes with metallic ions and thus preventing such ions from exerting their catalytic effect.

Salts and Acids. The rancidity accelerating effects of metals combined as salts either in solution in the fats and oils or dissolved in an aqueous phase in contact with the fats, has also been studied. Lea (42) reports that as little as 1 part per 100 million of copper salts had a detectable influence on the rate of oxidation, which increased rapidly at first with increasing concentration of the metal, but later appeared to approach a limiting value.

Hydrochloric acid has been found to have an accelerating effect on the oxidation of oils. Hilditch and Slightholme (27) found that boiling

olive oil with dilute hydrochloric acid reduced the induction period from 260 to 20 hours. Watterman and Van Vlodrop (77) found that gaseous hydrogen chloride and sulfur dioxide promoted the polymerization of linseed oils.

Qualitative Methods of Measuring Oxidative Rancidity

There have been numerous methods developed for the detection of oxidative rancidity in fats. The earlier ones were qualitative tests only and were usually applied to oils which had been under natural storage conditions. They attempted to measure the state of rancidity of oils in contrast with more recent accelerated tests which have been developed to measure the susceptibility of oils to become rancid.

Organoleptic. The earliest and simplest test applied to fats and oils for the detection of rancidity was based on odor and flavor. This has been called the organoleptic test. A criticism of this test is that it is not very sensitive. It is also impossible to express the results of the test on a numerical basis.

Kreis Color Test. One of the earliest and

most used chemical tests for oxidation rancidity of fats is the Kreis test (38). It is carried out by shaking 1 ml. of the oil with 1 ml. concentrated HCl, followed by further shaking 1 minute with 1 ml. 0.1% solution phloroglucinol in ether. The lower layer becomes red if the oil is rancid. Many studies of this test have been made, one of the most thorough being that of Powick (61). He concluded that the presence of heptylic aldehyde in rancid fats accounts for their characteristic odor. He also found that the compound responsible for the Kreis test is epihydrin aldehyde, $CH_2\underset{\underset{O}{|}}{CHCHO}$. This was not originally present in the rancid fat in the free state but was formed when the oxidized fat was brought in contact with concentrated hydrochloric acid.

Several procedures have been suggested for placing the Kreis test on a more quantitative basis. Kerr (34) carried out the test on samples by gradually diluting them more and more with non-reactive oil until no color was obtained. Holm and Greenbank (28) diluted the sample with petroleum ether after the test had been carried out until the color obtained matched a standard methyl red solution. A still later method was developed by Richardson (63) for the American Oil

Chemists' Society. He measured the red color produced in the test with the Lovibond tintometer.

Peroxide Test. A more recent method for detecting oxidative rancidity in oils is the peroxide test. This measures the loosely bound, or active oxygen which is formed in the early stages of rancidity. The early technic of the test (52) was not sufficiently sensitive to pick up the concentrations of peroxides found in slightly rancid oils. Several modifications have been suggested (71), (78), one of the most successful being that of Lea (43). This method is rather laborious and has been modified by Banks (2) to give a test which is much simpler to carry out, yet gives results essentially as accurate.

Schiff and Bisulfite Tests. The Schiff reaction has long been used as a test for oxidative rancidity in fats and oils (6). It consists in the interaction of acid fuchsin with the aldehydes, formed in the early stages of rancidity, giving a red color. A closely related test is the reaction of aldehydes with bisulfite (41). Lea has worked this out so that the small amount of aldehydes in rancid fat, which are considered responsible for the objectionable odor and flavor, can be titrated directly with bisulfite. This method has been used by Hamilton and Olcott (25) as one means of following

the Oxidative breakdown of oleic acid and related compounds.

Quantitative Methods of Measuring Rancidity

While all of these criteria give information concerning the degree of rancidity of a fat or oil, they are not very helpful in predicting the keeping quality of fresh fats. Many methods have been developed to accomplish this latter purpose, usually employing as criteria one or more of the qualitative reactions mentioned above. The principle involved in all such tests for susceptibility to oxidation of the fat or oil is the measurement of the induction period. The various methods of doing this usually employ specific standardized conditions. They differ only as to the methods used to produce these standardized conditions and the means of following the breakdown reaction.

Weight Increase. Since oils take up oxygen during autoxidation and therefore gain weight during the reaction one of the most obvious tests is that of determining the gain in weight of samples of the oil when exposed in thin films to

the air, usually at elevated temperatures. This method has been used extensively with drying oils, and has also been applied to edible oils (11), but it fails to detect the early stages of rancidity and does not measure the volatile products of autoxidation.

Oxygen Absorption. Another and more sensitive method of measuring the oxygen involved in the autoxidation reaction is to carry out the observations in a closed system containing either air or oxygen. A manometer is connected with this system so that any change of pressure can be observed. This is the method used by Moureu and Dufraisse (53) in their pioneer work on antioxidants. Mattill and Crawford (50) also used it in their earlier studies on the stabilization of fats by antioxidants. The reaction has usually been accelerated by maintaining the samples at an elevated temperature, and sometimes using agitation. However, Triebold, Webb and Rudy (72) compared the results of controlled oxidation of lard in a closed system at temperatures ranging from 70 to 95°, with and without stirring, and found that all these variations of the method gave the same relative results. French, Olcott and Mattill (16)

have shown that for small amounts of fat (5 gms. or less), the rate of oxidation is independent of the quantity used, indicating that the oxygen diffuses completely through this size sample. This indicates that stirring during the determination of the induction period is unnecessary. The method has been much refined by Mattill and coworkers (10) (25), for the purpose of studying the reactions taking place during the oxidation of oils. The method in general is too complicated, however, for routine examination of oils.

Aeration. One of the most practical methods of determining the susceptibility of oils to autoxidation is the aeration test developed by Wheeler (78). It consists in blowing air or oxygen through the oil, which is usually maintained at an elevated temperature, at a constant rate until rancidity develops. The progress of the breakdown is followed by making some chemical test, such as the peroxide determination on small samples drawn at intervals from the heated oil. This method has been standardized by King, Roschen and Irwin (37) and is known as the "Swift Stability Test". It consists in blowing washed air at a controlled rate through 20 ml. samples of oil in test tubes,

maintained at 100° C, until the oil has become rancid, as determined by peroxide tests made on small samples withdrawn periodically from the tubes. A preliminary indication of rancidity is obtained when the air emitted from the tubes of oil has a rancid odor.

Although acceleration of the aeration test by heating at 100° is commonly practiced, other modifications have been proposed (66). In these the test is further modified by irradiation with light or by the introduction of metallic catalysts. Accelerated tests of this nature, carried out at high temperature have been criticized by Evans (14) who claims that certain antioxidants are inactive at temperatures above 65°, and therefore these tests do not reflect actual conditions.

Incubation. One of the simplest, and yet most accurate, methods of determining the induction period of oils is the incubation test. This consists in exposing samples of the oil to the air at an elevated temperature and examining a sample at intervals by any one of a number of methods to determine the progress of rancidity. In one of the first applications of this test (23), a 100 gm. sample of oil was exposed in an open glass vessel at 60°, and examined at

intervals for odor and flavor to detect the onset of rancidity. This method was criticized because it was too slow and involved a large personal error in the detection of rancidity. These objections have been overcome in later modifications of the test by exposing smaller samples and expressing the degree of rancidity numerically by means of some chemical test. The method has been especially applicable to fish liver oils, and other vitamin A-containing oils, since they have a naturally short induction period.

Various modifications of the incubation test for stability of oils have been used. Greenbank and Holm (22), exposed samples of a variety of animal and vegetable oils (size not stated) for 10 days at 42°, then determined peroxides. Evans (14) employed 100-gm. amounts of cottonseed oil, plus a catalyst, and exposed these to pure oxygen at room temperature. Samples were withdrawn at intervals and peroxide determinations made. Holmes, Corbet and Hartzler (30) exposed small samples of several grams of fish liver oils to an atmosphere of oxygen at 96°. Vitamin A determinations were made on test samples of the oil

removed at intervals. The drop in vitamin A indicated when breakdown of the oil occurred. In the stability test used by Lowen, Anderson and Harrison (46) 30 ml. portions of halibut liver oils were exposed at room temperature. They measured both peroxide-formation and vitamin A-breakdown and found a correlation between the two. Simons, Buxton and Colman (67) exposed 2 ml. samples of fish oils in a dust-free cabinet at 34.5°. At intervals a sample was removed and a vitamin A test was made on 1 gm. and a peroxide test on the remainder. The breakdown rate was found to run parallel in both cases.

Control of Rancidity

Protection from Light and Air. Since autoxidation is dependent upon a supply of oxygen and is catalysed by light, an obvious protection against autoxidative rancidity of fats and oils is to enclose them in dark glass or opaque containers. However, it has been shown (35) that only a very small amount of oxygen is necessary to initiate the oxidative reaction, which may then proceed to the stage of rancidity even through the concentration of peroxides never becomes very great. The reason for this is that

the peroxides first formed are consumed in the progressive reactions which lead to rancidity. Under such circumstances the peroxide test, or other tests, indicative of beginning stages of rancidity, may be misleading as to the actual degree of rancidity (35), (60).

Use of Antioxidants

Discovery. The inherent stability of natural oils is due to traces of foreign substances, called antioxidants. This is also true of other organic compounds which tend to autoxidize. Moureu and Dufraisse (53) found that a trace of hydroquinone or certain other phenols inhibited the oxidation of acrolein and benzaldehyde. This was the first study of antioxidants reported. The authors concluded that only easily oxidizable substances are effective as antioxidants.

Theories of Antioxidant Action. The two outstanding theories of the action of antioxidants are those of Moureu and Dufraisse (53) and of Christiansen (7). The former deals exclusively with the inhibition of autoxidation, whereas the latter is known as the chain theory.

According to the theory of Moureu and Dufraisse, oxidant A unites with oxygen to form a peroxide, AO_2. This peroxide oxidizes the antioxidant B with the formation of a peroxide, BO, at the same time the oxidant being transformed to a lower oxide, AO. AO and BO are antagonistic and mutually react to regenerate the three original molecules A, B and O_2 in their original state.

$$A + O_2 \longrightarrow AO_2$$
$$AO_2 + B \longrightarrow AO + BO$$
$$AO + BO \longrightarrow A + B + O_2$$

According to this theory, the antioxidant behaves as a true catalyst and consequently is not used up in the reaction. However, it has been shown (4), (16), (24) that the antioxidant is destroyed in the oxidation reaction, so that this theory is not entirely consistent with the facts.

Christiansen's theory of antioxidants, on the other hand, accounts for the fact that the antioxidant is destroyed during the oxidation. It states that an activated molecule of the autoxidizable substance and a molecule of oxygen unite with the production of a peroxide and the liberation of energy. This energy is passed on to another molecule of the

substance, thereby starting a reaction which may involve a thousand molecules, depending upon the efficiency with which the energy is transferred, and upon the presence of foreign substances--the antioxidants. When an activated peroxide molecule comes in contact with a molecule of antioxidant, the latter takes up the energy and is itself usually oxidized in subsequent collisions with oxygen. The peroxide molecule fails, however, in its turn to activate any further molecules of the autoxidizing substance, and thus the reaction chain is broken. The antioxidant, therefore, according to this theory, lengthens the induction period of the autoxidizable substance by reducing the length of the chain reactions associated with the oxidative process.

Mechanism of Antioxidant Action. It is evident from the different types of compounds that are effective as antioxidants, that the mechanism of their action is not the same in all cases. Moureu and Dufraisse (53) believed that the antioxidant always becomes oxidized during the induction period of the reactant substance

and therefore that only easily oxidizable substances would be effective. However, this is not the case with certain inhibitors, such as the acid type of antioxidants (22), (42). Additional evidence for the belief that more than one mechanism is involved in the protective action of antioxidants is the fact that the combined action of certain compounds is much greater in effect than would be anticipated from their individual effects (30), (58).

Evans (14) has suggested that antioxidants increase the stability of oils by forming chemical complexes with peroxidants, such as traces of metals, etc., thus preventing the latter from hastening the oxidation process. This seems to be true in certain cases. For instance, certain compounds act as inhibitors under some conditions but not under others. Thus, Lea (42), found that potassium cyanide acted as an antioxidant if the oil contained a trace of metallic catalyst, but not in a fresh oil free from metals.

It is assumed that all antioxidants act in the same manner to the extent that none of them is effective after the induction period has ended (16). This is apparently due to the fact that in partially

oxidized oils peroxides or oxidation catalysts are present in concentrations sufficiently large to prohibit any further protection by the antioxidant.

Early Work on Antioxidants. Moureu and Dufraisse (53) studied the inhibiting effect of hydroquinone upon the oxidation of acrolein and benzaldehyde. Anderegg and Nelson (1) obtained probably the first evidence of natural inhibitors when they found that the oxidative destruction of vitamin A in experimental diets was greatly retarded when animal fats, such as lard or cod liver oil, were replaced, partially or wholly, by vegetable oils, even though the latter were of equal or greater saturation.

Types of Antioxidants

Hydroquinone. Huston and Lightbody (32) showed that small amounts of hydroquinone incorporated in the diets of rats increased protection against ophthalmia. They suggested that the hydroquinone acted by preventing oxidation of the protective vitamin A. Huston, Lightbody and Ball (33) proved that hydroquinone in a concentration of 1 in 2000 was capable of delaying the oxidation of milk fat

and of preserving the vitamin A in the fat and in cod liver oil. Wagner and Brief (75) studied the effects of antioxidants in linseed oil and found that hydroquinone was the best of all those tried. Marcus (47) noted that vitamin A adsorbed on finely divided solids such as lactose oxidized in a few hours. The presence of 1% hydroquinone in the vitamin A concentrate adsorbed on the solids delayed this destruction up to 15 to 45 days. Holmes, Corbet and Hartzler (30) studied the stabilizing effect of hydroquinone on halibut and cod liver oils.

Other Hydroxy Compounds. Mattill (49) demonstrated that the development of rancidity and the breakdown of vitamins A and E in unsaturated animal fats was retarded by substances containing hydroxyl groups, which he concluded might be sterols. He found that wheat germ oil prolonged the induction period of cod liver oil. Greenbank and Holm (22) studied hydroxy compounds, such as catechol, resorcinol, pyrogallol and phloroglucinol, as antioxidants in cottonseed oil. Their effect was found to depend on the location of the hydroxyl groups on the benzene ring. Olcott and Mattill (55)

obtained a crystalline compound from the unsaponifiable portion of the lipids of lettuce which contained one hydroxyl group and was an active antioxidant. Later (57) these workers suggested the name "inhibitols" for the antioxidant obtained from lipid fractions of vegetables and vegetable oils. Inhibitols were found present in lettuce, tomatoes, carrots, alfalfa, spinach; in the oil from wheat germ, cottonseed, corn, sesame, palm, soybean, and peanut but not in the oil from olive, cod liver, palm kernel or castor bean. The properties of these inhibitols and those of vitamin E were so similar that it was found impossible to separate the two. Both were found to contain hydroxyl groups. The activity was destroyed by reagents which react with the hydroxyl group. Strangly enough, the isolated inhibitols were found to protect lard and purified fatty acids and esters but not the vegetable oils from which they came.

Evans, Emerson and Emerson (15) isolated three individual compounds possessing vitamin E activity from wheat germ and cottonseed oils. These were designated α, β and γ tocopherol.

The relative effectiveness of these three compounds as antioxidants for lard at 75° C was investigated by Olcott and Emerson (59), who found them to be increasingly effective in the following order: α , β and γ .

Phospholipids. Evans (14) found vegetable lecithin to be a good antioxidant at 0.05 to 0.1% concentration in vegetable oils. Holmes, Corbet and Hartzler (30) also found crude lecithin to be an effective antioxidant for vitamin A. They used lecithin prepared from soy beans as an antioxidant for halibut and cod liver oils. On the other hand, Olcott and Mattill (56) claimed that commercial lecithins had only moderate antioxigenic action on cottonseed oil, little on lard and none on a mixture of lard and cod liver oil. They found these materials contained little true lecithin, the little effect there was being due to their content of cephalin.

Acids, Salts and Amines. A variety of acids and amines have been found effective as antioxidants. Hilditch and Sleightholme (27) found treatment of olive oil with concentrated sulfuric acid increased its induction period. Greenbank and Holm (22) studied the antioxidative

effect of various organic acids on cottonseed oil. The most active acid found was maleic, while its isomer, fumaric, was practically inert. Other acids having a fair degree of activity were citraconic, itaconic and citric. The effectiveness of maleic acid as an antioxidant for a number of oils was investigated. Its effectiveness was about the same in all cases except corn oil, where it was considerably greater.

The antioxidative effect of various water-soluble substances was investigated by Lea (42). The antioxidants were dissolved in water, which was in contact with lard. The aliphatic hydroxy acids, such as lactic and glycollic, the ethanol-amines, and maleic acid were moderate, while polybasic hydroxy acids, such as tartaric and citric acids, were powerful antioxidants. The aliphatic amino-acids were all powerful anti-oxidants. Proteins also had considerable protective ability. It was noted that these substances were effective antioxidants even when the water content of the fat was only 0.25%. Also, the salts of acids, such as citrates, lactates, malonates, etc., were active as antioxidants, as well as the acids themselves.

Olcott and Mattill observed (58) that the crude esters of vegetable oils were stabilized by oxalic and maleic acids and by sulfuric and phosphoric acids and their salts. Citric and pyruvic acids were also protective. Salts and esters of dicarboxylic acids were inactive, in contrast to the findings of Lea, indicating that the carboxyl groups must be free.

Complexes. In addition to the individual chemical compounds mentioned above, many complex substances have been used as antioxidants. Musher (54) patented the use of a great number of finely divided vegetable materials, such as oat, barley or soy bean flour, as antioxidants. Lowen, Anderson and Harrison (46) have also studied the use of oat flour as an antioxidant. They found it to be effective for lard and vegetable oils, but rather ineffective for fish oils.

Olcott and Mattill have made a preliminary classification of antioxidants (58), dividing them into three types: the acid type inhibitors in the first type, inhibitols and hydroquinone in the second, and phenolic inhibitors other than hydroquinone, in the third. The acid type was found active in vegetable oils and crude vegetable oil

esters, but inactive in lard and lard esters, and in purified fatty acids and esters. The second type of inhibitors was active in the latter group, but not in the former. The phenolic type was active in all the vehicles mentioned.

Synergism of Antioxidants. A further observation noted in this work was that any type I inhibitor, when used with any type II or III compound prolonged the induction period of certain fats and oils longer than the summation of their effects would be expected to do. This synergism of antioxidants was also noticed by Holmes, Corbet and Hartzler (30). The latter workers found that while either hydroquinone or lecithin afforded protection alone to halibut or cod liver oil, a combination of the two was better than would be expected from their computed additive effects.

Attempts at Isolation of Antioxidants. Several attempts have been made to isolate antioxidant compounds from natural products. Royce (66) suggested that gossypol, a polyhydroxy phenolic compound was the chief antioxidant in cottonseed oil. Olcott and Mattill (55) prepared a crystalline antioxidant from the unsaponifiable fraction of the lipids from lettuce. This substance seemed to be distinct from vitamin E, since it was

soluble in 92% methyl alcohol, while the latter was preferentially soluble in petroleum ether.

Hilditch and co-workers in England (27), (3), (20) have attempted to isolate the natural antioxidants of olive, linseed, tung, and other oils. They found that saponification practically eliminated the induction periods of these oils, that is, alkalies were detrimental. Dilute hydrochloric acid also destroyed the antioxidant. They found that the natural antioxidants of olive and linseed oils were apparently removed by boiling with water; at least the induction period of the oils disappeared. However, they were unsuccessful in recovering the antioxidants from the water extracts. Working with linseed oil and linseed press cake, they found the latter much richer in antioxidants than the former. This was also true with soybean oil. They found that the inhibitor present in soybean press cake could be extracted with acidulated methyl alcohol and was soluble in acetone. The final concentrate obtained in this manner was a viscous oil which readily reduced ammoniacal silver nitrate and Fehling's solution, but not iodine. It did not give a coloration

with ferric chloride. This concentrate therefore corresponded in properties to the inhibitol fractions of Mattill and coworkers.

Olcott and Mattill (57) isolated antioxidant fractions in the form of viscous oils from the unsaponifiable portions of the lipids of a number of substances including lettuce, tomatoes, wheat germ oil, cottonseed oil, corn oil, etc. They suggested the name, inhibitol, to designate this type of natural antioxidant. With the exception of the inhibitol from lettuce, which was a crystalline solid distinguishable from vitamin E fractions by its solubility, all were inseparable from vitamin E concentrates prepared from the same sources. This fact became understandable when three tocopherols, all having vitamin E activity but in different degree, were isolated from wheat germ oil by Evans and coworkers (15). All were later shown to have antioxidant activity (59), although not in direct ratio to their vitamin E activities.

Thus it appears that the large group of inhibitols are in reality mixtures of the naturally occurring tocopherols. From recent work of Hickman (26), using a newly developed method of molecular

distillation of oils, it appears that the natural antioxidants present in vegetable oils are largely of this type.

METHODS

Development of the Induction Test

Although many tests have been suggested for evaluating the breakdown periods of oils, it was felt that since vitamin A-containing oils were to be studied a test employing the actual breakdown of vitamin A as a criterion would be most satisfactory. The first stability studies were carried out by blowing oxygen through 25-gram samples of fish liver oils contained in deep test tubes heated in a steam bath at 100° C. At intervals a small sample was withdrawn and its vitamin A content determined by the antimony trichloride color test. Plotting these vitamin A values against time gave a curve representing the breakdown of the vitamin A in the oil. The effect of adding various inhibitors could be evaluated easily in this manner by comparing the breakdown curves for an oil before and after addition of a particular inhibitor.

This method had several disadvantages. It was difficult to keep the flow of oxygen through the oil constant, only a very few oil

samples could be studied at once without complicated equipment, and oils containing phospholipids could not be handled this way at all because of excessive foaming.

It was found that a much simpler method of determining the relative stability of vitamin A-containing oils consisted in exposing small, fairly uniform samples of the oils to air at elevated temperatures. At intervals one sample of the series was removed and a determination made of the vitamin A remaining in it. These determinations were made in the earlier studies by means of the antimony trichloride color method and later with the Hilger Vitameter--the latter method being considerably more accurate. Since this method of determining stability offered many possibilities, studies were made of its characteristics and limitations.

Effect of Surface Area and Temperature. Samples of equal weight of halibut liver oil were placed in open, cylindrical containers of different cross sections and exposed to the air, in the dark, at 27.5° and 37° C respectively. At intervals a sample was removed, mixed thoroughly and its vitamin A content determined. The induction period of a particular sample of oil was designated as the

time required for its vitamin A content to drop to 80% of the original value. The data are given in table 1.

TABLE 1.

Effect of Surface Area and Temperature on the Induction Period of Halibut Liver Oil.

Sample	Surface Area (Sq. Cm.)	Wt. Sample (Gms.)	Induction Period, Hours		Ratio
			37°	27.5°	27.5°/37°
(a)	12.6	0.82	8	15	1.9
(b)	7.07	0.82	12	17	1.42
(c)	3.14	0.82	18	40	2.22
				Average	1.85

This study indicates that the induction period varies inversely with the area of the exposed sample, and a decrease in temperature of 10° approximately doubles the length of the induction period.

Effect of Depth of Sample. A series of 4.6 gm. (5 ml.) amounts of halibut liver oil was weighed into cylindrical containers of the same size so that the column of oil in each was 2 cm. in diameter and 2.8 cm. deep. These were exposed to the air, in the dark, at 37°. At proper time intervals one of the containers was removed and a sample carefully taken from the top surface of

the oil. Next a sample was removed from the bottom. This was done by submerging the tip of a pipet (on the other end of which was a rubber bulb) to the bottom of the oil, gently blowing out the drop of oil which had risen into the pipet, then drawing up the sample. The vitamin A was then determined in these two samples.

TABLE 2.

Effect of Depth of Samples on the Induction Period of Halibut Liver Oil.

Hours at 37°	% Original Vitamin A	
	Top	Bottom
284	84.0	86.0
330	82.5	85.1
379	81.1	82.9
450	73.4	78.5
501	69.3	77.0
672	61.0	66.4

These results indicate that diffusion of air into oil is sufficiently rapid that, even in deep samples the rate of breakdown is approximately the same throughout.

Agitated vs. Stationary Samples. Some workers have suggested that in induction tests of oils the samples must be agitated during exposure. Therefore the following comparison of methods was

made. Vitamin A-breakdown tests were run on a group of various types of oils by two methods. One method was that mentioned above, consisting of exposing approximately 0.2 gm. samples in Erlenmeyer flasks at 37°. The other involved agitating 3 ml. samples of the oils in T tubes in a specially constructed rocking device, according to a method suggested by Dr. K. Hickman. The arms of the T tubes were each two inches in length so that the oil traveled over a distance of four inches and back during each revolution of the driving device, which operated at four revolutions per minute. The whole apparatus was operated in a 37° room. The open ends of the T tubes were loosely plugged with cotton during operation, to permit free access of air. At intervals samples of about 0.25 gm. of oil were withdrawn, dropped into tared flasks to be weighed and diluted for vitamin A determinations by the Vitameter. The endpoint adopted for the induction test by both methods was the same--namely length of exposure required to reduce the vitamin A content to 80% of the original value.

TABLE 3.

Comparative Induction Tests with Agitated and Stationary Samples.

No.	Type of Oil	Induction Period (Hours) Agitated	Stationary
32089	Distillate	59	60
32099	Distillate	115	124
32109	Fish Liver Oil	57	51
32119	Fish Liver Oil	54	49
32129	Concentrate	4	4.5
32139	Concentrate	4	5.5
32149	Reference Cod Liver Oil	100	100

The two methods of measuring stability of oils gave closely agreeing results in this series. The method employing stationary samples was much simpler to carry out, and therefore preferable.

Peroxide Formation in Relation to Vitamin A-Breakdown. A series of approximately 0.25 gm. samples of halibut liver oil in 50 ml. Erlenmeyer flasks was exposed at 37°. At intervals a sample was removed and its vitamin A and peroxide content determined. The data are shown in table 4, where the vitamin A determinations are expressed as per cent original vitamin A remaining and the peroxides as peroxide numbers. By peroxide number is meant the number of ml. of 0.002 N thiosulfate per gm., or millimoles of peroxide oxygen per kilogram of oil.

TABLE 4.

Relation of Peroxides to Vitamin A-Breakdown.

Hrs. at 37°	% A Remaining	Peroxide Number
0	--	3.4
16	100.0	28.0
18.2	89.5	55.0
20	73.0	80.0
22	50.4	131.0
24	38.3	162.0

These data are presented in graphic form in Figure 1, where the upper curve represents vitamin A-breakdown and the lower one, the increase of peroxides. The data indicate that peroxides develop in the samples at about the same rate at which the vitamin A breaks down. That is, both the vitamin A-breakdown and the increase of the production of peroxides make suitable criteria for the induction tests on fish liver oils.

Change of Ultra-violet Absorption of Vitamin A During Breakdown. In order to study the induction test more thoroughly, the following experiment was performed. A series of approximately 0.25 gm. samples of halibut liver oil in open 50 ml. flasks was exposed to the air at 37°. At intervals, beginning at the end of 12 hours, one of the samples was removed and an absorption spectrogram

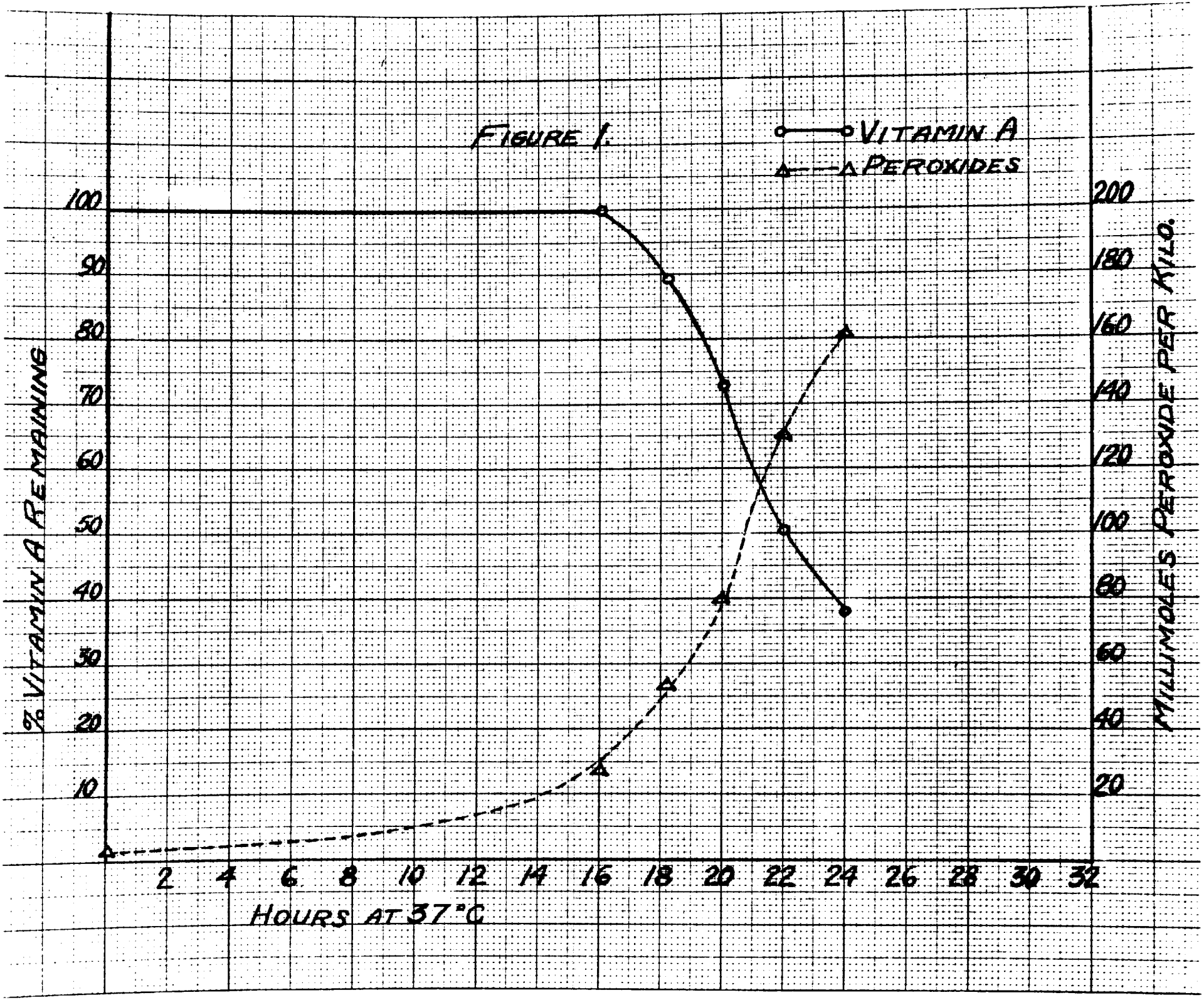


FIGURE 1.
VITAMIN A
PEROXIDES
% VITAMIN A REMAINING
MILLIMOLES PEROXIDE PER KILO.
100
90
80
70
60
50
40
30
20
10
200
180
160
140
120
100
80
60
40
20
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
HOURS AT 37°C

in the ultra-violet region was made on the medium quartz spectrophotometer.* These absorption curves are presented in Figure 2. The numbers on the curves represent the respective number of hours exposure at 37°. It will be seen that as the peak of absorption drops with time, there is also a gradual shift of the peak to the left, beginning at about the twentieth hour. The small curve at the left is the induction curve of the same halibut liver oil, obtained by plotting the extinction at various periods of exposure, as determined on the spectrophotometer, against time.

Outline of Induction Test Method. The method finally adopted for induction tests of oils, and used throughout the experiments to be reported, was carried out as follows:

A series of 0.2 to 0.25 gm. samples of the vitamin A-containing oil was accurately weighed into 50 ml. Erlenmeyer flasks. The flasks chosen were of uniform design with bottoms as flat and smooth as possible. Each sample of oil was introduced into the flask from a pipet, allowing it to drop squarely in the center of the bottom so as to permit it to spread out in a uniform, thin layer.

*Spectrograms made by Dr. J. M. Vandenbelt in the Section of Physical Chemistry at the Kedzie Chemical Laboratory.

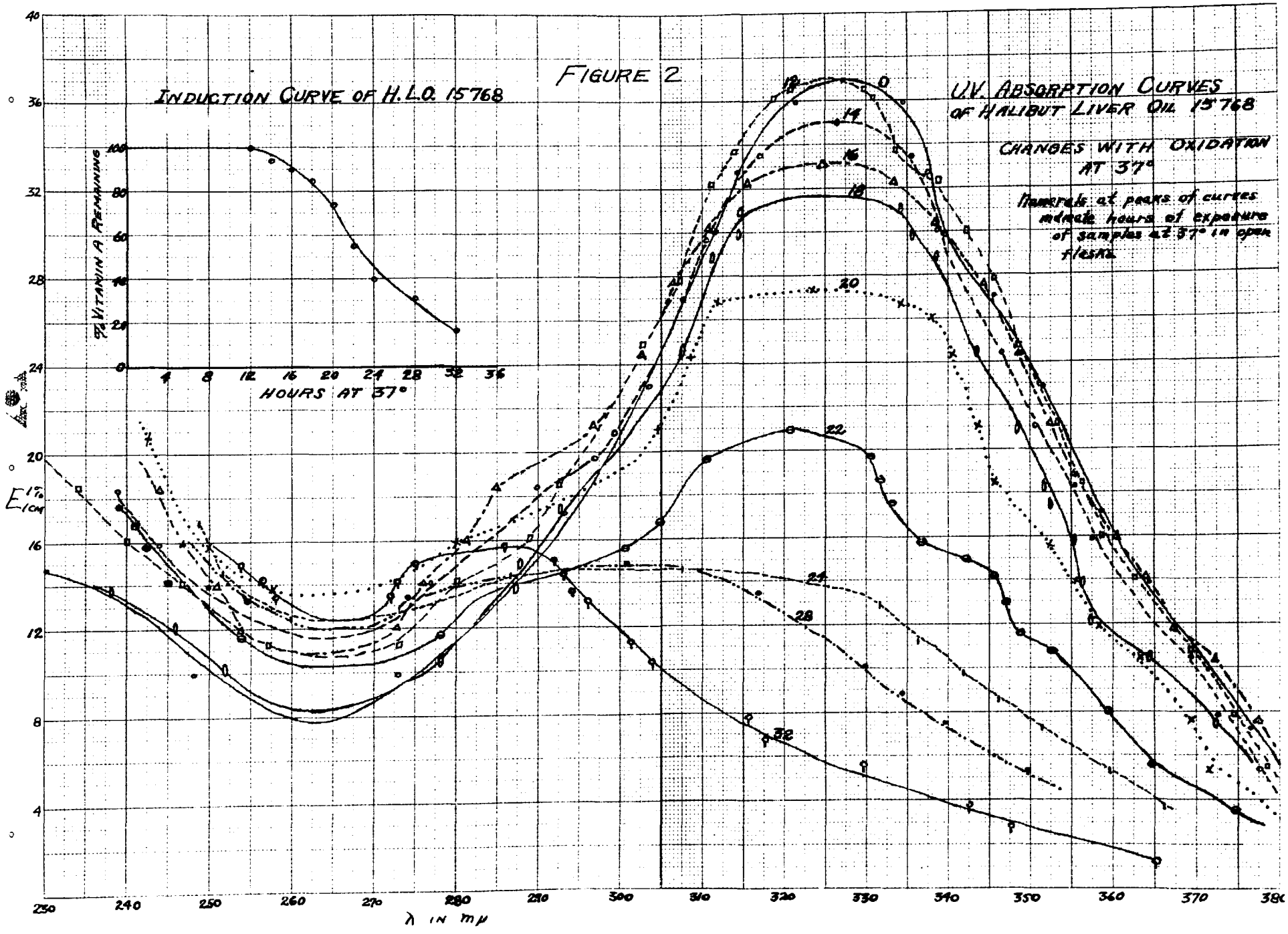


FIGURE 2
INDUCTION CURVE OF H.L.O. 15768
% VITAMIN A REMAINING
HOURS AT 37°
U.V. ABSORPTION CURVES OF HALIBUT LIVER OIL 15768
CHANGES WITH OXIDATION AT 37°
Numerals at peaks of curves indicate hours of exposure of samples at 37° in open flasks.
$E^{1\%}_{1cm}$
λ IN mμ

The flasks were then placed in an incubator at 37°C, care being taken to see that they were all on the same level so as to insure uniform temperature. The flasks were left open for free circulation of air. At intervals one flask from the series was removed from the incubator. If the vitamin A, only, was to be determined the sample was dissolved in isopropyl alcohol and the assay made on the Vitameter. In case the peroxides were to be determined simultaneously, the sample was dissolved in chloroform and diluted to 10 ml. with the same solvent. A 1 ml. sample was taken from this solution and diluted further for the vitamin A determination. To the remaining 9 ml. of solution were added 18 ml. glacial acetic acid and then two drops saturated potassium iodide solution. The mixture was shaken and allowed to stand 10 minutes in the dark. Fifteen ml. 15% potassium iodide were then added and the liberated iodine titrated with freshly diluted 0.002 N thiosulfate. A blank was carried out under the same conditions. This is based on the Banks modification of the Lea Peroxide test.

In carrying out the induction test, the first few vitamin A determinations generally checked those of the unexposed oil. As succeeding samples

were tested, however, they showed a slight falling off, and finally a sudden drop. The peroxide content rose at the same time. The point where the vitamin A-breakdown curve reached 80% of the original vitamin A activity was arbitrarily taken as indicating the end of the induction period. This was chosen since it was considered that a difference of 20% was beyond the experimental error of the method and represented a significant drop in vitamin A.

Relation of Induction Test to Natural Conditions

In order to establish a relationship between the above described induction test and breakdown of vitamin A under actual conditions, an experiment was carried out to determine the effect of the amount of oil sample on the induction period. Samples ranging from 0.3 to 5.0 ml. of oil were placed in 7 ml. bottles, one series tightly capped and the other open. At intervals one of the bottles of each series was removed, the whole sample mixed and the vitamin A determined. The data are presented in table 5 and illustrated in Figure 4.

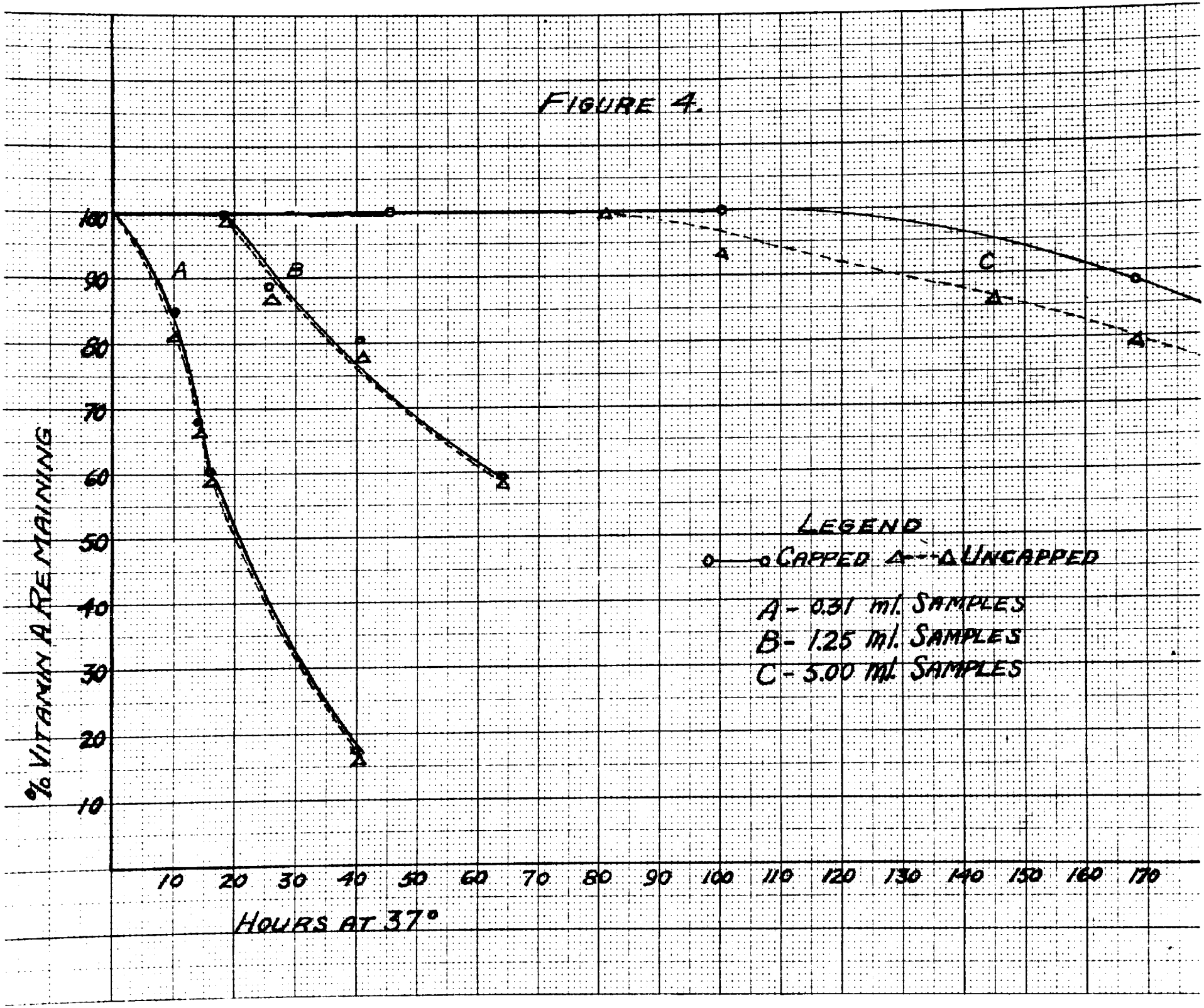


FIGURE 4.
% VITAMIN A REMAINING
100
90
80
70
60
50
40
30
20
10
10 20 30 40 50 60 70 80 90 100 110 120 130 140 150 160 170
HOURS AT 37°
A
B
C
LEGEND
o—o CAPPED Δ---Δ UNCAPPED
A - 0.31 ml. SAMPLES
B - 1.25 ml. SAMPLES
C - 5.00 ml. SAMPLES

TABLE 5.

Effect of Volume of Sample on the Induction Period of Halibut Liver Oil.

Sample (Ml.)	Induction Period, Hours	
	Open	Capped
0.313	10	11
1.25	38	40
5.0	160	230

From these data it is evident that the induction period is directly proportional to the volume of oil in the open containers; also that there is no appreciable difference between the induction periods of the oil in open or closed containers, except in the case of the 5 ml. samples.

The induction period of this halibut liver oil was $5\frac{1}{2}$ hours as determined by the standardized method described above. Since the induction period of a 5 ml. sample in a capped bottle at 37° was 230 hours, it would be approximately twice this, or about 500 hours, at room temperature. Thus there is a factor of approximately 100 which represents the ratio between the value obtained by the induction test and the expected breakdown time of the same oil stored in 5 ml. amounts in practically full, capped bottles at room temperature.

EXPERIMENTAL RESULTS

Stability of Various Types of Natural Vitamin A-Containing Oils

Using the induction test described above, data on the stability of several types of fish oils containing vitamin A were collected over a period of several years. Some typical data are recorded below in tables 6 and 7. The halibut liver oils, and mixed fish liver oils, were prepared by alkali-digestion of the livers, followed by gravity separation of the oil. The cod liver oil was prepared by the ordinary commercial process consisting of steam treatment of the livers to separate the oil, which rises to the top and is drawn off. The tuna liver oils were prepared by extraction from the livers with oils, etc., following a preliminary heating to coagulate the liver protein. The solvent-extracted oils were prepared by coagulating the livers by heat, followed by exhaustive treatment of the residue with either ethyl ether or petroleum ether. Part of the viscera oils were prepared by alkali digestion and part by solvent-extraction.

TABLE 6

Induction Periods of Fish Oils Prepared by Alkali Digestion or Steam Rendering.

Halibut Liver Oils

Sample Number	Induction Period, Hours
76405	7.5
79365	10
79375	30
81036	16
82246	10
83896	16
88726	19
88756	25
93587	14
1878	18
2028	12
2978	25
10388	18
10398	12
10438	21
Average (15)	16.9

Tuna Liver Oils (Oil-Extracted)

80006	80
80016	70
80066	120
80076	75
80166	90
80266	40
80276	50
80756	70
80876	42
80886	60
82206	50
82216	70
82406	32
82436	60
82446	72
Average (15)	65.4

TABLE 6 - Continued

Cod Liver Oils

Sample Number	Induction Period, Hours
82506	100
82516	100
99777	200
32149	100
Average (4)	125

Mixed Fish Liver Oils

79255	24
81576	31
82226	16
82236	10
82796	20
82806	20
83896	16
84026	48
88706	12
88766	17
11918	32
11948	17
11968	18
13818	21
13888	19
Average (15)	20.7

Fish Viscera Oils

13088	12
13808	4.5
13198	7.5
15208	12
15618	12
15748	4.5
15758	7.5
16899	5
Average (8)	8.1

TABLE 7

Induction Periods of Fish Oils Prepared by Solvent Extraction.

Number	Type	Induction Period, Hours
82396	Halibut Liver Oil	168
6058	Halibut Liver Oil	81
5268	Halibut Viscera Oil	100
5498	Halibut Viscera Oil	125
5598	Halibut Viscera Oil	82
5648	Halibut Viscera Oil	100
6758	Halibut Viscera Oil	65
10948	Black Cod Viscera Oil	164
	Average (8)	111

The data indicate that cod liver oils and solvent-extracted oils are the most stable, while alkali-digested oils, as a class, are least stable. Intermediate between the two is tuna liver oil. This can be accounted for by the method used in the extraction of the tuna livers, even though the latter are first digested with alkali.

Conditions Affecting Stability of Vitamin A in Oils

Free Fatty Acids. In table 8 are presented certain data collected in connection with experiments on the oils obtained from fish viscera. They indicate the entire lack of correlation between free fatty acids and stability. Although these solvent-extracted viscera

oils, with a few exceptions, are extremely stable, they are also high in free fatty acids. The fatty acid content seems to be a function of the particular lot of viscera, rather than a factor influencing the stability of the oil. This is indicated by the fact that each pair of oils, representing oils from the caecum and intestines respectively of a particular lot of viscera, are similar in free fatty acid content.

TABLE 8

Relation of Free Fatty Acids to Stability of Vitamin A in Fish Viscera Oils.

Viscera Oil No.	Source	Vitamin A U.S.P. u/gm.	Per Cent Free Acid #	Induction Period, Hours
0127	Halibut Caecum	750,000	25.6	50
0187	Halibut Intestine	600,000	27.3	7.5
0757	Ling Cod Caecum	65,000	10.9	100
0777	Ling Cod Intestine	21,100	9.9	180
0997	Black Cod Caecum	153,000	18.3	113
1007	Black Cod Intestine	120,500	18.9	168+
1247	Tuna Caecum	46,200	62.6	70
1257	Tuna Intestine	37,400	61.3	66
1387	Mackerel Caecum	27,000	24.5	8
1397	Mackerel Intestine	15,700	25.1	7
1608	Halibut Caecum	67,500	36.6	140
1818	Halibut Intestine	15,660	34.7	120+

Calculated as oleic acid.

Effect of Peroxides and Dilution of Oil Medium. Other workers have found that when peroxides are added to vitamin A-containing oils

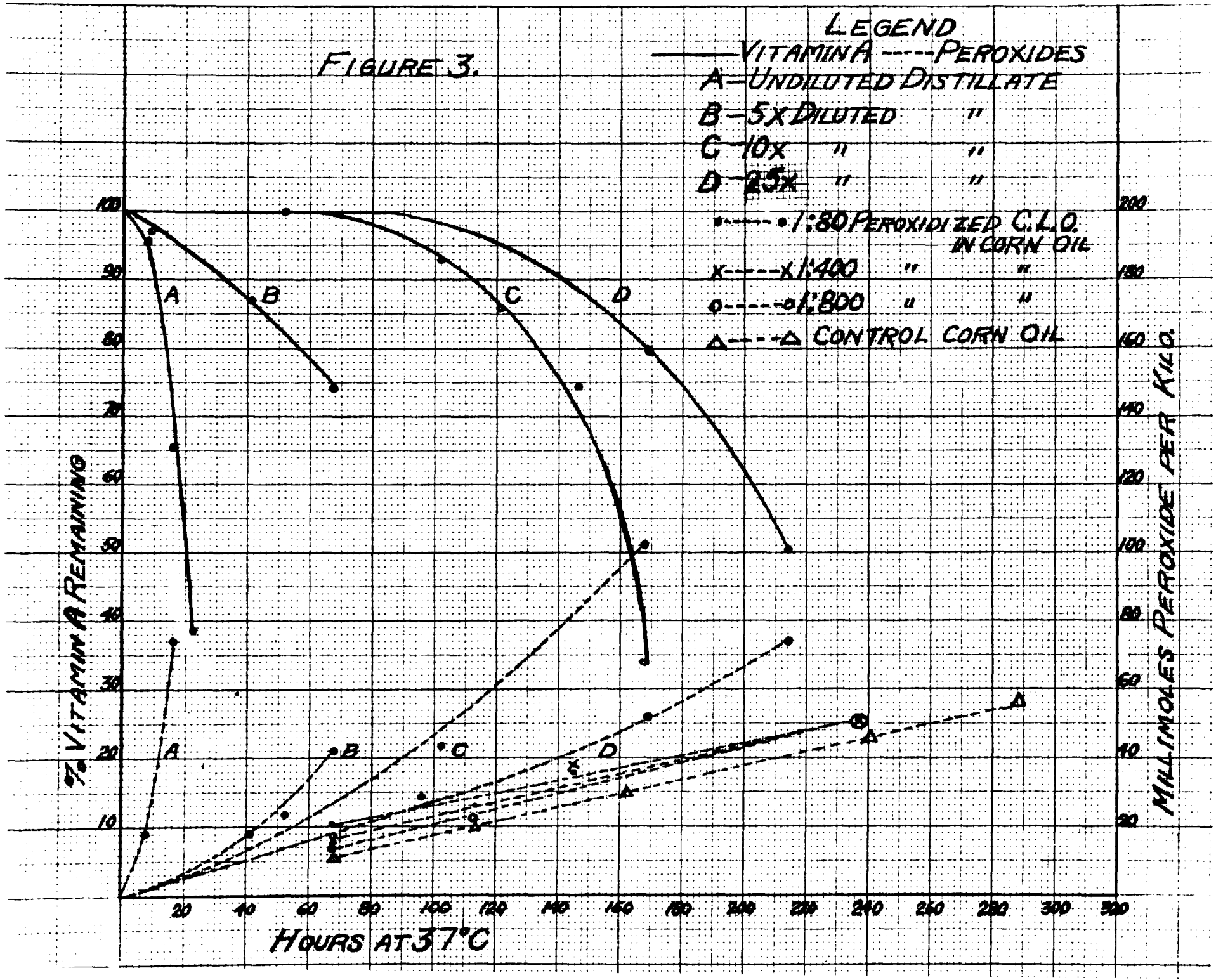
the vitamin A breaks down at a rate proportional to the concentration of peroxides present. In a preliminary experiment a high-potency vitamin A concentrate was diluted at different concentrations in corn oil. The vitamin A-breakdown as well as peroxide-formation were followed in these dilutions of vitamin A, while only the peroxide-formation was followed in the corn oil diluent. It was found that the rate of peroxide-formation and vitamin A-breakdown were proportional to the concentration of vitamin A, but in all cases were much greater than the rate of peroxide-formation in the corn oil diluent alone. It was thought this might possibly be due to the peroxide content of the vitamin A concentrate, which unfortunately was rather high.

Another experiment was therefore carried out in which the source of vitamin A was a vitamin A distillate of practically negligible peroxide content (kindly supplied by Dr. K. Hickman). This had an $E^{1\%}_{1\ cm.}$ of 264 at 328 Mu. Induction tests, involving both vitamin A-breakdown and peroxide-formation, were carried out on this distillate and on 5, 10 and 25-fold dilutions of the same in corn oil. Peroxide tests were made on the

diluent corn oil and also on a peroxidized cod liver oil, having a peroxide number of 280, diluted 1:80, 1:400 and 1:800 with this diluent oil. The vitamin A induction curves and peroxide curves are shown in Figure 3. They indicate, as in the preliminary experiment above that the rates of vitamin A-breakdown and peroxide-formation are inversely proportional to the dilution factor. The addition of peroxides to the corn oil in fairly large amounts did not hasten the peroxidation of the latter. Therefore it is evident that the autoxidation of these dilutions of vitamin A was affected more by the vitamin A itself than by the peroxides introduced with the vitamin A.

Effect of Different Oil Media. In table 9 are presented data on the induction periods obtained for 1:80 dilutions of a highly purified vitamin A concentrate in different oil media. This illustrates the inherent difference in the protective effect of various vegetable oils for vitamin A; also the increased protective effect of one of these oils when a small amount of hydroquinone is added to it. This method provides a means for measuring the relative antioxidant content of a natural oil by dissolving a vitamin A concentrate in it and

FIGURE 3.


LEGEND
VITAMIN A ---- PEROXIDES
A—UNDILUTED DISTILLATE
B—5X DILUTED "
C—10X " "
D—25X " "
•----• 1:80 PEROXIDIZED C.L.O. IN CORN OIL
x----x 1:400 " "
o----o 1:800 " "
△----△ CONTROL CORN OIL
% VITAMIN A REMAINING
MILLIMOLES PEROXIDE PER KILO.
HOURS AT 37°C
100
90
80
70
60
50
40
30
20
10
200
180
160
140
120
100
80
60
40
20
20
40
60
80
100
120
140
160
180
200
220
240
260
280
300
A
B
C
D

comparing its breakdown period with a standard.

TABLE 9

Effect of Type of Oil Used as Diluent on the Stability of Vitamin A.

Diluent	Induction Period, Hours
Corn Oil with 0.05% Hydroquinone	140
Cottonseed Oil	34
Linseed Oil	30
Wheat Germ Oil	30
Corn Oil	27.5
Peanut Oil	15.5
Cocoanut Oil	7

Effect of Oxidation Catalysts. (a) Salts.

In an experiment (to be described later) aimed at separating the phospholipid fraction from a halibut viscera oil a procedure was employed in which an alcoholic solution of calcium chloride came in contact with the oil. When an induction test was made on this calcium chloride-treated oil it was found to be extremely unstable. Next, the effects of various salts on the stability of the same viscera oil were studied. This was an attempt to find whether the calcium ion or chloride ion caused the effect noted above, although the series included many other compounds, such as the salts of cobalt, copper and iron.

The procedure consisted in allowing ten gram samples of the oil to stand overnight in contact with 10% of their weight of a particular salt, which had been finely powdered in a mortar. The next morning the oil was filtered to remove the salt and an induction test carried out as described above. The data are presented in table 10.

TABLE 10

Effect of Various Salts on the Induction Period of Fish Viscera Oil.

Treatment	Induction Period, Hours	Per Cent of Control
Cobalt Nitrate (6 H_2O)	2.5	3.0
Copper Nitrate (3 H_2O)	2.5	3.0
Calcium Chloride (Anhyd.)	3	3.7
Magnesium Chloride (6 H_2O)	3	3.7
Stannous Chloride (2 H_2O)	4.5	5.5
Manganous Sulfate (4 H_2O)	15	18.3
Ferric Chloride (6 H_2O)	16	19.5
Barium Chloride (2 H_2O)	23	28.0
Sodium Chloride	30	37.0
Cadmium Chloride (2 H_2O)	32	39.0
Magnesium Carbonate	39	47.5
Ferric Citrate	42	51.3
Calcium Carbonate	48	58.5
Magnesium Sulfate (Anhyd.)	60	73.0
Sodium Acetate (Anhyd.)	62	75.6
Ammonium Chloride	62	75.6
Ammonium Phosphate, Monobasic	63	76.8
Sodium Nitrate	63	76.8
Calcium Phosphate, Monobasic	64	78.0
Calcium Citrate	65	79.3
Ammonium Nitrate	70	85.4
Sodium Phosphate, Dibasic	73	89.0
Magnesium Citrate	75	91.4
Sodium Sulfate (Anhyd.)	82	100.0
Control Oil Alone	82	100.0

The data show a great range of effect; from almost complete elimination of the induction period with the cobalt and copper nitrates to no effect at all with sodium sulfate. It is hard to detect any trend of effectiveness, although the chlorides seem to have the strongest action as a group. This is interesting in view of the later findings with hydrochloric acid.

(b) Acids. Following this study of salts, an experiment to determine the effect of acids on the induction period of vitamin A oils was carried out. In order to obtain maximum and reproducible contact between the oil and the acid, the oil was dissolved in petroleum ether and washed with 80% methyl alcohol in which 1% of the acid was dissolved. The method of treatment was well standardized and was used later in studies of the antioxidant content of various oils. A control test had to be made with 80% methyl alcohol alone, since this had some effect. The results obtained by treatment with common acids are presented in table 11.

TABLE 11

Effect of Acids on the Induction Period of Halibut Liver Oil.

Number	Treatment	Induction Period, Hours
6058	Control Halibut Liver Oil	81
19899	(6058) Washed with 80% Methyl Alcohol	50
24139	Same as 19899 plus 1% Acetic Acid	50
27509	Same as 19899 plus 1% Phosphoric Acid	35
28169	Same as 19899 plus 1% Hydrochloric Acid	3
24129	Same as 19899 plus 1% Sulfuric Acid	126

It is evident from these data that hydrochloric acid greatly accelerates the breakdown of vitamin A in halibut liver oil. On the other hand, sulfuric acid has a protective effect. Acetic and phosphoric acids have very little effect.

The effect of hydrochloric acid was studied further. In table 12 are data showing the effect of treatment of the oil with various amounts of this acid. In this case the acid was added directly to the oil; the 0.5% level being added undiluted, while for the 0.05% and 0.005% levels the acid was first diluted 10-fold and 100-fold respectively with absolute ethyl alcohol.

TABLE 12

Effect of Various Amounts of Hydrochloric Acid on the Stability of Vitamin A in Halibut Liver Oil.

		Induction Period, Hours
37020	Control Oil	60
37020-A	With 0.5% HCl	1.5
37020-B	With 0.05% HCl	29
37020-C	With 0.005% HCl	45

These data indicate that treatment of halibut liver oil with as little as 0.005% hydrochloric acid has an appreciable detrimental effect on its stability.

An induction test, in which both vitamin A-breakdown and peroxide-formation were measured, was carried out on a sample of halibut liver oil to which 0.5% hydrochloric acid had been added. This indicated that the presence of the acid not only hastened the destruction of vitamin A, but also increased the production of peroxides. The data are in table 13.

TABLE 13

Effect of Hydrochloric Acid on the Development of Peroxides in Halibut Liver Oil.

Treatment	Peroxide Number	% Vitamin A Remaining
1. Control Halibut Liver Oil 37020	5.4	100
2. Control Halibut Liver Oil plus 0.5% HCl	9.2	100
3. Same as (2) after 1 hr. at 37°	14.4	71
4. Same as (2) after 2 hrs. at 37°	15.5	67
5. Same as (2) after 3 hrs. at 37°	33.4	58

To find if hydrochloric acid also accelerates the autoxidation of non-vitamin A oils, an experiment was carried out in which samples of corn oil were exposed in the incubator at 37° in exactly the same manner in which the vitamin A induction tests were carried out. Part of the samples had 0.5% hydrochloric acid added. Peroxide tests were made on these samples at intervals. The data, presented in table 14, indicate that hydrochloric acid has a decided accelerating effect on the accumulation of peroxides in corn oil.

TABLE 14

Effect of Hydrochloric Acid on the Development of Peroxides in Corn Oil.

Hours at 37°	Peroxide Number
Control Corn Oil	
73	2
150	9.5
195	17.8
245	15.8
264	20.7
313	38.4
384	28.8
435	32.4
600	40.5
Corn Oil plus 0.5% Hydrochloric Acid	
0	6.1
16	8.9
24	17.6
70	63.7
122	234
213	310

Certain experiments were next carried out to study the mechanism of the action of hydrochloric acid on oils. Some representative data from these experiments are presented in table 15. It was found that the effect was the same whether the oils were washed with methyl alcohol containing hydrochloric acid, or were treated directly with the concentrated acid. Also the result was the same when the oil was treated with dry hydrogen chloride gas. The action proved to be a reversible one, since the stability of oils which had been treated with hydrochloric acid was restored practically to the original value when they were treated with potassium hydroxide, either in the form of alcoholic potash or powdered, solid potassium hydroxide. It was found that a certain optimum amount of alkali was necessary to restore the stability of such oils; less than this amount apparently reacted only with the free fatty acids of the oils.

TABLE 15

Effect of Hydrochloric Acid and Potassium Hydroxide on the Induction Period of Halibut Liver Oil.

Number	Treatment	Induction Period, Hours
6058	Control Halibut Liver Oil	81
19899	(6058) Washed with 80% Methyl Alcohol	50
29179	Same as 19899 plus 1% Hydrochloric Acid	1.5
31769	(29179) Washed with 80% Methyl Alcohol plus 1% KOH	55
33589	(6058) Treated with 0.5% Hydrochloric Acid	2
33599	(33589) Treated with Dry KOH	70
38990	Control Halibut Liver Oil #2	28
42670	(38990) Treated with Dry Hydrogen Chloride Gas	5

Since a majority of the salts which were active as oxidation catalysts for vitamin A oils were chlorides, it was thought this might be related to the similar activity of hydrochloric acid. Experiments showed this to be the case. The data from some typical experiments are presented in table 16. When the control halibut liver oil stood in contact with dry calcium chloride its induction period dropped from 60 hours to less than one hour. However, when this extremely unstable oil was treated with dry sodium hydroxide in an amount slightly in excess of that required to neutralize the free fatty acids present the induction period was restored to 36 hours.

The similarity of action of hydrochloric acid

and calcium chloride in reducing the induction period of oils would indicate that the action of the latter might be due to interaction with the free fatty acids of the oil to produce free hydrochloric acid. There is evidence for this supposition in the data presented in the latter part of table 16. The control viscera oil, 5598, was first treated with aqueous sodium hydroxide to remove free fatty acids. This treatment reduced its induction period from 82 to 30 hours, due to the solvent effect of the water on the antioxidant. Five-gram amounts of the control oil and the acid-free oil were dissolved in 25 ml. amounts of ether and 0.5 gm. anhydrous calcium chloride added to each. After 24 hours at room temperature the ether solutions were filtered free from calcium chloride and the ether removed by distillation. The effect of the calcium chloride on the induction period of the acid-free oil was much less than on the control oil containing free fatty acids.

TABLE 16

Relation of Calcium Chloride and Free Fatty Acids to the Induction Periods of Fish Oils.

Number	Treatment	Induction Period, Hours
37020	Control Halibut Liver Oil	60
39220	(37020) Treated with Calcium Chloride	0.5
39230	(39220) Treated with Sodium Hydroxide	36
5598	Control Halibut Viscera Oil	82
8658	(5598) Treated with Calcium Chloride	2
8668	(5598) Treated to Remove Free Fatty Acids	30
8508	(8668) Neutral Oil Treated with Calcium Chloride	12

(c) Metallic Soaps. Among the commonest of the oxidation catalysts for oils are the metallic soaps, such as those of cobalt which are used as driers in paints. This type of oxidation catalyst may be introduced in the production of fish oils and concentrates due to their contact with metal during processing. Experiments along this line were carried out in which fish oils were purposely contaminated with cobalt linoleate and other similar catalysts. Also, means were investigated for eliminating such catalysts from oils in which they had been incorporated. Data from these experiments are presented in table 17.

TABLE 17

Effect of Thioglycolic Acid and Ammonia on Fish Oils Containing Metallic Oxidation Catalysts.

Number	Treatment	Induction Period, Hours
6058	Control Halibut Liver Oil	81
19149	(6058) plus 0.1% Cobalt Linoleate	24
19159	(19149) plus 1% Thioglycolic Acid	74
14458	Fish Viscera Oil plus 1% Cobalt Linoleate	1.5
14458-A	(14458) Treated with Ammonia Gas	11
2028-II	Control Halibut Liver Oil #2	18
22289	(2028-II) plus 1.2% added Tuna Liver Oil Concentrate (Containing Trace of Copper)	1.5
23539	(22289) plus 1% Thioglycolic Acid	19

Among a number of substances used in attempting to stabilize oils containing metallic oxidation catalysts, the most successful were thioglycolic acid and ammonia, especially the former. Halibut liver oil, 19149, had its induction period reduced from 81 to 24 hours by the addition of 0.1% cobalt linoleate. Treatment with 1% thioglycolic acid restored the induction period of the oil nearly to its original value. Fish viscera oil, 14458, which had had its induction period reduced to 1.5 hours by the addition of 1% cobalt linoleate, was stabilized to the extent of an 11 hour induction period by bubbling ammonia gas through the oil.

The second control halibut liver oil, 2028-II, was a typical alkali-digested oil. Its induction period was reduced from 18 hours to 1.5 by mixing with it 1.2% of a certain tuna liver oil concentrate. Upon spectrographic examination of this tuna liver oil concentrate, it was found to contain a trace of copper. This oil and concentrate mixture was stabilized to more than its original induction period value by the addition of 1% thioglycolic acid.

(d) Peroxides. Even more important than metallic soaps as oxidation catalysts in fish oils are peroxides. These are always formed rapidly at the time the vitamin A content of an oil begins to break down, and are even present to some extent in comparatively fresh oils. It is probably this small initial amount of peroxides which initiates and autoxidizes the vitamin A content of fish oils when they break down. Therefore it would be quite important to be able to stabilize a vitamin A-containing oil which already had a substantial peroxide content. Thioglycolic acid and amines were the only compounds found in this study which were effective in stabilizing fish oils containing appreciable amounts of peroxides. Data showing the

stabilizing effect of thioglycolic acid and ethylene diamine are presented in table 18.

TABLE 18

Effect of Thioglycolic Acid and Ethylene Diamine on Peroxidized Halibut Liver Oil.

Number	Treatment	Induction Period, Hours
38990	Control Halibut Liver Oil	28
47691	(38990) Plus Peroxides (Per.No., 18.7)	15
48211	(47691) Plus 1% Thioglycolic Acid	70
50941-D	(38990) Plus Peroxides (Per.No., 20)	13
52291	(50941-D) Plus 0.1% Ethylene Diamine	38

The action of the thioglycolic acid in these experiments is worthy of note. The peroxidized oil to which it had been added (48221) underwent a slight, rather abrupt loss of about 10 to 12% of its vitamin A content during the first 20 hours' exposure of samples in the incubator. Following this there was a continuous but very slow drop with time. This drop continued as nearly a straight line as long as the test was continued, namely until the vitamin A level reached 43% at 264 hours. The nominal induction period (80% of original vitamin A) was 70 hours, although there was not a true induction curve for this oil. Aside from the preliminary slight drop at 20 hours, the main

reaction was not in the nature of an autoxidation with its characteristic increase in rate of oxidation with time, but rather a simple oxidation at a constant rate. This fact might indicate that the thioglycolic acid was destroying the peroxides as rapidly as they were formed. Probably this is not the general mechanism, however, since peroxidized oil, 52291, containing added ethylene diamine and with an original peroxide number of 20, still had a peroxide number of 17 after standing a month.

Effect of Antioxidants. The relative effectiveness of various compounds as antioxidants for vitamin A was determined by incorporating them in halibut liver oil. The induction period of the oil was determined before and after the addition of the antioxidants so as to give an estimation of the improvement in stability. Some typical results of these studies are given in table 19. The compounds listed in the upper part of the table were dissolved in the respective halibut liver oils in the concentrations shown. The salts and acids were powdered in a mortar, from one to three

per cent was added to the oil and then mixed well. After several hours' standing, with occasional shaking, the solids were filtered off and induction tests made on the clear oils. The acetic acid and acetic anhydride were added directly to the oils and dispersed by shaking. The clear oil was decanted off for testing.

TABLE 19

Effect of Various Antioxidants on the Induction Periods of Halibut Liver Oils.

Treatment	Induction Period, Hours	
	Before	After
0.05% Hydroquinone	5.5	34
0.1 % Hydroquinone	5.5	120
0.05% Hydroquinone	19	169
0.5 % Lexinol*	5.5	30
0.5 % Lexinol	12	24
0.5 % Lexinol	28	47
0.5 % Phospholipid from Fish Viscera Oil	12	30
0.1 % Ethanolamine	12	60
0.1 % Ethanolamine	81	122
0.01% Ethylenediamine	12	75
0.1 % Ethylenediamine	12	112
0.1 % α Tocopherol	19	32
0.5 % α Tocopherol	19	65
1.0 % α Tocopherol	19	104
1.0 % Corn Oil Non-Sap. Fraction	19	33
0.1 % Condensate of NH_3 and Acetone	19	53
Maleic Acid	5.5	35
Maleic Acid	12	36
Citric Acid	5.5	34
Citric Acid	12	26
Tartaric Acid	5.5	22
Succinic Anhydride	12	20.5
Fumaric Acid	12	19
Succinic Acid	12	18
Ascorbic Acid	5.5	8.5
Benzoic Acid	5.5	4.5
Acetic Acid	5.5	4.5
Acetic Anhydride	5.5	3.5

* Vegetable lecithin prepared from soybean oil.

These results indicate that hydroquinone is the most active antioxidant of those studied, while the amines are nearly as active. Alpha tocopherol is also a very good antioxidant for vitamin A but has to be used in relatively large amounts. Among the organic acids maleic acid is the most effective, while citric and tartaric acids are only a little less active. It is interesting that fumaric acid, the naturally occurring isomer of maleic acid, is considerably less active than the latter. Benzoic and acetic acid, and acetic anhydride had a detrimental effect on the stability of halibut liver oil.

In the earlier experiments with organic acids as antioxidants the oils were triturated with rather large amounts of the powdered acids--usually about three per cent. Since it was obvious that very little if any of these dry acids actually dissolved in the oil, it seemed desirable to find a lower limit of concentration of acid which would still be active as an antioxidant. Powdered citric acid was added to 10 gm. lots of halibut liver oil in amounts equivalent to 1, 0.1, and 0.01%. These were shaken occasionally during a period of several hours and then filtered.

Even in the case of 0.01% acid there seemed to be practically as much remaining as was originally added. The results of induction tests on these oils are shown in table 20. They show that the stabilizing effect is accomplished with a very low concentration of added acid, and that larger amounts are not much more effective.

TABLE 20

Effect of Various Amounts of Citric Acid on The Induction Period of Halibut Liver Oil.

	Induction Period, Hours
Control Halibut Liver Oil	5.5
H.L.O. plus 1% Citric Acid	26
H.L.O. plus 0.1% Citric Acid	26
H.L.O. plus 0.01% Citric Acid	21

Synergism of Antioxidants. A synergistic action of hydroquinone and vegetable lecithin as antioxidants has been reported by Holmes, Corbet and Hartzler (30). A study was made here of the synergistic relations of hydroquinone, lexinol (vegetable lecithin) and citric acid. The data are given in table 21. They show that the combination of

hydroquinone and citric acid have almost as great a synergistic effect as does the combination of hydroquinone and lexinol--that is, when paired together a much greater protective effect results than would be expected from their additive effects. Also, when a combination of hydroquinone, citric acid and lexinol is used an even greater effect is shown. On the other hand, a combination of lexinol and citric acid shows no synergistic effect.

TABLE 21

Synergism of Antioxidants.

Treatment	Induction Period, Hours
Control Halibut Liver Oil	5.5
H.L.O. plus Citric Acid	34
H.L.O. plus 0.05% Hydroquinone	34
H.L.O. plus 0.05% Hydroquinone plus Citric Acid	275
H.L.O. plus 0.5% Lexinol	30
H.L.O. plus 0.5% Lexinol plus Citric Acid	36
H.L.O. plus 0.5% Lexinol plus 0.05% Hydroquinone	340
H.L.O. plus 0.5% Lexinol plus 0.05% Hydroquinone plus Citric Acid	400

Studies on the Nature of Natural Antioxidants in Fish Liver Oils

During the course of studies of the stability of various fish oils it became apparent that different chemical treatments of the oils greatly affected their stability. Therefore experiments were set up to investigate the chemical nature of the natural antioxidants present in these oils by applying various chemical treatments and determining the fate of the antioxidants by means of the induction test. In most cases the oils studied were solvent-extracted halibut liver oils.

Effect of Acids. In table 11 were shown data which indicate the effect of several common acids on the stability of vitamin A in a halibut liver oil. Acetic acid had no effect, while phosphoric and hydrochloric acids were detrimental--especially the latter. On the other hand, sulfuric acid enhanced the stability of the oil.

These results were obtained when the halibut liver oil, which was dissolved in petroleum ether, was washed with 80% methyl alcohol containing 1% of the acid in question. The effect

of aqueous sulfuric acid was next investigated, as was also the effect of the acid salt, sodium acid sulfate. The results of these experiments are summarized in table 22. They show that in all cases where sulfuric acid comes in contact with halibut liver oil the induction period is increased. This effect is only slight in the case of aqueous sulfuric acid, probably due (as later experiments show) to the solvent effect of water for the antioxidant fraction of the oil.

TABLE 22

Effect of Sulfuric Acid and Sodium Acid Sulfate on the Induction Period of Halibut Liver Oil.

Number	Treatment	Induction Period, Hours
6058	Control Halibut Liver Oil	81
19899	(6058) Washed with 80% Methyl Alcohol	50
24129	Same as 19899 plus 1% Sulfuric Acid	126
25559	(6058) Washed with Aqueous Sulfuric Acid	95
38990	Control Halibut Liver Oil #2	27.5
40930	(38990) Treated with Sodium Acid Sulfate	40

Effect of Alkalies. (a) Aqueous Alkali.

As the data of tables 6 and 7 show, induction periods of alkali-digested fish oils are much

lower than those of solvent-extracted oils. Since alkali-digested oils are treated during preparation with large volumes of aqueous alkali it was thought this might be a factor in their reduced stability. Therefore the effect of aqueous alkali on a solvent-extracted halibut liver oil was investigated. The data are shown in table 23. Ten gram amounts of halibut liver oil, 6058, were dissolved in minimum amounts of a mixture of equal parts ethyl alcohol and ether and to these solutions were added aqueous sodium hydroxide equivalent to half, equal, and double the amount of free fatty acid (12.8 per cent estimated as oleic) present in the oil. These mixtures were warmed and shaken for about an hour, when the ether layer was separated and the ether removed from the oil by distillation. The same treatment was followed for 17769, the last sample in the table, except that the phospholipids had been removed from the oil, prior to the alkali treatment, by precipitation with cold acetone. The aqueous ammonia treatment was carried out in the same manner as the treatment with aqueous sodium hydroxide.

TABLE 23

Effect of Aqueous Alkali on the Induction Period of Halibut Liver Oil.

Number	Treatment	Induction Period, Hours
6058	Control Halibut Liver Oil	81
18399	(6058) plus Half Theoretical Amount NaOH	23
18179	(6058) plus Theoretical Amount NaOH	25
18409	(6058) plus Twice Theoretical Amount NaOH	17
18839	(6058) plus Aqueous NH_4OH	33
17709	(6058) After Removing Phospholipids	78
17769	(17709) plus Theoretical Amount NaOH	4

It is evident from these results that there is little correlation between the amount of alkali used and the amount of reduction of the induction period. However, when the phospholipid fraction is removed from the oil the alkali has a much more severe effect on the antioxidant. Treatment with aqueous ammonia, representing an amount of ammonia theoretically required to neutralize the free fatty acids of the oil, had a slightly less detrimental effect than treatment with sodium hydroxide.

(b) Dry Alkali. The effect of dry alkali on the stability of fish oils was determined by treating halibut liver oil with ammonia gas and sodium ethylate respectively. In the case of the

former, an excess of ammonia gas was passed through 25 gm. halibut liver oil, 6058. Three volumes acetone were then added, which brought down a voluminous precipitate. This precipitate was filtered off and the acetone removed from the filtrate by distillation. There was no reduction in free fatty acids--the ammonia therefore had not neutralized any of them.

The treatment with sodium ethylate was as follows: To 12 gm. halibut liver oil, 6058, was added 37 ml. 0.79N sodium ethylate (about half the theoretical amount necessary to completely saponify the oil), causing an immediate precipitation of soap. After standing overnight, the soap was extracted with acetone, filtered and the acetone distilled under vacuum. More soap separated so the acetone extraction was repeated. The resulting limpid oil had an $E^{1\%}_{1\ cm.}$ value of 108.5, compared to 72 for the original oil. The data from stability tests on these dry alkali-treated oils are presented in table 24.

former, an excess of ammonia gas was passed through 25 gm. halibut liver oil, 6058. Three volumes acetone were then added, which brought down a voluminous precipitate. This precipitate was filtered off and the acetone removed from the filtrate by distillation. There was no reduction in free fatty acids--the ammonia therefore had not neutralized any of them.

The treatment with sodium ethylate was as follows: To 12 gm. halibut liver oil, 6058, was added 37 ml. 0.79N sodium ethylate (about half the theoretical amount necessary to completely saponify the oil), causing an immediate precipitation of soap. After standing overnight, the soap was extracted with acetone, filtered and the acetone distilled under vacuum. More soap separated so the acetone extraction was repeated. The resulting limpid oil had an $E^{1\%}_{1\ cm.}$ value of 108.5, compared to 72 for the original oil. The data from stability tests on these dry alkali-treated oils are presented in table 24.

TABLE 24

Effect of Dry Alkali on the Induction Period of Halibut Liver Oil.

Number	Treatment	Induction Period, Hours
6058	Control Halibut Liver Oil	81
18309	(6058) plus Ammonia Gas	81+
24169	(6058) plus Sodium Ethylate	81+

These results indicate that treatment with dry alkali has no destructive effect on the natural antioxidants in halibut liver oil. Apparently losses of antioxidant content from treatment with aqueous alkali has been due to the water content of the alkali solutions.

(c) Alcoholic Alkali. The first treatment of halibut liver oil with alcoholic alkali was made by shaking a solution of the oil in petroleum ether in a separatory funnel with 80% methyl alcohol to which potassium hydroxide had been added. This combination of petroleum ether and 80% methyl alcohol was used since the two phases could be intimately mixed by shaking but would separate at once on standing. A control treatment of the petroleum ether solution of the oil with 80% methyl alcohol

had to be made, since this alone had some effect. The data, shown in table 25, indicate practically no effect of alcoholic potassium hydroxide.

Treatment of halibut liver oil with alcoholic ammonia was as follows. A 2.86 N solution of ammonia was prepared by bubbling the gas through absolute ethyl alcohol. Three and a half ml. of this solution were added to 10 gm. halibut liver oil, 6058, shaken, and allowed to stand overnight at room temperature. The oil was then taken up in ten volumes of acetone and the precipitate that formed filtered off. The solvents were then distilled off and a stability test made on the resulting oil. The results were so outstanding that a further similar treatment was given an alkali-digested halibut liver oil, both with and without the supplementary acetone-treatment given the ether-extracted halibut liver oil, 6058, used above.

It is evident from the data that treatment of halibut liver oil with alcoholic ammonia greatly enhances its stability. This is especially true in the case of an oil prepared by solvent extraction such as 6058. The effect is even more pronounced when acetone is added to

the ammonia-treated oil.

TABLE 25

Effect of Alcoholic Alkali on the Induction Period of Halibut Liver Oil.

Number	Treatment	Induction Period, Hours
6058	Control Halibut Liver Oil	81
19899	(6058) Washed with 80% Methyl Alcohol	50
28179	(6058) Washed with 80% Methyl Alcohol with Added KOH	53
18779	(6058) plus Alcoholic Ammonia with Acetone	550
2028-II	Control Halibut Liver Oil #2	18
23489	(2028-II) plus Alcoholic Ammonia Alone	48
23499	(2028-II) plus Alcoholic Ammonia with Acetone	73

Effect of Water. The above experiments indicated that it was the water content of aqueous alkali which had the solvent effect on the anti-oxidants of halibut liver oil. It is difficult to extract oil with pure water, due to emulsions which form. However, it was possible to extract halibut liver oil, 6058, with a hot solution of 33% ethyl alcohol and get a separation of the aqueous and oily layers. The oil was then dissolved in ether, dried over sodium sulfate and the ether distilled.

A second experiment dealing with water

solubility of the antioxidant was also carried out. Ten grams of halibut liver oil, 6058, were dissolved in 40 ml. petroleum ether and this solution was extracted twice with 40 ml. amounts of a 10% aqueous solution of sodium sulfate. The sodium sulfate has been shown to have no effect on the antioxidant and was simply added to prevent emulsions. The petroleum ether was then distilled from the oil. Induction tests were carried out on the resulting oils from these two experiments. The data are presented in table 26.

TABLE 26

The Solvent Effect of Water on the Antioxidant of Halibut Liver Oil.

Number	Treatment	Induction Period, Hours
6058	Control Halibut Liver Oil	81
19139	(6058) Washed with Aqueous Alcohol	40
31709	(6058) Washed with 10% Sodium Sulfate	16

This data indicates that the antioxidant is more soluble in water than in alcohol since washing the oil with a water solution containing sodium sulfate reduces its stability more than washing it with 33% alcohol.

Effect of 80 Per Cent Methyl Alcohol.

Many of the chemical treatments given the fish oils whose antioxidant content was being studied were carried out by washing the oil first dissolved in petroleum ether, with an 80% solution of methyl alcohol to which the particular chemical was added. This necessitated knowing the solvent effect of the methyl alcohol alone for the antioxidant. The solvent effect was determined by dissolving 10 gms. of the oil in 40 ml. petroleum ether; this solution being given two thorough extractions in a separatory funnel with 40 ml. portions of 80% methyl alcohol. The petroleum ether layer was then carefully separated and the solvent evaporated.

To find the effect of additional extraction, another experiment was carried out the same as above except the oil was extracted with alcohol a total of five times. These two alcohol-extracted oils were then put on induction tests. The data are shown in table 27. They show that although a portion of the antioxidant is removed by extraction with 80% methyl alcohol, no significantly greater amount is removed by continued extraction.

TABLE 27

The Solvent Effect of 80% Methyl Alcohol on The Antioxidant of Halibut Liver Oil.

Number	Treatment	Induction Period, Hours
6058	Control Halibut Liver Oil	81
19899	(6058) After Two Washes with 80% Methyl Alcohol	50
20409	(6058) After Five Washes with 80% Methyl Alcohol	46

Effect of Various Chemicals. (a) Dinitrobenzoyl Chloride. Halibut liver oil was treated with various chemicals which react characteristically with certain chemical groupings. This was an attempt to establish which chemical groupings are important in the action of the natural antioxidants of these oils. First, 1 gm. finely powdered 3,5-dinitrobenzoyl chloride was added to 10 gm. halibut liver oil, 6058. This mixture stood overnight, was then shaken occasionally for 2 hours and filtered by suction.

(b) Formaldehyde. Ten grams halibut liver oil, 6058, were dissolved in 40 ml. petroleum ether and washed twice with 40 ml. portions of a solution composed of 80 ml. methyl alcohol and 20 ml. 40% formaldehyde. The petroleum ether layer was carefully separated and the solvent distilled from it.

(c) Phthalic Anhydride. Ten grams halibut liver oil, 6058, were dissolved in 40 ml. of a saturated solution of phthalic anhydride in petroleum ether (very slightly soluble) and allowed to stand in this solution for half an hour at room temperature. The petroleum ether was then removed by distillation.

A second experiment was carried out in which 5 gm. halibut liver oil, 6058, were dissolved in 45 ml. ethyl ether which contained 50 mg. phthalic anhydride. This solution stood in the dark at room temperature for 23 hours, when the ether was removed by distillation.

Induction tests were carried out on all the chemically-treated oils described under (a) to (c). The data from these tests are presented in table 28.

TABLE 28

The Effect of Various Chemicals on the Anti-oxidant of Halibut Liver Oil.

Number	Treatment	Induction Period, Hours
6058	Control Halibut Liver Oil	81
28269	(6058) Plus Dinitrobenzoyl Chloride	11
19899	(6058) Washed with 80% Methyl Alcohol	50
27979	(6058) Washed with 80% Methyl Alcohol Containing 8% Formaldehyde	29
27969	(6058) Plus Phthalic Anhydride in Petroleum Ether	22
28199	(6058) Plus Phthalic Anhydride in Ethyl Ether	6

The action of dinitrobenzoyl chloride on the activity of the antioxidant would tend to indicate the activity of hydroxyl groups in the latter. However, the possible breakdown of benzoyl chloride to form hydrochloric acid must not be overlooked. It has been shown that only a very small amount of hydrochloric acid is necessary to effectively reduce the stability of halibut liver oil.

Formaldehyde appears to be moderately reactive toward the antioxidant under the conditions employed. This would indicate that the antioxidant may be a phenolic type compound. This reasoning is also in harmony with the observed action of phthalic anhydride on the antioxidant activity of oils. This compound, which the data shows to be so readily destructive of the antioxidant in halibut liver oil, characteristically reacts with phenols and amines.

Relation of Chemical Treatment to Added Antioxidants. Various types of antioxidants were added to a given halibut liver oil and the effects of different chemical treatments on these added antioxidants were investigated. This was

to gain some information concerning the nature of the natural antioxidants in halibut liver oils by noting the effect of known chemical treatments on the added antioxidants.

To 30 gm. amounts of halibut liver oil were added 0.1% ethanolamine, 1.0% ∝ tocopherol, and 0.05% hydroquinone respectively. First, the induction periods of these three samples were determined to serve as a baseline, then part of each sample was treated as follows:

(a) Extracted with 80% methyl alcohol.
Ten grams of the oil were dissolved in 40 ml. petroleum ether and washed twice with 40 ml. portions of the 80% methyl alcohol, after which the petroleum ether was evaporated from the oil layer.

(b) Extracted with 80% methyl alcohol containing hydrochloric acid.
Same procedure as (a) except the 80% methyl alcohol contained an added 1% hydrochloric acid.

(c) Treated with phthalic anhydride.
Five grams were dissolved in 45 ml. ethyl ether in which had been dissolved 50 mg. phthalic anhydride. After standing overnight the ether was evaporated. (The sample containing ethanolamine gave a fine white precipitate when the ether solution of phthalic anhydride was added).

The induction periods of all these samples were determined. The data are presented in table 29.

TABLE 29

Effect of Chemical Reagents on Different Types of Antioxidants Added to Halibut Liver Oil.

Number		Treatment	Induction Period, Hours
	2028 II	Control Halibut Liver Oil	19
I	28379	0.1% Ethanolamine	56
	28819	(28379) plus 80% Methyl Alcohol	17
	28409	(28379) plus 80% Methyl Alcohol with HCl	15½
	28529	(28379) plus Phthalic Anhydride	57
II	28389	1.0% α Tocopherol	105
	28829	(28389) plus 80% Methyl Alcohol	90
	28419	(28389) plus 80% Methyl Alcohol with HCl	110
	28539	(28389) plus Phthalic Anhydride	105
III	28399	0.05% Hydroquinone	169
	28839	(28399) plus 80% Methyl Alcohol	3½
	28429	(28399) plus 80% Methyl Alcohol with HCl	11
	28549	(28399) plus Phthalic Anhydride	170

These results indicate that the induction periods of halibut liver oils stabilized by the addition of ethanolamine or hydroquinone were greatly reduced by extraction with 80% methyl alcohol, either alone or with added hydrochloric acid. However, as might be expected, this treatment has no effect on an oil stabilized by the addition of alpha tocopherol. Phthalic anhydride had no effect on oils stabilized

by any of the above treatments, although it had been found very destructive of the natural antioxidant of halibut liver oil.

Attempts at Isolation of Active Antioxidant Fractions of Fish Oils

Experiments were carried out whereby various fractions of stable fish oils were separated by appropriate treatments. The object was to find in which of these fractions the stabilizing factor, or antioxidant, was to be found. This was studied by two methods. The first was to add these separated fractions to another fish oil of known degree of stability and determine whether it was made more stable by the addition of these fractions. The second method was to determine the stability of an oil after a certain fraction had been removed, then find if the original stability of the oil could be attained by adding the isolated fraction back to the residue from which it had been separated.

Cold Acetone Precipitation. One method of fractionation employed was the separation of an acetone-insoluble fraction. Typical of several

such experiments is the following.

Thirty grams of a solvent-extracted ling cod viscera oil were dissolved in 30 ml. ethyl ether and poured slowly into a liter of ice-cold acetone with continuous stirring. A small amount of flocculent precipitate which separated was filtered off quickly on a Büchner funnel. The yield after drying in a vacuum was 0.54 gm., or 1.8%. The acetone was removed from the filtrate by distillation. Part of the isolated acetone-insoluble fraction was redissolved at a concentration of 1.8% in some of the residual acetone-soluble oil. The induction periods of the original ling cod viscera oil, of the residual oil after removal of the acetone-insoluble fraction, and of the reconstituted oil were determined. The data are in table 30, and are presented in graphic form in Figure 5.

TABLE 30

Effect of Cold Acetone Precipitation on the Antioxidant of Fish Viscera Oil.

Number	Treatment	Induction Period, Hours
0757	Ling Cod Viscera Oil	100
2268	Acetone-soluble Fraction	36
2448	(2268) plus Phospholipid Fraction	80

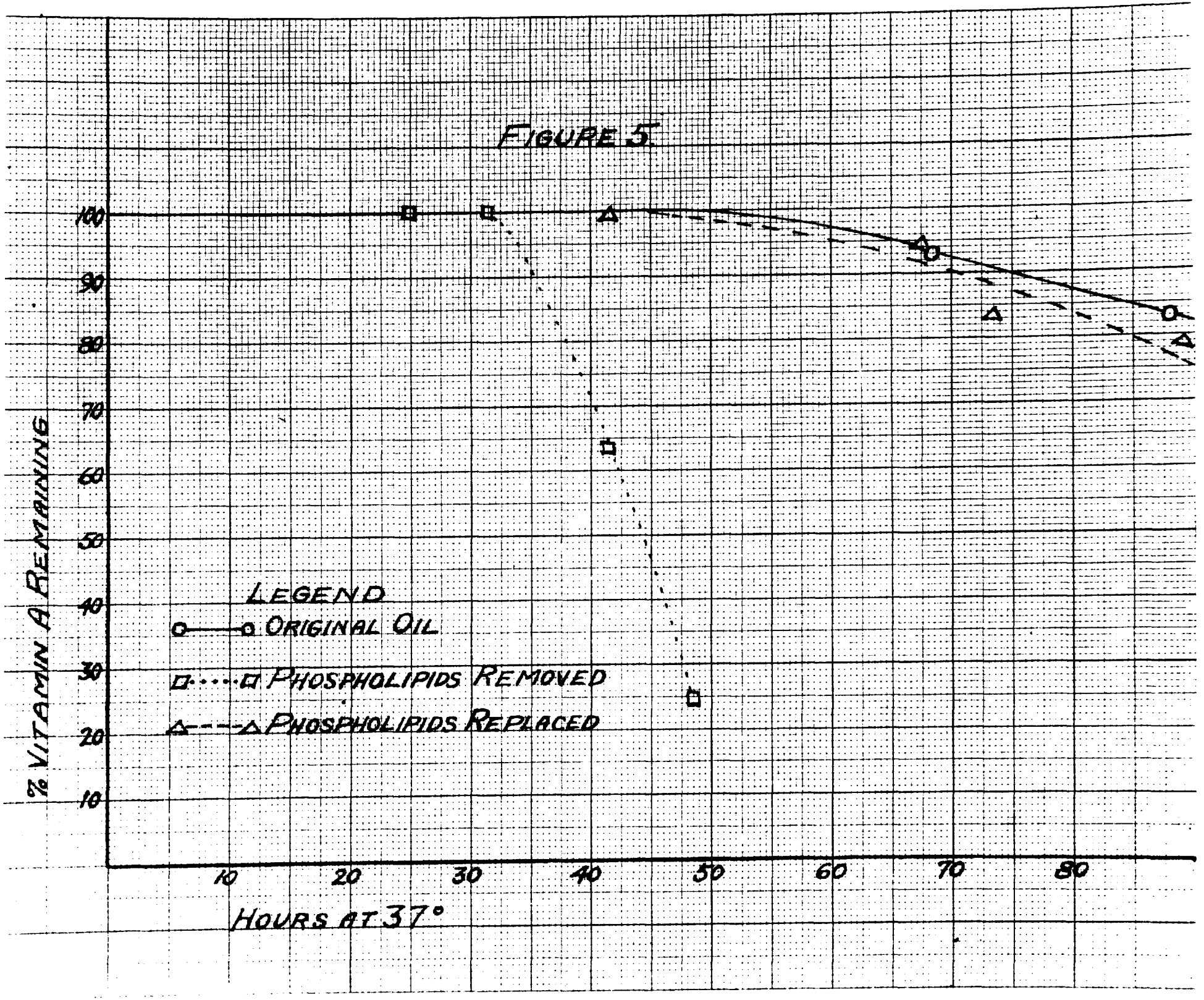


FIGURE 5.
% VITAMIN A REMAINING
100
90
80
70
60
50
40
30
20
10
10
20
30
40
50
60
70
80
HOURS AT 37°
LEGEND
ORIGINAL OIL
PHOSPHOLIPIDS REMOVED
PHOSPHOLIPIDS REPLACED

The induction period data, and especially the curves of Figure 5, illustrate what a large proportion of the stabilizing fraction of this oil was present in the acetone-insoluble, or phospholipid fraction. There is no doubt that this acetone-insoluble fraction was a phospholipid since a phosphorus determination on fractions of a similar oil, prepared in an identical manner, indicated 1.58% phosphorus in the acetone-insoluble fraction, and only 0.012% in the acetone-soluble oil. However, this type of antioxidant is not found in fish oils prepared by alkali-digestion since these are entirely soluble in acetone.

Extraction with an Aqueous-Alcoholic Solution of Alkali. Ten grams of a solvent-extracted halibut liver oil, which had been freed from its phospholipid content by precipitation with cold acetone, was treated with the theoretical amount of aqueous N/10 sodium hydroxide to neutralize the free fatty acids present. After warming on the steam bath the resulting neutral oil separated as an upper layer. The aqueous layer was drawn off from below in a separatory funnel and washed with ether to remove all traces of the neutral oil. It was then acidified to pH 5.0 with acetic acid and the fatty

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acids thereby released were extracted with ether. This ether extract was dried over anhydrous sodium sulfate and the ether removed by distillation. The yield was 1.05 gm. of fatty acids which were partially solid at room temperature.

To 4.5 gms. of halibut liver oil prepared by the method of alkali-digestion was added 0.502 gm. of the fatty acid fraction described above. The data from induction tests on this composite oil and the fractions mentioned above are presented in table 31.

TABLE 31

Stabilizing Effect of a Fraction of Halibut Liver Oil Extracted by Aqueous Alkali.

Number	Treatment	Induction Period, Hours
17709	Control H.L.O. (Solvent-extracted)	78
18369	(17709) Extracted with Aqueous NaOH	4
2028	Control H.L.O. (Alkali-digested)	12
18389	(2028) plus Fraction from 17709	16½

It is evident that dilute aqueous alkali either destroys or extracts the antioxidant fraction of halibut liver oil very completely. The fact that

the aqueous alkali extract contained a substance which when acidified possessed some antioxidant effect indicates that it was partly a matter of extraction.

Another experiment was carried out in which 10 gm. of the same halibut liver oil as above were dissolved in 20 ml. of an equal mixture of alcohol and ether. To this were added 50 ml. of N/10 aqueous sodium hydroxide and the mixture shaken occasionally for a half hour. Then 20 ml. additional ether were added to separate the two layers. To the water layer were added 60 ml. N/3 sulfuric acid and the solution was extracted with ether. This ether extract was washed once with water and dried over anhydrous sodium sulfate. The ether was evaporated, yielding 1.62 gm. of a fatty acid fraction. The ether was evaporated from the solution of neutral oil, yielding 8.05 gms. of oil. Part of this isolated fatty acid fraction was re-combined with some of the neutral oil in the proportions in which the two fractions were originally present. Induction tests were run on these samples. The results are shown in table 32.

TABLE 32

Partial Recovery of the Antioxidant Fraction of Halibut Liver Oil after Extraction with Dilute Alkali.

Number	Treatment	Induction Period, Hours
17709	Control H.L.O. (Solvent-extracted)	78
27499	(17709) Less Fatty Acid Fraction	6
27539	(27499) Plus Fatty Acid Fraction	14

These data show that part of the antioxidant removed from halibut liver oil by aqueous alkali extraction can be restored to the residual oil by returning the fatty acid portion removed by the alkali extraction.

Partition Between 80 per cent Methyl Alcohol and Petroleum Ether. Ten grams solvent-extracted halibut liver oil, 6058, were dissolved in 40 ml. petroleum ether and thoroughly extracted with two 40 ml. portions of 80% methyl alcohol. To the combined methyl alcohol extracts were added 75 ml. ether, and then water until a separation of layers took place. The aqueous layer was extracted twice more with ether. The ether was distilled off from the combined ether extracts,

yielding 0.36 gm. of an oily extract. Half of this oily residue was re-combined with half of the petroleum ether-soluble fraction, after the petroleum ether had been evaporated. The induction period of the alcohol-extracted oil was determined before and after the addition of the fraction soluble in 80% methyl alcohol. The data are shown in table 33.

TABLE 33

Separation of Antioxidant from Halibut Liver Oil by Methyl Alcohol Extraction.

Number	Treatment	Induction Period, Hours
6058	Control Halibut Liver Oil	81
19899	(6058) Extracted with 80% Methyl Alcohol	50
20179	(19899) Plus Ether-Soluble Portion of Methyl Alcohol Extract	64

The data from this experiment show that at least part of the antioxidant removed from halibut liver oil by extraction with 80% methyl alcohol is soluble in ether and can be separated in a definite fraction. Restoring this to the fraction from which it came restores a share of the original stability.

Extraction with Aqueous Sodium Sulfate.

Past experiments have indicated that the antioxidant fraction of halibut liver oil is more soluble in water than in alcohol. Therefore an experiment was carried out in which an extractant of as nearly pure water as possible was used. Ten grams halibut liver oil, 6058, were dissolved in 40 ml. petroleum ether and extracted thoroughly with two 40 ml. portions of a 10% solution of sodium sulfate (pure water caused an unbreakable emulsion). The solvent was evaporated from the petroleum ether layer, yielding 8.8 gm. residual oil. The aqueous sodium sulfate solution was extracted three times with ether. This ether extract was dried and the ether evaporated. There resulted 0.69 gm. of a sharp smelling oil. To 0.35 gm. of this were added 4.65 gm. of the residual oil. Table 34 gives data on the induction tests of the extracted and reconstituted oils.

TABLE 34

Attempted Separation of the Antioxidant of Halibut Liver Oil by Extraction with Aqueous Sodium Sulfate Solution.

Number	Treatment	Induction Period, Hours
6058	Control Halibut Liver Oil	81
32669	(6058) After Washing with Na_2SO_4	14
32679	(32669) Plus Na_2SO_4 Extract	16

These data indicate that the antioxidant fraction is much more completely soluble in 10% aqueous sodium sulfate than in 80% methyl alcohol. However, none of the antioxidant removed by the sodium sulfate solution could be recovered from it by extraction with ether. This indicates either that it was destroyed by the sodium sulfate solution or rendered ether-insoluble by it.

RESUME

Development of Induction Test. A method has been developed for studying the stability of oils containing vitamin A. This consisted of exposing small, uniform samples to the air at a temperature of 37° C. under standardized conditions. The vitamin A-breakdown in these samples was measured either by frequent vitamin A determinations using the Vitameter, or by peroxide determinations.

Studies were made of the effect of temperature, area, and depth of exposed samples on this induction test. It was found that the induction period varied inversely with the area of the sample exposed, and a decrease in temperature of 10° approximately doubled the length of the induction period. The depth of sample was relatively unimportant, although the weight of oil exposed largely determined the rate of vitamin A-breakdown. Agitation of samples proved unnecessary, since almost identical results were obtained with small, unagitated samples as with larger samples which were constantly agitated during exposure.

When titrations of peroxides as well as vitamin A determinations were made on samples of oils exposed in the induction tests, it was found that as vitamin A began to break down peroxides always increased at the same time. Thus either determination was satisfactory as an index in the test, but vitamin A determinations usually proved easier.

A critical examination of the breakdown of vitamin A during the induction test was also made by means of spectrophotometric measurements of exposed oil samples. A complete absorption curve in the ultra-violet was made for each sample of a particular halibut liver oil as it was removed from the constant temperature room during the induction test. This study indicated a drop in the peak of absorption with time, following the end of the induction period, and eventually a shift of the peak toward the lower ultra-violet.

An attempt was made to correlate results obtained by this accelerated induction test and actual breakdown of vitamin A under certain conditions of storage. These correlation studies showed that a factor could be used to translate the induction period obtained by the above method

into time required for the beginning of vitamin A-breakdown in storage. The studies also emphasized the importance of well-filled containers for storage, and the fact that breakdown time is largely a matter of the bulk of oil being considered.

Stability of Natural Vitamin A Oils. This standardized induction test was used to investigate the stability of a number of vitamin A oils of various sources and methods of preparation. It was found that cod liver oils and solvent-extracted oils were most stable, having average induction periods of 125 hours and 111 hours respectively. Tuna liver oils were intermediate in stability, with an average of 65 hours. Oils prepared by aqueous alkali digestion were much less stable as a class. These ranged from 21 hours and 17 hours, respectively, for averages of mixed fish liver oils and halibut liver oils, to 8 hours for alkali-digested viscera oils.

Conditions Affecting the Stability of Vitamin A in Oils. A study was made of the conditions affecting the stability of vitamin A in oils. There was found to be no correlation between the free fatty acid content of a group

of oils and their inherent stability. Peroxides, however, had an important effect on the induction periods of oils. With varying dilutions of vitamin A in a given vegetable oil, induction periods were in a direct ratio to the dilution factor. But regardless of the length of the induction period vitamin A dropped and peroxides rose at the same time. Addition of peroxides alone to the vegetable oil used for a diluent caused no such accelerated induction period. Therefore it was concluded that vitamin A is of the nature of an oxidation catalyst and stimulates autoxidation of oils in which it is dissolved.

The effect of adding various oxidation catalysts to vitamin A oils was studied. Among these were salts, acids, metallic soaps and peroxides. There was a great range of effect among the salts, being greatest with those which are known to be strong oxidation catalysts, namely salts of cobalt and copper. However, it was found that calcium and magnesium chlorides were just as active, and a number of other chlorides were also very active.

Among the acids, none was very active as an oxidation catalyst except hydrochloric, which

was extremely active. It was found that this proxidant effect of hydrochloric acid and chloride salts could be counteracted by the action of strong alkalis. This made it seem that the mechanism of action of the chloride salts might be their reaction with the free fatty acids of the oils in which the salts were placed, liberating free hydrochloric acid.

Metallic soaps were found to be extremely active oxidation catalysts, but their effect could be neutralized by thioglycolic acid or ammonia. The strong proxidant effect of added peroxides could be neutralized by only two methods--the addition of thioglycolic acid or amines.

Use of Induction Test for Evaluating Antioxidants. One of the greatest uses of the induction test was in evaluating the effect of the addition of various inhibitors of autoxidation, or antioxidants, to vitamin A oils. A great number of these was studied, among them being hydroquinone, phospholipids, organic acids, amines, tocopherols, various complexes, and ammonia. Of these, hydroquinone was found to be the most active when acting alone. However, certain combinations of two or more acting together showed outstanding synergistic effects.

Studies of the Nature of Natural Antioxidants in Fish Oils. Numerous studies were carried out to determine the nature of the natural antioxidants present in fish oils. These were made by subjecting the oils to various chemical treatments and evaluating the effects on the antioxidants in the oils by means of induction tests. Aqueous alcohol and aqueous alkalis extracted the antioxidants from oils. Water had an even more pronounced effect. On the other hand, dry alkalis or nearly anhydrous alcohol had no effect. Dinitrobenzoyl chloride, formaldehyde and phthalic anhydride had a pronounced destructive effect on the antioxidants, indicating perhaps their phenolic or amine nature.

Attempts at Isolation of Antioxidant Fractions. Several attempts were made at isolation of active antioxidant fractions from fish oils. The most successful was the separation of a phospholipid fraction by cold acetone precipitation. The fraction separated in this way restored almost completely the stability of the residual oil from which it was separated.

Extraction of oils with aqueous alkali or 80% methyl alcohol removed a good share of the

antioxidant fraction but evidently a part was destroyed in the process, since the original stability of the oils were not restored by returning these fractions.

Extraction of oils with water, to which sodium sulfate was added to avoid emulsions, was even more destructive of the antioxidant since restoring the extract to the water-extracted residual oil did not increase its stability significantly.

CONCLUSIONS

Studies leading to the development of an induction test for vitamin A oils demonstrated that oxygen diffuses through oils quickly and to a considerable depth. Thus it is unnecessary to agitate the samples of oil, as many workers have done, during exposure to the air. The measurement of either vitamin A or peroxides is a satisfactory method for following the course of the induction test.

As a general rule, fish oils prepared by solvent extraction, derived either from livers or viscera, are much more stable than those prepared by alkali-digestion. This is for two reasons. First, because solvent-extraction carries the phospholipid content of the livers into the oil, thus tending greatly to increase its stability. Second, large quantities of water are used in the preparation of alkali-digested oils, thus tending to extract the antioxidants from the oils. This lowered stability of alkali-digested oils can be remedied by inclusion of antioxidants, and by improvements in methods of digestion.

Among the components of fish oils which may affect their stability adversely, oxidation catalysts such as peroxides and metallic soaps are the most important. Peroxides develop in the oils during processing. Metallic soaps may also be introduced during processing by the interaction of free fatty acids in the oils with metallic containers. Certain acids--especially hydrochloric acid--have been found to cause rapid breakdown of vitamin A oils. Calcium chloride, sometimes used as a dehydrating agent during the processing of fish oils, is a very active oxidation catalyst. The data indicate that in all cases where chloride salts cause a rapid production of rancidity in oils, the probable cause is the development of free hydrochloric acid by the reaction of the chlorides with the free fatty acids in the oils.

Vitamin A itself may act as an oxidation catalyst. This is concluded from the fact that when a very pure vitamin A distillate, free of peroxides, is diluted in corn oil the rate of breakdown is always proportional to the concentration of vitamin A. On the other hand, adding

only a peroxidized oil to the same corn oil causes no such increase in rate of breakdown with increasing concentration.

Many types of compounds are active antioxidants for fresh fish oils, which are relatively free from peroxides and other oxidation catalysts. Among these, hydroquinone is the most active, while amines are almost as effective. Tocopherols and various phospholipids are quite effective. Certain organic acids, especially citric, maleic and tartaric, are active antioxidants in fish oils, although they have been stated to be inactive in animal oils when used alone, their only effect being to enhance the effect of tocopherols and inhibitols in such oils.

The only antioxidants studied which are effective in oils containing peroxides are thioglycolic acid and amines. Their action is not due to a reduction of the peroxides in the oils, since the peroxides are practically the same after the addition of these agents. Oils containing metallic oxidation catalysts are stabilized only by thioglycolic acid and

ammonia, among the compounds studied. There is evidence that the stabilizing effect of these two compounds is due to the formation of complexes with the metallic catalysts in the oils.

From studies of the nature of the natural antioxidants present in fish oils it is concluded that several types of compounds are involved. With regard to oils prepared by solvent-extraction, phospholipids are of major importance in viscera oils but not in liver oils. Another fraction, active as an antioxidant, is removed from halibut liver oil by extraction with 80% methyl alcohol; however, continued extraction with this solvent does not remove all the antioxidant. On the other hand, water removes practically all the antioxidant. Chemical reactions indicate that both amines and phenolic compounds may be involved.

Separation of relatively pure antioxidant fractions was only possible in the case of phospholipids. In other cases a good deal of destruction accompanied the separation of the fractions, since they could not be re-combined with the residual oils from which they came. This indicates the extremely labile character of the compounds involved.

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BIOGRAPHY

Orson David Bird was born at Romulus, Michigan, March 18, 1905. His elementary school education was obtained at Hayti district school, Romulus township, and he was graduated from Wayne High School in 1921. After earning a teacher's cretificate at Michigan State Normal College, he entered Michigan State College in 1924. He received a B. S. degree in 1926. From June, 1926, to June, 1928, he held a half-time Calumet Baking Powder Co. fellowship at the Agricultural Experiment Station, East Lansing, working on the determination of Aluminum in plants. During this time he did graduate work at Michigan State College, obtaining in June, 1928, the M. S. Degree in Chemistry with a minor in Bacteriology. The title of his thesis was "A Study of Carbohydrate Derivatives."

In June, 1928, he accepted a position in the Research Department of Parke, Davis and Co., where he is located at the present time.

In June, 1941, he presented to the Graduate School of Michigan State College, a thesis entitled: "A Study of Antioxidants with Respect to Vitamin A in Fish Oils."

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