The Stability of Vegetable Oils III. Investigation of the Effect of Radiation On the Methylene Blue-Oil System

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IN A PREVIOUS publication (1) an apparatus and method were described for determining the rate and total time of dye reduction of various methylene blue-oil systems. Certain factors, such as the substrate, temperature, dissolved gases, aldehydes, and peroxides were examined in an effort to evaluate their effect on the rate of dye reduction. With one exception the incident light intensity was maintained at constant value, and no effort was made to determine the mechanism by which the light catalyzed the reaction or what components of the oil-dye system were involved in the methylene blue reduction.

Previous investigators applied the methylene blue method to the determination of the stability of various fat systems and advanced several hypothesis regarding the nature of the reacting components and the mechanism by which they functioned to bring about dye reduction.

Davies (2), for example, devised a methylene blue stability test for fats making use of a "reductase" which was found to be present in nonsterile skim milk. The test was conducted as follows: One ml. of a fat or oil was placed in a test tube and shaken with 5 ml. of skim milk and 5 ml. of water, after which 1 ml. of a standard solution of methylene blue was added and the mixture incubated at 37° to 40° C. until the blue color disappeared. When the dye was completely reduced it was assumed that no peroxide or dissolved oxygen was present, and all samples were presumed to be at the same initial oxidation potential. The tubes were then vigorously shaken for 15 seconds in order to introduce oxygen into the system. After standing for 2 minutes the intensity of the blue color which developed was taken as a measure of the oxidation capacity of the fat or oil. Only a faint blue color developed with freshly refined oils, whereas the intensity of the color with aged oils was found to be proportional to their keeping quality. Davies concluded that a "reductase" present in the skim milk during incubation brought about the reduction of the added methylene blue and peroxides and the simultaneous utilization of the dissolved oxygen of the system. The intensity of the blue color which developed when the system was shaken with air was, therefore, considered as a measure of the state of oxidation of the individual fat.

Greenbank and Holm (3) observed that an alcoholic solution of methylene blue in a fat would become reduced upon exposure to light, and this behavior was also made the basis of a photochemical stability test. The authors assumed that they were measuring the variation in the oxidation potential of fats and oils through the action of an oxidation-reduction indicator (methylene blue) when catalyzed by light. At about the same time, Whitehead (4) published an account of the fading of methylene blue in sterile milk under the influence of sunlight. He found that skim milk under the same conditions did not induce fading in the methylene blue and therefore concluded that the fat was involved in the dye reduction. In order to determine what components of the fat were responsible for the observed behavior, experiments were carried out using aqueous solutions of sodium oleate and sodium palmitate containing methylene blue. It was determined that the double bond was involved in the mechanism since aqueous sodium oleate induced the fading under irradiation, while the aqueous sodium palmitate system remained blue when exposed to light. It was therefore concluded that the unsaturated constituent of the fat was oxidized and the dye functioned as a hydrogen acceptor. The oxidizing agent was not specified, and the question was left open whether the methylene blue was essential to the progress of the reaction or was merely functioning as an oxidation-reduction indicator.

Aikins and Fay (5) investigated this factor by means of oxidation-reduction potential measurements in accordance with Whitehead's suggestion. Their work indicated that the methylene blue was involved in the reaction in an irradiated system because changes in dye concentration affected the fading time, more dye was required to produce a given blue intensity when the fat content of the system was increased, and with 40 per cent cream the change in potential drift toward negative values was greater in the presence of methylene blue than in its absence. However, the exact manner in which the dye was functioning was still obscure.

Davies (6) in discussing the work of Whitehead (4) and of Aikins and Fay (5) came to the conclusion that dissolved atmospheric oxygen was an important factor. He concluded that "The light rays act as a pro-oxygenic catalyst to fat oxidation and the concentration of dissolved oxygen in the milk is lowered to such an extent that the E_h consistent with the point of total reduction of the methylene blue is quickly reached, the dye being reduced."

It should be noted at this point that the above-mentioned investigations were carried out primarily in an aqueous-fat system.

Royce (7) applied the methylene blue method to the study of fats in a nonaqueous system and compared the rate of dye reduction with the rate of formation of peroxides and aldehydes. From the analysis of the methylene blue fading time, Kreis number, and peroxide number curves of a cottonseed oil subjected to accelerated oxidation at 100° C., Royce concluded that they "may be interpreted to indicate that the oxidative decomposition products causing the Kreis reaction are responsible for the methylene blue reduction, whereas the concentration of peroxide as measured by Wheeler's (8) method is better adapted to register the termination of the induction period, when fat samples are aged by aerating at high temperature."

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However, an analysis of Royce's curves indicates that although the fading time decreases, and the Kreis number and peroxide number of an aerated oil increase with time, it cannot be inferred that the three phenomena are related in any simple manner. Actually the plots of the peroxide and Kreis numbers are represented by inverse sigmoid curves whose second derivatives are opposite in sign, while the plot of the methylene blue fading time indicates it to be a logarithmic function of the aeration time, as is clearly evident when the data represented by the latter curve are replotted on a semi-log scale.

It is shown in the present investigation that none of the above explanations are sufficient to account entirely for the behavior of methylene blue in nonaqueous fat systems activated by light, but that the three factors, namely, oxygen, unsaturation, and dye, must be considered as operating collectively and in conjunction with other substances occurring naturally in oils and fats. It is also shown that bands of light of definite frequencies in the visible spectrum catalyze specific reactions which determine the course and velocity of the reduction of the dye in the methylene blue-fat system. Furthermore, it is shown from a study of the fluorescence of methylene blue that the dye is actually reacting with the oil or substances formed in the oil during irradiation.

EXPERIMENTAL

Effect of light on the methylene blue-oil system: In order to evaluate the effect of various spectral frequencies on the reactivity of the methylene blue-oil system, a number of experiments were carried out with freshly deodorized soybean oil to which alcoholic methylene blue chloride had been added. The samples were subjected to isolated bands of light, as well as to the full solar spectrum, for various lengths of time, and observations were made of the effect of irradiation on the fading of the dye. The experiments were carried out in the following manner.

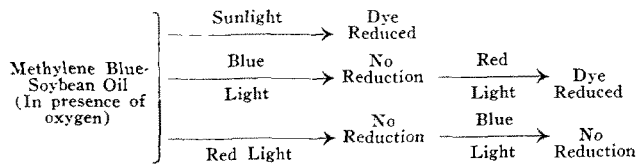
Six 10-ml. portions of a freshly deodorized soybean oil of good quality were placed in 20-ml. stoppered pyrex glass test tubes, A to F (see table I). To each sample there was added 1 ml. of 0.05 per cent methylene blue chloride in absolute ethyl alcohol. The amount of methylene blue added was sufficient to impart an intense blue coloration to the oil. A control sample, G, was prepared by adding 1 ml. of the alcoholic methylene blue solution to 10 ml. of absolute ethyl alcohol. Tubes A, B, and G were exposed to the full solar spectrum of an August midday. Samples C and D were exposed to blue light of λ 3400 to 4400 secured by interposing a blue-purple Corex filter between the sun and the samples. Tubes E and F were exposed to red light of λ 6300 and longer secured by interposing a Corning lighthouse-red filter between the sun and the samples.

Reference to table I, in which the results of these irradiation experiments are summarized, indicates that the blue color of samples A and B, which were exposed to direct sunlight, faded within 20 minutes.

On the other hand, 50 minutes' exposure of samples C and D to the blue light, and of E and F to the red light showed no evidence of dye reduction. When, however, tube C, which had previously been exposed to blue radiation for 50 minutes, was subsequently exposed to red radiation the blue color faded rapidly. But when tube E, which had previously been exposed to red radiation for 50 minutes, was subsequently exposed to blue light no fading (dye reduction) was observed after 50 minutes.

The control sample G which contained no soybean oil did not fade during irradiation, thus proving once more that methylene blue in pure alcoholic solution is quite stable toward light.[3] It was shown in a previous communication of this series (1) that methylene blue in absolute ethyl alcohol or ethylene glycol is perfectly stable at a temperature of 50° C. under prolonged radiation of the incandescent tungsten filament.

Analysis of the behavior of the methylene blue-soybean oil system exposed to the various spectral bands and combinations thereof as mentioned above, and as



EQUATION 1

shown schematically by equation 1, indicates that if methylene blue is to be reduced, the following conditions must obtain: A reaction which is catalyzed by light of wave lengths below about λ 5,500 (i. e., below the region of maximum absorption of methylene blue) must precede or occur simultaneously with another reaction in which methylene blue is a reactant.

In order to isolate the light catalyzed reaction, ab-

[3] This is in contrast to its behavior in acetone solution (9). Reduction of the dye in acetone solution is probably due to the action of diacetyl which is formed by the photochemical reaction of this solvent (10).

TABLE I.—EFFECT OF IRRADIATION ON THE METHYLENE BLUE-SOYBEAN OIL SYSTEM

Tube	Sample	First irradiation			Second irradiation		
		Light	Irradiation time	Apparent color change	Light	Irradiation time	Apparent color change
			Minutes			Minutes	
A	Methylene blue-soybean oil	Sunlight	20	Dye reduced	—	—	—
B	Methylene blue-soybean oil	Sunlight	20	Dye reduced	—	—	—
C	Methylene blue-soybean oil	Blue light λ 3400-4400	50	None	Red light > λ 6300	Very short	Dye reduced
D	Methylene blue-soybean oil	Blue light λ 3400-4400	50	None	—	—	—
E	Methylene blue-soybean oil	Red light > λ 6300	50	None	Blue light λ 3400-4400	50	None
F	Methylene blue-soybean oil	Red light > λ 6300	50	None	—	—	—
G	Methylene blue-alcohol	Sunlight	50	None	—	—	—

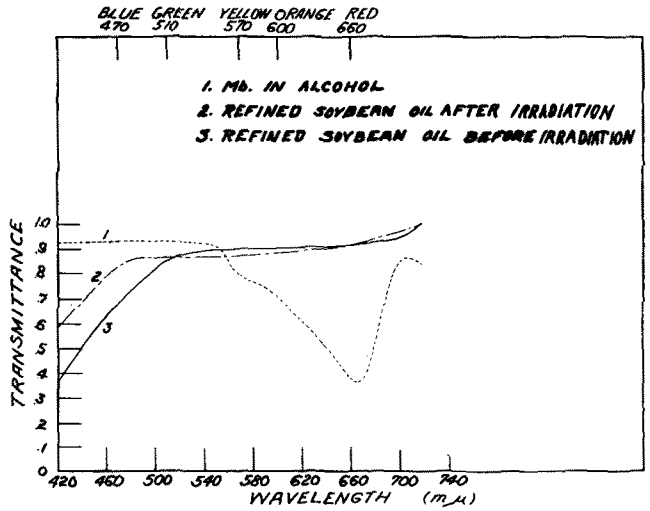


Figure 1. Absorption Spectra

sorption spectra were made of a methylene blue-alcohol solution and of a refined soybean oil prior to and following irradiation by direct sunlight. Reference to curve 1 of figure 1 shows that the maximum absorption of visible light by methylene blue occurs in the region of λ 6600.[4] The low transmittancy of the soybean oil in the blue region, prior to irradiation (curve 3, figure 1) has previously been attributed to the presence of carotenoid pigments (11). These carotenoid pigments are partially destroyed, upon irradiation, as is indicated by the average of 15 per cent greater transmittancy of the irradiated sample in the region λ 4200 to 5000 (curve 2, figure 1). This destruction of pigment apparently resulted from the reaction of the carotenoids with the dissolved oxygen of the oil during irradiation. No appreciable change in the transmittancy of the irradiated and nonirradiated oil is observable in the region λ 6000 to 7000 in which methylene blue absorbs strongly.

From the nature of the absorption spectra of the systems reproduced in figure 1, it might be inferred that the phenomena observed on irradiation by selected bands of light of tubes A to F containing methylene blue and soybean oil may have resulted from changes in the concentration of dissolved oxygen during irradiation.

Thus, it may be assumed that a reaction occurred between the carotenoids or other unsaturated substances and dissolved oxygen in the soybean oil in tube C when the system was irradiated with blue light, although the methylene blue was apparently unaffected and no reduction in color intensity could be detected. Following the reduction of the concentration of oxygen to a small value as a result of the reaction with the carotenoids or other unsaturated substances of the oil, the methylene blue is apparently reduced to the colorless or leuco form when the system is subsequently irradiated with red light. Furthermore, it would appear from the color reactions observed with tube E, that the reaction between carotenoids or other unsaturated substances and dissolved oxygen does not occur under the influence of irradiation with red light, and consequently no reduction of methylene blue occurs in this case.

To provide further evidence regarding the possible effect of dissolved oxygen on the photocatalyzed reactions of the methylene blue-soybean oil system, additional experiments were carried out in the presence and in the absence of oxygen, but under otherwise similar conditions to those obtaining above.

Effect of radiation and dissolved oxygen: Four 10-ml. portions of the same freshly deodorized soybean oil mentioned in table I were placed in 20-ml. pyrex glass test tubes H to K (see table II). To each sample there was added 1 ml. of 0.05 per cent alcoholic methylene blue and 0.5 ml. of absolute ethyl alcohol. As before, the samples were colored intensely blue by the dye.

Samples H and I were stoppered and stored in the dark. Samples J and K were frozen with liquid air, rolling the tubes at the start so that thin oil films were formed on the walls. The two frozen samples were evacuated under an oil pump for 60 minutes, after which the temperature was allowed to rise gradually until the alcohol boiled, thus insuring the complete removal of dissolved air. The tubes containing the samples were then sealed off while still under vacuum. During all these operations the samples were protected from light. Samples H (containing dissolved air) and J (evacuated) were placed in blue light as described in the experiments recorded in table I. Samples I (containing air) and K (evacuated) were placed in red light. The results of these experiments are summarized in table II. It should be noted that sample K, which contains no oxygen, was promptly reduced under red light. No visible change could be detected in the remaining samples during a period of 50 minutes' exposure under the conditions recorded in table II and shown schematically by equation 2.

Methylene Blue-Soybean Oil (In absence of oxygen)
- Blue Light → No Reduction
- Red Light → Dye Reduced

EQUATION 2

It is evident from these experiments that the reduction of methylene blue occurs only when the system is irradiated with red light (wave length greater than 6300) and, further, that oxygen is an important factor since the dye is reduced only in the absence of oxygen.

The results of all the foregoing irradiation experiments may be summarized as follows: Light in the region λ 3400 to 4400 catalyzes the reaction of carotenoids or unsaturated substances with dissolved oxygen of the oil (photoöxidation) but does not effect the reduction of methylene blue. Irradiation with light of λ 6300 or longer catalyzes the reaction of the reduction of methylene blue but only in the absence of oxygen. Light of this wave length apparently is not able to catalyze the reactions between oxygen and the carotenoids or other unsaturated constituents of the oil. Since the oxygen tension has not been lowered by a prior reaction, the reduction of methylene blue cannot be effected by red light. These results corroborate the assumptions made by the authors (1) in the preceding article of this series and identify the reactions which occur when the methylene blue-soybean oil system is irradiated with sunlight. Furthermore, they are in agreement with similar observations which have been reported by other workers.

For example, Greenbank and Holm (12) found that light λ 3400 to 4200 was the most effective for the production of peroxides in corn oil and that the degree of effective peroxide formation is inversely proportional to the wave length of the light used for irradiation.

Windaus and Brunken (13) have shown that ergosterol undergoes photoöxidation in 95 per cent

[4] Values for various wave lengths (λ) of light are expressed in Angstroms (Å). 10 Å = 1 mμ.

ethyl alcohol containing eosin, erythrosin, methylene blue, chlorophyll, or hematoporphyrin, when the system is irradiated by light from a powerful filament lamp. Ergosterol peroxide is formed under these conditions as was demonstrated by isolation and identification of the reaction product.

Euler and Adler (14) investigated the influence of light on the system alcohol and hexosemonophosphate-dehydrogenase in the presence of methylene blue as a hydrogen acceptor. They found the system to be most effectively activated (greatest velocity of dye reduction) in red light (λ 6450 to 6750) which corresponds to the absorption maximum of methylene blue. Green and blue light had no influence on the reaction velocity. They concluded that the activating effect of light must, therefore, occur through the intervention of the methylene blue.

Lasareff (15) and Gebhard (16) have found that methylene blue in a gelatin plate containing glycerol is reduced much more rapidly during irradiation in an oxygen-free atmosphere than it is when air is present. All of these observations indicate, as stated above, that blue light (spectral region of ca. λ 4700) is quite efficacious in producing photochemical oxidation of fats and related lipides, whereas red light, which is ineffective in photooxidation, is active in catalyzing the reduction of methylene blue in the oil-dye system.

Fluorescence spectra: It is obvious that any photochemical reactions occurring in the oil-methylene blue system must necessarily be accompanied by changes in the energy relations of the involved reactants. That certain energy changes actually do occur will be evident from the following experiments on the fluorescence of methylene blue.

In order to ascertain the fluorescent spectral behavior of the various components of the methylene blue-oil system, spectrograms were made of (1) an alcoholic solution of methylene blue, (2) soybean oil, and (3) soybean oil-methylene blue solution. These spectrograms were made by focusing on the fluorescence tubes the light from a quartz mercury arc filtered through a Wratten K-2 filter, and exposing at right angles to the incident light an Eastman infrared plate in a three-glass prism Steinheil spectrograph. These exposures were 30 minutes in length and were made in the presence of air.

The *unfiltered* spectral lines of the mercury arc are shown in plate I, spectrum 4. This spectrogram was made by focusing the light from the source on the photographic plate for a very brief exposure at low intensity. It is to be noted that the K-2 filter passes only those lines of the mercury arc above λ 5000. The various spectral lines reproduced in plate I are considerably less intense than those which may be observed on the original plate. The loss of clarity is the result of the many operations required to reproduce the plate in the printed form.

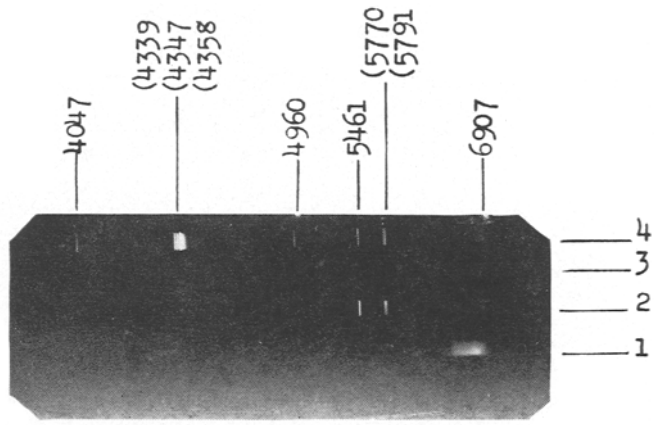


PLATE 1

Fluorescence Spectra

Spectrum 1. Alcoholic methylene blue solution.
Spectrum 2. Soybean oil + alcohol.
Spectrum 3. Soybean oil + alcohol + methylene blue.
Spectrum 4. Unfiltered mercury arc.

Spectrum 1 was made by focusing the light into an absolute ethyl alcohol solution of methylene blue in the manner described above. A strong fluorescence is observed in the region of λ 6500 to 6700. This region represents the strong absorption region of methylene blue (curve 1, figure 1) and is where the strongest fluorescence may be expected to occur.

Spectrum 2 was made by passing the light into refined edible soybean oil containing 1 ml. of absolute ethyl alcohol. In this case the spectral lines of the mercury arc above λ 5000 are transmitted, as evidenced by the appearance of scattered light at λ 5460, λ 5770, λ 5790, and λ 6907. However, no evidence of fluorescence is visible. The green line (λ 5460) and the yellow doublet (λ 5770, λ 5790) appear to have been absorbed by methylene blue in the system represented by spectrogram 1. This behavior is to be expected since methylene blue exhibits an absorption in these regions (figure 1, curve 3).

Spectrum 3 was made by passing the light into a solution of the same soybean oil as was used by spectrum 2, to which there was added alcoholic methylene blue. The concentration of methylene blue was the same as that used in taking spectrum 1. The only line visible in spectrum 3 is λ 6907 of the mercury arc, the green and yellow lines having been absorbed by the methylene blue. In this case it is to be noted that the fluorescence in the region of λ 6500 to 6700, which is clearly visible in spectrum 1, has been quenched.

The significance of this experiment becomes evident when it is considered on the basis of the known behavior of photochemically excited molecules. It is well known that an excited molecule of this type may either emit its energy by fluorescing, or liberate it by virtue of a chemical reaction with other species of molecules

TABLE II.—EFFECT OF DISSOLVED AIR ON THE METHYLENE BLUE-SOYBEAN OIL SYSTEM DURING IRRADIATION

Tube	Sample	Gas present	Irradiation		
			Light	Irradiation time	Color change
				Minutes	
H	Methylene blue-soybean oil	Air	Blue light λ 3400-4400	50	None
I	Methylene blue-soybean oil	Air	Red light > λ 6300	50	None
J	Methylene blue-soybean oil	Evacuated	Blue light λ 3400-4400	50	None
K	Methylene blue-soybean oil	Evacuated	Red light > λ 6300	50	Dye reduced

present in the system. That a chemical reaction of the latter type actually occurs in the irradiated methylene blue-soybean oil system is demonstrated by the fact that the methylene blue fluorescence is quenched, as has been shown in the above experiment.

A similar photochemical behavior has recently been reported by Coe (17), who studied the quenching effect of cottonseed oils on the fluorescence of chlorophyll. Coe assumed that the quenching of the chlorophyll fluorescence was induced by an "unnamed compound" which was presumed to be present in the cottonseed oil. When Coe's observations are reconsidered in the light of the known photochemical behavior of chlorophyll, it is possible to obtain an idea of the probable nature of the compound which is responsible for the quenching of the chlorophyll fluorescence.

As previously mentioned, an excited molecule such as irradiated methylene blue, chlorophyll, or other fluorescent dye, can follow one of the two usual alternative courses; namely, it can transfer its energy of excitation by reacting chemically with another molecule, or it may return to its original ground state and emit its energy as fluorescence.

According to Franck and Wood (18) the only photochemical reactions that can be induced in organic solutions by chlorophyll are photoöxidations. Consequently, the quenching of the chlorophyll fluorescence may take place by oxygen directly attacking the excited chlorophyll, or if oxygen acceptors (RH) are present, as may well be the case with vegetable oils, the chlorophyll (H-Chph) may be adsorbed on these acceptors, forming a complex H-Chph-RH. In the second case, the excited chlorophyll molecule can then transfer its energy of oxidation to the oxygen acceptor (RH) and the H is split off, thus consuming the energy. The remaining adsorption-complex, H-Chph-R, is readily attacked by oxygen.

If, however, no oxygen is present in the reacting system, the resulting adsorption-complex can again absorb light and, since it is protected from collision by the R, the chlorophyll can readily fluoresce since its H is not likely to split off, as can be shown from energy considerations. This condition would result in increased fluorescence instead of a quenching effect. From these considerations it would appear that the "unnamed compound" which Coe presumes to be active in quenching the fluorescence of chlorophyll is the dissolved oxygen in the oil under test.

Thus, a fresh oil rich in free oxygen would quench the flourescence of chlorophyll quite readily. An aged oil in which most of the dissolved oxygen has been consumed in autooxidation would have considerably more difficulty in quenching the fluorescence, which is in agreement with Coe's experimental observations. This conclusion is further confirmed by results reported by Bryson (19), who states that "The phenomena of quenching may be used to detect oxygen. The minutest trace of oxygen (down to pressure of 0.001 mm. of O_2) can be detected by its quenching action on phosphorescent compounds (e. g., chlorophyll)."

It may be assumed that the energy exchange reaction occurring in the oxidation of chlorophyll by irradiation is analogous to the reduction and fading phenomena which occur when the methylene blue-soybean oil system is irradiated. In the latter system the presence of oxygen inhibits the reduction of the methylene blue. Thus, the methylene blue, when activated by irradiation with light of appropriate wave lengths, red light for instance, will fluoresce unless the system contains unsaturated constituents. In the latter case the unsaturated components act as acceptors for the activation energy of the excited dye molecules and produce conditions of reduction which are necessary for the formation of the colorless leuco form of methylene blue.

SUMMARY AND CONCLUSIONS

In a previous communication a stability apparatus was described which employed the principle of methylene blue reduction in a fat-dye system exposed to filtered radiation of a tungsten filament lamp. The effect of such factors as the nature of the substrate, temperature, dissolved gases, aldehydes, and peroxides on the rate of dye reduction in the methylene blue-oil system was investigated. It was tentatively concluded that the method served as a measure of the system's ability to consume its dissolved oxygen.

No evidence was advanced to indicate the function of the light in the dye reduction or the reaction mechanisms by which the final reduction was attained. The experimental observations which have been described and discussed in the present communication have served to confirm the previous tentative conclusion concerning the role of oxygen in the irradiated oil-dye system and have clearly demonstrated the function of the light in the dye reduction.

The results of the present investigation may be summarized as follows:

1. In the methylene blue-soybean oil system, blue light apparently catalyzes a reaction which consumes oxygen without affecting the dye. This reaction probably takes place between the dissolved oxygen and the carotenoid pigments or other unsaturated constituents of the oil.

2. In the presence of soybean oil, methylene blue is reduced in red light when oxygen is absent.

3. In the presence of soybean oil, methylene blue is not reduced in red light when oxygen is present, probably because the reduced dye (leuco base), if formed, is reoxidized.

4. That methylene blue is a fluorescent dye has been clearly demonstrated and its fluorescent spectrum photographed.

5. In the presence of soybean oil, the fluorescence of irradiated methylene blue is quenched, thereby demonstrating that a reaction actually occurs between the activated dye and the oil.

Thus, it is evident that light, oxygen, and unsaturation are co-reactants and must be present simultaneously during the determination of the stability of an oil by the methylene blue method.

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