The Fluorescence of Chlorophyll in Fats in Relation to Rancidity[1]

C. S. FRENCH and W. O. LUNDBERG
University of Minnesota, Minneapolis

Introduction

Chlorophyll dissolved in organic solvents exhibits a deep red fluorescence when irradiated with ultraviolet or visible light. If an oil with an intense white fluorescence is gradually added to such a solution under ultraviolet illumination the red color of the chlorophyll fluorescence changes through various shades of pink until the chlorophyll fluorescence is supplanted by the white fluorescence of the oil itself. This effect was observed and developed by Coe [2] into a test for oxidative rancidity in fats. He reported that the more nearly rancid the fat the more chlorophyll was required to bring the mixture to a certain pink hue.

Briefly, the procedure in Coe's test is to titrate under ultraviolet illumination 0.25 ml. of a solution of chlorophyll (1.5 g/l) in non-fluorescing mineral oil with the oil or fat to be tested until the pink color due to chlorophyll fluorescence just disappears. The volume of oil required to produce a color match with an arbitrary standard is termed the "chlorophyll value" and is taken to be a measure of the keeping quality of the oil.

Coe theorized that the striking color change is due to the "quenching" [3] of the chlorophyll fluorescence by a transfer of excitational energy from chlorophyll molecules to acceptor molecules contained in the fat. The acceptor substance is apparently considered to bear some close relation to the keeping quality of the fat, presumably in the role of antioxidant. The quenching reaction is, moreover, considered by that author to be stoichiometric; hence the observance of an "endpoint" at which the chlorophyll fluorescence just disappears. The data published by Coe deal exclusively with cottonseed oils, but apparently the method and theory are considered to be applicable to other oils and fats.

Attempts in this laboratory to utilize the test in measuring the keeping qualities of lards were unsuccessful. Further experiments were therefore performed using lard and cottonseed oil, in an effort to clarify the principles underlying the method. It became immediately apparent that the variations in chlorophyll values in each of the oils studied depended greatly on two factors which seem to have been ignored by Coe in his discussion. When an oil is added to a chlorophyll solution under ultraviolet irradiation, the disappearance of the chlorophyll fluorescence as observed by eye is largely governed by (1) the magnitude of the masking effect exerted by the natural blue-white fluorescence of the oil itself, and (2) the extent of the absorption of the irradiating wavelength by the oil thus reducing the amount of light absorbed by the chlorophyll. These two factors, rather than chemical quenching by a reactive substance present, seem to be mainly responsible for the observed fluorescence color changes in chlorophyll solutions to which oils are added.

Equipment and Reagents

The source of radiation used to produce fluorescence throughout these experiments was a type H4 high pressure mercury vapor lamp equipped with appropriate glass lenses and filters to produce substantially monochromatic radiation of the desired wavelengths.

Some of the fluorescence observations were made with the naked eye and others were made through a Corning Signal Red No. 241 or a Jena RG2 filter, both of which were opaque to the exciting radiation, but transmitted freely the red fluorescence of chlorophyll. For quantitative comparisons of chlorophyll fluorescence, the fluorescent light was passed through the Corning Signal Red filter to a red sensitive photocell (RCA 917). The photocell current was amplified by a D.C. single stage amplifier and read on a box galvanometer.

The data on the transmission of light by the oils and fats studied were obtained with a Coleman Universal Spectrophotometer using distilled water as a control. When lard was used it was measured at 50°C.

The chlorophyll "solution" was prepared by dissolving 0.3 g. of chlorophyll, American Chlorophyll Co. 5 ×) in 50 cc. of diethyl ether, filtering, and adding the filtrate to 200 cc. of a high quality medium viscosity mineral oil. The ether was removed with the aid of a vacuum pump, leaving the chlorophyll partly in solution and partly as a fine suspension in the mineral oil. This was supposed to duplicate Coe's chlorophyll "solution A," but some of our results suggest that it contained considerably more chlorophyll than the solution used by him. Even when filtered through a double layer of filter paper to remove most of the suspended chlorophyll, our results were not appreciably altered.

In some of the experiments the "A" (1.5 g/l) solution was found to be too concentrated and a second solution, "A/10," was prepared by diluting a portion of the unfiltered "A" solution to 0.15 g/l with mineral oil.

The two organoleptically fresh cottonseed oils used were ordinary commercial grades whose history was unknown. The fresh lard used in most of the experiments was a specially prepared sample with unusually good keeping qualities. A sample of an ordinary commercial steam rendered lard was also used in a few experiments. The rancid samples of cottonseed oil and

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[2] Coe, M. R., Oil & Soap *18*, 227 (1941).

[3] It has been emphasized by Franck and Livingston (J. Chem. Phys. *9*, 184, 1941) that chlorophyll belongs to the class of fluorescent dyes that do not necessarily have their fluorescence quenched appreciably by the addition of reducing substances that are capable of sensitized photochemical oxidation. They state (Footnote 15, p. 188, referring to Franck and Levi, Z. f. physik. Chemie B27, 409, 1934), "This experimental result has been confirmed by the present authors (unpublished work) who found that the fluorescent yield of chlorophyll in a 10^{-5} M solution saturated with air and containing allylthiourea at a concentration of 0.5 M was 80 per cent (or 85 per cent) of the yield observed in a 10^{-5} M solution in pure air-free solvent (acetone)." Allylthiourea has been found by Gaffron (Ber. *60*, 755, 1927) to be oxidized photochemically by chlorophyll with a quantum yield of one.

lard were prepared by simply heating portions of the fresh samples on a steam bath for several days in the presence of air.

Experiments and Discussion

If light activated chlorophyll molecules reacted with acceptor molecules in the fat and lost their energy in this way rather than by fluorescing there might possibly be a stoichiometric relation (as Coe assumes) resulting in very little chlorophyll fluorescence until the concentration of chlorophyll was greater than the equivalent concentration of the acceptor molecules. From this point on as the chlorophyll concentration was increased there should then be a red fluorescence whose intensity would depend on the quantity of extra chlorophyll added. To test this idea the fluorescence intensity was followed both visually and with a photocell, as chlorophyll was added slowly to various oil samples. If there is an "endpoint" or critical ratio of chlorophyll concentration to oil concentration it should be observed equally well by varying either the amount of chlorophyll added to a definite quantity of oil or, as Coe prefers, by adding the oil to a definite quantity of chlorophyll solution. In neither case was any discontinuity found in plotting chlorophyll fluorescence against concentration (Fig. 1) nor was any critical concentration of either substance noticed beyond which the yield of fluorescence changed sharply.

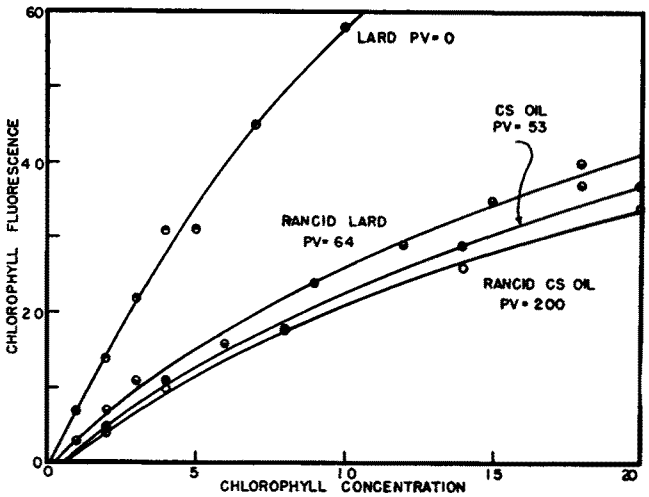

FIG. 1. The intensity of chlorophyll fluorescence in various fats as a function of chlorophyll concentration. The fluorescent light was measured by a photocell behind a red filter while chlorophyll in mineral oil was added dropwise. The concentration is given in drops of a solution (containing 1.5 g. of chlorophyll per liter) added to 20 cc. of the fat.

1. *The fluorescence of chlorophyll in fresh and rancid lards.*

Five cc. each of fresh (No. 1) and rancid (No. 2) lards, having peroxide values of 0.2 and 64 milliequivalents of peroxide per kilogram, respectively, were compared at about 50°C. (to keep the lard fluid) in porcelain crucibles under ultraviolet radiation of 365 mμ. Chlorophyll solution "A/10" was added dropwise with thorough mixing to each of the samples and the changes in their fluorescence were followed visually with and without the aid of a red filter.

Without any added chlorophyll, No. 1 fluoresced a strong blue white and No. 2 a dull blue gray. Through the red filter a very slight fluorescence was observable in No. 1 and none in No. 2; the addition of one drop (about .025 cc.) of chlorophyll solution "A/10" to No. 2 was sufficient to produce the same intensity of red fluorescence through the red filter as was observed in No. 1 without any added chlorophyll. A very slight pink was discernible by the naked eye in both samples after two more drops had been added to each. This changed to a salmon pink after 10 drops and to a brick red after 25 drops. However, although the colors were the same, No. 1 appeared to be brighter than No. 2, probably because of the brighter fluorescence of the fresh lard itself.

To the naked eye, therefore, the fluorescence of chlorophyll in the two lards appeared to be about the same for equal amounts of added chlorophyll. The effect of the higher transmission by No. 1, which caused greater chlorophyll fluorescence, was cancelled by the greater natural fluorescence of that oil sample, thus causing the mixed red and white fluorescence shade of both to appear about the same.

Observations through the red filter yielded quite different results. Accurate comparisons were difficult because through the filter the red fluorescence appeared quite intense. But, roughly, about 25 drops of "A/10" chlorophyll solution were required to make No. 2 fluoresce with the same intensity as No. 1 containing only 3 drops.

Thus when the solution is observed through a red filter just the reverse of Coe's effect was found. Here less of the rancid fat than of the fresh fat was re-

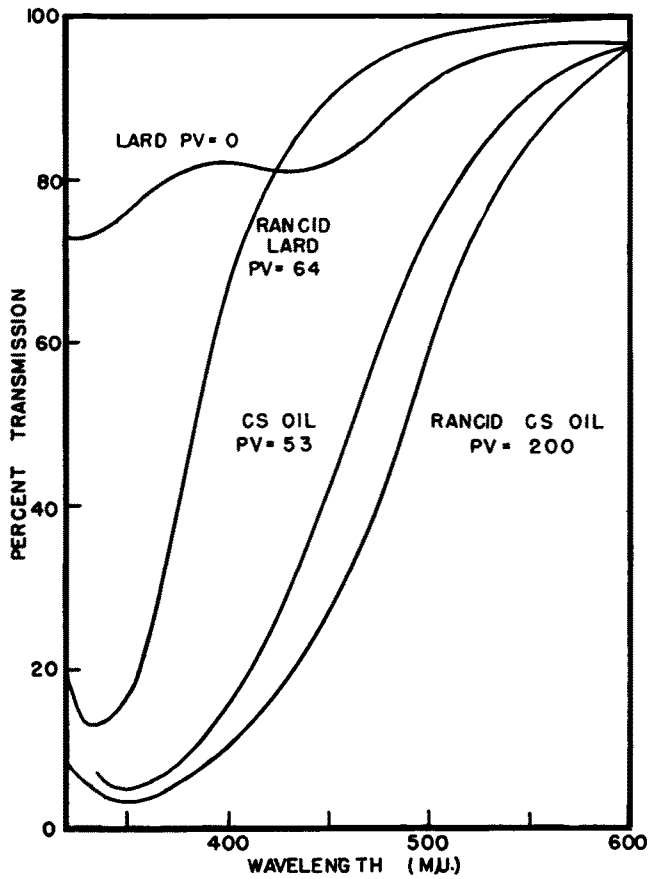

FIG. 2. Approximate transmission curves of the different fats used. Since different filters for the removal of stray light were used above and below 400 mμ the data gave discontinuous curves (except for the fresh lard) at that point. The points are therefore not shown and the curves were drawn so as to average out this discrepancy.

quired to reduce the chlorophyll fluorescence to the intensity match point.

To obtain quantitative measurements, the previous experiment was repeated using the photocell to measure the fluorescence coming through the Signal Red filter from the front of a $5 \times 5 \times 1$ cm. glass cell. The undiluted solution "A" was added dropwise to 20 cc. samples of No. 1 and No. 2 contained in glass cells, and galvanometer readings were made. No effort was made to maintain a constant temperature and occasionally, when the lard samples began to solidify it was necessary to reheat them, causing slight variations in the galvanometer readings. The data are reproduced in the two upper curves of Figure 1. There is no evidence whatever of any abrupt change of slope of the curve which would correspond to an endpoint in the titration of the oil with chlorophyll. The different intensities of the chlorophyll fluorescence in the various oils can be attributed to the different absorption of the oils themselves for the wavelength 365 mμ used. This conclusion is substantiated at least qualitatively by the measurements of the transmission of the different oils presented in Figure 2 and Table 1. There it is seen that the fresh lard has a high transmission and gives a correspondingly high value of fluorescence from chlorophyll dissolved in it since it does not itself absorb much of the incident light, thus allowing the latter to be absorbed by the chlorophyll and resulting in a high red fluorescence intensity.

TABLE I

The Fluorescence of Chlorophyll and of Fats in Relation to the Peroxide Value and the Light Absorption of the Fat

1	2	3	4	5
Sample	% Transmission of 365 mμ (1 cm. cell)	Fat Fluorescence (Galvanometer Reading)	Drops of "A/10" chlorophyll solution required to make pink color appear. (Observed without filter)	Drops of "A" required to give 30 mm. deflection, Signal Red Filter
No. 1. Fresh lard P.V. = 0.2	79%	4	2	4.5
No. 2. Rancid lard P.V. = 64	24	1.5	3	12.5
No. 3. Cottonseed oil P.V. = 2.2	9	....	20	....
No. 4. Cottonseed oil P.V. = 53	6	14	26	14.5
No. 5. Rancid cottonseed oil P.V. = 200	4	12	18	16.5
Fresh steam lard P.V. = 2.0	....	....	6	....
Pump oil	....	81	....	....
Untreated corn oil	....	24	....	....
Rancidified corn oil	....	11	....	....

A sample of fresh steam lard with a peroxide value of about 2.0, but with much poorer keeping quality than sample No. 1, was observed under ultraviolet radiation of 365 mμ without a filter. Somewhat more chlorophyll "A/10" was necessary than in sample No. 1 to make a slight pink fluorescence appear. The natural fluorescence of this sample was about the same as that of No. 1, but its transmission of 365 mμ was lower.

The differences between the chlorophyll fluorescence observed in the fresh special lard and fresh steam lard therefore appears to be accounted for by the same two factors, and the great difference in their keeping qualities appears to have played no role.

2. *The fluorescence of chlorophyll in fresh and rancid cottonseed oils.*

A.) Following the directions given by Coe cottonseed oil was added to 0.25 cc. of the chlorophyll solution A in mineral oil in a porcelain evaporating dish and observed with light from a type H4 mercury lamp with a glass lens and a Woods glass filter presumably producing mainly light of a wavelength of 365 mμ. The first few drops of oil changed the deep red color to a pink that then turned to white as the oil was added. The endpoint was rather indefinite, requiring about 2.8 cc. of the untreated cottonseed oil, peroxide value, 48, and 2.6 cc. of the rancidified oil of peroxide value 201, a difference smaller than the indefiniteness of the match point. There would seem to be no benefit (except as to reproducability in any one set of replicate titrations) in Coe's procedure of making up an arbitrary endpoint standard for each oil and titrating until it is exactly matched. When the pink color had disappeared from the bulk of the solution it was faintly seen around the edges of the solution or in a thin film spread on the side of the dish. This was taken to indicate that the chlorophyll at least in the outside thin layer still fluoresced in this solution, but its color was more than overbalanced by the bright white fluorescence in the main body of the solution. In this thin layer there was comparatively less of the white fluorescence from the oil than where the layer was deeper.

B.) Samples No. 3, No. 4, and No. 5 were cottonseed oils having peroxide values of 2.2, 53, and 200, respectively. The first two were organoleptically fresh and the last was rancid. All three were observed visually under ultraviolet radiation of 365 mμ in the same manner as the lards but at room temperature. Observed without a filter, they required the addition of about 20, 26, and 18 drops of chlorophyll solution "A/10," respectively, before a slight pink color could be seen. No sharp endpoint was discernible and the further addition of chlorophyll merely increased the pink color gradually. About 100 drops of "A/10" were required to give a salmon pink color.

Using the Signal Red filter, it was found that all three samples fluoresced slightly red without any added chlorophyll, and the intensity of this red color increased progressively from the beginning with the addition of each drop of chlorophyll solution. As with the lards, comparisons were difficult because of the apparent intensity of the red color, but equal amounts of added chlorophyll produced about equal intensities of red fluorescence in all three samples.

In the case of the cottonseed oils, the chlorophyll fluorescence is not visible to the naked eye until considerably greater amounts of chlorophyll have been added than in the lards (see Table I). This result is explained by both the greater natural fluorescence and the poorer transmission of the irradiating light in the cottonseed oils.

Some brilliantly fluorescent chlorophyll in mineral oil was observed with the above ultraviolet lamp. A 1 cm. layer of cottonseed oil was then placed between the lamp and the chlorophyll. It absorbed all the light so that no red fluorescence whatever was obtained from the chlorophyll. When the 1 cm. layer of cottonseed oil was diluted 20 times with an alcohol ether

mixture it let light through so that bright chlorophyll fluorescence was to be seen.

The transmissions of samples No. 3, No. 4, and No. 5 compared with distilled water in 1 cm. cells at room temperature and for 365 mμ are recorded in Table I. Because of the low transmission of these samples, the data for them are only approximations.

In comparing cottonseed oils No. 4 and No. 5 it is found that their own natural fluorescence is about the same and their transmissions are nearly equal; consequently, assuming that no other important factors are involved, the chlorophyll fluorescence should be similar in both of them when observed with or without a red filter. This was found to be the case. The slight differences are accounted for, of course, by the small variations in fat fluorescence and transmission of light in the two samples.

Measurements were also made on No. 4 and No. 5 using the Signal Red filter and the photocell. The results are contained in the two lower curves of Figure 1.

3. *The fluorescence of the fats themselves.*

Since we believe that the fluorescence of the oils themselves (or some substances present as impurities) have a large effect in determining the amount of oil necessary to mask the chlorophyll fluorescence the relative intensities of the fluorescence from several of the oils used in the other experiments was measured. This was done by illuminating a $5 \times 5 \times 1$ cm. glass absorption vessel with light of wavelengths of 407, 405, and 365 mμ from a mercury arc and measuring the illumination falling on a photocell placed near the front face but having a Noviol filter placed in front of it so that none of the exciting light fell on the photocell. This data is given in Table 1, column 3.

4. *The fluorescence of chlorophyll and cottonseed oil mixtures in blue light and in ultraviolet.*

Since the ultraviolet is so strongly absorbed by the cottonseed oil, blue light was used for further studies of the effect of cottonseed oil on chlorophyll fluorescence. The light from the same lamp was filtered through Corning filter No. 038 and through a blue glass thus giving monochromatic radiation of wavelength 436 mμ. Very weak fluorescence of the chlorophyll solution in mineral oil was observed when observed in this light. With a red filter in front of the eye the fluorescence was barely perceptible. Rancidified cottonseed oil was then gradually added until 2.5 cc. was present. During the addition the red fluorescence became progressively more brilliant. This increase in fluorescence is attributed merely to the further solution by the cottonseed oil of some of the finely dispersed chlorophyll particles present in the mineral oil. After 2.5 cc. of the cottonseed oil had been added the mixture was observed in light of 365 mμ which gave a white fluorescence around the edges and where the solution had been spread over the surface of the porcelain dish. It was returned to the blue light and more oil added. The mixture still showed red fluorescence even with 12 cc. oil, four times the amount needed to "quench" the fluorescence when observed with ultraviolet. A control diluted with mineral oil showed less fluorescence.

The dish with diluted chlorophyll in mineral oil showed bright red fluorescence in ultraviolet light but was weak with blue light. That with the cottonseed oil was brilliant white in ultraviolet but red in blue light. These experiments may be taken to mean that the apparent fluorescence quenching is not a chemical reaction but an optical phenomenon dependent on the internal filter action of the oil and on its own fluorescence.

5. *Intensity measurements of the red fluorescence of chlorophyll with or without cottonseed oil.*

To provide a more critical test for the question of fluorescence quenching approximate relative measurements of the intensity of the red chlorophyll fluorescence in a more dilute mineral oil solution were made and then the cottonseed oil was added to it. Green and blue light together were required to produce sufficient intensity for the measurement and avoid any appreciable absorption by the cottonseed oil. The addition of this oil caused a small increase rather than a quenching of the red chlorophyll fluorescence. This experiment appears also to be sufficiently definite evidence for the rejection of the quenching theory at least in the case of the sample of cottonseed oil used in our experiments.

The details of the procedure were as follows: The same mercury lamp was used with a Jena GG 5 light yellow filter and 5 cm. of nearly saturated $CuSO_4$ to remove all red light. This light of wavelengths (578), 546, (491) and 436 mμ fell on a test tube holding the chlorophyll solution. Some of the red fluorescent light emitted from the solution passed through a Corning signal red filter No. 241 which removed practically all the reflected and scattered blue and green exciting light from the beam which fell on the red sensitive photocell. About 5 cc. of a diluted solution of our chlorophyll in mineral oil gave a galvanometer deflection due to its red fluorescence of 9 mm. A few drops of cottonseed oil increased this fluorescence to 15 mm. though a control tube of the cottonseed oil gave no measurable red fluorescence under these conditions but did show a red fluorescence much weaker than the chlorophyll solution, when examined visually through a red filter. Another solution of the chlorophyll in mineral oil diluted to about 5 cc. with alcohol-ether mixture gave a galvanometer deflection of 12 mm. due to its fluorescence and increased up to 20 mm. when about 2 cc. of cottonseed oil was added with shaking (the solution separated into two phases).

6. *Quantitative correlation of chlorophyll and fat fluorescence and light absorption with the "chlorophyll number."*

Attempts were made to relate the concentration of chlorophyll necessary to give a barely perceptible pink color with the fat absorption coefficient, relative fat fluorescence, and chlorophyll absorption coefficient. However because of the variation of the fluorescence color of the different oils, reabsorption of the red chlorophyll fluorescence by dissolved chlorophyll and the variable thickness of the solution layers we were unable to treat the present data in a quantitative way.

It is a pleasure to thank Professor George O. Burr for the suggestion of the problem and for much helpful advice. For the construction of the amplifier we are indebted to Mr. Caldwell of the Physics Department. We also wish to thank Professor Bryce Crawford of the Chemistry Department for his help in deriving an equation relating fat fluorescence and

chlorophyll fluorescence with the absorption coefficients of the constituents of the mixture and with its thickness.

Summary

1.) Difficulties in applying the "chlorophyll value" test to fat samples has led us to investigate the apparent "quenching" of chlorophyll fluorescence in mineral oil solution when cottonseed oil or lard is added to it. The disappearance of chlorophyll fluorescence in ultraviolet light caused by the addition of cottonseed oil appears to be due to the absorption of the light by the cottonseed oil and to the intense white fluorescence of the oil itself rather than to a chemical reaction of some constituent of the oil with the excited chlorophyll.

2.) There was no evidence of a stoichiometric quenching reaction between chlorophyll and acceptor substances in the fats used in this study and, in consequence, no "endpoint" was observed in any of the titrations.

3.) A lack of correlation between either the peroxide value or the stabilities measured in conventional ways and the amount of chlorophyll fluorescence of several fats makes the "chlorophyll value" test appear to have doubtful value as a generally applicable test for fat rancidity or stability.

4.) The crude absorption curves here presented suggest that the greater absorption of near ultraviolet light by oxidized fats may be related to their content of fat peroxides.

Qualitative Test for Bicarbonate Ion In Soap Products

EUGENE W. BLANK and LILLIAN E. UTTER
Research and Development Department, Colgate-Palmolive-Peet Co.
Jersey City, N. J.

The usual qualitative tests available for ascertaining the presence of bicarbonate in a mixture of carbonate and bicarbonate yield ambiguous results in the presence of alkaline salts such as sodium pyrophosphate, silicate and others.

Mellor (1) suggests four tests for detecting the bicarbonate ion, one of which gave promising results in preliminary experiments. The test described in this paper is based upon the fact that bicarbonates will liberate hypochlorous acid from hypochlorites. In the presence of potassium bromide, bromine will be liberated by the hypochlorous acid and can be extracted by chloroform or carbon tetrachloride. The depth of color obtained gives an approximation of the amount of bicarbonate present.

Reagents

(a) *Hypochlorite Solution.* Dissolve 50 grams of chlorinated lime, U. S.P. XII reagent grade, in 500 ml. of water. An appreciable residue will remain. Stir vigorously at intervals and after an hour or longer filter into a 1000-ml. volumetric flask. Make to volume with water at room temperature, and mix. This solution keeps well if stored in a cool, dark place.

Instead of the above, 20 to 25 grams of true calcium hypochlorite may be dissolved directly in water in a 1000-ml. flask, made up to volume at room temperature, and mixed.

(b) *Potassium Bromide Solution.* Dissolve 75 grams of potassium bromide in 500 ml. of water. Filter into a 1000-ml. volumetric flask and bring to volume with water at room temperature.

Procedure

The test should be run on alcohol-insoluble material obtained in the usual manner (2) except that the alcohol-insoluble material need only be air dried by drawing air through the filter.

Transfer 25 ml. of the hypochlorite solution and 25 ml. of the potassium bromide solution to a 60 ml. cylindrical separatory funnel. Add 5 ml. of carbon tetrachloride. Add 0.5 to 5 grams of the alcohol-insoluble material (depending upon the amount of $NaHCO_3$ expected to be present) in small quantities at a time.

Stopper the separatory funnel and shake vigorously. Allow the carbon tetrachloride to settle and observe for any brown or pink coloration. View against white paper as a background.

Liberation of bromine with subsequent coloration of the carbon tetrachloride layer indicates the presence of sodium bicarbonate.

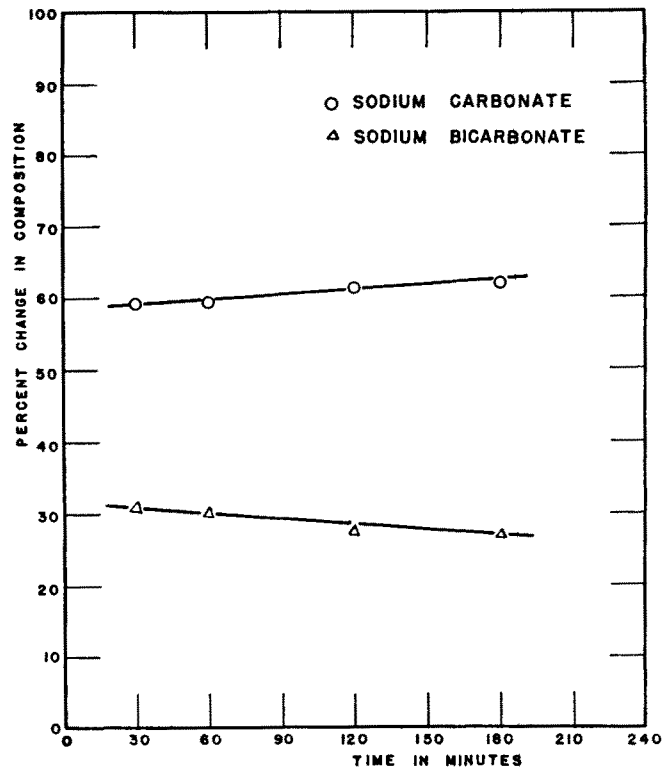

FIG. 1. Change in composition of a mixture of sodium bicarbonate and sodium carbonate in ethyl alcohol at the boiling point.