[CONTRIBUTION FROM THE BAKER CHEMICAL AND ROCKEFELLER PHYSICAL LABORATORIES OF CORNELL UNIVERSITY]

THE ABSORPTION SPECTRA OF FLUORESCEIN, FLUORAN AND SOME RELATED COMPOUNDS[1]

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The following investigation on the absorption of fluorescein was undertaken in the course of our study of triphenylmethane derivatives.[3] The absorption of fluorescein has been studied extensively in the past but the recorded data show wide variations. In the earlier work,[4] only solutions of alkali salts of fluorescein were employed. Meyer and Fischer[5] prepared a violet solution of fluorescein by boiling it with a concentrated solution of alkali. They called attention to the similarity of its absorption spectrum with that of phenolphthalein in dilute alkaline solution. Medhi and Watson[6] and Howe[7] compared the absorption of neutral and alkaline solutions of fluorescein in the visible and ultraviolet regions, respectively. Howe also reported on the absorption of fluoran in neutral and alkaline alcoholic solutions. Moir[8] studied the visible absorption of fluorescein in concd. sulfuric acid solution. Other reports on the absorption in the visible region were made by Nichols and Merritt,[9] Formánek and Knop[10] and Holmes.[11]

The lack of concordant results can be ascribed to two causes: (1) using impure materials, such as commercial preparations or extracts made directly from the fusion mixture; (2) neglecting to mention concentrations, both of fluorescein and alkali in the case of alkaline solutions, for

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[1] The assistance of a grant made to the first two authors from the Heckscher Research Foundation of Cornell University which enabled us to make the measurements described in this report is gratefully acknowledged.

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[3] (a) Orndorff, Gibbs and McNulty, THIS JOURNAL, **47**, 2767 (1925); (b) Orndorff Gibbs and Shapiro, *ibid.*, **48**, 1327 (1926); (c) Orndorff, Gibbs and McNulty, *ibid.*, **48**, 1994 (1926); (d) Orndorff, Gibbs, McNulty and Shapiro, *ibid.*, **49**, 1541 (1927).

[4] (a) G. Krüss, *Ber.*, **18**, 1426 (1885); (b) *Z. physik. Chem.*, **2**, 312 (1888); (c) Vogel, *Wied. Ann.*, **43**, 449 (1891); (d) P. Krüss, *Z. physik. Chem.*, **51**, 257 (1905); (e) Meyer and Marx, *Ber.*, **40**, 3603 (1907); (f) Stark and Meyer, *Physik. Z.*, **8**, 248 (1907); (g) Meyer and Marx, *Ber.*, **41**, 2446 (1908); (h) Meyer and Fischer, *Ber.*, **44**, 1944 (1911); (i) Massol and Faucon, *Bull. soc. chim.*, **13**, 217 (1913).

[5] Meyer and Fischer, *Ber.*, **46**, 70 (1913).

[6] Medhi and Watson, *J. Chem. Soc.*, **107**, 1579 (1915).

[7] Howe, *Phys. Rev.*, **8**, 674 (1916).

[8] Moir, *Trans. Roy. Soc. So. Africa*, **7**, 5 (1918).

[9] Nichols and Merritt, THIS JOURNAL, **36**, 707 (1914).

[10] Formánek and Knop, *Z. anal. Chem.*, **56**, 273 (1917).

[11] Holmes, THIS JOURNAL, **46**, 2770 (1924).

not only is the transition from the neutral state to the alkaline state a gradual one but also, as has been found in this Laboratory, hydrolysis effects are very marked.

The fluorescein used in this investigation was an analytically pure sample prepared by Dr. A. J. Hemmer under the direction of the senior author. He has shown[12] definitely that fluorescein exists in only two physical modifications—red and yellow. Various attempts were made to determine spectroscopically whether these might be chemically distinct individuals, that is, the red form, quinoid, and the yellow, lactoid. However, the result of examining both forms in a number of solvents showed conclusively that they give identical absorption and that both exist in solution in the quinoid state. This last conclusion is based on the fact that compounds closely related to fluorescein but known definitely to possess the lactoid structure, such as fluoran, dichlorofluoran and fluorescein diacetate, show a characteristic absorption which is entirely different from that of fluorescein in neutral alcoholic solution. Again, fluorescein crystallizes from a pyridine solution in the form of bright yellow crystals, but a pyridine solution shows exactly the same type of absorption bands, up to its limit of transmission at about frequency number 3000, as a neutral alcohol solution, which deposits red crystals on concentration.

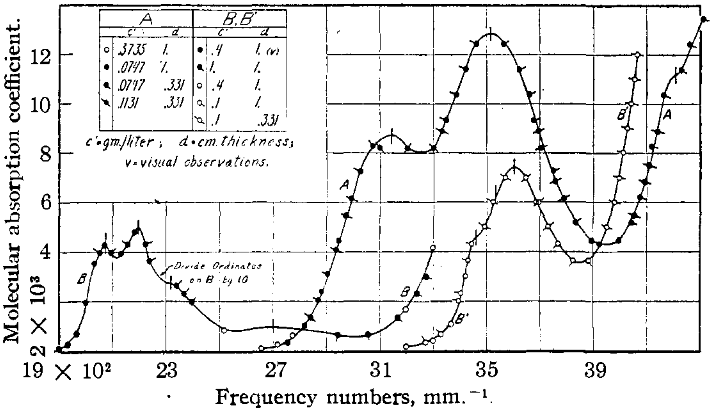

Fig. 1.—Neutral absolute alcohol solution: A, 2,4-dihydroxy-*o*-benzoylbenzoic acid; B,B', fluorescein.

As crystals large enough to use for observing their absorption spectrum could not be obtained, some experiments were made on the selective reflection of the powdered red and yellow forms. The results, while indicating some differences between the two modifications of fluorescein, do not seem sufficiently decisive to warrant presentation here in detail.

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[12] Orndorff and Hemmer, THIS JOURNAL, 49, 1272 (1927).

Fig. 1 shows the absorption in neutral absolute alcohol of fluorescein and of 2,4-dihydroxybenzoyl-*o*-benzoic acid, the intermediate product in the formation of fluorescein by the condensation of phthalic anhydride and resorcinol. 2,4-Dihydroxybenzoyl-*o*-benzoic acid, Curve A, has two bands at frequency numbers 3144 and 3515, and a shoulder at 4208. There is no apparent similarity to the absorption of fluorescein, despite the synthetic relation between the two compounds. However, the authors expect to make a further study of the intermediate acids, which are diphenylmethane derivatives, for the purpose of comparison with the corresponding phthaleins, which in turn are derivatives of triphenylmethane. The absorption spectrum of fluorescein, curve B,B′, shows two bands in the visible region at frequency numbers 2075 and 2198 and two bands in the near ultraviolet at 2319 and 2700. These four bands are similar in shape to the first four bands in resorcinolbenzein,[3b] although the intensity of these bands in fluorescein is only one-twentieth of that for the corresponding bands in resorcinolbenzein. In the further ultraviolet, fluorescein possesses bands at 3460, 3533 and 3604, but in this region there appears to be no close resemblance to resorcinolbenzein.

Some observations on the absorption of fluorescein in neutral aqueous solution were made, although no quantitative measurements on the absorption coefficient were attempted because of the extremely low solubility of fluorescein in water. A saturated solution, prepared by allowing ammonia-free distilled water to stand over finely divided fluorescein in a quartz[13] flask, showed two bands in the visible at 2090 and 2203, with several more in the ultraviolet which could not be accurately located because of the high transmissivity of the solution in this region. However, the general character of the absorption is entirely similar to that in alcoholic solution. In this respect fluorescein does not behave like some of the colored sulfonephthaleins,[14] which give different types of absorption in neutral alcohol and water solutions. It is a well known fact that, when these sulfonephthaleins are used as indicators, the color changes do not occur at the same values of P_{H} in alcoholic and aqueous solutions.

Fig. 2 shows the absorption of fluoran, 4,4′-dichlorofluoran and fluorescein diacetate in neutral alcoholic solution. The three substances show very similar absorption and, though they all contain the pyrone ring, there appears to be a very close resemblance to the absorption of diphenylphthalide, phenolphthalein (*p p*′-dihydroxydiphenylphthalide) and isophenolphthalein (*o,p*′-dihydroxydiphenylphthalide).[3c] Since fluoran may be regarded as anhydro *o,o*′-dihydroxydiphenylphthalide, this

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[13] Glass vessels yield sufficient alkali to water on prolonged standing to bring out the fluorescence characteristic of alkaline solutions of fluorescein and to change the absorption markedly.

[14] Unpublished data.

relation was to be expected. However, the introduction of hydroxyl groups meta to the pyrone oxygen (and apparently in the meta position only) radically changes the character of the absorption, as is shown by fluorescein in Fig. 1. Hydroquinolphthalein, in which the hydroxyl groups are para to the pyrone oxygen, is a colorless compound and its absorption[14] in neutral solution is closely related to that of fluoran. The frequency numbers of the absorption bands of fluorescein and the fluoran derivatives are given in Table I.

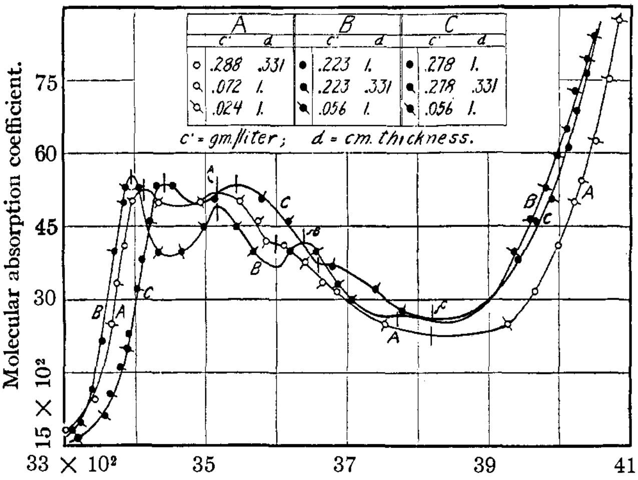

Fig. 2.—Neutral absolute alcohol solution: A, fluoran; B, 4,4'-dichloro fluoran; C, fluorescein diacetate.

TABLE I

FREQUENCY NUMBERS OF BANDS IN NEUTRAL ALCOHOLIC SOLUTIONS

Fluorescein	Dihydroxy-benzoyl-o-benzoic acid	Fluoran	4,4'-Dichloro-fluoran	Fluorescein diacetate
2075	....	....	....	....
2198	....	....	....	....
2319	....	....	....	....
2700	....	....	....	....
3460	3144	3413	3394	3441
3533	3515	3517	3516	3544
3604	4208	3597	3638	3660
....	....	3818	3771	3820

When either the red or yellow forms of fluorescein are dissolved in 75% formic acid, only the yellow form is obtained on crystallization. The absorption of such a solution, Curve C, Fig. 3, is, however, entirely similar to the absorption of fluorescein in concentrated sulfuric acid, Curve B, Fig. 3. The conclusion to be drawn is that the fluorescein while in solution is present in both cases as the salt of the respective acids. The sulfate of fluorescein has been isolated from a 35% sulfuric acid solution by A. J. Hemmer.[15] Formic acid solutions on concentration, however, yield not the formate but fluorescein itself. The absorption of fluorescein in concd. sulfuric acid is characterized by a strong band at 2317 and a prominent double band in the ultraviolet at 3906 and 4005, with weaker bands at 3039, 3215 and 3541. It is entirely analogous to the absorption of resorcinolbenzein[3b] in concd. sulfuric acid. Fluoran dissolves in concd. sulfuric acid to form a yellow solution with a faint green fluorescence. In the visible region, Curve A, there are three weak bands at 2135, 2239 and 2343, while in the ultraviolet there is a prominent band at 2646 with a shoulder on its short wave length side at 2765 and in the further ultraviolet a shoulder at 3605, a very intense band at 3862 and a shoulder at 3945. It is possible to account for this difference in the absorption of fluoran and fluorescein in sulfuric acid solution by assuming that in the former the pyrone oxygen carries the basic properties while in the latter the quinoid oxygen has the greater basicity.

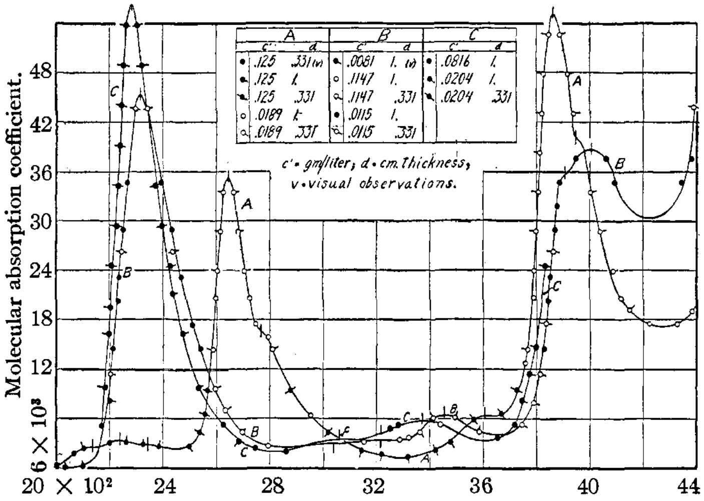

Fig. 3.—Concentrated (93%) sulfuric acid solution: A, fluoran; B, fluorescein. 75% formic acid solution: C, fluorescein.

Fig. 4 shows the effect on the absorption of fluorescein due to the addition of various amounts of dry hydrogen chloride gas to the neutral alcoholic solution. With four molecules of hydrogen chloride to one of fluorescein, Curve A, there is an increase of absorption at frequency number 2254 while the band at 2085 has become much weaker, the remainder of the spectrum being still very similar to that of neutral fluorescein. In-

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[15] Hemmer, *Dissertation*, Cornell University, 1926.

creasing the hydrogen chloride content to 155 molecules, Curve B, and to 960 molecules, Curve C, completely changes the character of the absorption, the bands for the latter solution being located at 2258, 3240, 3349, 3790 and 4018. The latter two bands appear to be approaching the type of double band found in the concd. sulfuric acid solution at 3906 and 4005, a behavior analogous to that of resorcinolbenzein under the same conditions,[3b] thus giving evidence of the presence of a hydrochloride of fluorescein, which has been isolated and analyzed by Gattermann.[16]

Fluorescein, when dissolved in alcoholic alkali, exhibits the phenomenon that we have previously termed reversion,[3b] an hydrolysis-like effect; that is, at low concentrations of alkali, dilution of the solution causes the absorption to revert from the characteristic alkaline type to the neutral type. To avoid this complication, we have chosen to measure the absorption of the alkaline solutions at constant concentrations of fluorescein, but at different thicknesses. In so far as possible, the concentrations were kept approximately the same for the various ratios of potassium hydroxide employed in order to follow better the progressive change in absorption with increase of alkali. In Fig. 5, Curve A for one molecule of potassium hydroxide to one of fluorescein shows the same bands as fluorescein in neutral solution except that the visible bands at 2075, 2199 and 2318 are more than ten times as intense. There is also a new band at 3174. In Curve B for three molecules of potassium hydroxide, and Curve C for twenty molecules, the absorption of the dipotassium salt of fluorescein is more clearly brought out, the latter curve showing bands

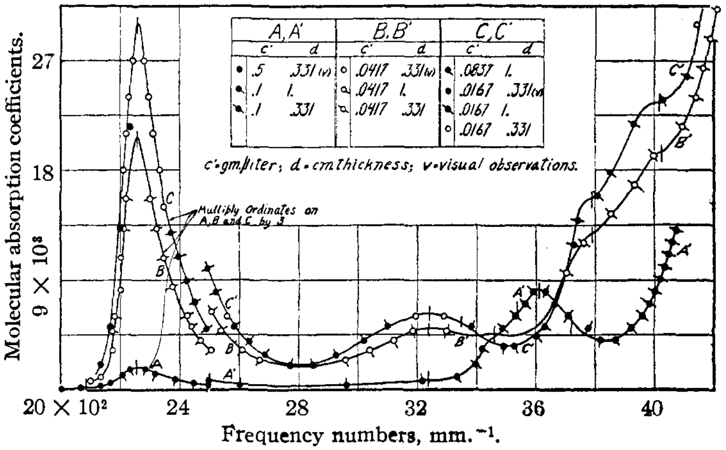

Fig. 4.—Fluorescein in alcoholic HCl solution: A,A', 1 molecule:4 molecules of HCl; B,B', 1 molecule:155 molecules of HCl; C,C', 1 molecule:960 molecules of HCl.

absorption to revert from the characteristic alkaline type to the neutral

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[16] Gattermann, *Ber.*, **32**, 1135 (1899).

at 2005, 2153, 2673, 3082, 3423, 3823 and 4138. Higher ratios of potassium hydroxide in alcohol show no further change in the absorption.

Fluorescein dissolves in cold 33% aqueous potassium hydroxide with a deep orange color and an intense green fluorescence. The absorption of the freshly prepared solution is given in Fig. 6, Curve A,A′. In the visible region there are two bands, a very weak one at 1715 and an extremely intense one at 2033, while in the ultraviolet there are bands at 2628, 3088 and 3502. With the exception of the first, these agree closely with the bands found in a solution of resorcinolbenzein[3b] in 5% aqueous potassium hydroxide. However, this strongly alkaline solution changes

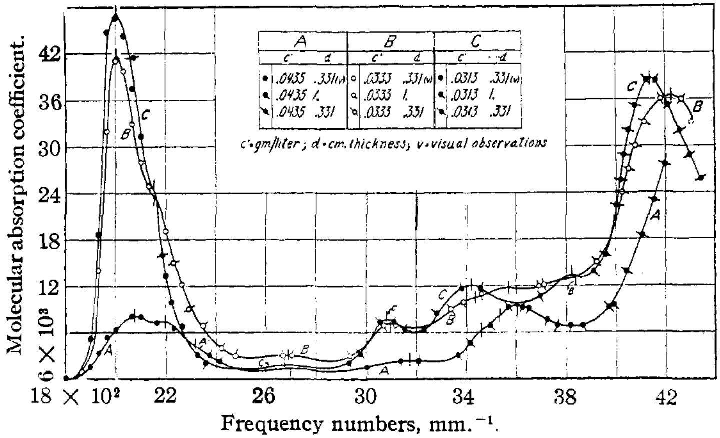

Fig. 5.—Fluorescein in alcoholic KOH solution: A, 1 molecule: 1 molecule of KOH; B, 1 molecule:3 molecules of KOH; C, 1 molecule:20 molecules of KOH.

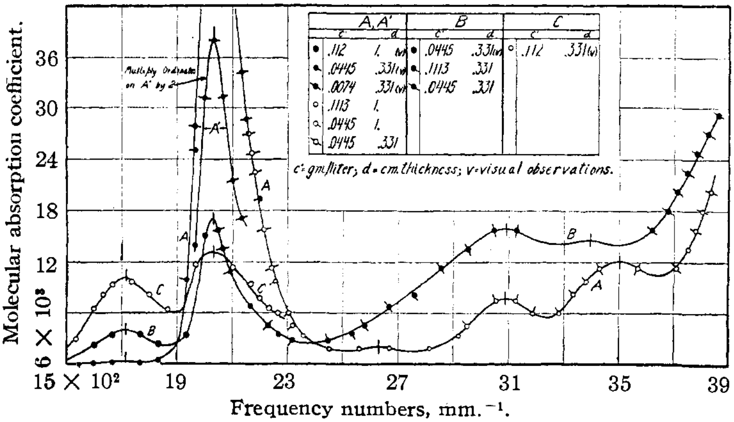

Fig. 6.—Fluorescein in cold 33% aqueous KOH: A,A′, fresh solution; B, solution A after standing for five weeks; C, solution A after standing for ten weeks.

over in a few weeks from an orange color to a deep purple and then gradually fades out over several months to a pale pink, even though kept in a dark room. Curve B shows an intermediate stage. A still later stage is shown in Curve C, the band at 1715 still growing and the one at 2033 still decreasing, which accounts for the observed change in color, from orange to purple. Baeyer[17] has called attention to a change of this kind when a solution of fluorescein in strong alkali is boiled and he has explained it by the rupture of the pyrone ring with the formation of a tetrabasic salt. Meyer and Fischer[5] have measured the absorption of such a solution and claim to have found a resemblance to the absorption of phenolphthalein in weak alkaline solution. A comparison of the data here with our results on phenolphthalein[3c] gives no indication of any simple relation between the two compounds. As a further check on these results, the purple solution was prepared by dissolving fluorescein together with a weighed amount of potassium hydroxide in the required volume of water, the heat of solution of the potassium hydroxide being sufficient to produce the purple solution instantaneously.

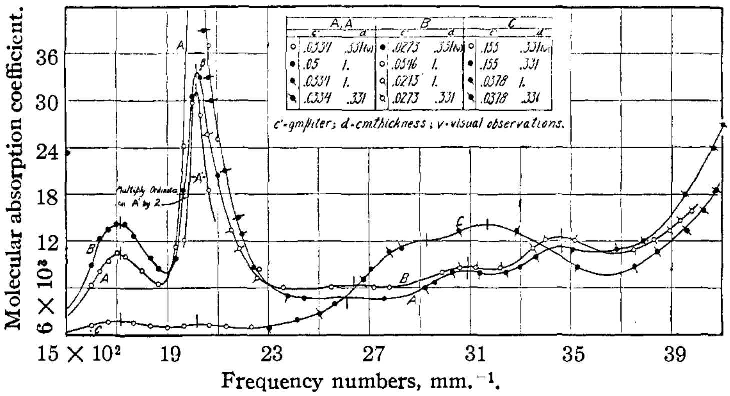

Fig. 7.—Fluorescein in hot 33% aqueous KOH: A,A′, fresh solution; B. solution A after standing for twelve hours; C, solution A after standing for seven weeks.

The absorption of this solution was measured as soon as it had cooled and it is shown in Fig. 7, Curve A,A′. The band at 1715 is quite intense, while that at 2018 is somewhat less intense than the corresponding one in the freshly prepared, cold solution of fluorescein. Twelve hours later the band at 1715 has increased by one-fourth and the one at 2018 has decreased about half, while the bands in the ultraviolet have all increased to some extent. At the end of six weeks, the solution had faded to a pale pink and the absorption, Curve C, shows the bands at 1715 and 2018 as very weak, while the ultraviolet absorption has changed in character,

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[17] Baeyer, *Ann.*, **372**, 108 (1909).

with two bands at 2926 and 3168. Neither Baeyer nor Meyer and Fischer have mentioned this progressive change in the color of the solution. There would thus appear to be two processes at work: (1) the rupture of the pyrone ring, represented by the growth of the band at 1715; (2) some other disruptive process preceded possibly by the formation of a carbinol, which causes both visible bands eventually to fade out, while producing a different type of absorption in the ultraviolet. The behavior of eosin and sulfonefluorescein is being studied under similar conditions.

In Table II the positions of the bands in the various acid and alkaline solutions of fluorescein are summarized.

TABLE II

FREQUENCY NUMBERS OF BANDS IN SOLUTIONS OF FLUORESCEIN

Neutral alcohol	Alcoholic HCl 4 m.	155 m.	960 m.	Concd. sulfuric acid	75% formic acid
2075	2085	..	..	..	..
2198	2254	2257	2258	2317	2285
2319	3236	3238	3240	3039	3062
2700	3462	3360	3349	3215	3219
3460	3536	..	..	3451	3372
3533	3609	..	..	..	..
3604	3719	3790	3790	3906	..
..	..	4024	4018	4005	..

Neutral alcohol	Alcoholic KOH			Aqueous 33% KOH Cold		Hot	
	1 m.	3 m.	20 m.	Fresh	Faded	Fresh	Faded
..	..	..	..	1715	1715	1715	1715
..	..	2005	2005	2033	2033	2018	2018
2075	2075	..	..	..	..	..	..
..	..	2151	2153	..	..	..	..
2198	2199	..	..	..	..	..	..
2319	2318	..	..	..	..	..	..
2700	2698	2688	2673	2628	..	2612	..
..	3174	3095	3082	3088	3090	3089	2926
3460	3460	3437	3423	..	3395	..	3168
3533	3535	3570	..	3502	..	3461	..
3604	3604	..	..	..	..	..	..
..	..	3840	3823	..	..	..	..
..	..	4225	4138	..	..	..	..

Summary

1. The absorption curves of fluorescein, fluorescein diacetate, fluoran and dichlorofluoran in neutral alcoholic solutions have been determined. It has been concluded that fluorescein exists in solution in the quinoid state only.

2. The absorption curves of fluorescein in concd. sulfuric acid, 75% formic acid and in alcoholic solutions of hydrogen chloride indicate that the fluorescein is present in each case as the salt of the respective acid.

3. The effect of the addition of various amounts of potassium hydroxide to the alcoholic solution of fluorescein, due to the formation of the dipotassium salt of fluorescein, has been studied.

4. The absorption of fluorescein in strong aqueous potassium hydroxide undergoes a progressive change, indicating the occurrence of two processes: (1) the rupture of the pyrone ring and (2) the possible formation of a carbinol followed by some other disruptive process.

ITHACA, NEW YORK

[CONTRIBUTION FROM THE SCHOOL OF CHEMISTRY, UNIVERSITY OF MINNESOTA]

THE DETERMINATION OF OXYGEN IN ORGANIC COMPOUNDS[1]

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Many methods for the determination of oxygen in organic compounds have been described.[4] In all these methods it is necessary to perform a *separate* experiment for the determination of oxygen. In the new method proposed here we determine the amount of oxygen consumed *during* the combustion of an organic compound *gasometrically* and are thus able to analyze for Carbon, Hydrogen *and* Oxygen in *one* operation.

We used the modification of Wise's[5] semi-micro combustion method which has been developed and used in this laboratory by Dr. W. M. Lauer

Apparatus and Procedure

The apparatus used is shown in Fig. 1. Oxygen was made from solid potassium permanganate and measured (N. T. P.) in a Ramsay buret. The combustion tube contained platinized asbestos only. The use of copper oxide was avoided at this time following a suggestion of Professor W. H. Hunter. It is possible that copper oxide wire reduced to copper during the combustion may not be reoxidized to the same extent as it had been originally. The sample was weighed on an ordinary analytical balance, as were the absorption tubes.

[1] This method was devised in examining the liquid product obtained in the course of the investigation on "the effect of electrical discharge on gaseous hydrocarbons," listed as research project No. 8 of American Petroleum Institute Research. Financial assistance for this work has been received from a research fund of the American Petroleum Institute, donated by the Universal Oil Products Co. This fund is being administered by the Institute with the coöperation of the Central Petroleum Committee of the National Research Council.

[2] American Petroleum Institute Research Associate.

[3] Professor of Chemistry, Colorado School of Mines.

[4] Older work: "Analyse, etc., org. Verbind.," Hans Meyer, 3rd ed., Julius Springer, Berlin, **1916**, p. 301; M. C. Boswell, THIS JOURNAL, **35**, 284 (1913); **36**, 127 (1914); R. Strebinger, *Z. anal. Chem.*, **58**, 97 (1919); H. Ter Meulen, *Rec. trav. chim.*, **41**, 509 (1922); **43**, 899 (1924); *Chem. Weekblad*, **23**, 348 (1926).

[5] Wise, THIS JOURNAL, **39**, 2055 (1917).