FLUORESCENCE ABSORPTION IN RESORUFIN.[1]

By Frances G. Wick.

THE relation between absorption of light by a fluorescent substance and emission during fluorescence is difficult to understand. Waves of one length are absorbed while those of another length are emitted, in apparent contradiction to Kirchhoff's law. At least a step toward an explanation of this difficulty has been made in the experiments of Burke[2] and in the more recent work of Nichols and Merritt,[3] who found that a change takes place in the absorbing power during fluorescence. Previous observations of absorption had been made when the body was not under excitation. The work of Burke upon uranium glass, and that of Nichols and Merritt upon fluorescein in water, eosin in alcohol, and resazurin in alcohol, showed that during fluorescence a substance acquires the temporary power of absorbing the same wave-lengths which it emits. M. Camichel,[4] who has since made measurements of a similar kind, failed to find any change in absorbing power during fluorescence, throwing doubt upon the existence of the phenomenon.

The subject thus brought into question seemed of sufficient interest to justify further study. At the suggestion of Professors Nichols and Merritt the following series of tests for fluorescence absorption was made upon Weselski's diazo-rezorufin. Some experimental work[5] just completed, in which a study had been made of the fluorescence and ordinary absorbing power of resorufin, suggested this as a desirable substance for further investigation.

A preliminary set of experiments showed that resorufin acquires a measurable increase in absorbing power during fluorescence. This phenomenon was then studied in its relation to :

[1] A paper presented at the Ithaca meeting of the American Physical Society, June, 1906.

[2] Burke, John, Philosophical Transactions, Vol. 191A, p. 87, 1898.

[3] Nichols and Merritt, Physical Review, Vol. XIX., p. 397, 1904.

[4] Camichel, M., Comptes Rendus, 140, p. 139.

[5] Wick, Frances G., Physical Review, Vol. 24, p. 356, 1907.

1. Intensity of transmitted light.
2. Intensity of fluorescent light.
3. Thickness of absorbing layer.
4. Wave-length.
5. Concentration.

METHOD AND APPARATUS.

The method used was essentially the same as that employed by Nichols and Merritt, the instrument used being the Lummer-Brodhun spectrophotometer. The fluorescent solution, contained in a rectangular glass cell R (Fig. 1), was placed in front of slit S.

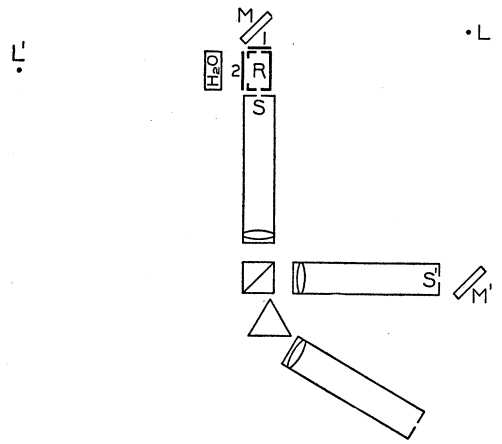

Fig. 1.

A bank of four acetylene flames L' was used as a source of fluorescence excitation. The source for transmission was an acetylene flame L from which light was diffusely reflected by a block of magnesium carbonate M. The intensity of transmission was adjusted by altering the angle of M. Screens 1 and 2 were arranged like shutters so that light from M or L' could be entirely cut off. For observations in which the intensity of fluorescence was varied, and those in which the thickness of the solution was changed at a constant wave-length, a glass cell containing a dilute solution of the fluorescent substance was placed between the flame L and the carbonate M. This acted as a screen to prevent fluorescence excitation by the transmitted light.

To determine the increase in absorption due to fluorescence three readings of the micrometer screw attached to S' were necessary.

1. *Transmission* (T). — Intensity of light transmitted from M, shutter 1 being open and 2 closed.

2. *Fluorescence* (F). — Intensity of the fluorescent light, shutter 2 being open and 1 closed.

3. *Transmission and Fluorescence combined* (C). — Intensity of light observed with 1 and 2 both open.

If the absorbing power were not changed during fluorescence the sum of the first two readings ($T + F$), would equal the third (C). This, however, did not prove to be the case. From actual observations

$$T + F > C.$$

An increase in absorption takes place which is measured by the expression

$$A_F = T + F - C.$$

This increase, A_F, is called by Nichols and Merritt "fluorescence absorption." The values of T, F, C and A_F were obtained in terms of the width of slit S' as read from the drum of its micrometer screw. Different sets of observations are not comparable since the comparison source was so adjusted as to produce the best possible match in color and intensity.

Difficulties and Sources of Error.

In all spectrophotometric work, especially determinations such as this, in which a small final result is obtained from the difference of two relatively large observations, the probability of error is great. This work was done in a dark room and special precautions were taken in the adjustment of screens and shutters to eliminate stray light. All possible care was taken in the arrangement of every part of the apparatus.

The most serious difficulty, however, was one of color match, and this could not be avoided by any mechanical adjustment. In making a set of observations extending through the fluorescence band the width of the collimator slit S (Fig. 1) had to be kept constant. A perfect color match was possible only when the intensities of the

two sources of light was such as to make slit widths S and S' (Fig. 1) equal. But as different wave-lengths were observed, keeping the comparison source L constant, the intensities of T, F and C, varied in such a way as to require the width of S' to be made in some cases greater and in others less than that of S.

Since the proper adjustment is difficult to make with accuracy it is essential that the observer's eyes be kept sensitive and that they be allowed to become perfectly restored after exposure to light before any observations are made. Care was taken in this work to have the periods of observations short, and results were rejected as inaccurate when the individual settings for any one measurement began to vary widely. An average of from three to six settings of the micrometer screw was taken for each of the values obtained.

FLUORESCENCE ABSORPTION AND INTENSITY OF TRANSMITTED LIGHT.

The first series of measurements was made to determine the relation between fluorescence absorption and the intensity of transmitted light. Tables I. and II. give the results obtained by a variation in the intensity of transmitted light, all other conditions being kept constant. Fluorescence absorption (A_F), it will be observed, is nearly constant for all values of transmission (T), in a given set of measurements.

According to Nichols and Merritt, as the intensity of the transmitted light increases from zero, fluorescence absorption must also increase to a certain maximum value, at which saturation is reached. At this point the intensity of transmitted light is so small as to make accuracy of measurement impossible. The tables here given serve as additional evidence of the truth of the conclusions drawn by Nichols and Merritt, that fluorescence absorption does not take place in accordance with the ordinary laws of absorption. An increase in the intensity of the incident light is not accompanied by a corresponding increase in absorption.

FLUORESCENCE ABSORPTION AND INTENSITY OF FLUORESCENCE.

In the next set of observations the intensity of the fluorescent light was varied by changing the distance of L' from the face of the cell (R) (Fig. 1).

TABLE I.

Dependence of Fluorescence Absorption upon the Intensity of the Incident Light. I., II. and III. indicate the Individual Observations from which the Average was Computed. The Measurements were made upon Concentration 1/32, *at Wave-length* .646 μ.

T				F				C				$T+F$	A_F
I.	II.	III.	Av.	I.	II.	III.	Av.	I.	II.	III.	Av.	Av.	Av.
25.2	24.8	24	24.7	12	11.4	11.3	11.57	34.3	34.5	33	33.93	36.27	2.34
36.4	36.5	35	35.97	11	11	11	11	44.5	44.7	44	44.4	46.97	2.57
56.2	60	59	58.4	11.3	10.8	11	11.03	65.4	66.5	66.2	66.03	69.43	3.4
68.3	67	67	67.43	10.6	10	11	10.53	75.5	75	76	75.5	77.96	2.46
74	73.8	74.8	74.2	10.6	11	11.2	10.93	82	82.8	82.4	82.4	85.13	2.71

TABLE II.

Dependence of Fluorescence Absorption upon the Intensity of the Incident Light.
Concentration 1/128. *Wave-length* .646 μ.

T	F	C	A_F
13	10.4	21.73	1.67
31.5	10.1	39.56	2.04
68.23	9.93	75.2	2.96
89.9	10.43	97.8	2.53
98.27	10.3	105.375	3.195
Concentration 1/64. *Wave-length* .646 μ.			
4.5	18.9	21.7	1.7
24.27	18.7	39.8	3.17
29.8	18.4	45.48	2.72
Concentration 1/128. *Wave-length* .646 μ.			
13.17	10.5	21.43	2.24
32.85	10.5	39.4	3.95
57.47	10.87	62.5	5.84
68.65	10.5	76.96	2.19
84.3	10.6	90.7	4.2
114.1	11	117	8.1

The relation between fluorescence and fluorescence absorption was thus determined. The results indicate that for small values an increase in fluorescence causes an increase in fluorescence absorption. At a certain point saturation is reached, further increase in the intensity of fluorescence producing no change in fluorescence absorp-

tion. This is shown in Table III., graphically represented in Fig. 2. In the last two sets of observations given, there are no observations

TABLE III.

Dependence of Fluorescence Absorption upon the Intensity of the Fluorescent Light.
Concentration 1/64. *Wave-length* .646 μ.

T	F	C	A_F
30.2	3.4	33.2	.4
30.95	6.2	35.2	1.95
31.45	8.2	38.2	1.45
30.95	13.45	41.45	2.95
31.7	17.2	46.37	2.53
31.2	23.5	51.2	3.5
32.8	27.95	56.8	3.95
32.46	33.7	61	5.16
31.95	46.63	72.2	6.38
31.6	60.6	87.4	4.8
31.4	66.5	90.9	7
31.8	94.83	120.3	6.33
31.6	83.07	109.26	5.41
32.3	140.7	168.12	4.88
	Concentration 1/64. *Wave-length* .646 μ.		
34.9	7.37	41.1	1.17
33.82	9.37	40.25	2.94
32.9	12.9	42.9	2.9
34.9	16.27	46	5.17
35.7	20.7	51.5	4.9
35.33	22.53	53.37	4.49
34.63	23.7	52	6.33
	Concentration 1/128. *Wave-length* .646 μ.		
38.9	12.92	40.71	11.11
37.6	13.6	43.8	7.4
39.18	15.4	44.7	9.88
38.65	19	44.9	12.75
38.83	26	55.98	8.85
40.1	35	61.65	13.45
	Concentration 1/128. *Wave-length* .646 μ.		
45.88	10.05	44.125	11.805
44.9	13.37	46.17	12.1
44.78	19.6	47.66	16.72
46.3	22.95	54.9	14.35

below the point at which saturation is reached. This same saturation effect was previously observed by Nichols and Merritt.

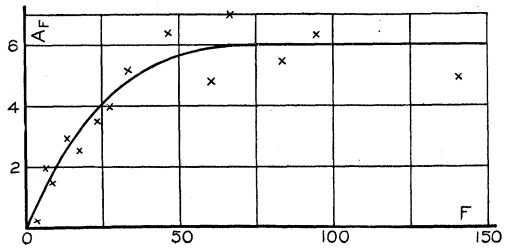

Fig. 2. Fluorescence absorption as a function of intensity of fluorescence.

Fluorescence Absorption With Variation in the Thickness of the Absorbing Layer.

In the previous experiments the section of the liquid excited to fluorescence through which the transmitted light passed was kept constant, *i. e.*, 5.45 cm. To find what effect a change in thickness has upon fluorescence absorption, the width of the section of the cell, *R*, exposed to the exciting light *L'* (Fig. 1) was varied. A section of the solution 1 mm. wide at the end of the cell next to the collimator slit *S* was first excited to fluorescence. The width of this section was gradually increased until it covered the whole face of the cell, readings being taken for each increase in width.

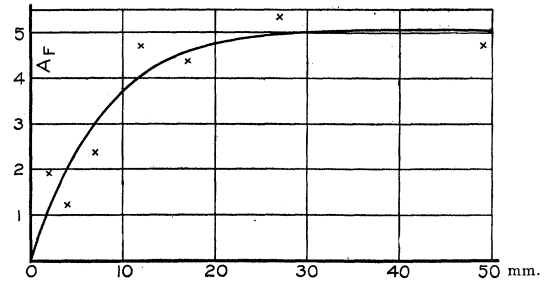

Fig. 3. Fluorescence absorption as a function of the thickness of the absorbing layer.

Fig. 3 shows the result. Fluorescence absorption increases with an increase in thickness up to a certain point, for this particular concentration about 1.25 cm. Further increase in thickness pro-

duces no change in fluorescence absorption. The complete set of curves from the first observations given in Table IV. is shown in Fig. 4.

As a check upon this work the same series of measurements was made beginning with the 1 mm. slit at the opposite end of the

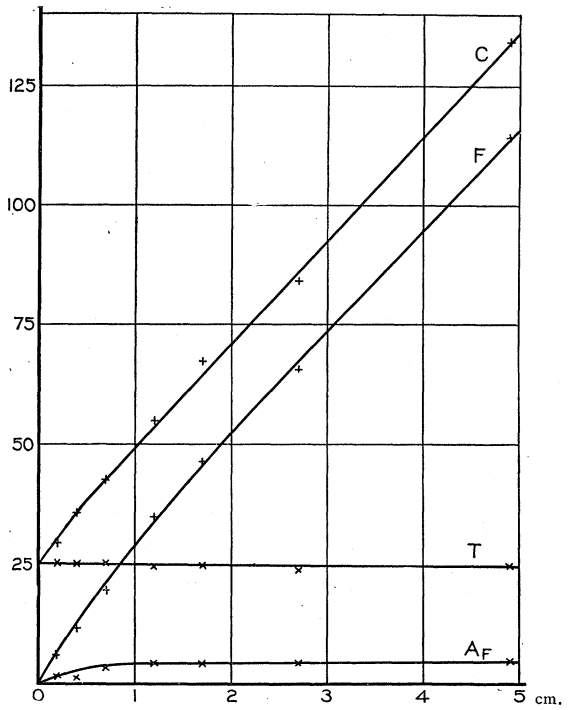

Fig. 4. Curves showing the effect of change in the thickness of the absorbing layer, the width of band being increased from the end of the cell next to the collimator tube toward the end next to the source of transmitted light.

F = observed fluorescence.
T = transmission.
C = fluorescence and transmission combined.
$A_F = T + F - C$ = fluorescence absorption.

cell, *i. e.*, the end next to M (Fig. 1), and gradually increasing the width of the opening toward the collimator tube until the whole face of the cell was excited. The results, given in the third set of observations in Table IV., and Fig. 5, were similar to those ob-

TABLE IV.

Dependence of Fluorescence Absorption upon the Thickness of the Absorbing Layer.
Concentration 1/64. *Wave-length* .646 μ.

Variation in thickness beginning at end of cell next to the collimator tube.

Thickness.	T	F	C	A_F
2 mm.	25.5	6.1	29.69	1.91
4	25.21	11.85	35.83	1.23
7	25.43	19.58	42.65	2.36
12	24.6	35	54.91	4.69
17	25.05	46.57	67.25	4.38
27	23.9	65.7	84.26	5.34
49	24.7	114.2	134.2	4.7

Concentration 1/64. *Wave-length* .646 μ.

1 mm.	28.9	3.95	31.94	.91
3	28.9	8.4	34.1	3.2
5	28.1	12.2	37	3.3
7	28.1	16	40.2	3.9
12	27.84	24.3	47.3	4.84
17	28.1	34.8	57.3	5.6
27	27.5	46.88	68.74	5.64
37	28.1	62.8	84.5	6.4

Concentration 1/64. *Wave-length* .646 μ.

Variation in thickness beginning at end of cell away from the collimator tube.

1 mm.	27.5	2.6	29.4	.7
3	27.6	6	32.1	1.5
5	27.63	8	33.6	2.03
7	26.58	12.8	35.3	4.08
12	26.9	20.37	42.7	4.57
17	26.2	28.45	49.6	5.05
22	26.2	37.3	54.45	9.05
32	26.2	46.9	67.2	5.9
42	26.2	59.7	80.7	5.2
54	26.2	71.5	91.8	5.9

tained from measurements in the other direction. This might be expected since the solution used was very dilute and at the wave-length studied ordinary absorption was insignificant.

In Fig. 5, the curve marked A_F shows the same saturation effect observed in Figs. 3 and 4. Curve F, Figs. 4 and 5, gives the observed fluorescence and curve C the fluorescence and transmis-

sio n combined. Curve F bends to about the point at which satu-

ration is reached in A_F then becomes a straight line. This is explained by the fact, that, at first, with increased thickness there is an increase in the fluorescence absorption. This decreases the intensity of the observed fluorescent light. After saturation has been reached the increase in the observed fluorescence is proportional to the increase in thickness, fluorescence absorption entering

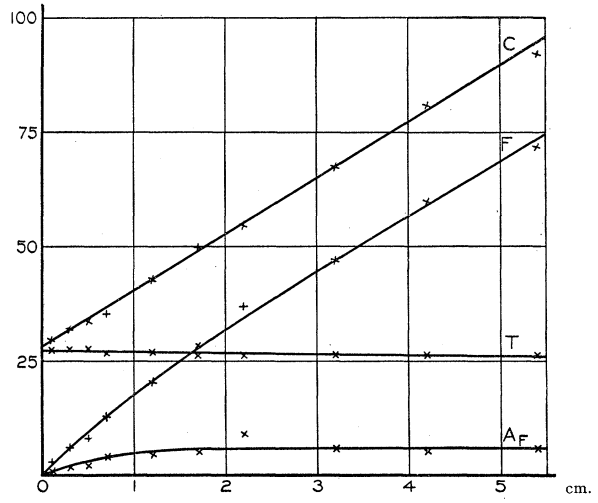

Fig. 5. Curves showing the effect of change in thickness of the absorbing layer, the thickness being increased from the end of the cell next to the source of transmission toward the collimator tube.

as a constant factor. Curve C is approximately a straight line. Other sets of measurements were made with similar results. In every case a saturation point was reached beyond which an increase in thickness produced no change in fluorescence absorption.

It thus appears that the energy absorbed from the transmitted light increases with increasing thickness up to a certain limit, beyond which fluorescence absorption is not changed by increasing the thickness of the fluorescent solution. A saturation point is reached similar to that observed in changing the intensity of fluorescence.

RELATION OF FLUORESCENCE ABSORPTION TO WAVE-LENGTH.

The relation between fluorescence absorption and wave-length was determined by shifting the telescope into different positions in the

fluorescence band and making readings of T, F, and A_F at different wave-lengths.

TABLE V.

Dependence of Fluorescence Absorption upon Wave-length. Concentration 1/16.

Wave-length.	T	F	C	A_F
.542 μ	.7	1.7	1.5	.9
.562	.38	2.3	2.55	.13
.589	7.76	15.75	22.9	.61
.614	31.97	26.31	54.7	3.58
.646	39.88	13.8	52.7	.98
.69	36.55	7.82	43.35	1.02
		Concentration 1/2.		
.589 μ	.55	4.325	4.9	.025
.614	11.3	20.63	29.575	2.355
.646	31.75	27.95	51.975	7.725
.69	40.4	15.9	52.375	3.92

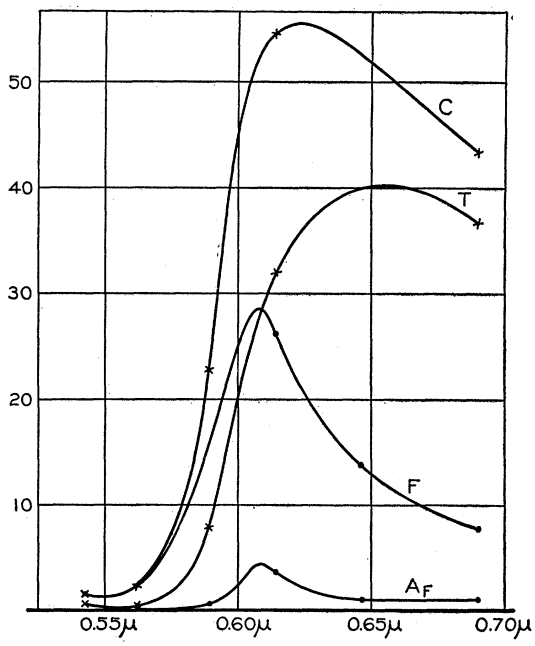

Fig. 6. Fluorescence absorption as a function of wave-length.

Table V., represented graphically in Fig. 6, gives the average of three sets of observations made upon concentration one sixteenth,

a screen of one sixty-fourth resorufin being so adjusted between L and M (Fig. 1) as to cut off those rays which might produce fluorescence and thus introduce error. The fluorescence absorption curve, A_F, follows the fluorescence curve, F, the maxima of the two corresponding in wave-length. Table V. gives also the average of three sets of observations made upon concentration one half.

Other measurements given in Table VI. were made earlier without the interposition of the resorufin screen. These indicate greater errors of observation but in general it will be noticed that the greatest fluorescence absorption takes place in the region of greatest fluorescence.

TABLE VI.

Dependence of Fluorescence Absorption upon Wave-length. Concentration 1/128.

Wave-length.	T	F	C	A_F
.524 μ	11.7	2.5	11.8	2.3
.542	11.56	2.7	11.73	2.43
.562	9.86	4.55	13.35	1.06
.589	14.5	16.6	29.75	1.35
.614	23.65	17.9	37.25	4.3
.646	32.9	10.4	39.35	3.95
	Concentration 1/64.			
.524 μ	10.25	1.95	10.3	1.9
.542	7.73	1.8	7.5	2.03
.562	6.47	5.07	9.27	2.27
.589	8.6	35.9	42.4	2.1
.614	21.73	47.7	65.23	4.2
.646	30.83	26.9	53.97	3.76
	Concentration 1/128.			
.542 μ	11.2	1.2	11.45	.95
.562	7	4.2	10.2	1
.589	14.45	29.8	40.7	3.55
.614	34.4	39.7	64.9	9.2
.646	46.6	22.5	55.4	13.7
.69	48.2	19.45	59.8	7.85

CHANGE IN CONCENTRATION.

The relation between fluorescence and fluorescence absorption is further brought out by a comparison of the results obtained from different concentrations. The positions of maximum fluorescence and fluorescence absorption correspond in each case.

Fig. 7 gives results from two concentrations. Curves F and A_F represent fluorescence and fluorescence absorption for concentration one sixteenth, the corresponding maximum being .61 μ. Curves F' and A_F' represent fluorescence and fluorescence absorption of concentration one half. The maximum of fluorescence, F', has shifted

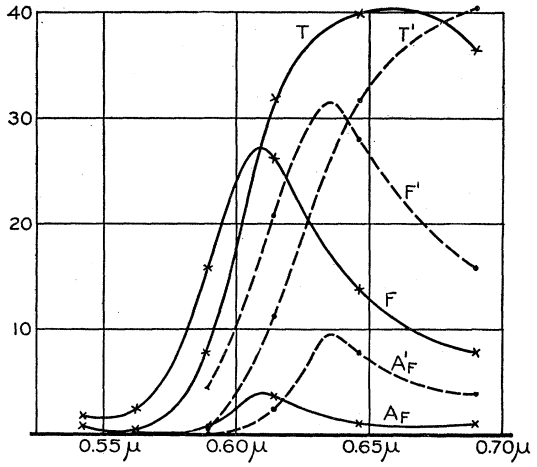

Fig. 7. Fluorescence absorption as a function of wave-length in different concentrations.

Curves T, F and A_F correspond to concentration $\frac{1}{16}$.
Curves T', F', and A_F' correspond to concentration $\frac{1}{2}$.

to about .635 μ—an effect due to increased absorption on the side of the band next to the violet. The maximum fluorescence absorption, A_F', has also shifted to about the same point.

Conclusion.

The experiments described in this paper serve as a confirmation of the work done by Professors Nichols and Merritt for whose encouraging interest the author wishes to express her grateful appreciation.

The fact that during fluorescence a substance acquires a temporary power of absorbing those rays emitted during excitation seems reasonable; but certain phenomena observed in connection with this increase baffle explanation. It seems natural to expect that with an increase in the intensity of transmitted light an increase in fluores-

cence absorption will take place according to the known laws of ordinary absorption, but such is not the case. The increased absorption seems independent of the intensity of transmitted light.

The saturation effect observed with increase in the thickness of the fluorescent solution is equally strange. After a certain thickness has been reached further increase in thickness has no effect upon fluorescence absorption.

Another surprising fact brought out is that the fluorescence absorption band corresponds in position with the *observed* fluorescence band. It might be expected that a phenomenon which seems to be an accompaniment of fluorescence should show a maximum effect not at the observed maximum of fluorescence, after absorption has taken place, but at the actual maximum fluorescence point found by correcting for absorption.

In this paper no attempt has been made at explanations, the object being simply to present the observational facts with the object of discovering whether or not any peculiar absorption phenomena accompany fluorescence in resorufin.

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