RESEARCH PAPERS

THE ESTIMATION OF FLUORESCEIN IN DILUTE SOLUTIONS

By E. Shotton and A. F. S. A. Habeeb

From the School of Pharmacy, University of London, 17 Bloomsbury Square, W.C.1

Received March 18, 1955

In a previous paper we have described the use of fluorescein as an indicating substance for the entrainment of liquid during the distillation of water[1]. The work described below was carried out to determine the conditions for the estimation of fluorescein in very dilute solutions.

The intensity of the fluorescence of an aqueous solution of fluorescein has been found to be constant from 0° to 80° C., the wavelength of the emitted light increasing with increasing temperature[2] the intensity of the fluorescence being maximum at room temperature[3]. The presence of the following ions I^-, Br^-, Cl^-, CNS^-, $S_2O_3^-$, SO_3^-, Ag^+, Cu^{++}[4,5,6] may cause quenching of the fluorescence but the cations Na^+, K^+, Ca^{++} and Ba^{++} are without effect. Oxygen has also been found to have no effect[7,8].

Boutaric and Roy[9] and Boutaric and Maraux[10] found that the fluorescence of fluorescein solutions was greatest at *p*H 8. According to Volmar[11] fluorescein gives an intense green fluorescence at *p*H 4·3 or above and a slight blue fluorescence below *p*H 3·8.

Concentration quenching is a general property of fluorescent substances and deviations from linearity occur if data are collected over a sufficiently wide range of concentration. Calibration curves are made to allow for concentration quenching. Cohen[12] obtained a linear relation for fluorescein solutions containing 0·125 to 6 μg./ml.

Fluorescein solutions exposed to direct sunlight undergo decomposition[13] seen by a decrease in the intensity of the fluorescence and a change in the absorption spectrum.

Experimental

The Spekker Fluorimeter (model H760)[14,15] was used with a Woods glass filter[16] on either side of the mercury vapour lamp. A heat absorbing filter was also inserted between the lamp and the left hand photocell but not on the right hand side as this reduced too greatly the intensity of the incident light and therefore the intensity of the fluorescence. With small galvanometer deflections a neutral filter (H.508) was inserted in front of the left hand photocell. Readings on the transmission scale of the drum were recorded.

The fluorescein solutions were prepared from Fluorescein Sodium B.P.C. dried at 105° C. to constant weight. The blank reading[17,18] for the cuvette filled with buffer solution was obtained by comparison with a solution of fluorescein containing 0·125 μg./ml. The blank with respect to the other standards was calculated and deducted from each observed fluorescence to give the net fluorescence. One cell was used throughout for the standard and another for the test solutions.

The effect of the p*H of the solution*

A series of solutions were prepared containing 1 μg./ml. of fluorescein buffered at the following *p*H values 4·4, 5, 5·5, 6, 6·5, 7, 8 and 9. Potassium chloride was omitted from the buffer at *p*H 9 to avoid the quenching effect of the chloride ion[19]. The *p*H of the fluorescein solutions was checked with a glass electrode. Preliminary experiments indicated that the solution at *p*H 6 gave the greatest fluorescence and this solution was therefore used as a standard to compare the other solutions.

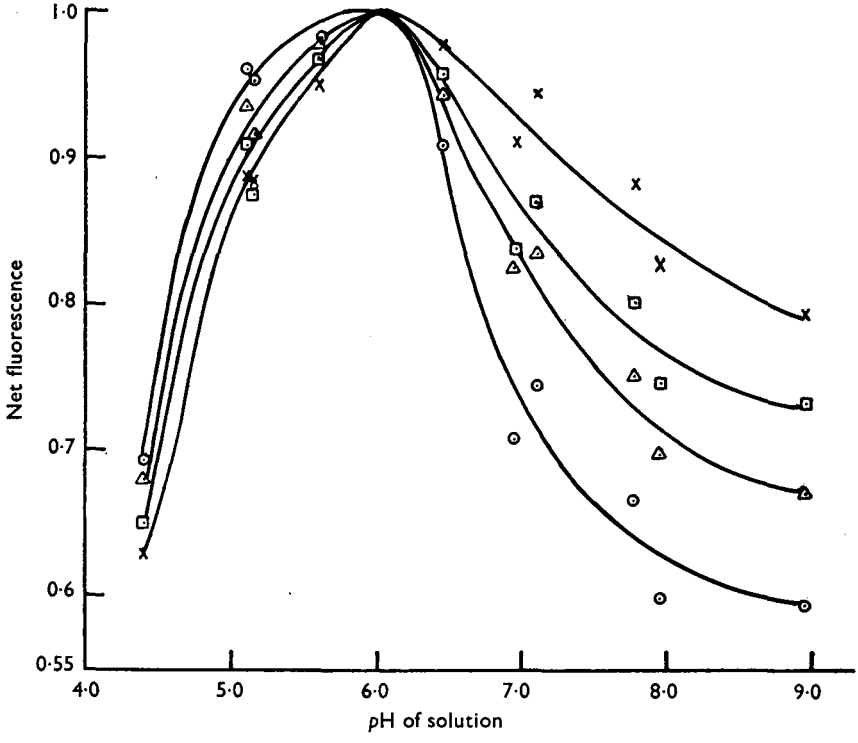

FIG. 1. Effect of *p*H on the fluorescence of fluorescein solutions.
× Kodak Filter F_6 maximum transmission at 5200Å°
⊙ ,, ,, F_7 ,, ,, ,, 5400Å°
△ Chance Filter OG_1 ,, ,, ,, 5300Å°
⊡ ,, ,, OB_2 ,, ,, ,, 4800Å°

Bertrand[20] reported that the fluorescence spectrum of fluorescein solutions exhibits a maximum at $\lambda = 5490$ Å for 0·01 molar solution and $\lambda = 5200$ Å for 0·0001 molar solution. Filters with a maximum transmission at these wavelengths were inserted between the fluorescing solution and the photocells.

The results are given in Fig. 1.

The gelatin filters F6 and F7 transmit only a fraction of the fluorescent radiation compared with the glass filters resulting in smaller galvanometer deflection and consequently greater errors in the determinations.

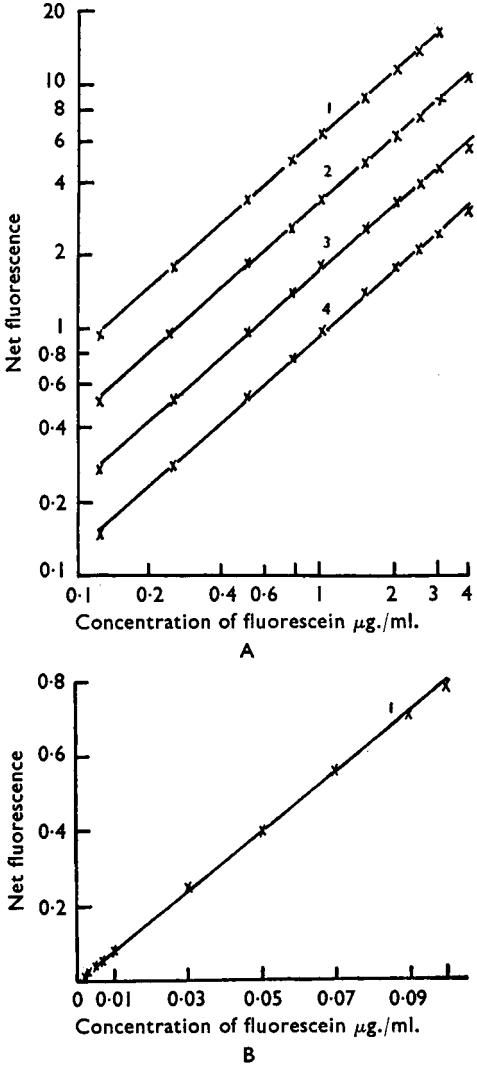

FIG. 2. The effect of concentration on the fluorescence of fluorescein solutions.

1. Standard solution containing 0·125 μg./ml.
2. ,, ,, ,, 0·25 ,,
3. ,, ,, ,, 0·50 ,,
4. ,, ,, ,, 1·0 ,,

Filter OG_1 was used for all subsequent work in preference to filter OB_2 as the cell blank was smaller indicating that only the fluorescent radiation was transmitted and light of unsuitable wavelength, such as that reflected from the cell lid and walls, was cut off.

Calibration of the Instrument

Experiments were carried out to obtain the calibration data and to determine the lowest concentration of fluorescein that could be estimated. A range of solutions were prepared, buffered to *p*H 6 and these were compared with four standard solutions containing 1, 0·5, 0·25 and 0·125 μg./ml. fluorescein in buffer of *p*H 6. The greatest accuracy in determining the concentration is considered to be obtained when the reading falls within the middle third of the scale[15,21].

The results are shown in Figs. 2A and 2B.

Fig. 2A represents the fluorescence-concentration relation plotted on a log-log scale for solutions 0·125 to 4·0 μg./ml. fluorescein using a 0·125, 0·25, 0·5 and 1·0 μg./ml. solutions of fluorescein as standards. It is seen that it follows a straight line suggesting the relation to be an exponential function of the formula $F = a\,c^n$

where a = value of F when c is unity which is 0·97, 1·78, 3·4 and 6·3 for standards 1, 0·5, 0·25 and 0·125 μg./ml. respectively.

c = concentration of fluorescein solution.

n = slope of the curve which was 0·88 in each case.

This value for n may apply only to the data obtained under our experimental conditions.

Fig. 2B shows that the data fall on a straight line for concentrations between 0·003–0·1 μg./ml.

Accuracy of the method

Test solutions of fluorescein buffered at *p*H 6 were prepared and their concentration was determined. For concentrations between 0·02–1·5 μg./ml. the error varied from −0·53 to −5·83 per cent. With more dilute solutions the error increased and for a solution containing 0·01 μg./ml. the error was −19 per cent.

The effect of heat on fluorescein solutions

Solutions containing 0·1, 0·01 and 0·001 per cent. w/v of fluorescein in distilled water were boiled under reflux for 80, 8 and 8 hours respectively. The fluorescence of the heated and unheated solutions suitably diluted at *p*H 6 was compared with standard solutions containing 0·5 and 1·0 μg./ml. of fluorescein.

Results are given in Table I.

TABLE I

THE EFFECT OF HEAT ON FLUORESCEIN SOLUTIONS

Concentration of standard	Nominal concentration of test dilution μg./ml.	Net fluorescence					
		0·1 per cent. w/v solution		0·01 per cent. w/v solution		0·001 per cent. w/v solution	
		Heated	Not heated	Heated	Not heated	Heated	Not heated
1·0 μg./ml.	0·25	0·278	0·267	0·283	0·288	0·277	0·288
	0·50	0·518	0·52	0·534	0·537	0·524	0·537
	1·0	1·02	1·0	0·99	0·99	0·97	0·99
	2·0	1·86	1·81	1·78	1·80	1·75	1·80
0·5 μg./ml.	0·25	0·51	0·50	0·525	0·532	0·515	0·532
	0·50	1·004	0·997	0·979	0·978	0·962	0·978
	1·0	1·90	1·83	1·81	1·81	1·77	1·81
	2·0	3·38	3·32	3·26	3·27	3·2	3·27

From these results it is evident that boiling has not affected the fluorescence of these solutions.

TABLE II

THE EFFECT OF LIGHT ON FLUORESCEIN SOLUTIONS

Concentration of standard	Nominal concentration of test dilution μg./ml.	Net fluorescence					
		0·1 per cent. w/v solution		0·01 per cent. w/v solution		0·001 per cent. w/v solution	
		Light	Dark	Light	Dark	Light	Dark
1·0 μg./ml.	0·25	0·282	0·291	0·279	0·278	0·276	0·278
	0·50	0·532	0·537	0·533	0·531	0·531	0·531
	1·0	0·985	0·989	0·986	0·99	0·982	0·99
	2·0	1·76	1·78	1·78	1·78	1·74	1·75
0·5 μg./ml.	0·25	0·531	0·556	0·524	0·523	0·516	0·524
	0·50	0·983	0·985	0·984	0·983	0·978	0·982
	1·0	1·82	1·82	1·81	1·83	1·8	1·82
	2·0	3·24	3·27	3·27	3·29	3·23	3·23

The effect of light on fluorescein solutions

Solutions containing 0·1, 0·01 and 0·001 per cent. w/v of fluorescein in distilled water were prepared and each divided into two portions. One portion was exposed to indirect sunlight for fourteen days and the other kept in the dark, both at room temperature. Dilutions buffered at *p*H 6 were prepared from each portion and their fluorescence was compared against standards containing 0·5 and 1·0 μg./ml. of fluorescein. Results are shown in Table II.

Exposure of the fluorescein solutions to indirect sunlight for 14 days in the presence of air did not materially affect their fluorescence.

Volatile fluorescent material in fluorescein

Two solutions in distilled water were prepared, solution A from fluorescein previously dried to constant weight at 105° C. and solution B from fluorescein which had not been dried. The concentration of each solution was 0·1 per cent. w/v of fluorescein calculated with respect to the dry weight. 100 ml. of the solutions were distilled at a very low rate using the same loading on the heating mantle in each case, the time to collect 25 ml. of the distillate being recorded. The concentration of fluorescein in the distillate was estimated by comparison with a standard containing 0·125 μg./ml. A quantity of distilled water, equal to the volume of the distillate collected was added to the still and the distillation repeated. Four such distillations were carried out with each solution. The results are recorded in Table III.

TABLE III

VOLATILITY IN STEAM OF THE DRIED AND UNDRIED FLUORESCEIN

	Solution A (fluorescein dried at 105° C.)				Solution B (fluorescein not dried)			
Successive runs using the same solution of fluorescein	1st	2nd	3rd	4th	1st	2nd	3rd	4th
Time in minutes to collect 25 ml.	136	135	147	128	194	200	224	194
Fluorescein in the distillate μg./ml.	1st 0·00294		2nd 0·00306		1st 0·00813		2nd 0·00692	
	3rd 0·0029		4th 0·00298		3rd 0·00558		4th 0·0029	

The concentration of fluorescein in the distillate from solution A was constant for successive distillations and this may be due to the volatility of fluorescein in steam. For solution B the concentration of fluorescein in the distillate was initially higher than that obtained from solution A but it decreased with successive runs and in the 4th distillation the concentration was the same as that obtained from solution A. This suggests that the fluorescein originally contains some volatile fluorescent material which is removed by drying at 105° C. to constant weight.

DISCUSSION

From Fig. 1 a solution of fluorescein 1 μg./ml. shows a maximum fluorescence at *p*H 6 which differs from the results reported by Boutaric

and others[9,10] that fluorescein exhibited a maximum fluorescence at *p*H 8. They used a solution containing 0·5 g./l. (500 μg./ml.) and this is greatly in excess of the concentration at which concentration quenching occurs where the yield of fluorescence decreases with increasing concentration.

Under our experimental conditions the fluorescence of fluorescein solutions containing from 0·125 to 4 μg./ml. was found to be an exponential function of the concentration according to the expression $F = a\,c^{0.85}$ where a = 0·97, 1·78, 3·4 and 6·3 for standards 1, 0·5, 0·25 and 0·125 μg./ml. respectively. Cohen[12] obtained a linear relation for fluorescein solutions containing 0·125 to 6·0 μg./ml. using a fluorimeter in which the galvanometer deflection was taken as a measure of the fluorescence. Pyke[22] and Hennessy and Cerecedo[23] whose fluorimeters were similar to that of Cohen[12] obtained a linear relationship between galvanometer deflection and concentration for concentrations up to 50 mg. and 20 μg. of aneurine respectively. Whereas Williams and Wokes[18] obtained a linear relationship at concentrations of $\frac{5}{16}$–$2\frac{1}{2}$ μg./ml. of aneurine using a fluorimeter similar to that used in this work. However, at concentrations below 0·1 μg./ml. of fluorescein we found that the relationship was linear. Fluorescein could be estimated down to a concentration of 0·02 μg./ml. and could be detected down to 0·001 μg./ml., the error of the estimation becoming high as the drum readings approach the extremity of the scale and the galvanometer deflections are very small.

SUMMARY

1. The Fluorescein Sodium B.P.C. used for this work contained some volatile fluorescent material which was volatile in steam but was removed by drying at 105° C. to constant weight.

2. The maximum intensity of the fluorescence of fluorescein solutions was found to be at *p*H 6.

3. The fluorescence of 0·1, 0·01 and 0·001 per cent. w/v fluorescein solutions was unaffected by boiling under reflux for 80, 8 and 8 hours respectively.

4. Exposure of the fluorescein solutions to indirect sunlight at room temperature for fourteen days did not affect their fluorescence.

5. Fluorescein solutions down to a concentration of 0·02 μg./ml. have been estimated with an error of less than 6 per cent. and fluorescein was detected down to a concentration of 0·001 μg./ml.

The authors wish to thank Dr. F. Wokes for his advice on the fluorimetric assays.

REFERENCES

1. Shotton and Habeeb, *J. Pharm. Pharmacol.*, 1954, **6**, 1023.
2. Bouchard, *J. Chim. Phys.*, 1936, **33**, 232.
3. Jenness, *Phys. Rev.*, 1929, **34**, 1275.
4. Rollefson and Stoughton, *J. Amer. chem. Soc.*, 1941, **63**, 1517.
5. Bouchard, *C.R. Acad. Sci., Paris*, 1933, **196**, 485.
6. Jette and West, *Proc. Roy. Soc.*, 1928, A**121**, 299.
7. Pringsheim, *Fluorescence amd Phosphorescence*, 1949.
8. Chechan, *C.R. Acad. Sci., Paris*, 1946, **222**, 80.

9. Boutaric and Roy, *ibid.*, 1939, **209**, 162.
10. Boutaric and Maraux, *Bull. Soc. chim. Fr.*, 1948, 5e series, 952.
11. Volmar, *Chim. et Ind.*, 1937, **37**, 446.
12. Cohen, *Rec. Trav. chim. Pays-Bas.*, 1935, **54**, 133.
13. Mathur and Bhatnagar, *Indian J. Phys.*, 1928, **3**, 37.
14. Isbell, *Analyst*, 1949, **74**, 618.
15. Lothian, *J. sci. Instrum.*, 1941, **18**, 200.
16. Umberger and La Mer, *J. Amer. chem. Soc.*, 1945, **67**, 1099.
17. Wokes and Slaughter, *Analyst*, 1949, **74**, 624.
18. Williams and Wokes, *Quart. J. Pharm. Pharmcol.*, 1947, **20**, 240.
19. Desha, Sherrill and Harrison, *J. Amer. chem. Soc.*, 1926, **48**, 1493.
20. Betrand, *C.R. Acad. Sci., Paris*, 1945, **220**, 525.
21. Weissberger, *Physical Methods of Organic Chemistry*, Vol. II, 2nd Ed., Interscience Publishers N.Y., 1949, p. 1409.
22. Pyke, *Biochem. J.*, 1937, **31**, 1958.
23. Hennessy and Cerecedo, *J. Amer. chem. Soc.*, 1939, **61**, 179.