CHAPTER II

MATERIAL AND METHODS

2.1 INTRODUCTION

The earliest report of a phthalein dye that possesses indicator property is due to Kruger [83]. In 1876 fluorescein was mentioned as a probable substitute for litmus. The very next year, Luck proposed the use of the most commonly used phthalein dye, phenolphthalein [84]. Since than a large number of phthalein dyes were synthesised and their uses in chemical studies were investigated. The search was mainly aimed at a vivid colour change at various pH transition intervals. During the last fifty years the study of fluorescence has been developed and the fluorescent substances have been used in a variety of problems. The compounds of this class are mainly prepared by coupling phthalic anhydride or o-sulphophthalic anhydride with substituted phenols. In our laboratory we have synthesised several phthalein dyes starting with pyromellitic acid dianhydride and coupling it with resorcinol and one more phenol so that on one hand there is coupling with resorcinol to give fluorescein-like property and on the other hand to the other half side there is coupling with phenol or a substituted phenol, so as to get phenolphthalein-like property. In this chapter synthesis of such compounds and their characterisation by conventional methods is described. In the further part, the esters of the analogues of fluorescein have been used in enzyme assay.

2.2 GENERAL METHOD OF PREPARATION OF PHTHALEIN DYES FROM PYROMELLITIC ACID DIANHYDRIDE

Pyromellitic acid dianhydride is coupled with resorcinol and one more phenol in presence of concentrated sulphuric acid or fused anhydrous zinc chloride [85].

(i) Phenol fluoromellitein

Preparation

Pyromellitic acid dianhydride 2.2 g, molten phenol 2 ml and resorcinol 2.2 g were mixed with fused zinc chloride 4.0 g in a conical flask and was heated in an oil bath with constant stirring at 180° C for 45 to 90 minutes, when a dark red brown paste was formed. When the temperature dropped down to 90° C, 40 ml water and 4 ml concentrated hydrochloric acid were added and then it was heated at 110° C, so that zinc chloride dissolves and gets removed on filtration. The very thick and viscous residue was treated with 10 % solution of sodium hydroxide when the dye gets dissolved. The deeply coloured fluorescent liquid was filtered out which on acidification gave a precipitate or a brown suspension, which is filtered out. By using similar procedures nine more fluoromellitein dyes were obtained.

Purification

The dye was purified by several reprecipitations by using sodium hydroxide for dissolution and hydrochloric acid for

precipitation. The precipitate was filtered out, dried by repeatedly triturating with dry diethyl ether. The solid was dried to give a yellowish brown coloured amorphous powder.
Molecular formula : $C_{34}H_{20}O_9$

Properties and Analysis

It is an amorphous, light weight, easy-flowing powder, very sparingly soluble in distilled water or dilute acids, soluble in alcohol-water mixtures and also in dilute solutions of weak and strong alkalies like sodium carbonate and sodium hydroxide. The material does not melt but gradually starts charring above 260° C.
Molecular weight 572.534;
Yield 6.0 g 90.9 % on the basis of acid anhydride.
Compound analyses to the formula : $C_{34}H_{20}O_9$.
C, 71.3 % found 71.1; H, 3.52 % found 3.2; and O (by difference) 25.2 %.

(ii) Difluoromellitein

Preparation and Purification

It was prepared by using 2.2 g pyromellitic acid dianhydride 4 g fused zinc chloride and 4.4 g resorcinol in place of the two phenols, when a yellowish brown product was obtained. Purification of this and all the subsequently described dyes was carried out as described in the case of the previous compound.

Properties and Analysis

It is almost insoluble in water and dilute acid and gives deep green fluorescence with alkali solution. Does not melt but chars above 250° C.

Yield 5.5 g, 83.3 % on the basis of acid anhydride.

Molecular weight 586.52.

Compound analyses to the formula : $C_{34}H_{18}O_{10}$.

C, 69.63 % found 69.2 %; H, 3.09 % found 2.9 % and

O (by difference) 27.28 %.

(iii) o-Cresol fluoromellitein

Preparation

It was prepared by using 2.2 g pyromellitic acid dianhydride, 4 g fused zinc chloride, 2.2 ml o-cresol and 2.2 g resorcinol when a brown coloured product was obtained.

Properties and Analysis

It is almost insoluble in water and dil. acids and gives deep green fluorescence with alkali solution. Does not melt but chars above 250° C.

Yield 4.2 g, 64.6 % on the basis of acid anhydride.

Compound analyses to the formula : $C_{36}H_{24}O_{9}$.

C, 72.0 % found 71.1 %; H, 4.03 %, found 3.92 % and

O (by difference) 23.97 %.

Molecular weight 600.588.

(iv) m-Cresol fluoromellitein

Preparation

It was prepared by using 2.2 g pyromellitic acid dianhydride, 4 g fused zinc chloride, 2.2 g resorcinol and 212 ml m-cresol. Fused melt obtained is not as much viscous as in other case.

Properties and Analysis

It is almost insoluble in water and dil. acids and gives deep green fluorescence with alkali solution. Does not melt but chars above 250° C.

Yield 4.0 g, 61.5 % on the basis of acid anhydride.

Compound analyses to the formula : $C_{36}H_{24}O_9$.

C, 71.99 %, found 71.1 %; H, 4.03 %, found 3.89 %;

O (by difference) 23.97 %.

Molecular weight 600.59.

(v) p-Cresol fluoromellitein

Preparation

It was prepared by the reaction between 2.2 g of pyromellitic acid dianhydride, 4 g fused zinc chloride, 2.2 g resorcinol and 2.2 ml of p-cresol, when a brown coloured product was obtained.

Properties and Analysis

It is insoluble in water and dilute acids. It gives deep green fluorescence with alkali solution. Does not melt but chars above 250° C.

Yield 3.3 g, 50.7 % on the basis of acid anhydride.

Compound analyses to the formula : $C_{36}H_{24}O_9$.

C, 71.99 %, found 71.4 %; H, 4.03 %, found 4.2 %; and

O (by difference) 23.97 %.

Molecular weight 600.588.

(vi) o-Chlorophenol fluoromellitein

Preparation

It was prepared by fusing 2.2 g pyromellitic acid dianhydride, 4 g fused zinc chloride, 2.2 g resorcinol and 2.53 ml o-chlorophenol, when a brown coloured product was obtained.

Properties and Analysis

It is almost insoluble in water and dilute acids and gives deep green fluorescence with alkali solution. Does not melt but chars above 300° C.

Yield 5.5 g, 83.5 % on the basis of acid anhydride.

Compound analyses to the formula : $C_{34}H_{18}O_9Cl_2$.

C, 63.65 % found 63.9 %; H, 2.83 found 2.9 %; Cl, 11.05 %, found 11.6 % and O (by difference) 22.47 %.

Molecular weight 641.43.

(vii) p-Chlorophenol fluoromellitein

Preparation

It was prepared by fusing 2.2 g pyromellitic acid dianhydride, 4 g fused zinc chloride, 2.2 g resorcinol and 2.53 ml p-chlorophenol, when a brown coloured product was obtained.

Properties and Analysis

It is almost insoluble in water and dilute acids and gives deep green fluorescence with alkali solution. Does not melt but chars above 300° C.
Yield 4.0 g, 60.6 % on the basis of acid anhydride.
Molecular weight 641.432.
Compound analyses to the formula : $C_{34}H_{18}O_9Cl_2$.
C, 63.65 % found 64.0 %; H, 2.83 found 2.7 %; Cl, 11.05%, found 11.3 %; and O (by difference) 22.47 %.

(viii) 1,2 dihydroxyphenolfluoromellitein

Preparation

It was prepared by fusing 2.2 g pyromellitic acid dianhydride, 4 g fused zinc chloride, 2.2 g catechol [1,2 dihydroxyphenol] and 2.2 g resorcinol. Dark red coloured product is obtained.

Properties and Analysis

It is almost insoluble in water and dil. acid and gives deep green fluorescence with alkali solution. Does not melt but chars above 260° C.

Yield 4.0 g, 60.6 % on the basis of acid anhydride.

Compound analyses to the formula : $C_{34}H_{20}O_{11}$.

Molecular weight 604.53.

C, 67.5 % found 67.2 %; H, 3.33 %, found 3.12 %, and

O (by difference) 29.11 %.

(ix) 1,2,3 trihydroxyphenol fluoromellitein

Preparation

It was prepared by fusing 2.2 g pyromellitic acid dianhydride, 4 g fused zinc chloride, 2.2 g resorcinol and 2.52 g pyrogallol. Product obtained is a dark red power.

Properties and Analysis

It is almost insoluble in water and dilute acid and gives deep green fluorescence but chars above 250° C.

Yield 4.2 g, 63.6 % on the basis of acid anhydride.

Compound analyses to the formula : $C_{34}H_{20}O_{13}$.

C, 64.1 % found 63.2 %; H, 3.16 %, found 3.32 %; and

O (by difference) 32.67 %.

Molecular weight 636.53.

(x) p-Nitrophenol fluoromellitein

Preparation

Reaction between 2.2 g resorcinol, 2.8 g p-nitrophenol, 2.2 g pyromellitic acid dianhydride and 4 g fused zinc chloride gave brown coloured product.

Properties and Analysis

It is almost insoluble in water and dilute acid and gives deep green fluorescence with alkali solution. Does not melt but chars above 240° C.

Yield 3.6 g, 50 % on the basis of acid anhydride.

Compound analyses to the formula : $C_{34}H_{18}O_{13}N_2$.

C, 61.6 %, found 61.9 %; H, 2.7 %, found 2.47 %; N, 4.23 %, found 4.4 %; and O (by difference) 31.39 %.

Molecular weight 662.53.

2.3 SYNTHESIS OF ESTERS OF MELLITEIN DYES

The butyric and acetic esters of the mellitein dyes were synthesized and used in the study of lipase activity. The details are given in Chapter 4.

2.4 CHEMICALS AND SOLUTIONS

All the chemicals used in the synthesis were Fluka or Koch Light make. Ethanol was purified by double distillation.

Conductivity water was prepared by distilling glass-distilled water containing potassium hydroxide and potassium permanganate, by collecting the distillate in polythene bottle. Every time, before use, the water was passed through monobed mixed resin column.

Substrates and enzyme preparations are described in Chapter 6.

2.4.1 Buffer Solutions

Ingradient for buffer solutions were of high purity, AR grade and were BDH/E. Merck make. Buffer solutions were prepared by using formulation given in Ju. Luries "Handbook of Analytical Chemistry" [86].

Stock solutions

Solution No.1 : Hydrochloric acid 0.1 M.

Solution No.2 : Monosodium citrate ($NaH_2C_6H_5O_7$) 0.1 M (21.014 g of $H_3C_6H_5O_7(H_2)$ + 200 ml of 1 N NaOH solution in one litre).

Solution No.3 : Sodium hydroxide 0.1 M

Solution No.4 : Dihydrogen potassium ortho phosphate (KH_2PO_4) 1/15 M

Solution No.5 : Disodium hydrogen ortho phosphate (Na_2HPO_4) 1/15 M

Solution No.6 : Tris [hydroxymethyl] aminomethane $NH_2C(CH_2OH)_3$ 12.11 g dissolved in water to give one litre solution.

Table 2.1

Buffer solutions of pH 1.10 to 4.96

Every given amount of the solution of sodium citrate [0.1 M] is made upto 100 ml with solution No.1

pH	0	1	2	3	4	5	6	7	8	9
1.1	4.8	5.6	6.4	7.1	7.8	8.4	9.0	9.6	10.1	10.6
1.2	11.1	11.6	12.1	12.5	13.0	13.5	14.0	14.5	14.9	15.4
1.3	15.9	16.2	16.6	16.9	17.3	17.6	17.9	18.3	18.6	19.0
1.4	19.3	19.6	19.9	20.2	20.5	20.8	21.1	21.4	21.6	21.9
1.5	22.2	22.4	22.7	22.9	23.2	23.4	23.6	23.9	24.1	24.4
1.6	24.6	24.8	25.0	25.2	25.4	25.6	25.8	26.0	26.1	26.3
1.7	26.5	26.7	26.9	27.0	27.2	27.4	27.6	27.7	27.9	28.0
1.8	28.2	28.3	28.5	28.6	28.8	28.9	29.0	29.1	29.3	29.4
1.9	29.5	29.6	29.7	29.9	30.0	30.1	30.2	30.3	30.4	30.5
2.0	30.6	30.7	30.8	31.0	31.1	31.2	31.3	31.4	31.5	31.6
2.1	31.7	31.8	31.9	31.9	32.0	32.1	32.2	32.3	32.4	32.5
2.2	32.6	32.7	32.8	32.9	33.0	33.1	33.2	33.3	33.4	33.5
2.3	33.6	33.7	33.8	33.8	33.9	34.0	34.1	34.2	34.3	34.4
2.4	34.5	34.6	34.7	34.8	34.9	35.0	35.1	35.2	35.2	35.3
2.5	35.4	35.5	35.6	35.7	35.8	35.9	36.0	36.1	36.2	36.3
2.6	36.4	36.5	36.6	36.7	36.8	36.9	37.0	37.1	37.1	37.2

Table 2.2 : Buffer solutions of pH 4.80 to 8.00
Every given amount of solution of disodium-orthophosphate [1/15 M] is made upto 100 ml with solution No.4.

pH	0	1	2	3	4	5	6	7	8	9
4.8	0.35	0.37	0.39	0.41	0.43	0.45	0.48	0.51	0.54	0.57
4.9	0.60	0.63	0.66	0.69	0.72	0.75	0.79	0.83	0.87	0.91
5.0	0.95	0.99	1.03	1.07	1.11	1.15	1.19	1.23	1.27	1.31
5.1	1.35	1.39	1.43	1.47	1.51	1.55	1.60	1.65	1.70	1.75
5.2	1.80	1.85	1.90	1.95	2.00	2.05	2.10	2.15	2.20	2.25
5.3	2.30	2.37	2.44	2.51	2.58	2.65	2.72	2.79	2.86	2.93
5.4	3.00	3.09	3.18	3.27	3.36	3.45	3.54	3.63	3.72	3.81
5.5	3.90	3.99	4.08	4.17	4.26	4.35	4.46	4.57	4.68	4.79
5.6	4.90	5.02	5.14	5.26	5.38	5.50	5.62	5.75	5.90	6.05
5.7	6.20	6.35	6.50	6.70	6.85	7.00	7.20	7.35	7.55	7.70
5.8	7.90	8.10	8.25	8.45	8.60	8.80	9.00	9.20	9.40	9.60
5.9	9.80	10.00	10.20	10.40	10.60	10.80	11.10	11.30	11.60	11.80
6.0	12.1	12.4	12.7	12.9	13.2	13.5	13.8	14.1	14.4	14.7
6.1	15.0	15.3	15.7	16.0	16.4	16.7	17.0	17.4	17.7	18.1
6.2	18.4	18.7	19.1	19.4	19.8	20.1	20.5	20.9	21.3	21.7
6.3	22.1	22.5	22.9	23.4	23.8	24.2	24.6	25.1	25.5	26.0

Table 2.3 : Tris buffer pH range 8 to 85

'x' ml of 0.1 M hydrochloric acid + 50.0 ml of 0.1 M tris diluted with water to 100 ml.

pH	0	1	2	3	4	5	6	7	8	9
8.0	29.2	28.9	28.7	28.3	27.95	27.65	27.35	27.05	26.7	26.4
8.1	26.1	25.75	25.45	25.15	24.8	24.5	24.15	23.85	23.55	23.2
8.2	22.9	22.6	22.35	22.05	21.75	21.5	21.2	20.9	20.65	20.3
8.3	20.05	19.75	19.5	19.2	18.9	18.65	18.3	18.05	17.75	17.5
8.4	17.2	16.75	16.5	16.25	16.0	15.8	15.55	15.3	15.05	14.82
8.5	14.6	14.35	14.1	-	-	-	-	-	-	-

Table 2.4 : Buffer solutions of pH 8.53 to 12.9
Every given amount of the solution of sodium hydroxide [0.1 M] is made upto 100 ml with solution No.7.

pH	0	1	2	3	4	5	6	7	8	9
8.5	-	-	-	5.00	5.11	5.22	5.33	5.44	5.56	5.68
8.6	5.80	5.92	6.04	6.16	6.28	6.41	6.54	6.68	6.82	6.96
8.7	7.10	7.24	7.38	7.52	7.66	7.81	7.96	8.12	8.28	8.44
8.8	8.60	8.77	8.94	9.12	9.30	9.48	9.66	9.84	10.02	10.21
8.9	10.4	10.6	10.8	11.0	11.2	11.4	11.6	11.8	12.0	12.2
9.0	12.4	12.6	12.8	13.0	13.2	13.4	13.6	13.8	14.0	14.3
9.1	14.6	14.8	15.1	15.3	15.6	15.8	16.0	16.3	16.5	16.8
9.2	17.0	17.2	17.4	17.6	17.9	18.2	18.5	18.8	19.1	19.4
9.3	19.7	19.9	20.1	20.3	20.5	20.8	21.1	21.4	21.7	22.0
9.4	22.3	22.5	22.8	23.1	23.4	23.7	24.0	24.3	24.6	24.9
9.5	25.2	25.4	25.6	25.9	26.2	26.5	26.8	27.1	27.4	27.7
9.6	28.0	28.3	28.6	28.9	29.2	29.5	29.8	30.1	30.4	30.7
9.7	31.0	31.3	31.6	31.9	32.2	32.5	32.8	33.1	33.4	33.6
9.8	33.8	34.1	34.4	34.7	35.0	35.2	35.4	35.6	35.8	36.0
9.9	36.2	36.5	36.7	36.9	37.1	37.3	37.5	37.7	37.9	38.1
10.0	38.3	38.5	38.7	38.9	39.1	39.3	39.5	39.7	39.9	40.05
10.1	40.2	40.4	40.55	40.7	40.9	41.05	41.2	41.4	41.55	41.75
10.2	41.9	42.05	42.2	42.4	42.55	42.7	42.85	43.0	43.2	43.35
10.3	43.5	43.65	43.75	43.9	44.0	44.15	44.3	44.4	44.55	44.7
10.4	44.8	44.9	45.0	45.1	45.2	45.3	45.4	45.5	45.6	45.7
10.5	45.8	45.9	46.0	46.05	46.15	46.25	46.35	46.45	46.5	46.6
10.6	46.7	46.75	46.85	46.9	47.0	47.05	47.1	47.2	47.25	47.35
10.7	47.4	47.45	47.5	47.6	47.65	47.7	47.75	47.8	47.9	47.95
10.8	48.0	48.05	48.1	48.15	48.2	48.25	48.3	48.35	48.4	48.45

Solution No.7 : Glycin (NH_2CH_2COOH) 0.1 M (7.507 g of glycin + 5.85 g of NaCl in one litre).

Compositions of the buffer solutions are given in Tables 2.1 to 2.4.

2.4.2 Apparatus

All the glassware was Corning make and volumetric apparatus was class B but calibrated in our laboratory [87].

2.5 EQUIPMENT

Spectrophotometric measurements were done by using Beckman DU2 Single beam UV-Vis spectrophotometer, using a matched pair of glass cuvettes.

The pH measurements were done on an Elico Digital Precision pH meter model 39A. The pH meter was calibrated by using 0.05 M potassium hydrogen phthalate buffer and standard borax buffer. The pH meter was periodically checked for stability and corrected for $\pm$ 0.01 pH unit. If distortion exceeded the above limit the observation set was repeated.

Infrared Spectra in KBr pellates were run on Beckman IR 20, ratio-recording Infrared Spectrophotometer over the range 4000-600 cm^{-1}.

Fluorescence spectral studies were carried out on Aminco Bowman double monochromator recording spectrofluorometer Model SPF, by using excitation wavelength of 330 nm. The spectra were run on a wide pH range and were finally corrected for constant excitation intensity. The curves were corrected for spectral response of the photomultiplier by using an electronic cam.

For enzyme assay a Czechoslovac make LP Fluorimeter was used. The exciting radiation was obtained from a mercury discharge tube and Jena-UV pass-visible cut off-filter and the emission was measured by using Jena-UV cut off-visible pass-filter followed by appropriate interference filter. There was fairly good agreement in the final results obtained with Aminco or LP instruments. Although the second instrument was of a lower quality compared with the first, its easy availability was the main purpose of its use.

2.6 PLAN OF RESEARCH WORK

Fluorescein has been extensively studied fluorometrically and substituted fluoresceins were also studied. The reported substituted fluoresceins alter the nature of fluorescence spectrum also as is shown by eosin [tetrabromo derivatives] or erythrosine β [iodo derivative]. Pyromellitic acid is 1,2,4,5 tetra carboxylic analogue of phthalic acid. We synthesize new fluorescein analogues by using half side of this molecule with resorcinol and the remaining half was coupled to a phenol so as to form a phthalein

dye analogue of phenolphthalein. These dyes on one hand possess almost identical fluorescence characteristics but colour transition pH interval varies with the substituents on the phenol. This novel type of fluorescein analogue may be converted into corresponding non fluorescent butyric or acetic acid esters and their fluorometric study in enzyme assay makes available a new refinement for the study of enzyme lipase. The thesis embodies the above mentioned aspects of fluorometry as applied to mellitein dyes. This report is an initiation in this field and subsequehtly extensive work is possible.