CHAPTER IV

FLUORESCENCE SPECTRA OF MELLITEIN DYES IN THEIR SCREENING FOR APPLICATIONS IN THE STUDY OF ENZYME ACTIVITY

4.1 INTRODUCTION

The theoretical aspects of fluorescence phenomenon has been discussed in Chapter I. This chapter presents the data on fluorescence spectra of the new mellitein dyes prepared in our laboratory and their applications.

4.2 NATURE OF ABSORPTION AND FLUORESCENCE SPECTRA

Typical spectra of a fluoromellitein dye is shown in Fig. 3.2. The excitation radiation from a monochromator has a sharp, symmetric band in the ultraviolet region and the emission of fluorescence occurs at long wavelength low energy region. In fluorescein this band is quite symmetric and intense. The intensity of fluorescence depend on several experimental conditions such as pH and in fluorescein optimum pH condition is between pH values 4 and 6. The energy and the intensity of the excitation band determines the nature and intensity of fluorescence as shown in Fig. 4.1. Eight spectra are recorded here to show the effect both of excitation wavelength and pH. The first set of the spectra 1,2,3,4 in the excitation part of the figure shows that in mellitein dyes somewhat longer wavelength

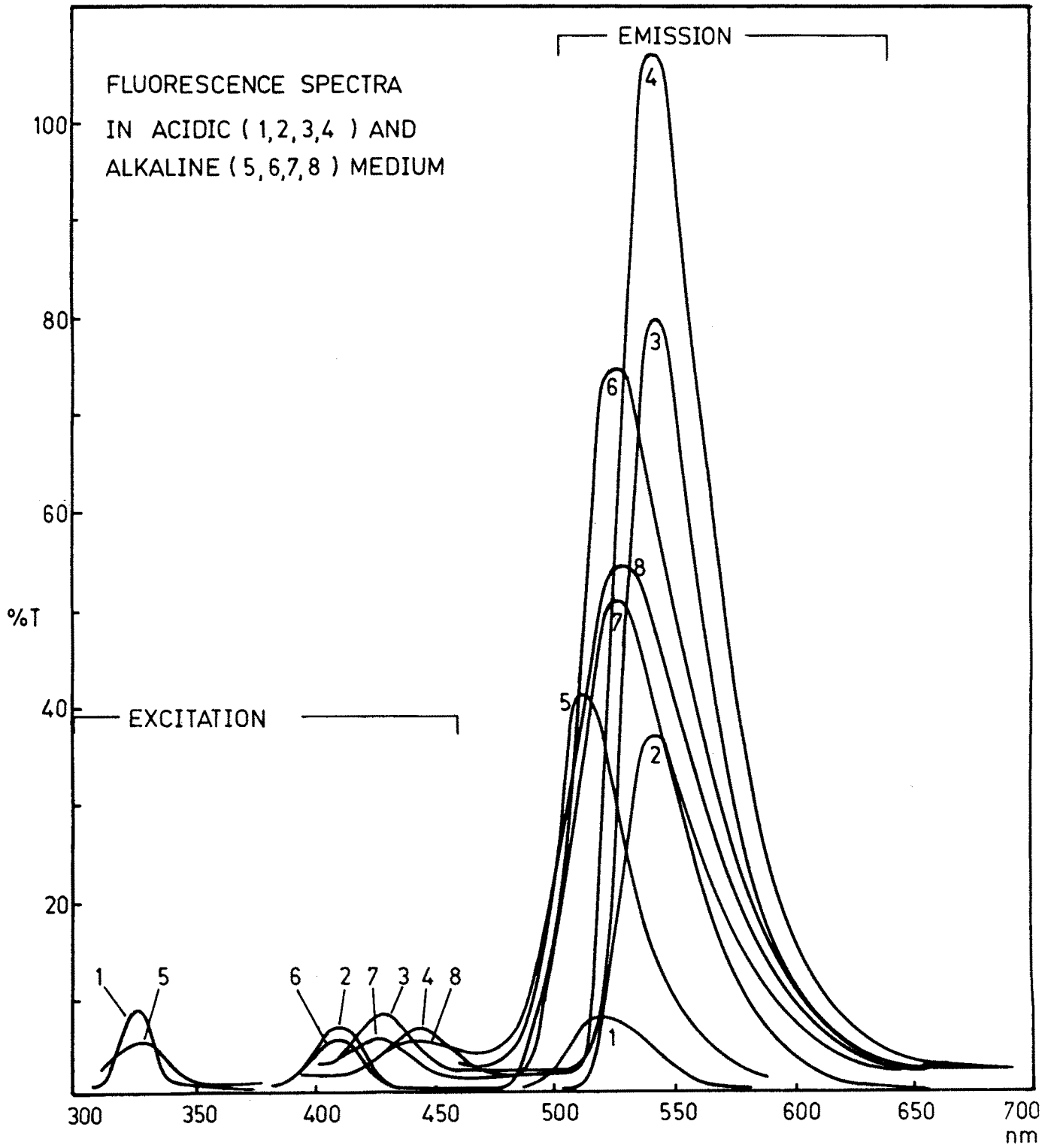
FLUORESCENCE SPECTRA
IN ACIDIC (1,2,3,4) AND
ALKALINE (5,6,7,8) MEDIUM
EMISSION
EXCITATION
%T
100
80
60
40
20
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Fig. 4·1

around 400 nm shows very strong fluorescence band, whereas the spectra in alkaline medium show that the emission intensity is less affected by the change in excitation wavelength.

The absorption spectrum in Fig. 3.2 shows strong pH dependent band which has a long wavelength absorption $\sim$ 520 nm in alkaline medium and in acidic medium it is at much shorter wavelength around 430 nm. In the present study much shorter excitation wavelength of 330 nm was used to facilitate the clear recording of the band in acidic condition also. The two forms of the dye in the extreme pH condition are in equilibrium and show well defined isobestic points corresponding to the two ionization equilibria involved.

4.3 EMISSION SPECTRA OF MELLITEIN DYES

Figs. 4.2 to 4.11 show the excitation and fluorescence spectra of the ten mellitein dyes prepared in the present work. The emission wavelength is not much altered with pH but the intensity is highly pH dependent. Hence pH is a very important parameter. The intensity data is given in Tables 4.1 to 4.10.

4.4 RESULTS

The excitation and emission intensities are depicted in Tables 4.1 to 4.10.

Table 4.1 : Fluorescence spectrum of Difluoromellitein

pH	Excitation Intensity	Emission Intensity
12.00 - 8.00	32	99
7.23	32	83
6.77	32	70
6.01	32	55
5.14	32	37
4.36	32	20
4.10	32	12

Table 4.2 : Fluorescence spectrum of phenolfluoromellitein

pH	Excitation Intensity	Emission Intensity
12.00 - 8.15	15	78
7.40	15	63
6.58	15	48
6.05	15	38
5.19	15	24
4.63	15	14
4.25	15	6

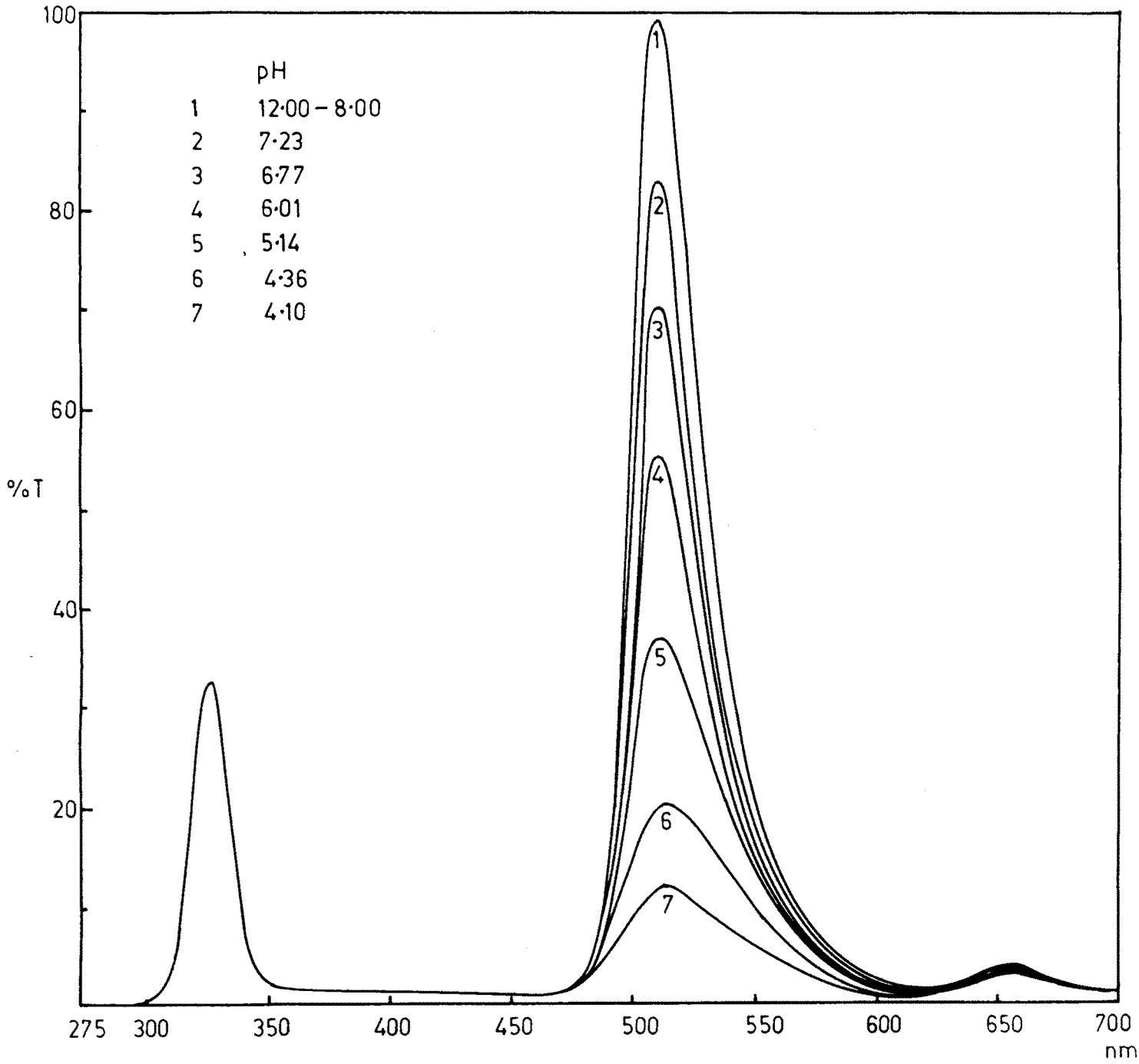


Fig.4·2 EXCITATION AND FLUORESCENCE SPECTRA OF DIFLUOROMELLITEIN AT pH 4·40 TO pH 12·00 . (CONC. 0·004 mg/ml)

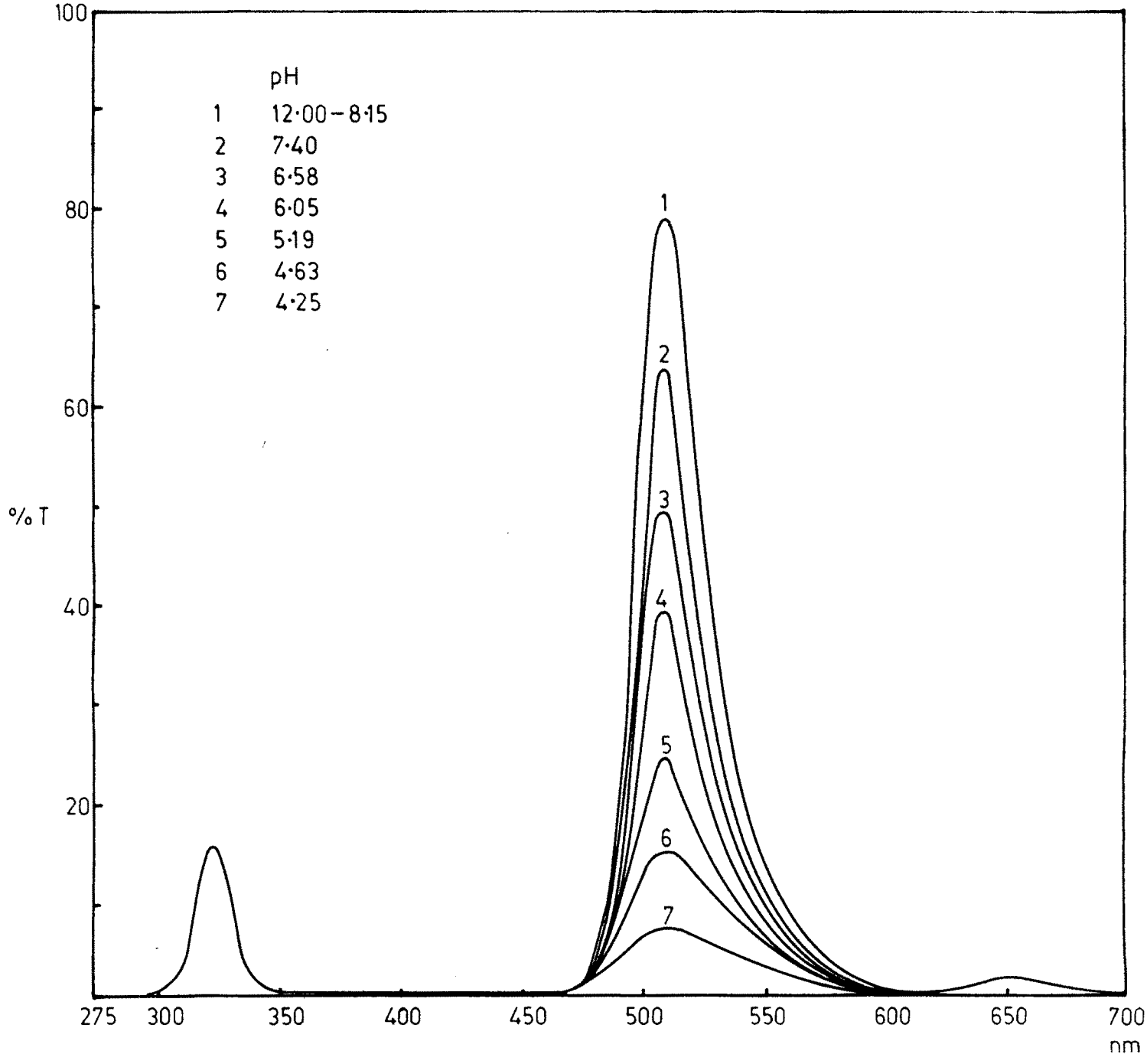


Fig.4·3 EXCITATION AND FLUORESCENCE SPECTRA OF PHENOL FLUOROMELLITEIN AT pH 4·40 TO pH 12·00 .(CONC. 0.004 mg/ml)

Table 4.3 : Fluorescence spectrum of 1,2 dihydroxyphenol fluoromellitein

pH	Excitation Intensity	Emission Intensity
12.00 - 8.20	14	92
7.78	14	76
6.45	14	60
6.05	14	47
5.41	14	34
4.40	14	17

Table 4.4 : Fluorescence spectrum of 1,2,3 Trihydroxyphenol fluoromellitein

pH	Excitation Intensity	Emission Intensity
12.00 - 8.15	34	75
7.13	34	59
6.43	34	46
5.82	34	37
4.89	34	20
4.23	34	7

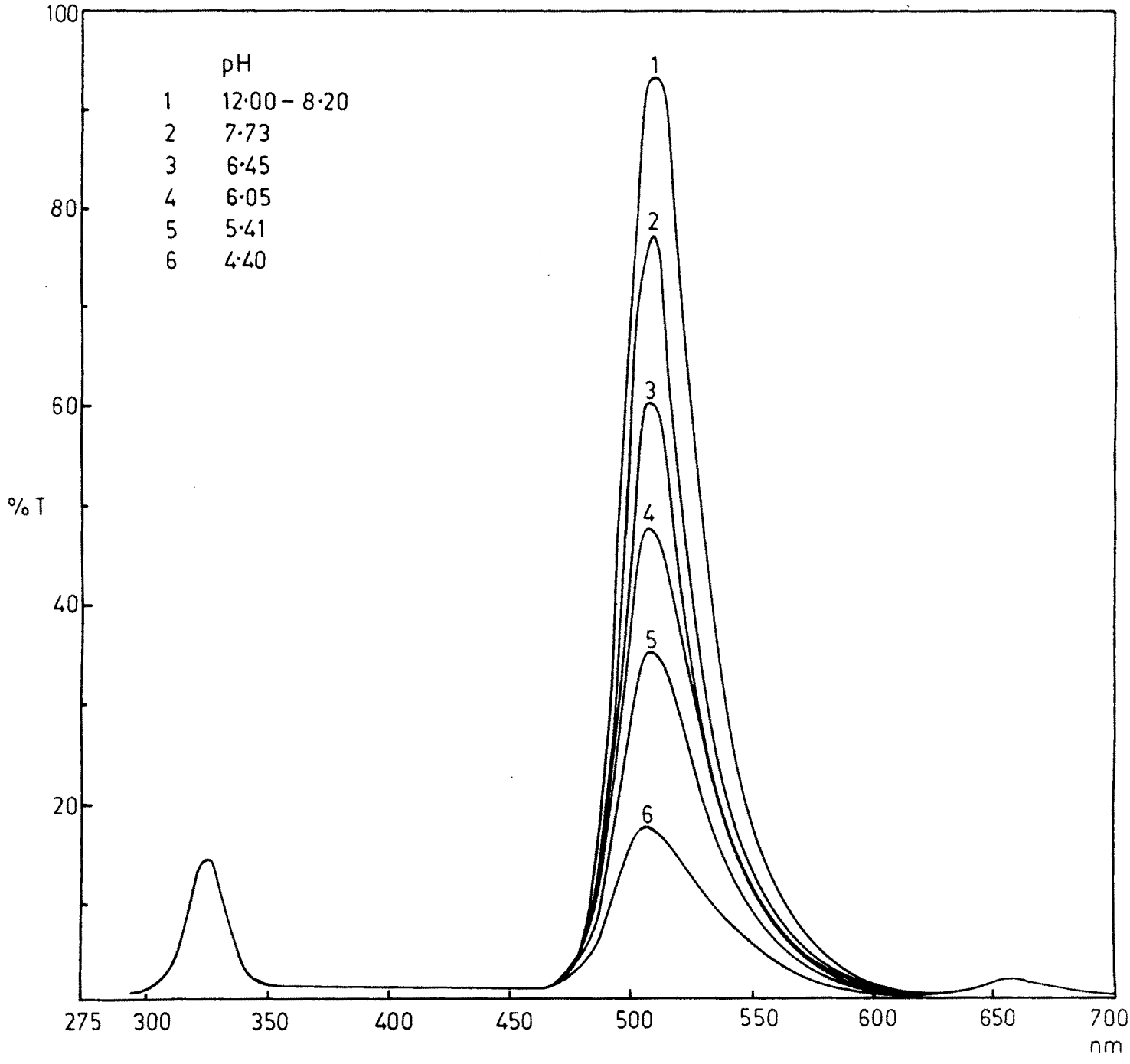


Fig.4·4 EXCITATION AND FLUORESCENCE SPECTRA OF 1,2 DIHYDROXYPHENOL FLUOROMELLITEIN. (CONC. 0·004 mg/ml)

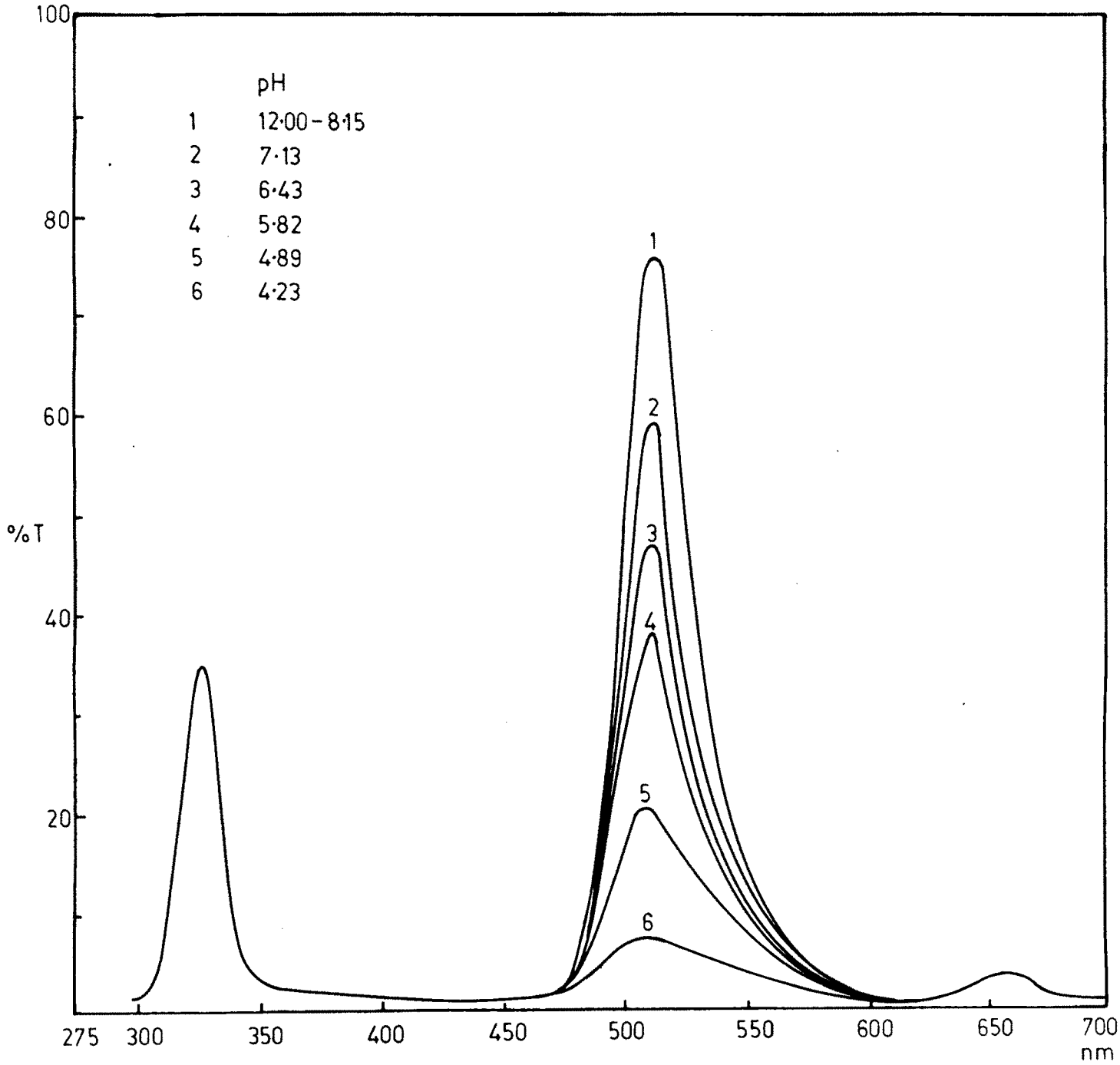


Fig.4·5 EXCITATION AND FLUORESCENCE SPECTRA OF 1,2,3 TRIHYDROXY PHENOL FLUOROMELLITEIN. (CONC. 0·004 mg/ml)

Table 4.5 : Fluorescence spectrum of o-Cresol fluoromellitein

pH	Excitation Intensity	Emission Intensity
12.00 - 8.05	14	100
7.15	14	84
6.23	14	63
5.66	14	50
5.15	14	33
4.52	14	20
4.10	14	11

Table 4.6 : Fluorescence spectrum of m-Cresol fluoromellitein

pH	Excitation Intensity	Emission Intensity
12.00 - 8.20	14	54
7.55	14	46
6.90	14	36
6.02	14	27
4.62	14	10
4.15	14	3

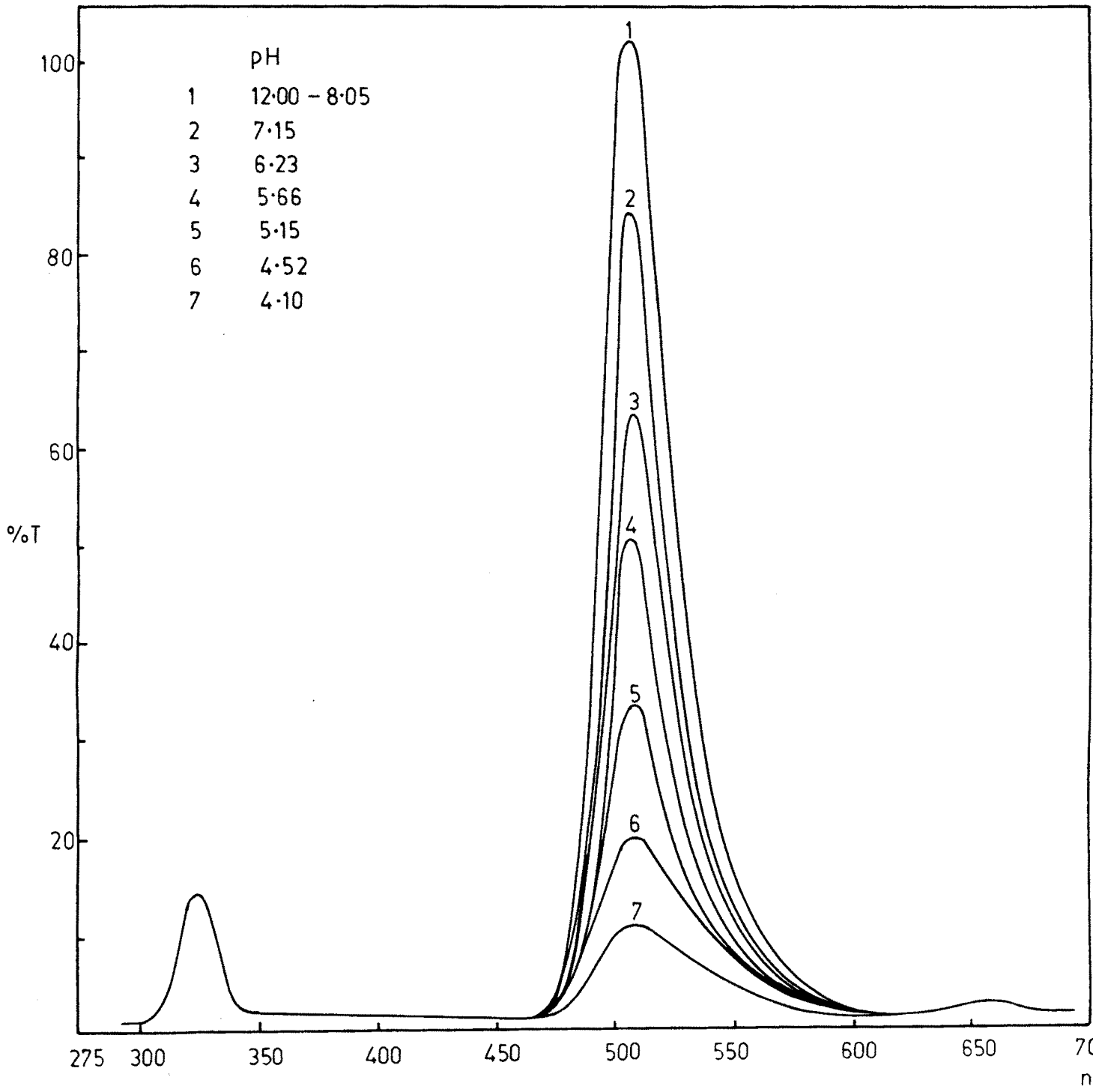


Fig. 4·6 EXCITATION AND FLUORESCENCE SPECTRA OF O–CRESOL FLUOROMELLITEIN
(CONC. 0·004 mg/ml)

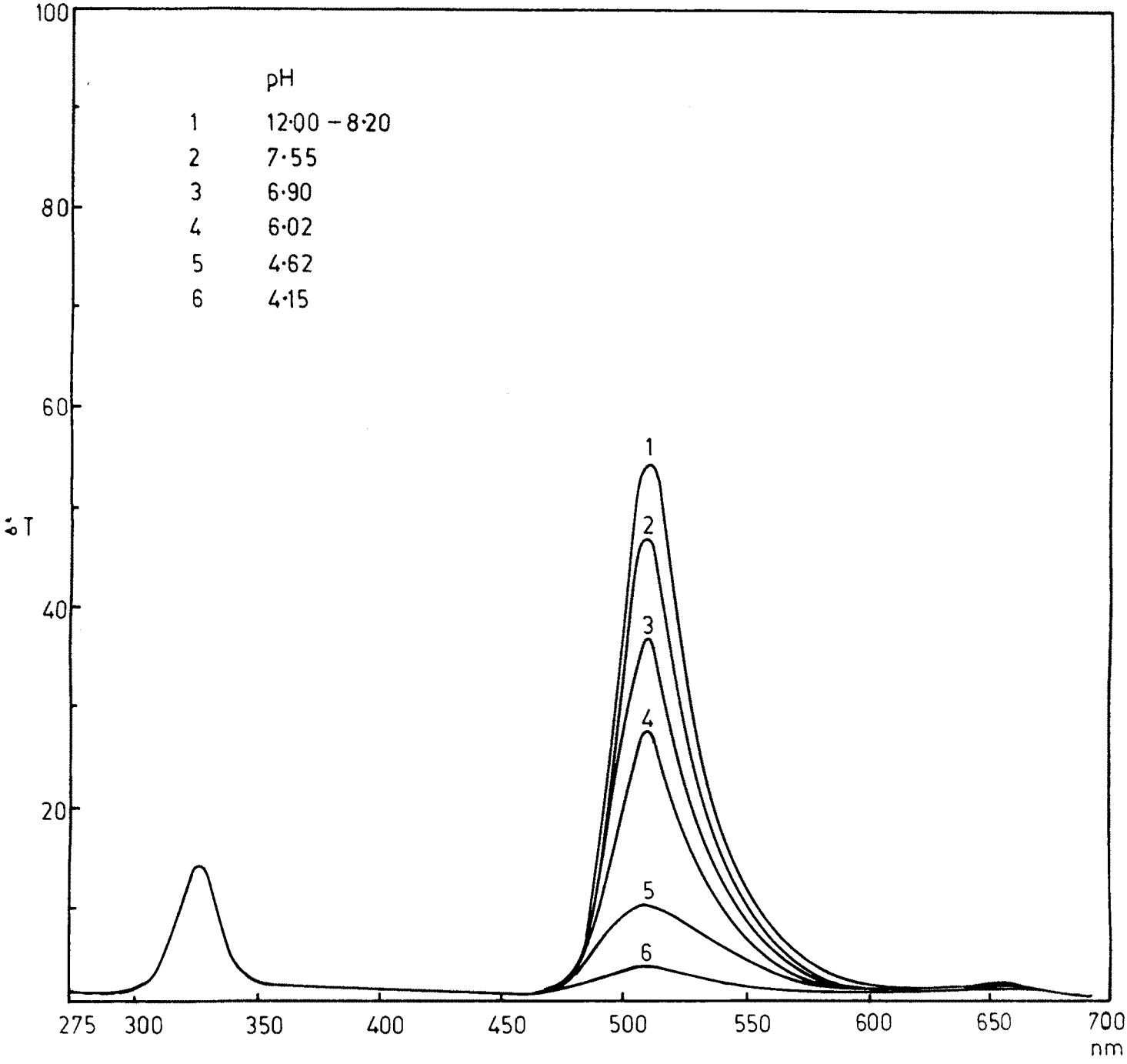


Fig.4·7 EXCITATION AND FLUORESCENCE SPECTRA OF M-CRESOL FLUOROMELLITEIN.
(CONC. 0·004 mg / ml)

Table 4.7 : Fluorescence spectrum of p-Cresol fluoromellitein

pH	Excitation Intensity	Emission Intensity
12.00 - 8.10	13	110
7.30	13	90
8.55	13	75
8.15	13	55
5.35	13	40
4.82	13	30
4.55	13	22
4.25	13	16
4.05	13	11

Table 4.8 : Fluorescence spectrum of p-Nitrophenol fluoromellitein

pH	Excitation Intensity	Emission Intensity
12.00 - 8.15	15	76
7.69	15	66
7.12	15	58
6.45	15	47
6.05	15	38
5.15	15	29
4.85	15	20
4.20	15	10

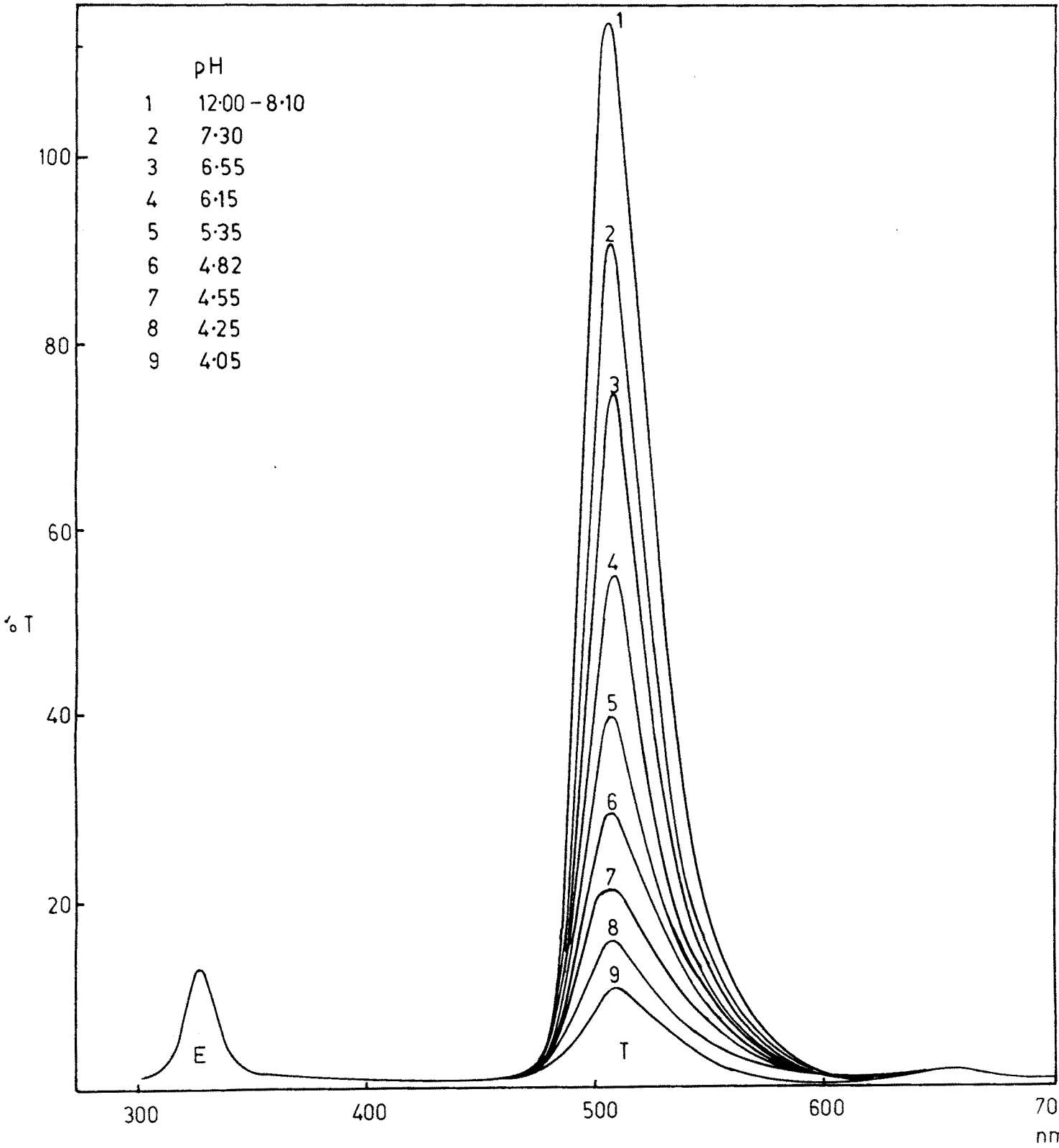


Fig.4·8 EXCITATION AND FLUORESCENCE SPECTRA OF P–CRESOL FLUOROMELLITEIN .
(CONC. 0·004 mg/ml)

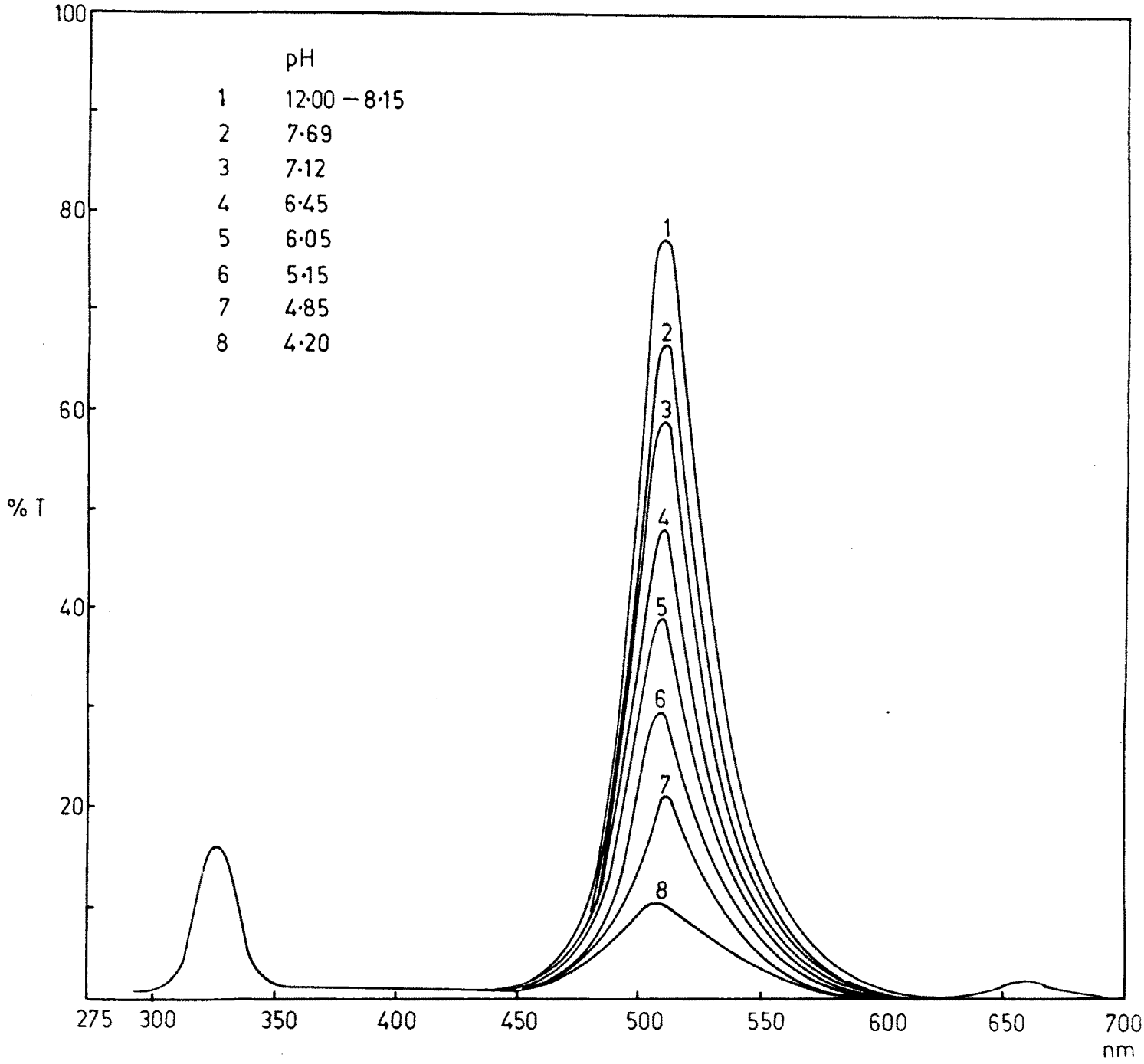


Fig. 4·9 EXCITATION AND FLUORESCENCE SPECTRA OF P-NITROPHENOL FLUOROMELLITE
(CONC. 0·004 mg / ml)

Table 4.9 : Fluorescence spectrum of o-Chlorophenol fluoromellitein

pH	Excitation Intensity	Emission Intensity
12.00 - 8.10	10	61
7.52	10	58
6.85	10	48
5.95	10	34
5.02	10	19
4.35	10	11
4.10	10	7

Table 4.10 : Fluorescence spectrum of p-Chlorophenol fluoromellitein

pH	Excitation Intensity	Emission Intensity
12.00 - 8.20	18	79
6.57	18	51
5.45	18	30
4.72	18	15
4.40	18	9

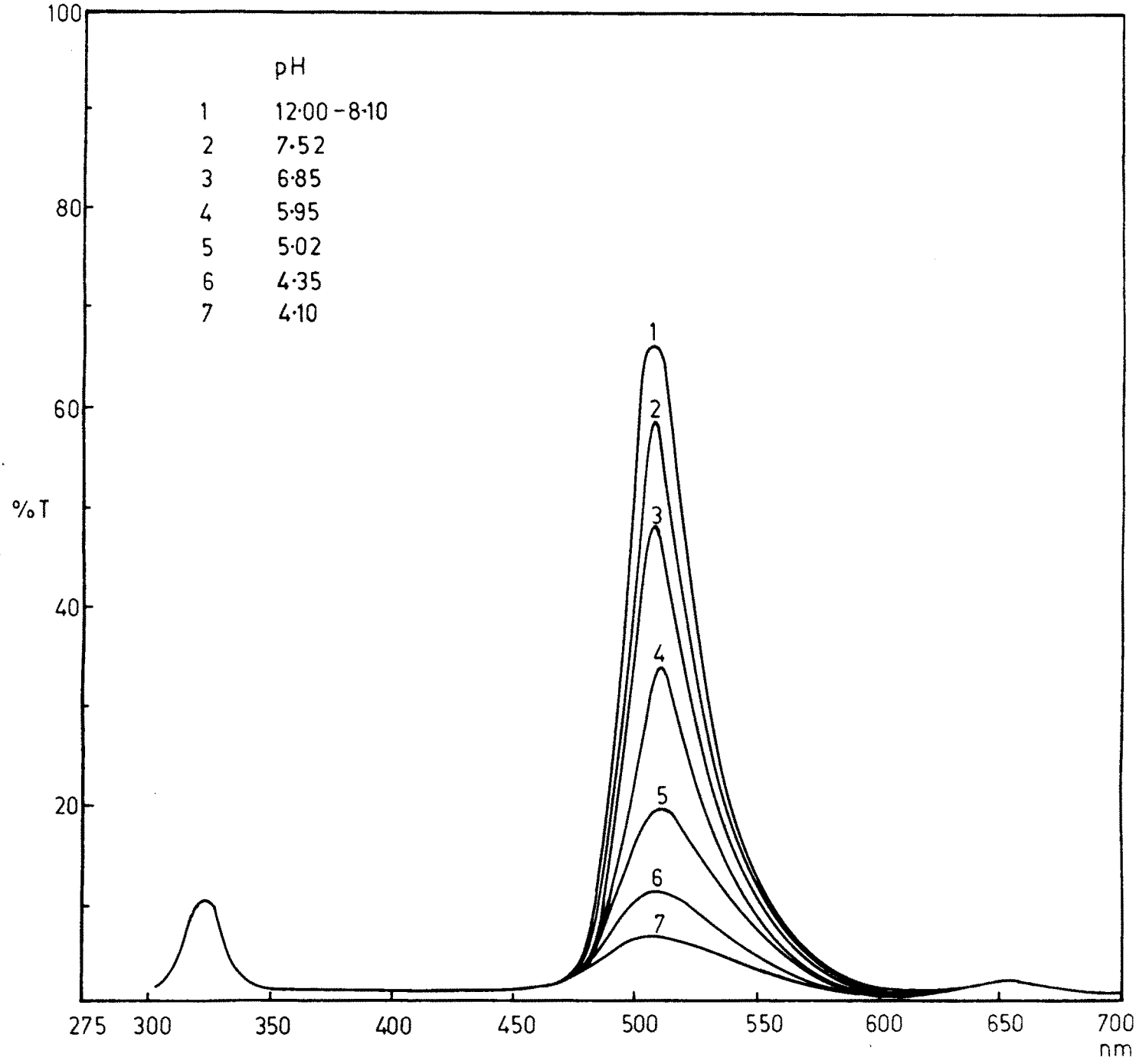


Fig.4·10 EXCITATION AND FLUORESCENCE SPECTRA OF O-CHLOROPHENOL FLUOROMELLITEIN. (CONC. 0·004 mg/ml)

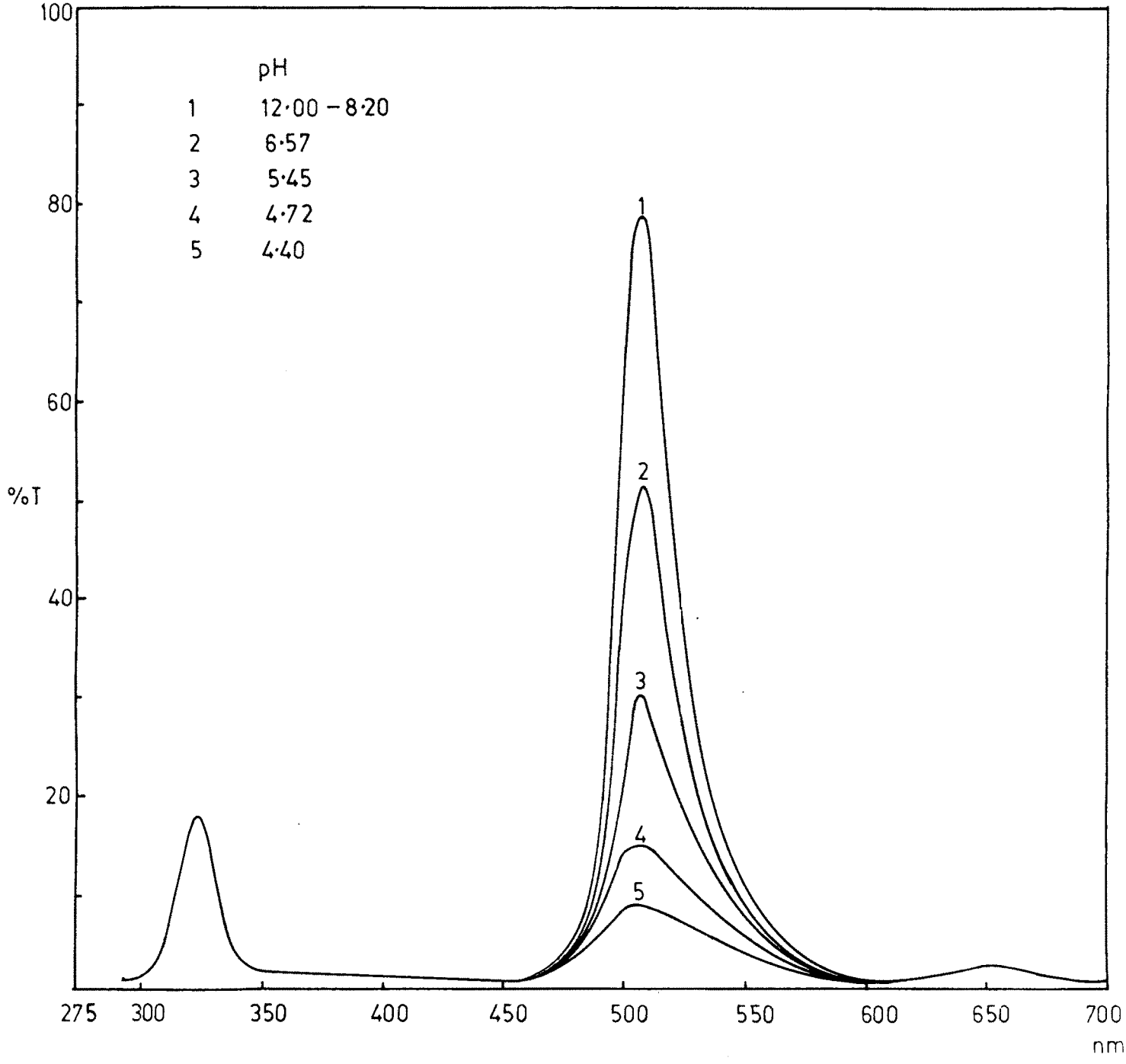


Fig.4·11 EXCITATION AND FLUORESCENCE SPECTRA OF P-CHLOROPHENOL FLUOROMELLITEIN. (CONC. 0·004 mg/ml)

4.5 KINETIC METHOD

It is now almost 25 years that kinetic methods of micro-analysis have gained recognition and come to stay as a worthy analytical micromethod [107]. The growth of new branches of science, industry and technology necessitate the development of new sensitive methods better than the known ones. The sensitivity of any method is a composite result of factors like the nature of the reaction that produces the necessary measurable change in the parameter and also the detection limit of the sensor. Here the specificity of enzyme reactions that induce fluorescence and the high sensitivity of fluorometric technique are the merits which have been exploited for enzyme assay.

4.6 ENZYMES

All living organisms synthesis their own systems of catalysts which are very active and specific. These catalysts, known as enzymes, are responsible for control of many physiological and metabolic activities. Enzymes are essentially proteins with high molecular weights, made up of chains of amino acid molecules connected by peptide bonds. The peptide chains are folded together and the catalytic activity depends on the precise conformational structure. Even a slight change in the conformation leads to denaturation. The earliest report of the enzyme activity as catalytic one is due to Berzelius [108] and therefore it can be said that the enzymes are known

for the last one and a half century. However, preparation of pure form of an enzyme, urease, by Sumner in 1926 marks the beginning of rapid growth of enzymology.

More than 2000 enzymes are known now. There is extensive work done so far on the preparation of pure forms of the enzymes. Some enzymes have low molecular weights of the order of 10000 but several others have molecular weights as high as 2000000. Some enzymes become active only in presence of additional compounds called cofactors. The protein part of the enzyme called the apoenzyme together with coenzyme or metal ion activator give rise to entire active complex or the holoenzyme.

$$\text{Apenzyme} + \text{Cofactor} \longrightarrow \text{Holoenzyme}$$

An enzyme catalyzes only a particular reaction of a particular substrate and gives rise quantitatively to a specific product. The concentration of the enzyme required is very small. Much more important is that the enzymatic reaction goes only in a particular way. Glucose can be determined by oxidation with ceric ion yielding gluconic acid and a host of otner products. But if glucose oxidase is used at pH 7, the product is almost one single compound. The catalytic activity and specific nature of the enzyme action prompted chemists to introduce their use as active analytical materials. Use of peroxidase for assay of peroxide was proposed by Osann in 1845 [109]. Warburg proposed colorimetric methods for the determination of coenzymes, nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine

dinucleotide phosphate (NADP) [110]. Since enzyme action is a catalytic one, its measurement is based on determination of rate of reaction. The rate method is much quicker since one need not wait for total equilibration.

Enzymes are classified on the basis of the type of catalytic role they play in typical reactions as given below [111].

1. Oxidoreductases :

Enzymes which catalyse the transfer of H atoms, O atoms or electrons from one substance to the other as by alcohol dehydrogenase.

$$R\ CH_2\ OH + NAD^+ \longrightarrow RCHO + NADH + H^+$$

2. Transferases :

Enzymes which catalyse the transfer of a group from one substance to the other as is done by hexokinase.

Glucose + ATP $\longrightarrow$ Glucose-6-phosphate + ADP.

3. Hydrolases :

Enzymes which cause hydrolysis. Esterases are the typical examples.

$$R\ COO\ R' + H_2O \longrightarrow R\ COOH + R'OH$$

4. Lyases :

These enzymes cause splitting of C-C, C-O, C-N or C-S bonds. Decarboxylases belong to this class.

$$R\ CH\ R'COOH \longrightarrow R\ CH_2\ R' + CO_2$$

5. Isomerases :

Enzymes which catalyse intramolecular rearrangement and produce different isomers.

L-amino acid $\xrightarrow{\text{racemase}}$ D-amino acid.

6. Ligases :

Enzymes which catalyse the formation of C-O, C-S, C-N or C-C bonds and simultaneously split ATP.

Amino acid + ATP + $_t$RNA $\longrightarrow$ Aminoacyl-$_t$RNA + Pyrophosphate.

The concentration of enzyme or the enzyme activity can't be expressed in usual manner as grams per litre or moles per litre or milligrams per ml, since the concentration in terms of weight is very low and as the activity per unit mass is very high. Therefore appropriate method of expression of concentration of the enzyme is developed. Such measurements are generally reported in enzyme activity units. Sumner units are used to express urease activity and Karmen units are used for GOT activity. In addition to this there are several other units named after Bodansky, Bucher, Wroblewski, Rosalki and Babson. To avoid the

confusion introduced by several differently defined units the Fifth International Congress of Biochemistry adopted the recommendations of the IUPAC and the International Union of Biochemistry Commissions of Enzymes for definition of an enzyme unit [112].

One unit (U) of any enzyme is defined as that amount which will catalyse the transformation of 1 μmole of substrate is per minute or where more than one bond of each substrate is attached, 1 μ equiv of the group concerned per minute, under defined conditions. The temperature should be stated end, wherever possible, 25° C has been recommended for use. The other conditions, including pH and substrate concentration, should be optimal wherever practical.

For the measurements of enzyme activity certain precautions must be taken. It is necessary to base the measurements upon the initial rates of reactions so that effect of complicated inhibitory products or reversibility of the reaction does not affect the results. The substrate concentration must be enough so that the kinetics approach zero order in substrate. The enzyme concentration is expressed in units per milliliter ($U\ ml^{-1}$) or in International units per liter ($U\ L^{-1}$). The specific activity of an enzyme is expressed as units of enzyme per milligram of protein.

4.7 ENZYME KINETICS OF HOMOGENEOUS SYSTEMS

Michaelis and Menten developed basic equations for enzyme kinetics assuming the following mechanism :

$$E + S \underset{K_{-1}}{\overset{K_1}{\rightleftharpoons}} ES \xrightarrow{K_2} E + P$$

Here substrate S combines with enzyme E and form enzyme substrate complex EF, which further breaks into product P and free enzyme E is set free. Using the steady-state assumption that $dE/dt = 0$ and $d(ES)/dt = 0$, along with the kinetic equations

$$\frac{dS}{dt} = K_{-1}(ES) - K_1(E)(S)$$

$$\frac{dE}{dt} = (K_{-1} + K_2)(ES) - K_1(E)(S)$$

$$\frac{d(ES)}{dt} = K_1(E)(S) - (K_{-1} + K_2)(ES)$$

$$\frac{dP}{dt} = K_2(ES)$$

and from the conservation equation for enzyme :

$$E_t = E - ES$$

the following equation can be derived :

$$v = \frac{K_2 E_t S}{\frac{(K_{-1} + K_2)}{K_1} + S}$$

This equation reduces to the Michaelis Menten equation :

$$v = \frac{VS}{K_m + S}$$

where $V = K_2 E_t$, $K_m = \frac{(K_{-1} + K_2)}{K_1}$ and S is the initial substrate concentration. V is known as maximum velocity (V_{max}) and K_m as the Michaelis constant.

This is based on the case $k_1 = k_{-1} = k_2$. It can be seen from the Michaelis-Menten equation that K_m equals S for $v = V/2$. However there are several other forms of Machelis-Menten equation, which can be used profitable for getting the values of kinetic constants K_m and V_{max} which are mentioned below.

(a) Lineweaver-Burk equation

$$\frac{1}{v} = \frac{K_m}{V} \cdot \frac{1}{S} + \frac{1}{V}$$

A plot of 1/v vs 1/S is a straight line with a slope of K_m/V and on intercept of 1/V on the 1/v axis and -1/K on the 1/S axis. Other widely used linear transformations and plots are the

(b) Hofstee plots

$$v = V - (v/S)K_m$$

(c) Hanes plot

$$\frac{S}{v} = K_m/V + (1/V)S$$

(d) Reiner equation

$$\frac{r}{1-r} = S/K_m$$

where $r = v/V$

In the above cases :

(a) A straight line plot with 1/S as abscissa and 1/v as ordinate are plotted to give a straight line with slope K/V. The line cuts ordinate at 1/V and cuts the abscissa at -1/K.

(b) From the plot of v/S as abscissa and v as ordinate we get a straight line that cuts x-axis and y-axis at V/K and v respectively and the slope of the line is -K.

(c) In this case a plot of S on x-axis and S/v on y-axis gives a slope is equal to 1/V and x and y-axis are cut at -K and and K/V respectively.

(d) In the fourth case a plot of S on x-axis and v/1-v on y-axis gives a straight line with a slope 1/K.

The factors which affects enzyme catalyzed reactions are (a) substrate concentration (b) Enzyme concentration (c) Presence of activators (d) Presence of inhibitors (e) temperature (f) pH and (g) ionic strength.

Enzyme analysis is a very important diagnostic tool in clinical chemistry. Enzymes occur in the entire body in different amounts in different organs such as heart, liver, kidney, brain, muscle, bone and in body fluids and secretions. In one organ a particular enzyme may be present or absent. In several organs a particular enzyme may occur in different percentage and in the others it may be absent. There are two principle groups of enzymes which are diagnostically important. The plasma significant enzymes are responsible for blood coagulation and nonspecific plasma enzymes are either secreted enzymes such as amylase and lipase or the intracellular metabolic enzymes, such as α-HBDH. When a particular organ or tissue is damaged there is a sudden release of the enzyme associated with it into the blood stream [113]. Enzyme tests can be used to prove the existance of one diseases rather than another. In case of various liver diseases enzyme tests are very useful [114] and it is possible to differentiation between obstructive jaundice and viral hepatitis. Several such cases can be quoted from literature on clinical chemistry [115 to 122].

4.8 EXPERIMENTAL TECHNIQUES IN ENZYMATIC METHODS OF ANALYSIS

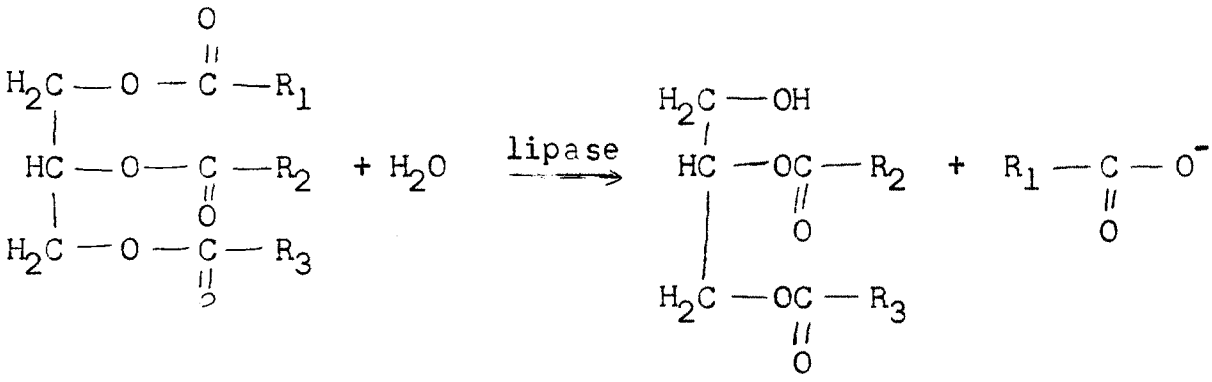
The methods can be divided in to equilibrium methods and kinetic methods which are useful for the determinations of participants in enzymatic reactions. In order to measure the progress of enzymatic reaction to follow the change with the time (kinetic method) or the total change in reactant or product (equilibrium

method). These methods may be monitored by using manometer, spectrophotometer, polarimeter, ion-selective meter, pH meter or fluorescence spectrometer, fluorescence methods superseed other methods since it is possible to detect very low concentrations of even 10^{-12} M as also the fluorometric analysis is very specific.

4.9 APPLICATIONS OF FLUORESCEIN DIACETATE AND DIBUTYRATE IN ENZYME ASSAY

LIPASE (Triacetyl glycerol acylhydrolase)

Lipase hydrolyses a triglyceride at optimum pH value of 8.6 - 9.0 (for human pancreatic lipase). The Michaelis constants for porcine pancreas with 4-methylumbelliferone as substrate at pH 7.5 in tris buffer is 7.3×10^{-6} [123]. Lipase catalyses the hydrolysis of emulsified substrates but it is not active when substrates are in the form of a solution. The reason for it is that the enzyme gets absorbed by the emulsified substrate and the rate of reaction is a function of the number of enzyme molecules, adsorbed at the interface. Esters formed from glycerol or similar alcohols with long chain fatty acids are hydrolysed by lipase. The effect of lipase on the substrate leads to the formation of 2 moles of fatty acids and one mole of β-monoglyceride per mole of substrate. The enzyme attacks the end ester bond of the glycerol ester



Ca^{++} acts as an activator as also Sr^{++} and Mg^{++}. Cystein thioglycollic acid and bile salts act as activators. Zn^{++}, Cu^{++}, Hg^{++}, I_2 and versene act as inhibitors. The enzyme can be stored in a refrigerator at 4^{o} C in dry condition.

Diagnostic Importance

Serum contains about 50-250 U L^{-1} of lipase. U of lipase catalyses the hydrolysis of 1 μ mole of fatty acid from a triglyceride in 1 hour at pH 8 at 37^{o} C. In acute pancreatitis and in case of pancreatic carcinoma serum lipase levels rise 50-200 times the normal value. In acute and chronic renal diseases also lipase level is raised.

Preparation of Lipase Powder from the pancreas of the animals

In the present study powdered enzyme preparation were prepared from the porcean pancreas, the sheep pancreas and the rat pancreas [124]. About 20 gm of fresh sheep pancreas glands are cooled at 0^{o} C within 4 to 6 hours of the animals death. By maintaining this low temperature the glands are freed

mechanically until liquid paste is obtained. This paste is shaken for 4 to 6 hours with 200 ml of acetone and is then centrifuged. The residue is reextracted three more times with same volume of acetone, twice with acetone-ether mixture (1:1) and twice with ether. The product is dried for 48 hours in vacuum and a stable, friable powder is obtained.

Standard sample : Standard sample of porcine pancrease lipase was obtained from Sigma . Its activity was 25000-50000 U mg^{-1} protein using olive oil.

Preparation of Dibutyryl esters of fluorescein analogues :

(a) Tetrabutyryl ester of Difluoromellitein :

Difluoromellitein, pyridine and butyric anhydride in the proportion of 1:1:6 was heated in a hot water bath for several hours. The resulting product was decanted, filtered washed with alcohol and finally crystallised from 95 % alcohol to give buff coloured powder.

(b) Dibutyryl ester of other mellitein dyes :

The above procedure was used for the preparation except that the proportion of the dye; pyridine and butyric anhydride was 1:1:3. The crystallised products were deep brown in colour.

Table 4.11 : Microanalysis of dibutyryl esters of mellitein dyes

Mellitein ester	Colour	Formula weight	Formula of butyryl ester	C %		H %		Cl %	
				Calc.	found	Calc	found	Calc	found
Tetrabutyryl difluoromellitein	White buff	966	$C_{50}H_{42}O_{14}$	62.11	61.3	4.35	4.1	-	-
Dibutyryl phenol fluoromellitein	Deep brick red	712	$C_{42}H_{32}O_{11}$	70.78	70.1	4.49	4.25	-	-
Dibutyryl o-Cresol fluoromellitein	Yellow	740	$C_{44}H_{36}O_{11}$	71.35	70.25	4.86	4.56		
Dibutyryl m-Cresol fluoromellitein	Pale green yellow	740	$C_{44}H_{36}O_{11}$	71.35	70.2	4.86	4.36		
Dibutyryl p-Cresol fluoromellitein	Greenish yellow	740	$C_{44}H_{36}O_{11}$	71.35	70.3	4.86	4.58		
Dibutyryl o-Chlorophenol fluoromellitein	Yellow	781	$C_{42}H_{32}O_{11}Cl_2$	64.53	62.1	3.84	3.55	9.09	9.1
Dibutyryl p-Chlorophenol fluoromellitein	White	781	$C_{42}H_{30}O_{11}Cl_2$	64.53	62.3	3.84	3.62	9.09	8.84

Table 4.12 : Colour change and fluorescence of mellitein dyes

Mellitein dye	Acidic	Basic
Difluoromellitein	Pale yellow	Green
Phenol fluoromellitein	Pale yellow	Red green
o-Cresol fluoromellitein	Pale yellow	Yellow green
m-Cresol fluoromellitein	Yellow	Green yellow
p-Cresol fluoromellitein	Yellow	Orange yellow green
o-Chlorophenol fluoromellitein	Yellow	Orange yellow green
p-Chlorophenol fluoromellitein	Yellow	Orange green

Reagents

Substrate solution : 2.5 x 10^{-5} M solution of tetrabutyryl difluorescence and 5 x 10^{-5} M solution of other dibutyryl melliteins were prepared by dissolving the esters in 5 ml methyl cellosolve and 95 ml of tris buffer of pH 8.0.

Tris buffer : M/10 solution of tris (hydroxy methyl) aminomethane buffer was prepared by dissolving $NH_2C(CH_2OH)_3$ M. wt. 121.14 i.e. 12.114 g L^{-1} in distilled water. M/10 HCl was used to adjust the pH at desired level.

Apparatus

LP fluorometer was used for all measurements. Excitation wavelengths were long wavelength total UV radiation obtained from low pressure Hg lamp and passed through a black UV pass-visible cut off filter. The 20 mm path length cell was illuminated from the top. The secondary radiation was passed through a 525 nm interference filter.

Procedure

4 ml of the substrate is taken in a cuvette and the fluorescence is adjusted to zero by the set zero knob. 0.2 ml of the solution of lipase is added and fluorescence is read at every 0.5 min. Plots of time vs fluorometer reading is obtained for lipase concentration of 0.01 unit.

From this curve $\Delta F/\Delta t$ values are obtained and the procedure is repeated for several concentrations upto 0.04 unit and a plot of $\Delta F/\Delta T$ vs [Lipase] in units is prepared. From this calibration curve an unknown concentration in a sample of pancreas can be determined.

4.10 DISCUSSION

For the determination of lipase activity several methods are available. In the titrimetric methods Cherry and Crandall used emulsified olive oil as substrate and measured after 24 hours at 37° C the liberated carboxylic acid by using sodium hydroxide. One unit of the enzyme liberates carboxylic acid requiring 1.0 ml of 0.05 M sodium hydroxide [125].

Henry *et al.* proposed pH metric detection of end point or use of thymophthalein [126]. It was shown that olive oil emulsion substrate in tris buffer gives better precision [127]. Many other parameters such as different emulsifying agents, pH, buffers and accelerators were further studied [128]. A photometric method for determination of lipase was developed by Vandermeers, Saifer and Perie used phenyl laurate for colorimetric estimation of lipase and β-naphthyl laurate was used by Nachlas and Blackburn [129]. Seligman *et al.* proposed 2-naphthyl myristate as a substrate.

Table 4.13 : Fluorometer reading F at time T for substrats

S.No.	Time T	1	2	3	4	5	6	7
	Lipase conc. [0.01] unit							
1	00 min.	0	0	0	0	0	0	0
2	30 sec.	10	9	11	11	8	10	11
3	60 sec.	21	18	21	22	17	20	22
4	90 sec.	29	25	33	32	24	30	33
5	120 sec.	40	34	43	44	31	39	45
6	150 sec.	49	43	55	56	40	51	56
	Lipase conc. [0.015] unit							
1	00 min.	0	0	0	0	0	0	0
2	30 sec.	15	12	16	15	12	15	16
3	60 sec.	30	24	32	30	24	30	32
4	90 sec.	44	36	49	46	37	45	48
5	120 sec.	60	49	65	60	49	61	63
6	150 sec.	74	60	80	74	60	76	78
	Lipase conc. [0.02] unit							
1	00 min.	0	0	0	0	0	0	0
2	30 sec.	21	17	22	21	16	20	22
3	60 sec.	40	35	43	42	32	40	44
4	90 sec.	59	52	64	64	49	59	68
5	120 sec.	80	69	88	86	62	79	68
6	150 sec.	100	85	-	100	79	98	-

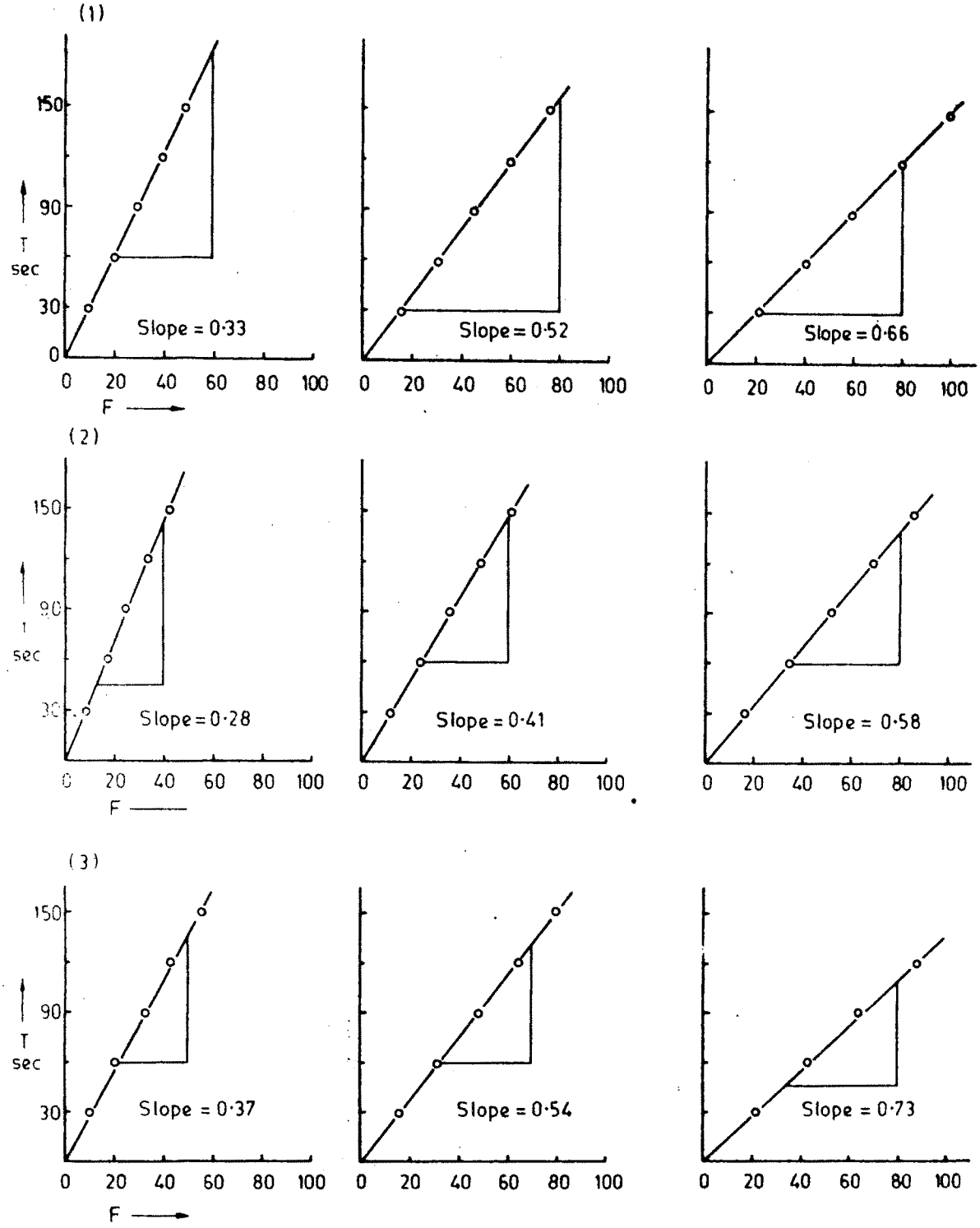


Fig 4·12 GRAPH OF T vs F FOR DIFFERENT LIPASE CONCENTRATION.

(I) TETRABUTYRYL DIFLUOROMELLITEIN.(II) DIBUTYRYL PHENOL FLUOROMELLITEIN.
(III) DIBUTYRYL O CRESOL FLUOROMELLITEIN.

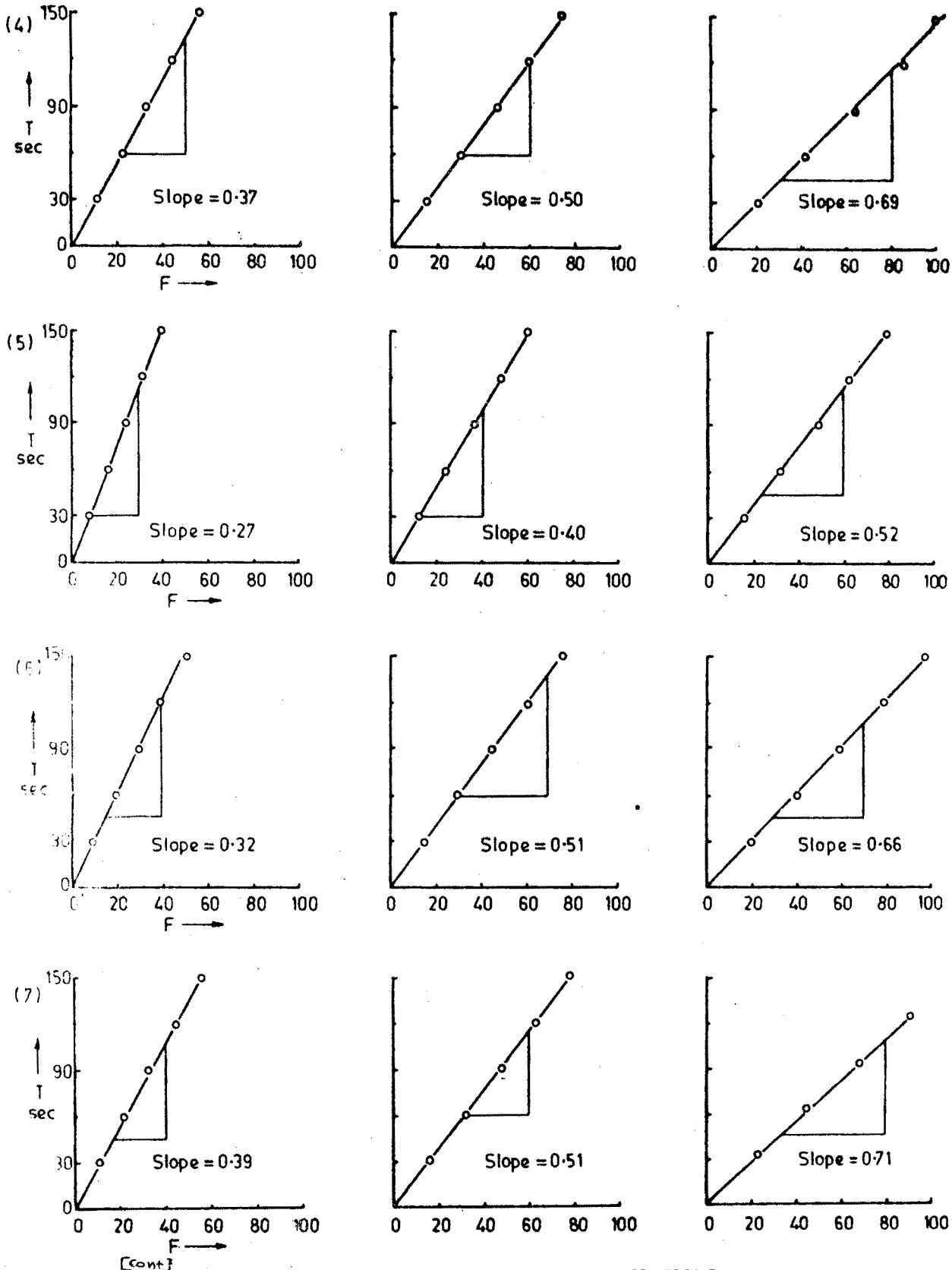


Fig 4·12 [Cont] GRAPH OF T vs F FOR DIFFERENT LIPASE CONCENTRATIONS.
(IV) DIBUTYRYL M-CRESOL FLUOROMELLITEIN. (V) DIBUTYRYL P-CRESOL FLUOROMELLITEIN.
(VI) DIBUTYRYL O-CLOROPHENOL FLUOROMELLITEIN (VII) DIBUTYRYL P-CLOROPHENOL FLUOROMELLITEIN.

Table 4.14

Sr. No.	Substrate	[Enzyme]	$\frac{F}{T}$
1	Tetrabutyryl difluoromellitein	0.010	0.33
		0.015	0.52
		0.020	0.66
2	Dibutyrylphenol fluoromellitein	0.010	0.28
		0.015	0.41
		0.020	0.58
3	Dibutyryl o-Cresol fluoromellitein	0.010	0.37
		0.015	0.54
		0.020	0.73
4	Dibutyryl m-Cresol fluoromellitein	0.010	0.37
		0.015	0.50
		0.020	0.69
5	Dibutyryl p-Cresol fluoromellitein	0.010	0.27
		0.015	0.40
		0.020	0.52
6	Dibutyryl o-Chlorophenol fluoromellitein	0.010	0.32
		0.015	0.51
		0.020	0.66
7	Dibutyryl p-Chlorophenol fluoromellitein	0.010	0.39
		0.015	0.51
		0.020	0.74

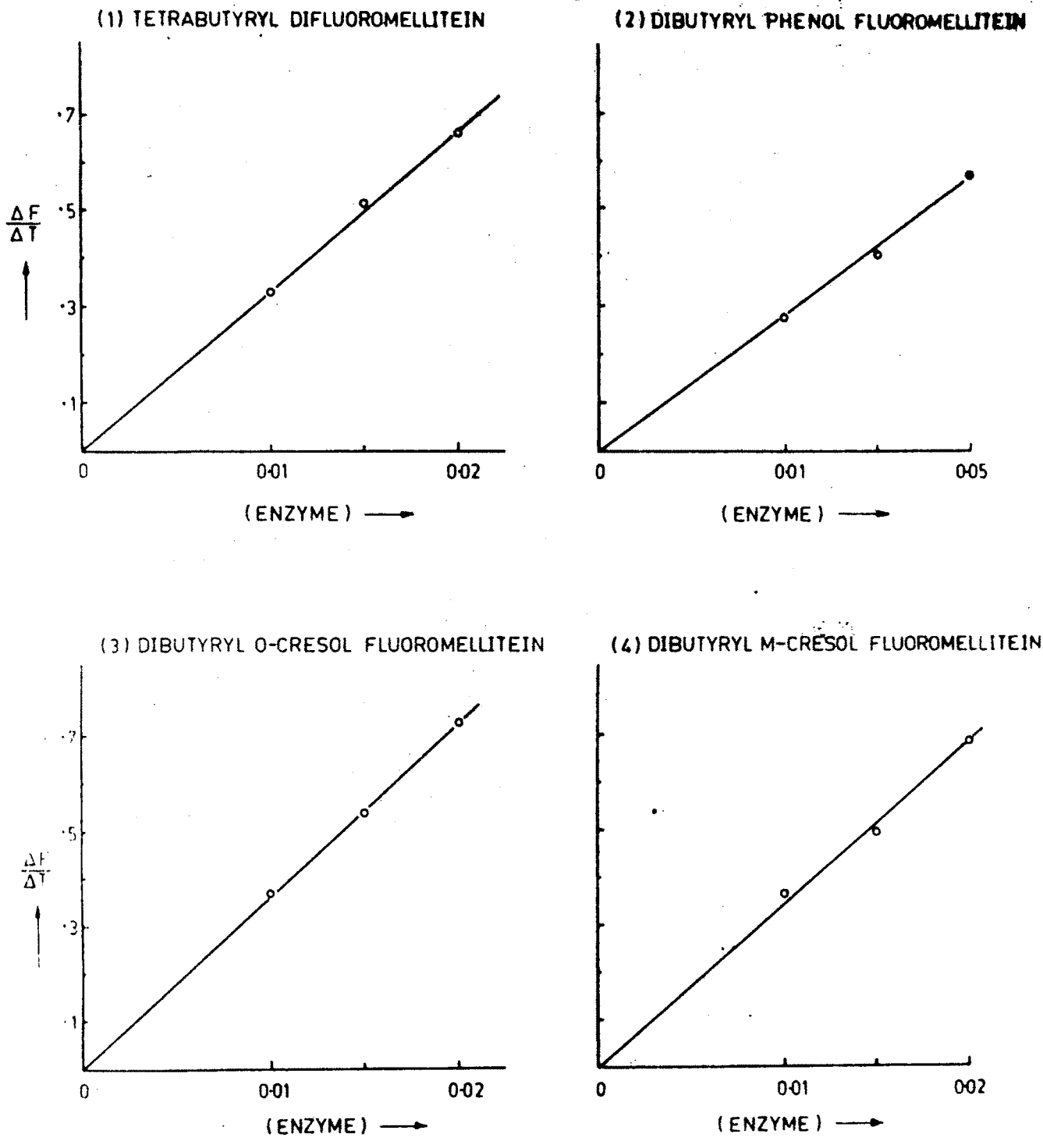
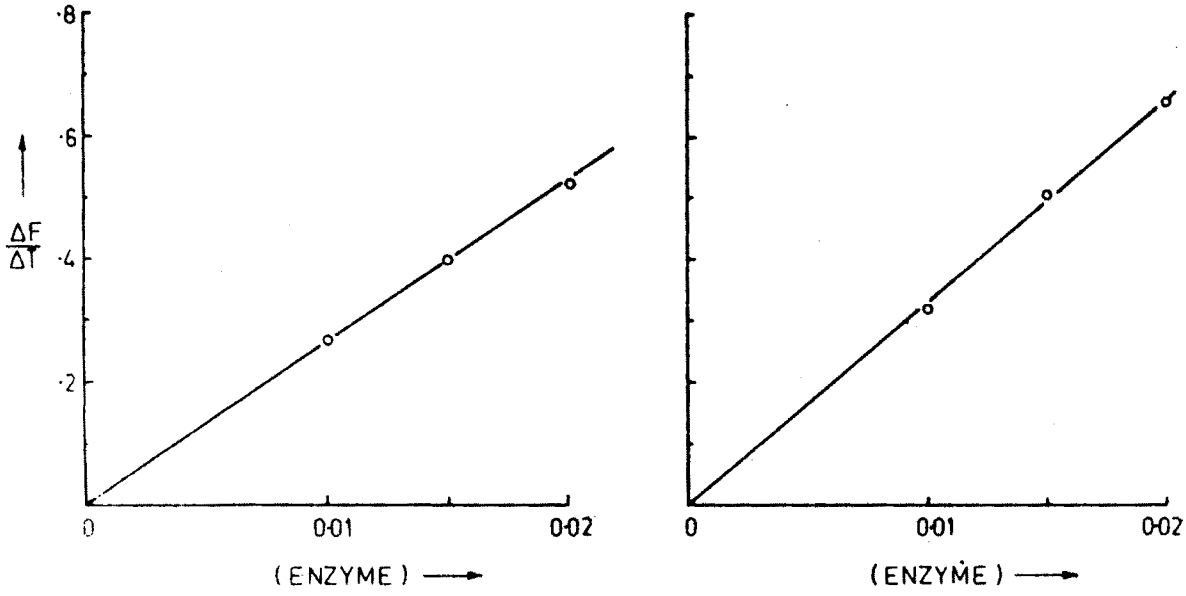


Fig. 4.13 PLOTS OF $\frac{\Delta F}{\Delta T}$ vs LIPASE CONCENTRATION (CALIBRATION CURVES).

(5) DIBUTYRYL P-CRESOL FLUOROMELLITEIN (6) DIBUTYRYL O-CLOROPHENOL FLUOROMELLITEIN



(7) DIBUTYRYL P-CLOROPHENOL FLUOROMELLITEIN.

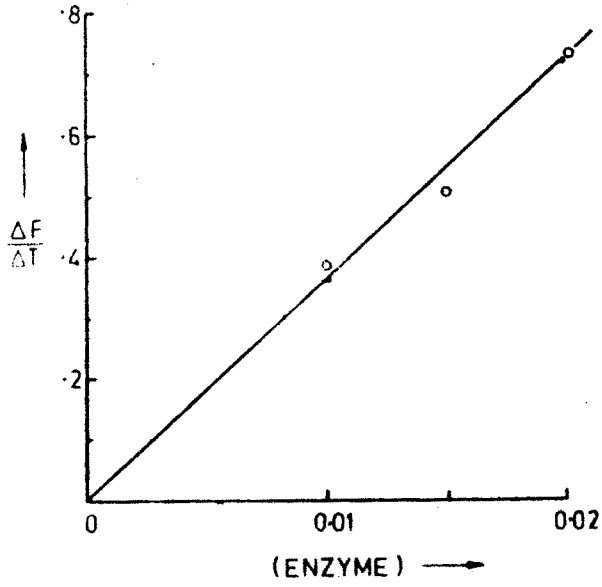


Fig 4·13 [Cont]
PLOTS OF $\frac{\Delta F}{\Delta T}$ vs LIPASE CONCENTRATION (CALIBRATION CURVES).

A rapid kinetic method was developed by Zwez [130]. Kramer and Guilbault described simple fluorometric method for the determination of lipase [131, 132]. In the present study several butyryl esters of fluoromellitein dyes were prepared and were screened for their use in lipase determination. The screening indicator that all the seven compounds studied possess more or less comparable substrate behaviour. Table 4.3 shows the plots of T vs F for different lipase concentrations studied by using the seven new substrates. Further measurements were done by using different concentrations of lipase. Table 4.4 is a tabulation of $\triangle F/\triangle T$ vs Enzyme concentrations and plots and calibration curves for the determination of lipase are obtained for all the seven substrates. Figs. 4.12 and 4.13 are the plots mentioned above. It has been observed that in most of the cases the results show good agreement with the expectations. Since the bond strength of the weakest bonds in fluoromellitein dyes may have variable energy in different substrates. It is necessary to use different lipase extracts from pancreases of various animals so that the applications will be precisely defined.

4.11 SUMMARY

The screening of seven new derivatives has been carried out. The results are as per expectations and encouraging. It is found that all the seven compounds can be used for fluorometric assay of lipase.