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FLUORESCEIN AND NAPHTHOL SUBSTRATES FOR PHOSPHATASE ASSAYS

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UNPUBLISHED PRELIMINARY DATA

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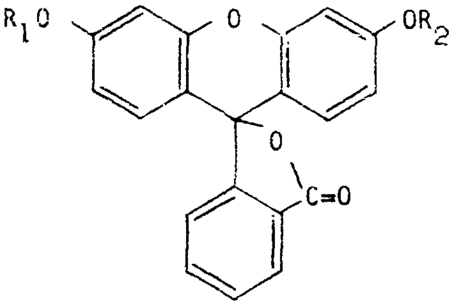
INTRODUCTION

In a previous report Hochstein[5] has shown that phosphatase activity in soils may be detected using a fluorometric assay procedure. The principle of the assay was based upon the hydrolysis of α-naphthyl phosphate to α-naphthol, which was measured by determining the fluorescence at 460 mμ. The phosphatase assays, however, were complicated by the presence of a "blank" fluorescence associated with the soils, which was quite considerable at this wave length. This problem of soil fluorescence suggested the use of alternative phenolic substrates in which the fluor is activated in the visible region of the spectrum. Because of its high quantum yield of fluorescence (0.92 in 0.1M $NaHCO_3$[1]), fluorescein and related compounds were selected as a suitable starting point of this investigation. The high quantum yield of fluorescein means that 10^{-10} to 10^{-11} molar solutions can be detected using a commercially available fluorometer. Furthermore, this sensitivity could certainly be extended by several orders of magnitude for special applications.

I. DISCUSSION

A preliminary literature survey on the chemistry of fluorescein indicated some doubts about the actual structure of the two known forms of fluorescein. A detailed physical chemical investigation using I.R., N.M.R. and mass spectrometry showed unequivocally that the red and yellow forms of fluorescein had the structures suggested by Orndorff and Hemmer[2], the only unexplained property being the yellow color of the labile lactone form of fluorescein compared to the colorless derivative - fluorescein diacetate - which is white.



I, $R_1 = R_2 = H$

II, $R_1 = H$, $R_2 =$ methyl

III, $R_1 = R_2 = PO_3^=$

IV, $R_1 = PO_3^=$, $R_2 =$ methyl

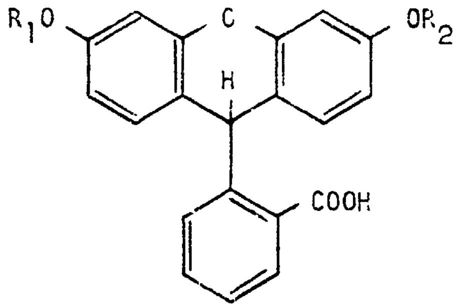
Phosphorylation of (I) and the related methylfluorescein (II), which has about half the quantum yield of fluorescence (I), then yielded the fluorescein diphosphate (III) and methylfluorescein phosphate (IV), respectively. The use of (II), which has only one phenolic group, greatly facilitates isolation and purification in the phosphorylation step. It should be noted, however, that the use of (II) introduces an asymmetric center into the molecule, and it is conceivable that the two optical isomers of (IV) could have significantly different properties as enzyme substrates.

The rate of spontaneous hydrolysis of the phosphates (III) and (IV) in aqueous solution at pH 7.5 was then determined. The results (Table I) showed that both compounds were too unstable for use as substrates in this assay procedure.

TABLE I

Compound	Code	Rate of Hydrolysis (% per hr.)
Fluorescein diphosphate (III)	FDP	0.11
Dihydrofluorescein diphosphate (VI)	HFDP	0.018
Methyl fluorescein phosphate (IV)	MFP	0.42
Methyl dihydrofluorescein phosphate (VII)	MHFP	0.08

This ready hydrolysis compares unfavorably with that observed by Hochstein[5] for α-naphthyl phosphate (0.003% per hour). It is suggested that this is due to the low pK_a of ~4.5 of the two fluors, as compared with the pK_a of 8 for α-naphthol. The suggestion that dihydrofluorescein might give more stable derivatives than fluorescein was originally formulated by Dr. Elie A. Shneour of the Department of Genetics, Stanford University. Dihydrofluorescein and methyl dihydrofluorescein phosphates (VI) and (VII) were prepared by known methods[3] and the rate of spontaneous hydrolysis measured at pH 7.5.



V, $R_1 = R_2 = H$

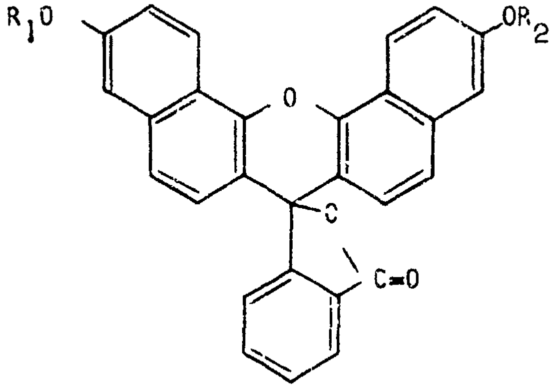
VI, $R_1 = R_2 = PO_3^=$

VII, R_1 = methyl, $R_2 = PO_3^=$

The results (Table I) showed that the dihydro derivatives have considerably greater stability than their parent compounds (II) and (IV). (The detailed work on these derivatives, most of which preceded and was independent of the work reported here, will be the subject of a separate report by Dr. Shneour.)

The development of a suitable assay procedure thus depended on the preparation of a fluor which was activated as far toward the red as possible and which formed a phosphate derivative of equal or better stability compared to α-naphthyl phosphate.

A survey of simple naphthalene derivatives indicated that 6,6-dihydroxynaphthofluoran (XV) (the naphthalene analogue of fluorescein) might have the desired properties.



XV, $R_1 = R_2 = H$
XVI, $R_1 = H$, $R_2 = PO_3^=$
XVII, $R_1 = R_2 = PO_3^=$

The compound was prepared by the method of Fischer and König[6]. When dissolved in alkali, a deep blue solution resulted which exhibited red fluorescence. Potentiometric titration using fluorescence as an indicator gave a pK_a of approximately 8. On phosphorylation a mixture of the mono (XVI) and diphosphate (XVII) was formed which was separated on a DEAE column. The monophosphate was found to hydrolyze at a rate of about 0.01% per hour, but the diphosphate showed no detectable hydrolysis within the limits of sensitivity of the apparatus used. When the mono- and diphosphate were used as substrates for alkaline phosphatase, it was found - rather surprisingly - that the fluor was released at a faster rate from the diphosphate than the monophosphate. For this reason and also its high stability, the diphosphate was used as a substrate for B. subtilis. Bacterial concentrations as low as 10^4 ml could be detected although the rate of hydrolysis by the bacteria was only about 1/50 the rate when p-nitro-phenyl phosphate was used as substrate.

It is clear that a detailed study of fluorescein chemistry is called for. This, then, would permit optimal choice of substrates based on chemical structure of the fluorescein used.

II. EXPERIMENTAL SECTION

A. Fluorescein Derivatives

1. Purification and Characterization of Fluorescein.

a. Purification of fluorescein[2].

Red fluorescein (Eastman P780) was acetylated by heating under reflux for 2 hours with 5 parts (w/w) of acetic anhydride and allowing to cool overnight. The resulting crystals were filtered off and recrystallized twice from ethyl acetate to constant m.p. 205°.

Fluorescein diacetate (5 g) was heated for 30 minutes in 1 M alcoholic potassium hydroxide (100 ml). The solution was evaporated to dryness and the residue dissolved in water (100 ml). Hydrochloric acid (1 M) was added at room temperature to precipitate a yellow amorphous powder which was filtered, washed and dried. This was assumed to be fluorescein lactone.

b. Ultraviolet absorption of fluorescein.

Compound	Solvent	λmax (mμ)	ϵmax
Eastman P780	0.01N.NaOH	489.5	70,000
Purified yellow fluorescein	0.01N.NaOH	489.5	86,500
Purified yellow fluorescein	Ethanol	455	4,200
Purified yellow fluorescein	Ethanol	480	4,500

c. Fluorescence.

This measurement was made on the Turner fluorometer (30 x scale) using a Wratten 2A-47B filter as primary and a Wratten 2A-12 filter as secondary filter. The solutions were 10^{-8} M in 0.01N aqueous sodium hydroxide.

Relative Fluorescence	
Eastman P780	540
Eastman 735 (disodium salt)	590
Purified fluorescein	690

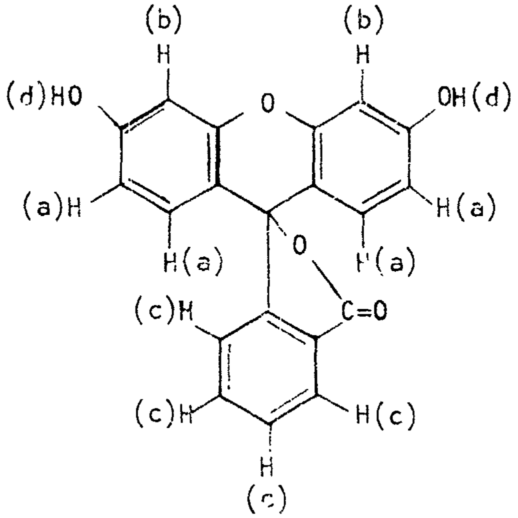
These results show that Eastman P780 is about 80% pure and the contaminants have negligible absorption or fluorescence.

d. Infrared spectra.

The infrared spectra were measured using potassium bromide discs and the carbonyl absorption of the two forms of fluorescein are compared to related classes of compounds as reported in the literature[7].

Compound	Wavenumber of Carbonyl Absorption
Red fluorescein	1670 cm^{-1}
Yellow fluorescein	1730 cm^{-1}
1,4-Quinones	1675 cm^{-1}
Aromatic carboxyl	1690 cm^{-1}
$\alpha\beta$ unsaturated γ lactones	1750 cm^{-1}

e. N.M.R. spectrum of yellow fluorescein in acetone-d_6.



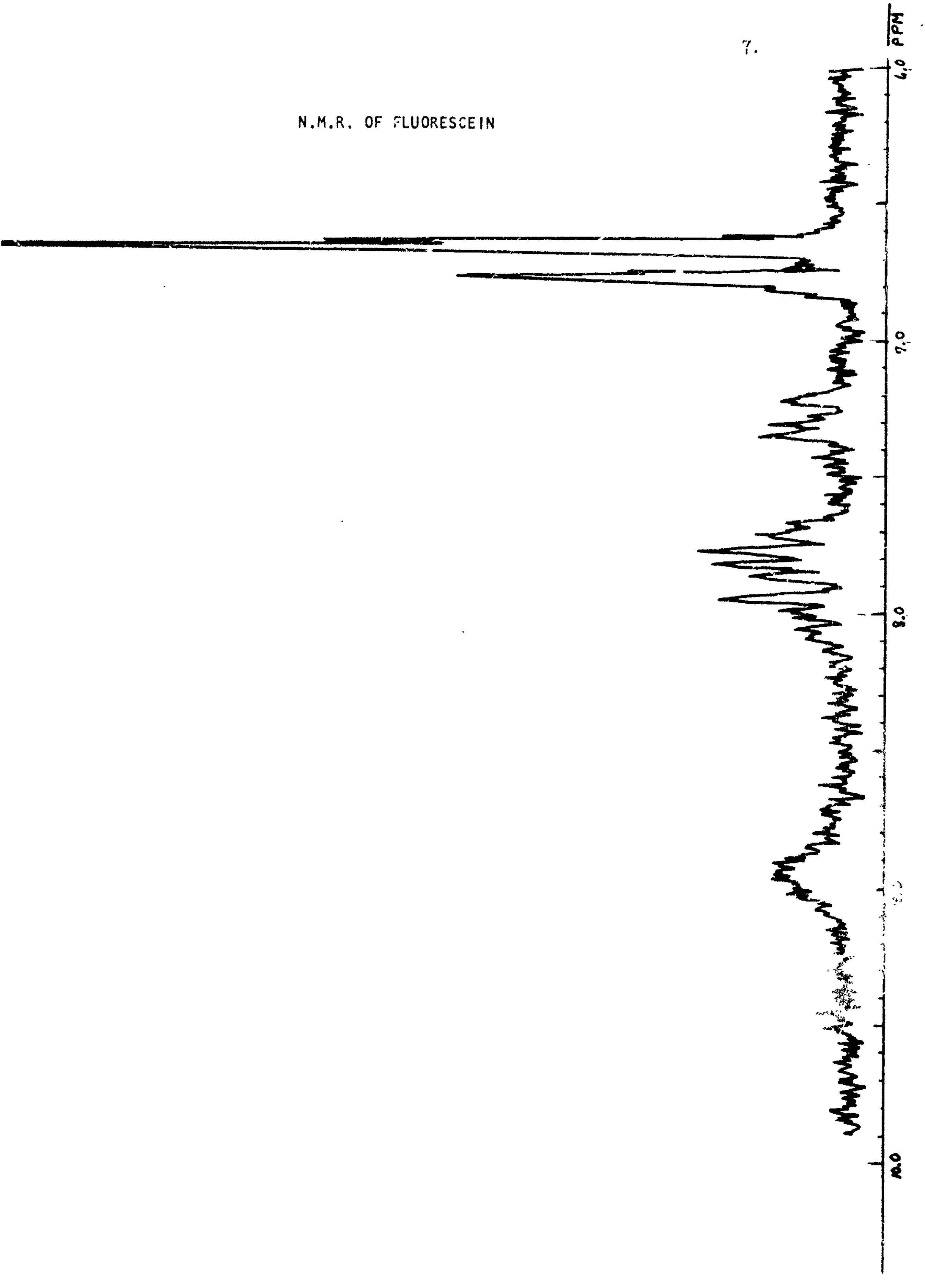
Sweep offset	200 & 0 CPS
Freq. response	2 CPS
Sweep time	250 secs
Spec. amp.	32

ASSIGNMENTS

a	6.67
b	6.79
c	7.2 8.2
d	8.95

Yellow fluorescein is quite soluble in acetone to give a colorless, non-fluorescent solution which shows no absorption in visible (450-500) and so is presumably stabilized in lactoid form. Addition

N.M.R. OF FLUORESCEIN



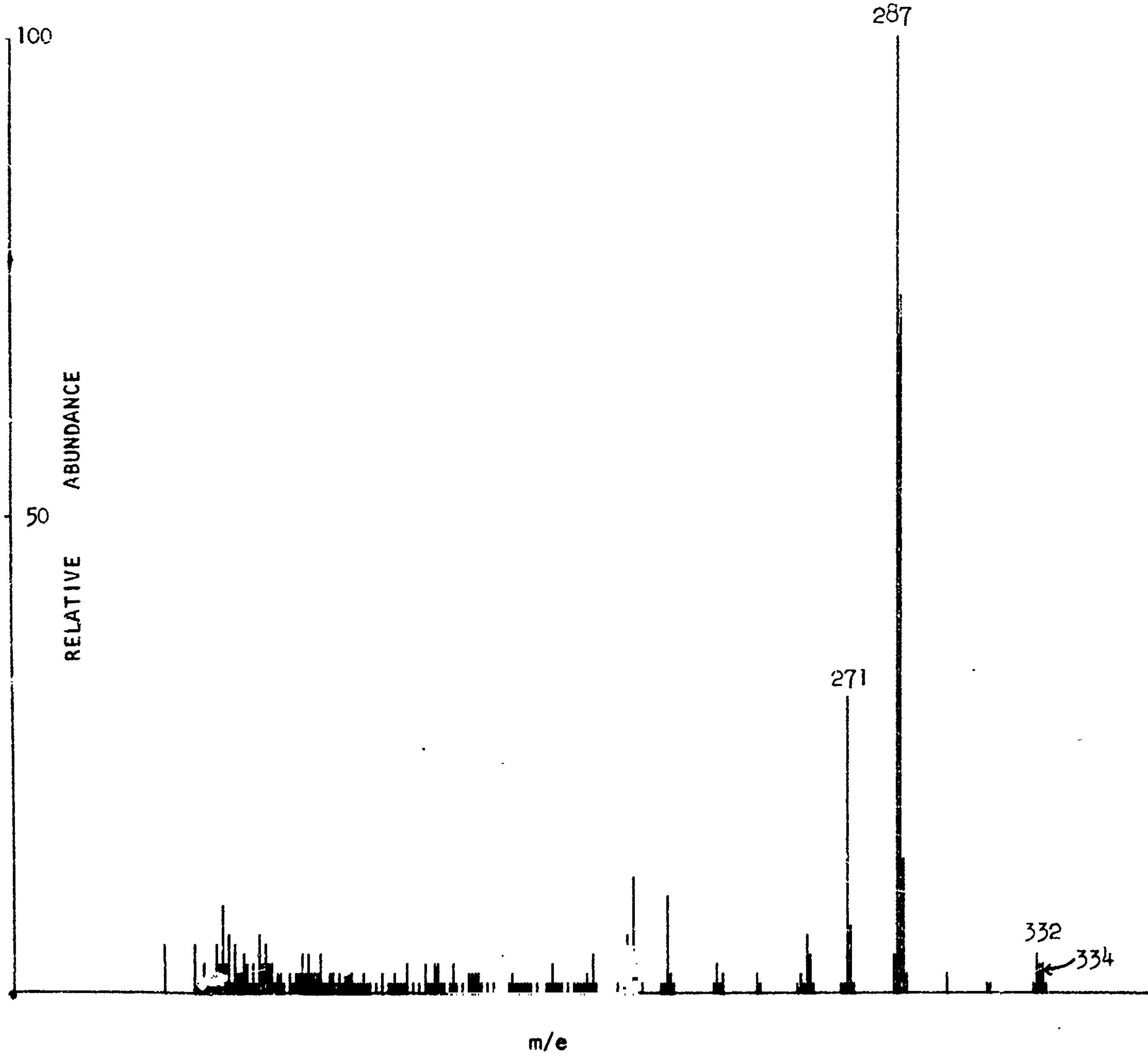
of water gives rise to characteristic yellow-green color and fluorescence.

It was hoped that addition of D_2O to the acetone $-d_6$ solution might effect a change in N.M.R. spectrum due to transformation to quinonoid form; however, there was virtually no change in chemical shift or coupling and only disappearance of (d) was observed. The N.M.R. spectrum of fluorescein diacetate was also run, but due to its surprisingly lower solubility in acetone than yellow fluorescein, the resulting spectrum had a high noise/signal ratio. A downfield shift in proton resonance of protons a,b compared to no change in c, helped in assignments, however.

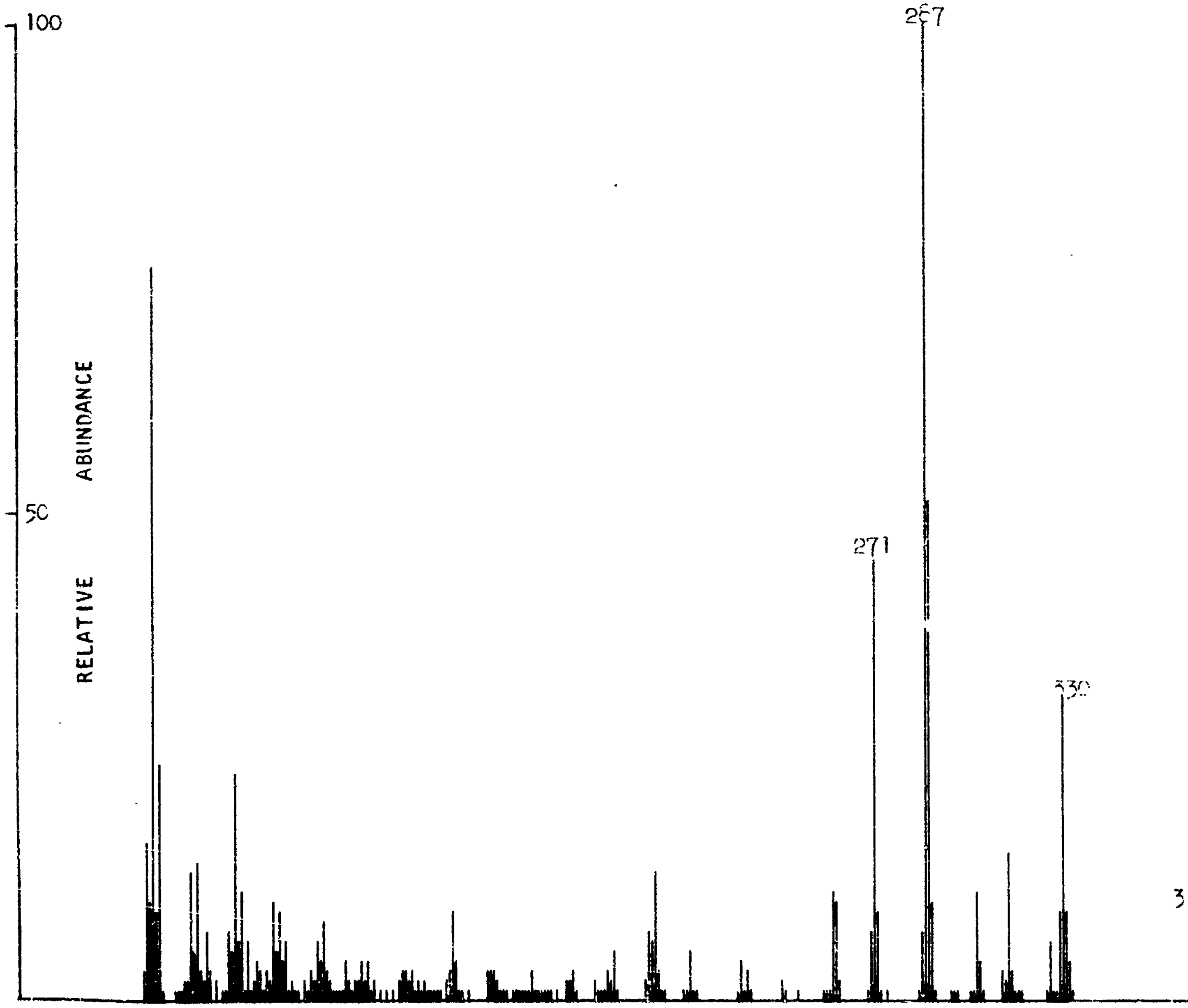
f. Mass spectrum of fluorescein.

In order to confirm the molecular weight of fluorescein as 332, mass spectrum of a sample of purified material provided by B. Rotman was run. A peak at m/e of 334 was observed at 2/3 the weight of m/e 332 peak, suggesting 40% contamination with dihydrofluorescein! However, the mass spectral fragmentation pattern of fluorescein diacetate, which had been recrystallized three times, was in full agreement with a molecular weight of 416 and hence a fluorescein molecular weight of 332.

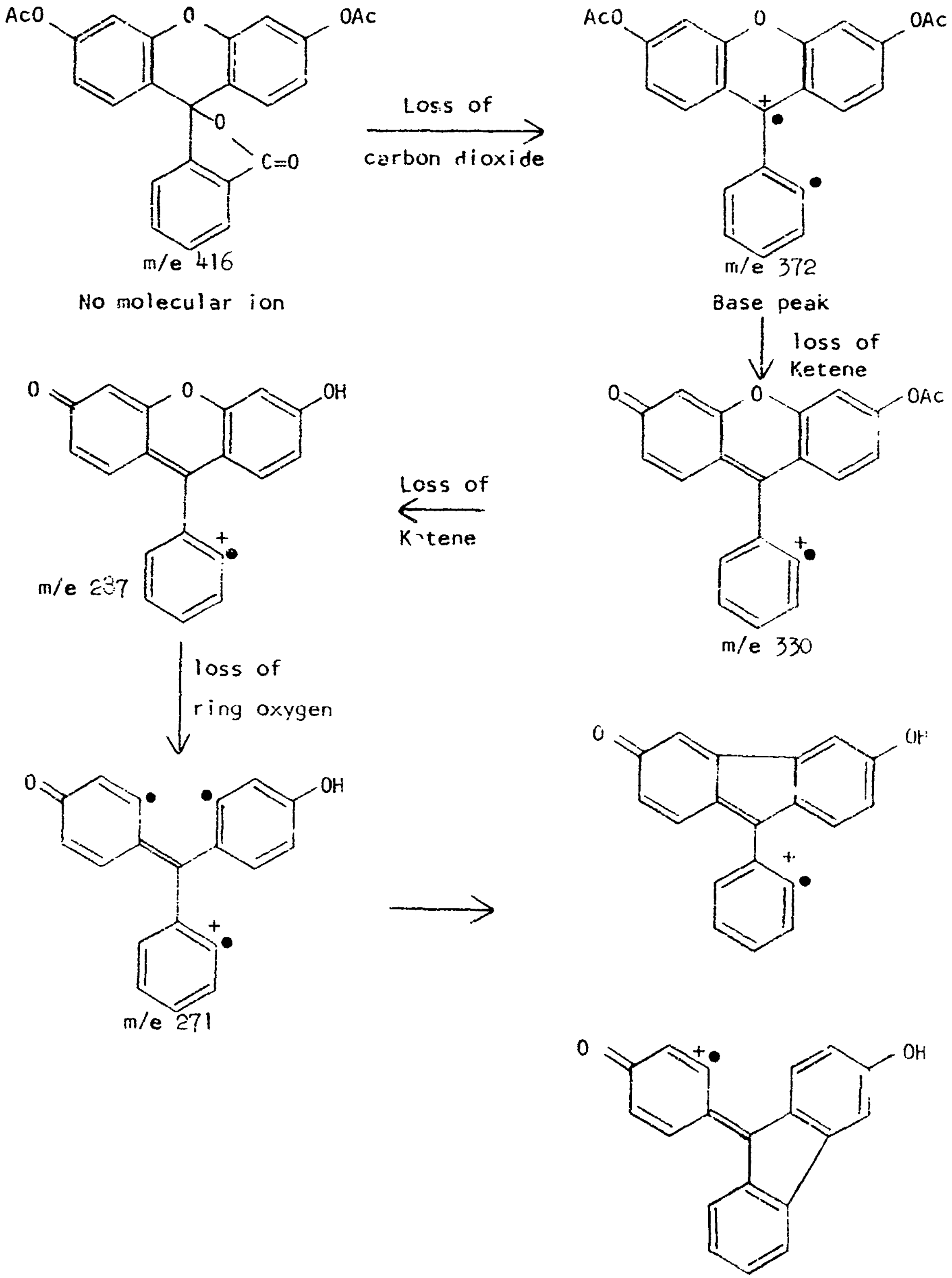
MASS SPECTRUM OF FLUORESCEIN



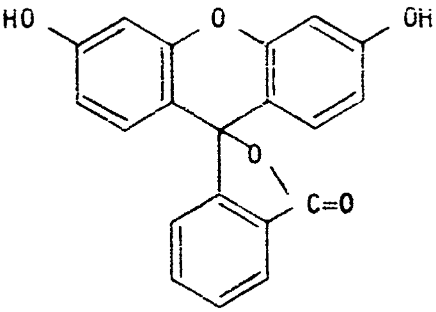
MASS SPECTRUM OF FLUORESCEIN DIACETATE



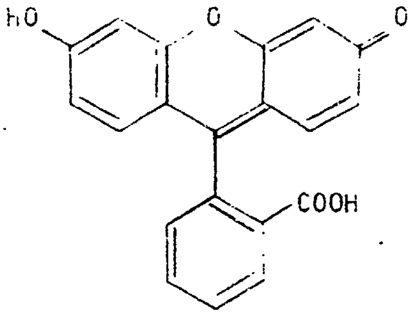
g. Mass spectral fragmentation of fluorescein diacetate.



The conclusion from these results is that fluorescein exists in two forms. The yellow lactone form is obtained by precipitation of a cold alkaline solution with acid, and the red quinonoid form is obtained by precipitation of a boiling alkaline solution with acid. The labile yellow form can be converted to the stable red form by heating at 250°.



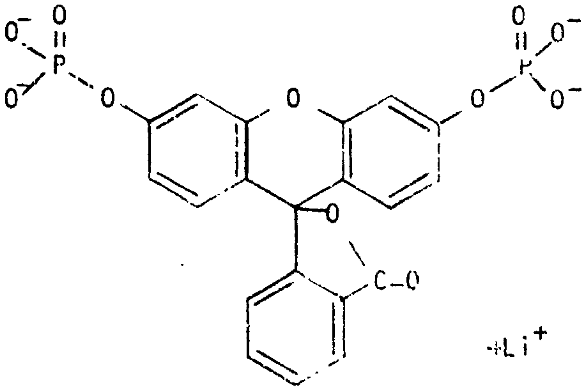
Yellow fluorescein



Red fluorescein

2. Fluorescein diphosphate.

Fluorescein was phosphorylated by a method described by Dr. Boris Rotman (private communication). A solution of fluorescein in pyridine was treated with 3 moles of phosphorus oxychloride in pyridine for one hour at 0°C. The mixture was poured into crushed ice and the pH adjusted to 7.2 with LiOH and evaporated to dryness. The residue was redissolved in water and LiOH was added to pH 12 to precipitate Li_3PO_4, which was centrifuged off. The supernatant was treated with Dowex 50 (acid form) and then LiOH to pH [illegible]. The solution was again evaporated to dryness and the residue dissolved in a minimum volume of water. The crude lithium salt of fluorescein diphosphate was obtained by precipitation on addition of methanol to the saturated aqueous solution.



The white lithium salt was hydrolyzed by heating in 0.1N. sodium hydroxide for 5 minutes and then diluting to a 1 x 10^{-5} M solution in 0.01N. sodium hydroxide.

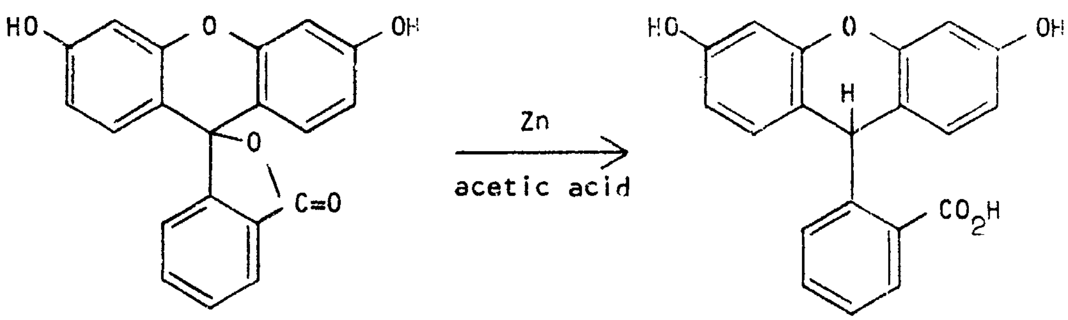
At 490 mμ, the solution had $\epsilon = 8{,}500$, suggesting the phosphate was approximately 10% pure. In order to check this value, a further sample of the phosphate was hydrolyzed with alkaline phosphatase (see figure) and hydrolysis followed on Turner fluorometer. Knowing that pure fluorescein has a fluorescence of 690 on 30x scale at 1 x 10^{-8} M, the amount of free fluorescein in the phosphate, the purity of the phosphate and rate of non-enzymatic hydrolysis could all be calculated:

Amount of free fluorescein in FDP = 1/690 = 0.14%
Purity of fluorescein diphosphate = 145/690 ≈ 21%
Rate of non-enzymatic hydrolysis ≈ 5/145 x 1/30 = 0.11%/hour.

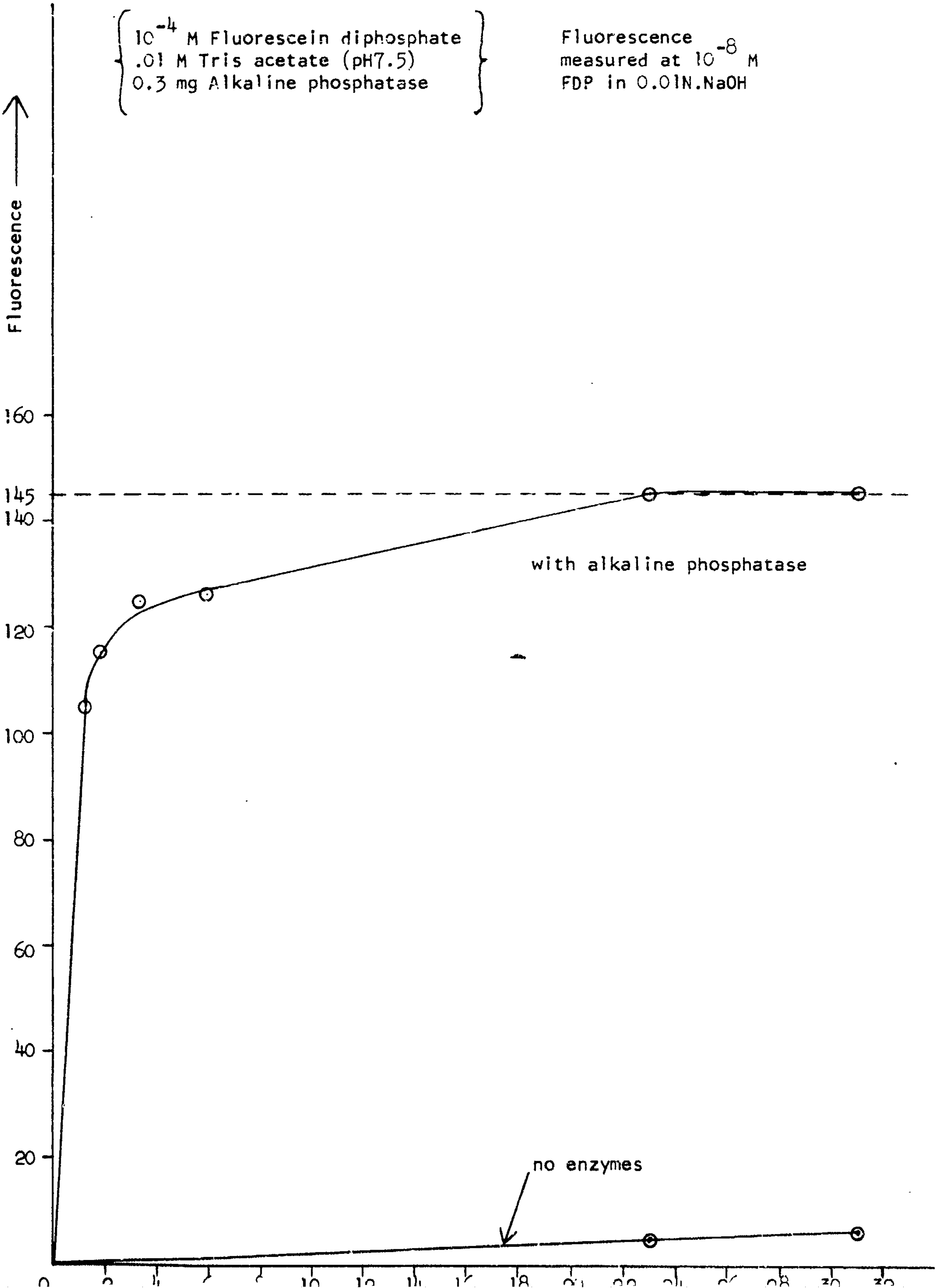
Dr. Hochstein reported[5] the rate of hydrolysis of fluorescein diphosphate at pH8 and 24° as 0.025%/hour, but he did not state the purity of his phosphate. If the two phosphates were of similar purity (21%) then the rates of non-enzymatic hydrolysis are consistent.

3. Dihydrofluorescein phosphate (HFDP)

a. Preparation of dihydrofluorescein.



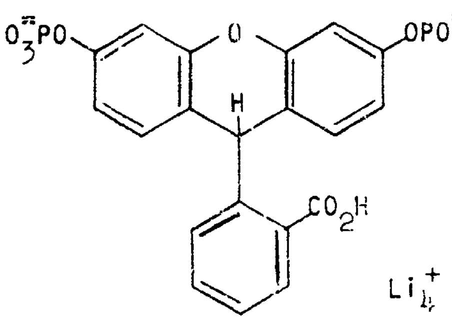
Fluorescein was reduced by the method of von Liebig[3] to dihydrofluorescein which crystallized from acetic acid as needles, m.p. 125-7°. After drying, the compound had an ϵmax at 490 mμ of 980,


10^{-4} M Fluorescein diphosphate
.01 M Tris acetate (pH7.5)
0.3 mg Alkaline phosphatase
Fluorescence measured at 10^{-8} M FDP in 0.01N.NaOH
Fluorescence
160
145
140
120
100
80
60
40
20
with alkaline phosphatase
no enzymes
0
10
12
18
28
30

suggesting contamination with about 1% fluorescein. This, however, was considerably better than the commercial product, supplied by K & K Laboratories, Inc., which contained 10% fluorescein. It was subsequently found that all fluorescein could be removed by silica gel chromatrography.

b. Phosphorylation of dihydrofluorescein (HFDP).

Prepared in identical manner to FDP:



Microanalysis

Calculated %C = 46%

Found %C = 14%

(Compound is therefore <30% pure)

c. Phosphatase assay.

As dihydrofluorescein is not fluorescent an oxidizing enzyme has to be added to convert HF to fluorescein. It was found that peroxidase was a much better oxidizing enzyme than catalase for HF:

Substrate	Conc.^{n}	ENZYME	FLUORESCENCE 0 hour	 2 hours
F	10^{-8} M		690	
HF	10^{-8} M	catalase	6	27
HF	10^{-8} M	peroxidase	0	520

The phosphatase assay was run, therefore, using peroxidase as the oxidizing enzyme.

Substrate	Conc.	ENZYME Phosphatase	 Peroxidase	FLUORESCENCE Time=0	 Time=18 hr.
FDP	10^{-8} M	✓		1	153
FDP	"	✓	✓	1	57
HFDP	"	✓		0.2	0.5
HFDP	"	✓	✓	0.2	6.7

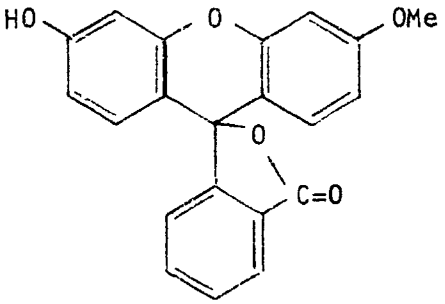
These results show that although peroxidase is a more efficient oxidizing agent, it causes a considerable decrease in the phosphatase activity. This is probably due to the presence of sodium peroxide.

If, at given time intervals, aliquots of an aqueous solution of dihydrofluorescein diphosphate are treated with peroxidase for ten minutes and subsequently their fluorescence measured in the Turner fluorometer, the rate of non-enzymatic hydrolysis can be determined. This was found to be 0.018%/hour.

4. Methyl Fluorescein Phosphate (MFP)

a. Methyl fluorescein (3-O-methyl-fluorescein).[8]

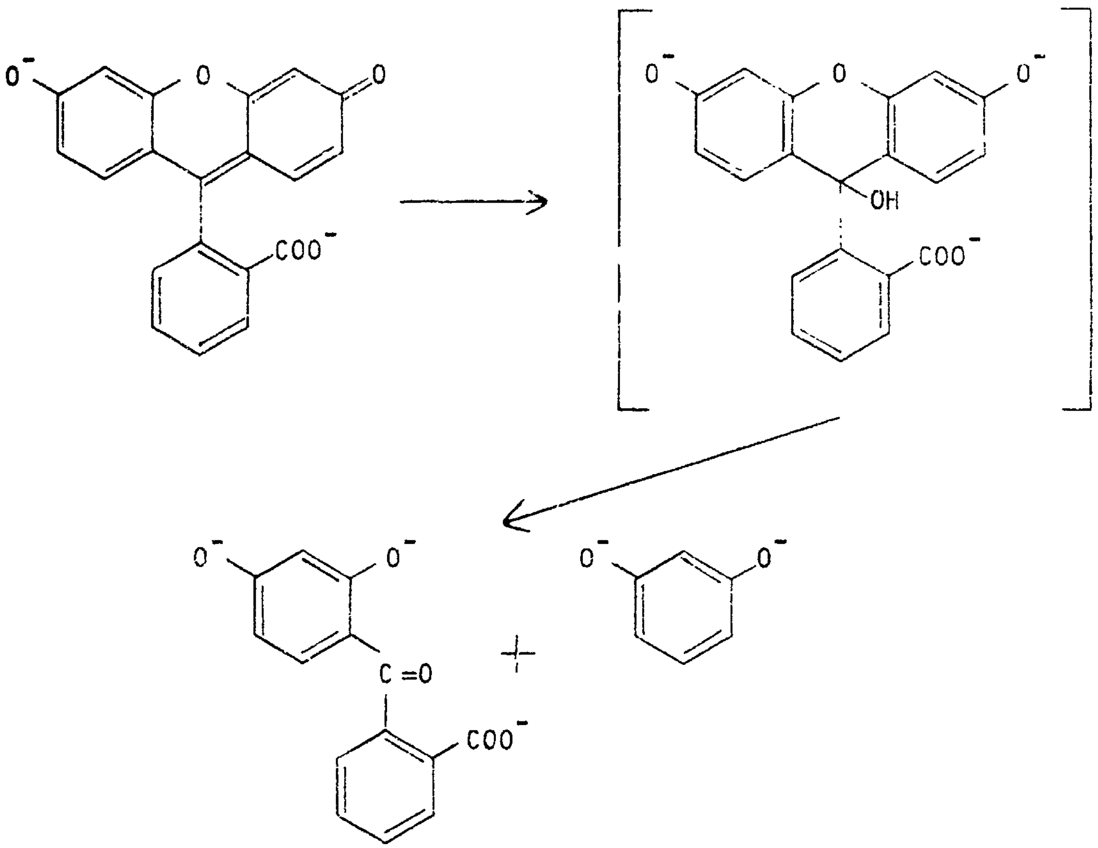
This material was obtained from Syntex Laboratories, Inc., Palo Alto, California; Lot No. 294-97-A:



Using the same filter system as was used for fluorescein, the fluorescence of a 10^{-8} M solution in 0.01N.NaOH measured on the Turner fluorometer was:

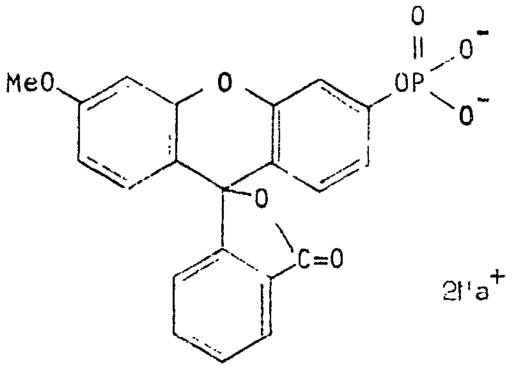
Methyl fluorescein (MF)	315
Purified fluorescein (F)	690

In both cases, F and MF, the fluorescence of the alkaline solutions showed a steady decrease with time due to decomposition of the fluor which probably proceeds by formation of the carbinol acid followed by retroaldolization and cleavage to resorcinol and O-(2,4-dihydroxybenzoyl) benzoic acid, since products of this type are obtained from fluorescein and 50% alkali at 120° C.[9]

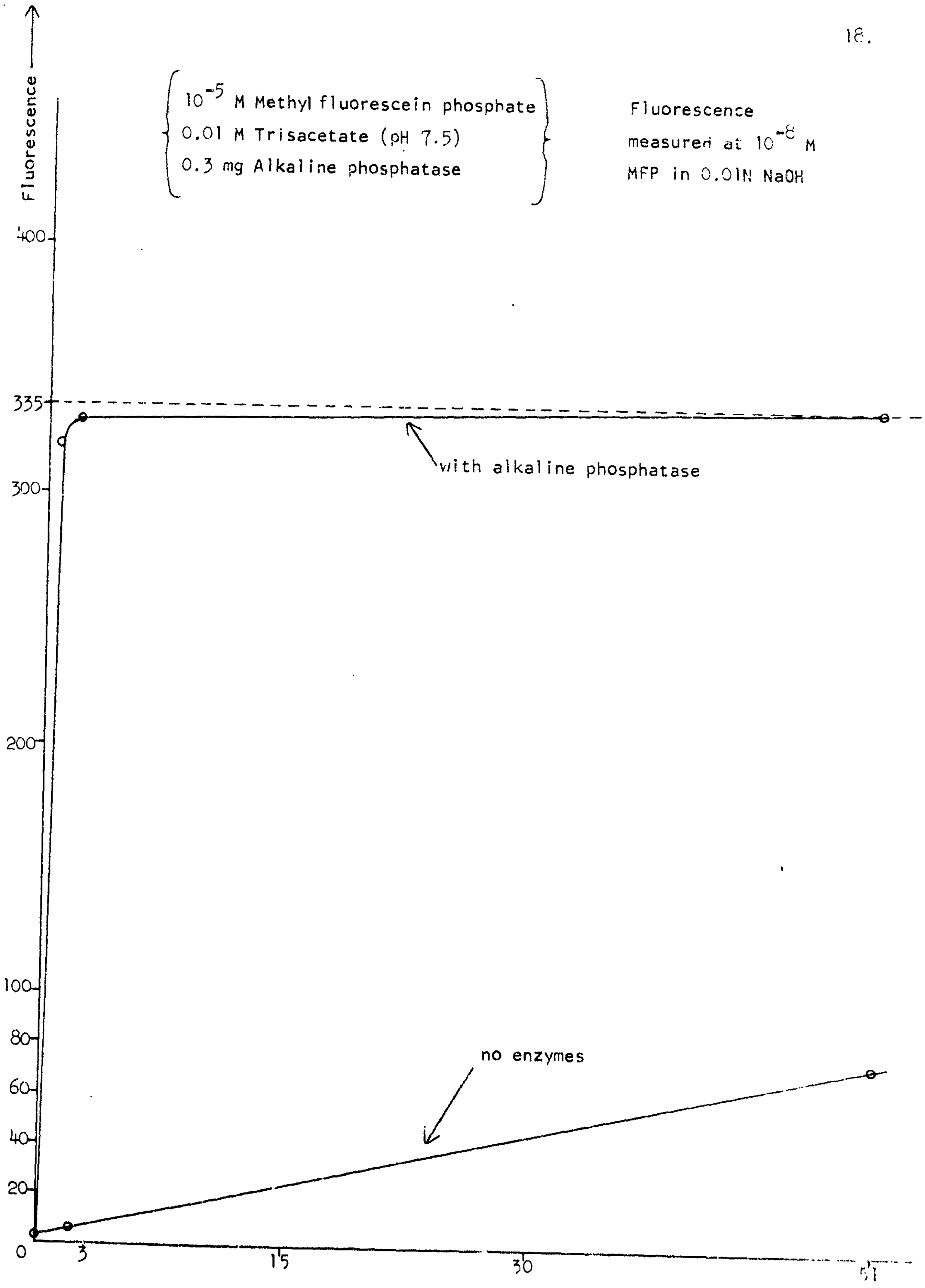


b. Preparation of methyl fluorescein phosphate (MFP).

Methyl fluorescein was phosphorylated by the method of Rotman and Zderic[10] and isolated as the sodium salt.



The purity of the compound and its rate of non-enzymatic hydrolysis were determined in an identical manner to that described for fluorescein diphosphate. The results are illustrated in the figure on page 18.

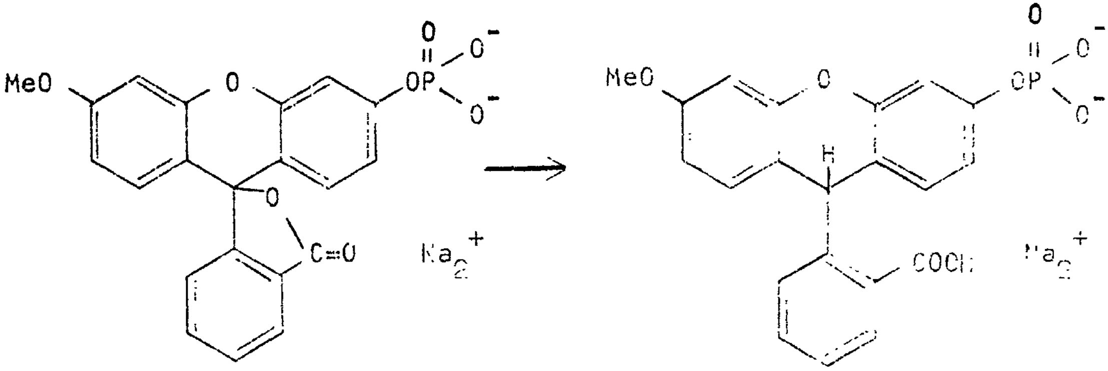

Fluorescence
10^-5 M Methyl fluorescein phosphate
0.01 M Trisacetate (pH 7.5)
0.3 mg Alkaline phosphatase
Fluorescence measured at 10^-8 M MFP in 0.01N NaOH
400
335
300
200
100
80
60
40
20
0
3
15
30
51
with alkaline phosphatase
no enzymes

In this case, the asymptotic value of the fluorescence (335) was greater than the fluor itself at the same concentration (315); and the most likely explanation for this is that the alkaline solution of the methyl fluorescein had already decomposed to some extent when the fluorescence reading was taken.

The rate of non-enzymatic hydrolysis in the case of MFP was found to be 0.42%/hour.

5. Methyl Dihydrofluorescein Phosphate (MHFP).

a. Preparation.



Methyl fluorescein phosphate suspended in ethanol ([illegible] ml) was spun down and dissolved in the minimum quantity of methanol and hydrogenated overnight, using 10% palladium on charcoal (100 mg). The catalyst was filtered off and the filtrate evaporated under vacuum. The sodium salt of methyl dihydrofluorescein phosphate was obtained as a white precipitate by the addition of ether to the concentrated methanolic solution.

b. Non-enzymatic Hydrolysis of Methyl Dihydrofluorescein Phosphate.

The stability of 10^{-8} M solution of the sodium salt of methyl dihydrofluorescein phosphate buffered at pH 7.5 was investigated in the following experiments.

Experiment 1: The increase in fluorescence of the solution was measured without the addition of any enzymes.
Rate of appearance of methyl fluorescein = [illegible]%/hour.

Experiment 2: Peroxidase and sodium peroxide were added one minute before each fluorescence measurement.
Rate of appearance of methyl fluorescein = 0.08%/hour.

Experiment 3: This experiment was to estimate the amount of phosphatase activity in the peroxidase. 10^{-5} M MHFP and 1 mg/ml of peroxidase were incubated in 10^{-4} M sodium peroxide at pH 7.5 and the rate of production of methyl fluorescein was found to be 5%/hour. This result suggests that the peroxidase contained a considerable amount of phosphatase activity. This does not invalidate the results in experiment 2, as each aliquot was treated for exactly the same time with peroxidase in this experiment and each time there was an increased fluorescence the longer the MHFP had been in solution.

6. Paper Chromatography of Fluoresceins

The best solvent for resolving the fluoresceins and their phosphate derivatives was found to be 2-propanol: ammonia: water (7·1:2). Rf values are given for Whatman no. 1 paper.

Compound	Rf
Fluorescein	0.55
Fluorescein monophosphate	0.26
Fluorescein diphosphate	0.08
Methyl fluorescein	0.80
Methyl fluorescein phosphate	0.61
Dihydrofluorescein	0.80
Dihydrofluorescein diphosphate	0.04
Methyl dihydrofluorescein	0.85
Methyl dihydrofluorescein phosphate	0.30

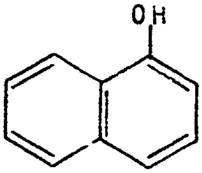
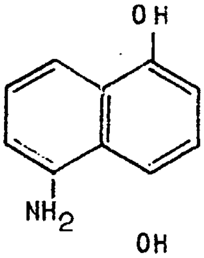
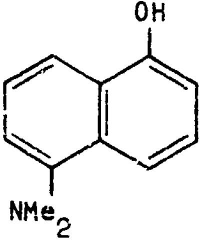
Fluorescein and methylfluorescein could easily be detected on chromatograms by their fluorescence when illuminated with ultra-violet lamp. The phosphates of these compounds could be detected by spraying with phosphatase; the reduced compounds were detected with peroxidase and subsequent illumination with ultra-violet lamp.

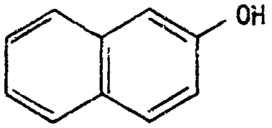
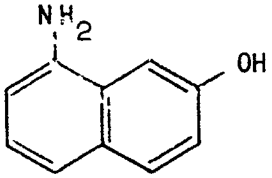
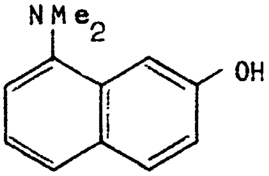
B. Naphthol Derivatives

1. Methylation of Amino-naphthols.

8-amino-2-naphthol and 5-amino-1-naphthol were methylated with dimethyl sulphate using the method of Hünig[11], and the resulting dimethylamino-naphthols were examined for absorption and fluorescence properties.

a. Ultra violet absorption (in 0.01N aqueous sodium hydroxide).

Compound	λmax (mμ)	ε max
	246 335	4[illegible],000 13,000
	240 342	[illegible]5,000 23,000
	240 320 338	53,000 20,000 20,000

Compound (cont.)	λmax (mμ)	ε max
 	236	63,000
	280	12,000
	350	3,000
 	229	51,000
	250	48,500
	304	12,000
	353	7,000
 	240	57,000
	290	12,000
	300	12,000
	340	8,000
	356	11,000

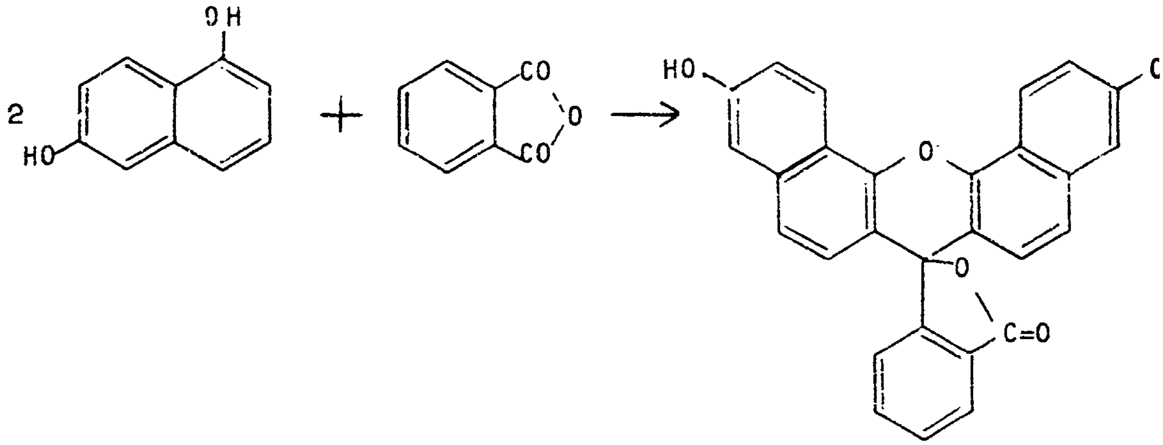
b. Fluorescence.

The fluorescence of the six naphthalene derivatives was measured on the Aminco-Bowman spectrophotofluorometer in aqueous alkali. The α-naphthols were excited at around 340 mμ and the β-naphthol derivatives at 350-360 mμ. An approximate value of the relative fluorescence of the compounds was obtained by calculating the ratio of the peak heights of the fluorescent light to the scattered light.

Compound	Wavelength of fluorescent light (mμ)	Relative Fluorescence
1-naphthol	480	2
5-amino-1-naphthol	430	0.5
5-dimethylamino-1-naphthol	460	10
2-naphthol	430	5
8-amino-2-naphthol	428	3.5
8-dimethylamino-2-naphthol	430	11

2. 6,6'-dihydroxy-naphthofluoran

a. Preparation.

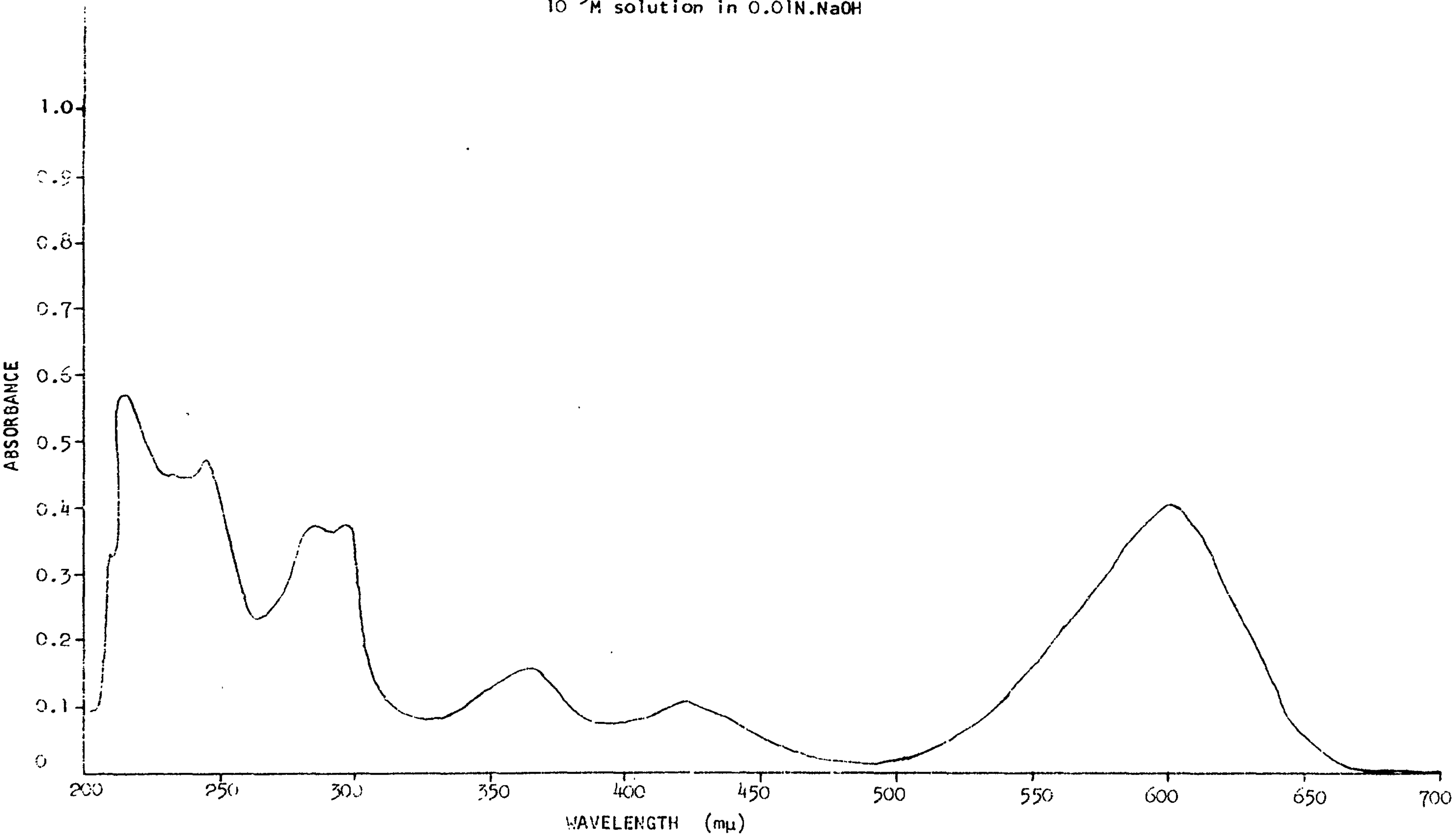


Equal amounts of phthalic anhydride (5 g) and 1,6 dihydroxynaphthalene (5 g) were ground up together to form an intimate mixture. The mixture was heated in a test-tube immersed in an oil bath. On heating, the mixture melted to a green liquid which at 150° turned red. The temperature was increased to about 190° and after 1/2 hour at this temperature the mixture thickened to a sticky solid. After heating for a further 1-1/2 hours at temperatures between 180-190°, the tube was allowed to cool and the resulting deep red solid crushed and dissolved in 1 N sodium hydroxide to give an intense blue solution. The solution was heated on the steam bath, filtered, and the product precipitated from the hot solution with glacial acetic acid. The red voluminous precipitate was filtered, washed until free of acid and then dried to give 5.5 g of red powder.

The compound was purified on a silica gel column using benzene-25% ethyl acetate as eluent. This removed dark brown polar material which remained on the column. The purified product crystallized from acetone or methanol as grey-white prisms which turned purple on drying.

b. Ultraviolet and visible absorption (see figure following).

Ultraviolet and visible absorption
of 6,6'-dihydroxy-naphtho fluoran
10^{-5}M solution in 0.01N.NaOH



UV was measured in 0.01N sodium hydroxide:

λmax (mμ)	ε max
243	55,000
290	42,000
360	17,000
422	11,500
595	40,400

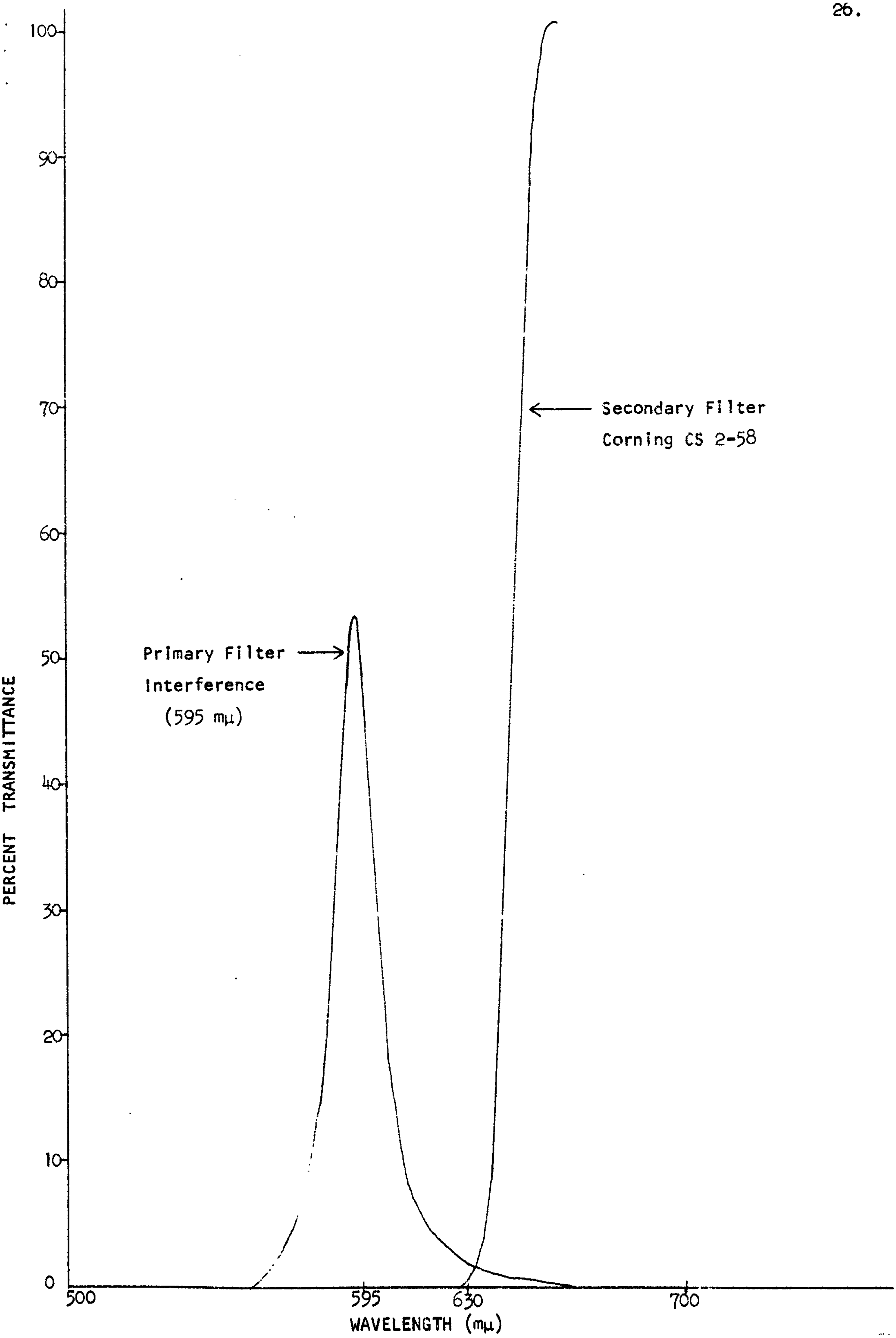
c. Fluorescence.

When an alkaline solution of 6,6'-dihydroxy-naphthofluoran (NF) was illuminated with ultraviolet light it was seen to have a strong red fluorescence. Measurement of the fluorescence on the Aminco-Bowman Spectrofluorometer showed a maximum at 660 mμ when excited at 595 mμ. In an experiment carried out with Dr. Lubert Stryer of the Stanford Department of Biochemistry, the quantum yield of fluorescence was found to be approximately 0.05.

For the Turner fluorometer, an interference filter centered at 595 mμ (from Optics Technology, Inc.) was used as the primary filter and a Corning CS 2-58 as the secondary filter (see figure following). With this filter system, NF buffered at pH 9 gave the following readings on 30x scale:

Concentration	Scale reading
1×10^{-7} M/litre	16
5×10^{-7} M/litre	80

Therefore, 1 scale unit is equivalent to 6×10^{-9} M. If 1 unit on the 30x scale of the Turner fluorometer represents the lowest limit of sensitivity, then the least amount of NF that can be detected is 0.006 μM/litre, which is about two orders of magnitude less than fluorescein. However, this is due in part to the filter system and the sensitivity of the photo tube.


100
90
80
70
60
50
40
30
20
10
0
PERCENT TRANSMITTANCE
500
595
630
700
WAVELENGTH (mμ)
Secondary Filter
Corning CS 2-58
Primary Filter
Interference
(595 mμ)

d. Potentiometric titration (see figure following).

NF at a concentration of 10^{-6} M was colorless at acidic pH, purple around pH 8 and blue in alkali. The colorless acid form showed weak yellow fluorescence, the purple mono-anion showed weak red fluorescence, and the dianion strong red fluorescence. This change in fluorescence was used as an indicator for a potentiometric titration using the Turner fluorometer and the filter system described above. The pK_a was found to be 7.95.

This showed that NF was much closer in basicity to α-naphthol (pK_a 8.0) than fluorescein (pK_a 4.5) and should, therefore, form a relatively stable phosphate.

e. Phosphorylation of 6,6'-dihydroxy-naphthofluoran.

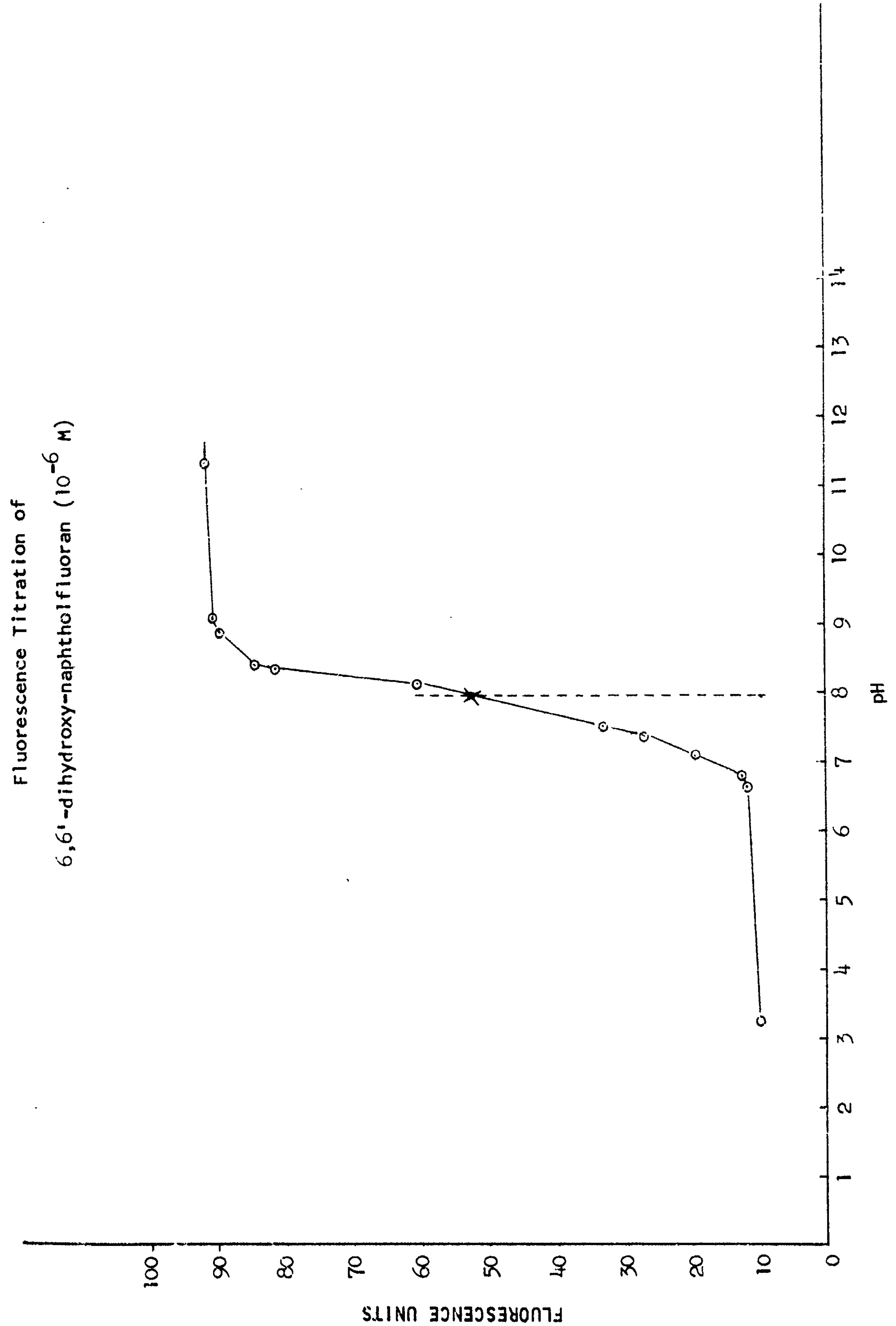
NF was phosphorylated in the same way as fluorescein and the resulting crude product, like FDP, was found to be approximately 20% pure. Paper chromatography using the 2-propanol: ammonia: water (7:1:2) solvent showed the product to contain a mixture of the diphosphate (NFDP) and monophosphate (NFMP).
NFDP exhibited a yellow fluorescence which, after spraying with phosphatase, changed to red fluorescence. The weak red fluorescence of NFMP was increased after spraying with phosphatase.

Compound	Rf
NFDP	0.16
NFMP	0.50
NF	0.56

Absorption spectrum of the crude product showed continuous end-absorption with shoulder at 300 mμ (ϵ = 5,000) and small peak at 500 mμ (ϵ = 1,000).

f. Purification of NFDP.

Crude phosphate (500 mg) was dissolved in water (25 ml) and found to contain 6000 O.D. units at 300 mμ. The pH of the purple solution was 7.8 and it had a strong, yellowish orange fluorescence.

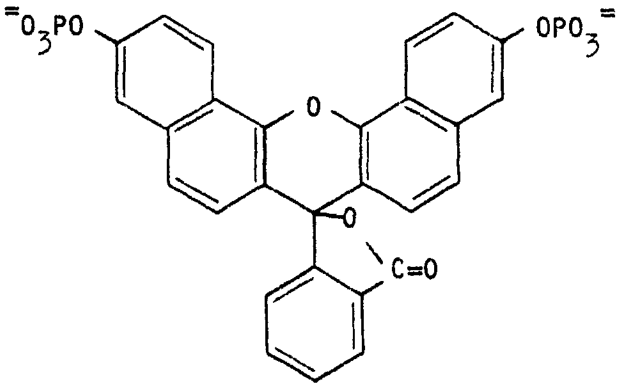


Fluorescence Titration of 6,6'-dihydroxy-naphtholfluoran (10^{-6} M)

The solution was applied to a DEAE column (40 x 3.8 cm) which had been prepared by washing with molar ammonium bicarbonate and then with water until free of bicarbonate (no precipitate with silver chloride solution). The column was eluted by gradient elution with 10 litres of aqueous triethylammonium bicarbonate (0.25-0.67 M). Soon after elution was started, a yellow fluorescent band could be seen travelling slowly down the column. The fact that the diphosphate was eluted before the monophosphate was due to its higher charge, and this was the reverse order to the mobilities on paper chromatography. Elution was followed by measuring the optical density of the fractions at 300 mμ and is illustrated in the following figure. NFDP was eluted in fractions 240-310 and the pooled fractions contained 1440 O.D. units. Fractions 310-340 were discarded as they contained a mixture of the two phosphates. Fractions 340-400 (240 O.D. units) were found to contain the monophosphate, NFMP.

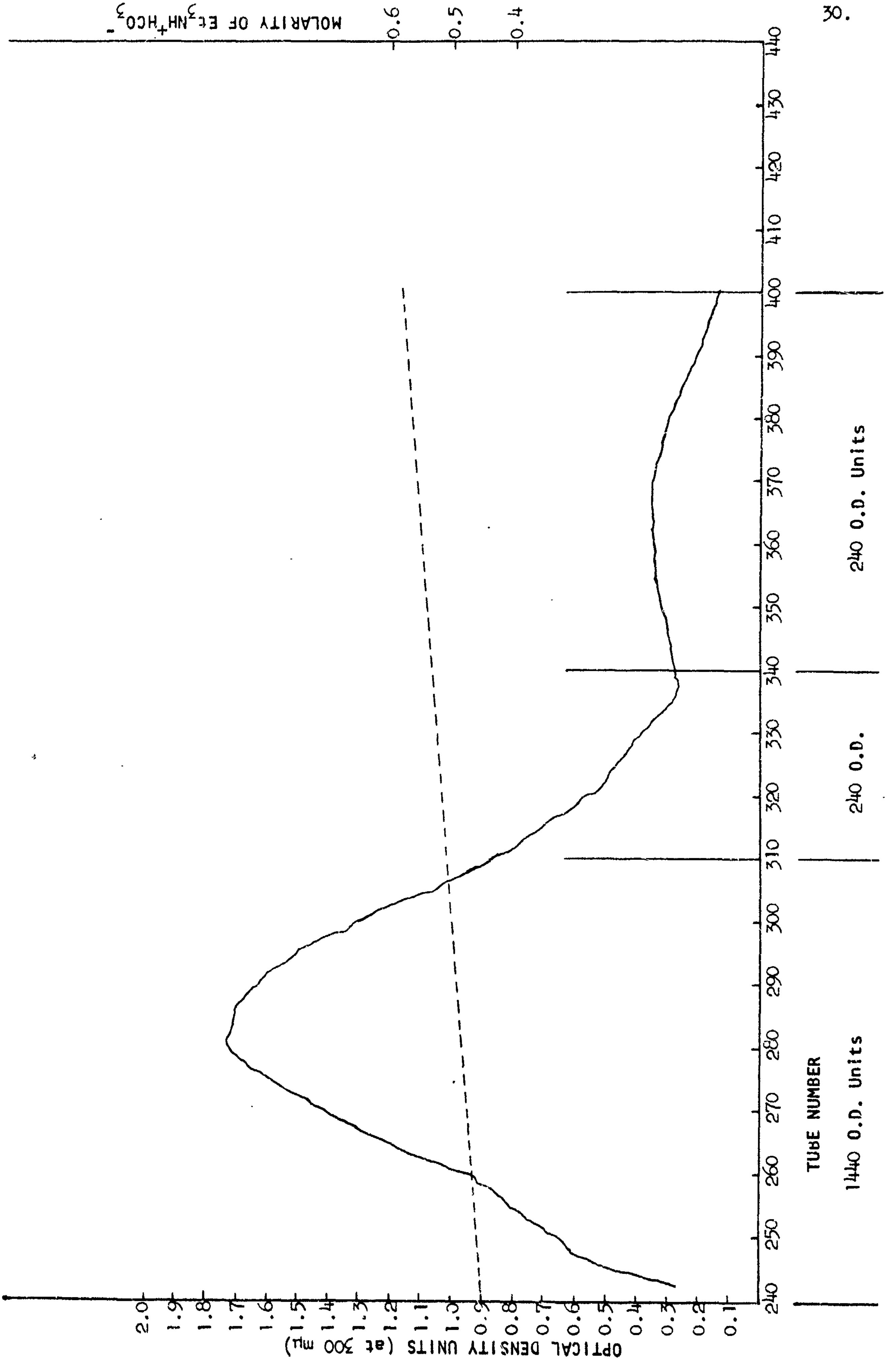
The pooled fractions were evaporated under vacuum at 30° and the triethylammonium bicarbonate was removed by dissolving the residue in methanol and evaporating the methanol. This operation was repeated three times and then the residue was again dissolved in methanol, the solution filtered and filtrate treated with excess ether to precipitate the product. In the case of NFDP, the product was an oil which changed to a straw colored amorphous solid (80 mg) on drying for 24 hours at 0.1 mm over phosphorus pentoxide. NFMP precipitated as a solid but the yield was so low (about 5 mg) that it was dissolved in water and the concentration determined enzymatically by conversion to NF.

As the compounds were eluted from the column with triethylammonium bicarbonate, they were in the form of the triethylammonium salt.



$[(C_2H_5)_3NH^+]_4$

M.W. 998


MOLARITY OF $Et_3NH^+HCO_3^-$
0.6
0.5
0.4
OPTICAL DENSITY UNITS (at 300 mμ)
2.0
1.9
1.8
1.7
1.6
1.5
1.4
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
TUBE NUMBER
240
250
260
270
280
290
300
310
320
330
340
350
360
370
380
390
400
410
420
430
440
1440 O.D. Units
240 O.D.
240 O.D. Units

Ten mg of the amorphous yellow solid was dissolved in 100 ml of water to give a 10^{-4} M solution which was used as a stock solution for the enzymatic assays.

g. Continuous recording of enzymatic assays.

In the earlier assays to determine the purity of FDP and MFP, the progress of the experiment was followed by taking aliquots of the reaction solution, diluting them in 0.01 N sodium hydroxide solution and then reading the fluorescence in the Turner fluorometer. In order to simplify the method, a Moseley Autograf Model 680 strip chart recorder was connected to the Turner fluorometer, and the assays were run at pH 9, which is the lowest pH at which NF shows maximal fluorescence. In order to run assays at lower pH, when looking for acid phosphatases for instance, the earlier method of taking aliquots at different times would have to be used.

h. Purity of NFDP.

A solution (4 ml) containing 5 x 10^{-7} M NFDP and 0.1 M tris acetate (pH 9.0) was treated with 0.12 mg of alkaline phosphatase (calf intestine, B grade) and immediately placed in the Turner fluorometer, set up for continuous recording. Within two minutes, the fluorescence reached an asymptotic value of 70 on 30 x scale. NF has a fluorescence of 80 units at this concentration. Therefore NFDP is 70/80 = 87.5% pure.

i. Absorption of NFMP, NFDP.

	λmax (mμ)	ε max
NFMP	284	18,000
	354	3,600
	565	4,500
NFDP	300 (shoulder)	23,000
	500	1,600

j. Fluorescence of NFMP and NFDP.

These measurements were made on the Aminco-Bowman Spectrofluorometer:

Compound	Excitation maximum	Fluorescent maximum
NF	595 mμ	660 mμ
NFMP	565 mμ	655 mμ
NFDP	500 mμ	565 mμ

NF, NFMP and NFDP, all buffered at pH 9, were compared on the Turner fluorometer using a 595 mμ primary interference filter and a Corning CS 2-58 secondary filter.

Compound	Concentration	Observed Fluorescence
NF	5×10^{-7} M	80
NFMP	1×10^{-5} M	70
NFDP	1×10^{-5} M	0

k. Non-enzymatic hydrolysis of NFDP.

After one week, there was no detectable increase in fluorescence of a solution of 10^{-5} M NFDP, which means the rate of conversion to NF was less than 0.00035%/hour.

l. Enzymatic hydrolysis of NFDP and NFMP.

Using the Turner fluorometer, set up for continuous recording, assays were run using a constant enzyme concentration (1.2 μg/ml) and various substrate concentrations. From these results, the substrate concentration was determined which gave rise to highest speed of hydrolysis. From a Lineweaver-Burk double reciprocal plot the Michaelis constant was calculated.

Results for NFDP (see figure following).

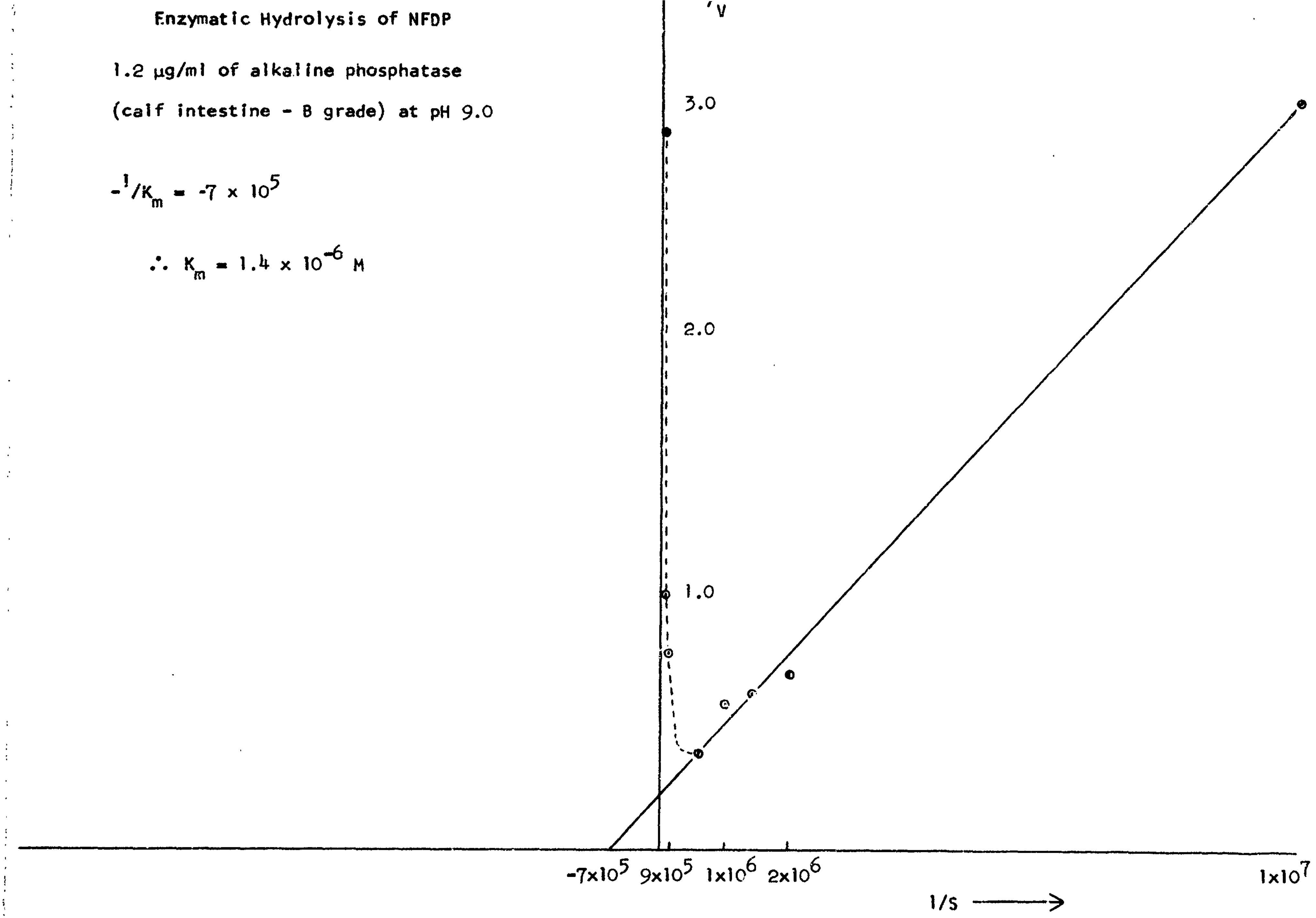
Enzymatic Hydrolysis of NFDP

1.2 μg/ml of alkaline phosphatase

(calf intestine - B grade) at pH 9.0

$-{}^{1}/K_m = -7 \times 10^5$

$\therefore K_m = 1.4 \times 10^{-6}$ M



Results for NFDP (cont.)

Substrate concentration (moles/litre) s	1/s	Rate of production of NF (μM/litre/hour) v	1/v
1×10^{-7}	1×10^{7}	0.33	3.0
5×10^{-7}	2×10^{6}	1.45	0.69
7×10^{-7}	1.4×10^{6}	1.63	0.61
1×10^{-6}	1×10^{6}	1.76	0.57
1.7×10^{-6}	6×10^{5}	2.64	0.38
1×10^{-5}	1×10^{5}	1.3	0.77
9×10^{-5}	1.1×10^{4}	1.0	1.0
1×10^{-3}	1×10^{3}	0.35	2.86

Michaelis constant, $K_m = 1.4 \times 10^{-6}$ M.

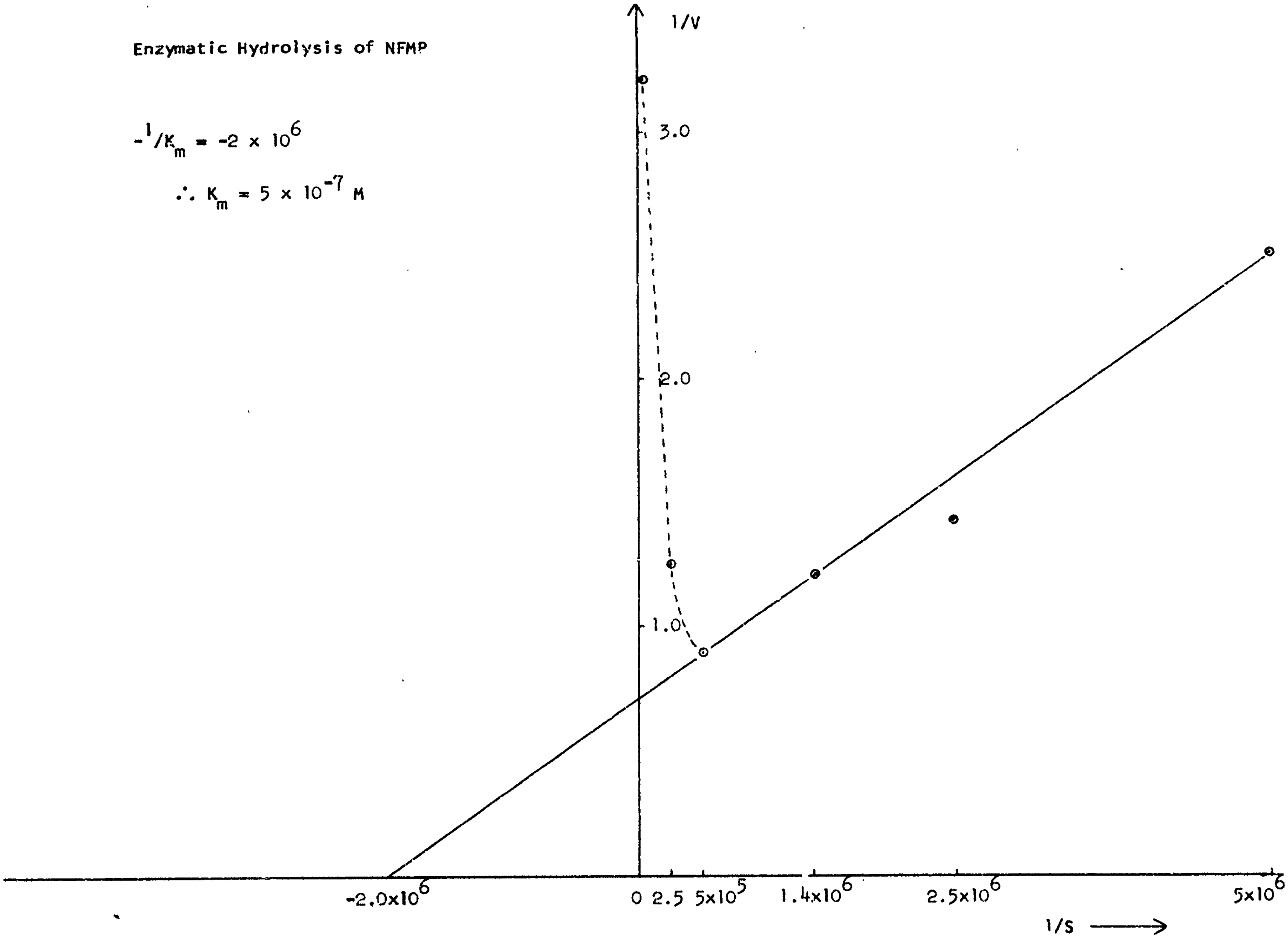
Results for NFMP (see following figure)

Substrate concentration (moles/litre) s	1/s	Rate of production of NF (μM/litre/hour) v	1/v
2×10^{-7}	5×10^{6}	0.40	2.5
4×10^{-7}	2.5×10^{6}	0.70	1.43
7×10^{-7}	1.4×10^{6}	0.84	1.19
2×10^{-6}	5×10^{5}	1.14	0.88
4×10^{-6}	2.5×10^{5}	0.79	1.26
3.6×10^{-5}	2.8×10^{4}	0.31	3.2

Michaelis constant, $K_m = 5 \times 10^{-7}$ M.

Conclusions

1. The optimal substrate concentration for enzymatic hydrolysis of NFDP or NFMP is about 2×10^{-6} M.
2. Concentrations of NFDP or NFMP greater than 2×10^{-6} M cause inhibition of enzyme activity.
3. Rate of production of NF is greater when NFDP is used as a substrate than when NFMP is substrate.



Enzymatic Hydrolysis of NFMP

$-{}^{1}/K_m = -2 \times 10^6$

$\therefore K_m = 5 \times 10^{-7}$ M

m. Bacterial hydrolysis of NFDP.

B. subtilis was grown under the conditions of phosphate deprivation described by Garen and Levinthal.[12] Viable counts were run concurrently with the phosphatase assays and the bacterial concentrations used in the experiments were determined. Bacteria were added to a solution containing 2×10^{-6} M NFDP and 0.1 M tris acetate (pH 9).

Bacterial concentration (per ml)	Rate of production of NF
4×10^{6}	0.033
4×10^{7}	0.84

There appeared to be a decrease in the relative rate at the lower concentration. It has been observed[13] that the addition of magnesium ion generally causes an activation of phosphatase. To the solution described above was added 10^{-2} M magnesium sulphate.

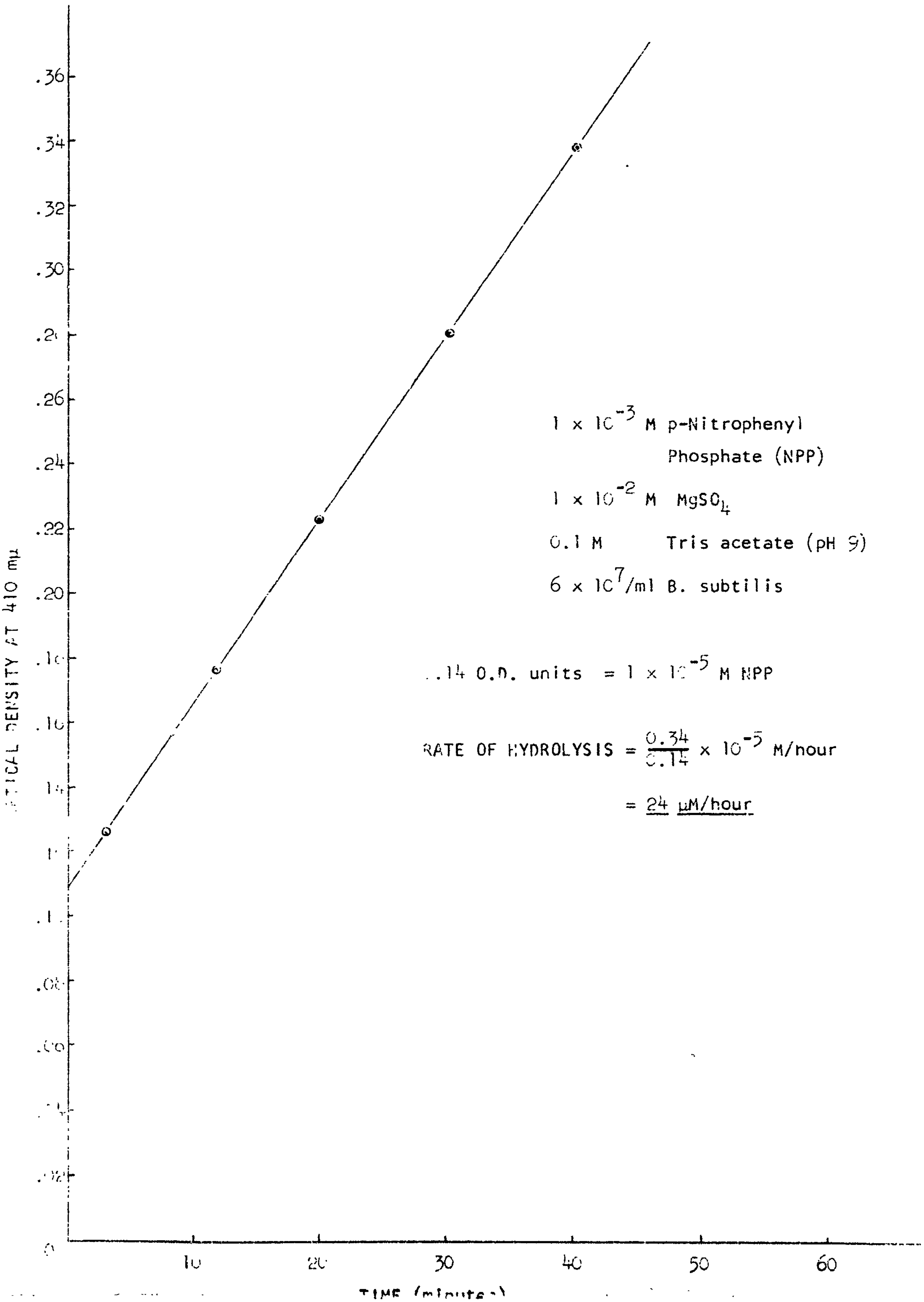
Bacterial concentration (per ml)	Rate of production of NF (μM/litre/hour)
6×10^{4}	0.002
7×10^{5}	0.01
7×10^{6}	0.10
6×10^{7}	0.48
7×10^{7}	0.72

The addition of magnesium ion appears to cause an increase in relative rates at lower bacterial concentrations and a decrease at higher concentrations.

n. Comparison of NFDP and p-nitrophenyl phosphate as substrates for phosphatase.

Phosphatase activity is usually assayed by following the color change of p-nitrophenyl phosphate.[14] A fluorometric assay using NFDP (2×10^{-6} M) was run simultaneously with a spectrophotometric assay using p-nitrophenyl phosphate (10^{-3} M) (see figure following) with the same bacterial concentration (6×10^7) and the rates compared.

Rate of production of p-nitrophenol = 24 μM/litre/hour.
Rate of production of 6,6'-dihydroxy-naphthofluoran = 0.48 μM/litre/hour.


.36
.34
.32
.30
.28
.26
.24
.22
.20
.18
.16
.14
.12
.10
.08
.06
.04
.02
0
OPTICAL DENSITY AT 410 mμ
10
20
30
40
50
60
TIME (minutes)
1 x 10^{-3} M p-Nitrophenyl Phosphate (NPP)
1 x 10^{-2} M MgSO$_4$
0.1 M Tris acetate (pH 9)
6 x 10^7/ml B. subtilis
0.14 O.D. units = 1 x 10^{-5} M NPP
RATE OF HYDROLYSIS = $\frac{0.34}{0.14}$ x 10^{-5} M/hour
= 24 μM/hour

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