STUDIES ON LUCIFERASE FROM *PHOTOBACTERIUM PHOSPHOREUM*

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INTRODUCTION

Bioluminescence, the emission of visible light from living organisms, is due to the catalytic action of an enzyme called luciferase. The bioluminescent system, what is called the luciferin-luciferase reaction, causes highly efficient conversion of chemical energy to light energy without production of any appreciable amount of heat. It is a matter of concern how such conversion of energy occurs in various bioluminescent systems, such as that in bacteria and much research has been reported on such reaction systems in nature.

For light emission *in vitro*, a luciferase from marine luminous bacteria requires molecular oxygen, reduced flavin mononucleotide($FMNH_2$) and a saturated, normal, long chain aliphatic aldehyde(RCHO).

$$FMNH_2 + O_2 + RCHO \xrightarrow{\text{Luciferase}} h\nu + \text{Products}$$

The main problems in investigations on bacterial bioluminescence are as follows.

(1) FMN, one of products during the luminescent reaction, is fluorescent and all bacterial luciferases so far obtained also have chromophores with similar fluorescence. The maximum wavelengths of fluorescence of these components are different from the maximum wavelengths of emitted bioluminescence *in vitro*. Accordingly, it is unknown which components are emitters or whether a third component, such as aromatic amino acid residues of the enzyme, acts as the emitter.

(2) The nature of the "excited intermediate" formed in the luminescent reaction is unknown.

The present work was carried out to clarify these problems.

First, an active luciferase (denoted as stripped luciferase) with no fluorescence was prepared and a new flavin (denoted as p-flavin), the chromophore which bound with luciferase non-covalently, was characterized. Using this active luciferase the mechanism of the luciferase reaction *in vitro*, and especially the natures of the intermediates were investigated spectrophotometrically and kinetically by the stopped flow method. This work is described in three parts in this paper.

Part I reports the purification of a bacterial luciferase(native) from *Photobacterium phosphoreum*. The stripped luciferase was prepared by reversible denaturation of this enzyme with guanidine-HCl, followed by gel filtration on Sephadex. Studies on the physicochemical properties and natures of the luminescent reactions of these preparations are also reported. The stripped luciferase did not show any absorption or fluorescence in the visible region but it had the same activity as native luciferase. Using this preparation it was possible to analyze the behavior of the reaction-intermediate, spectrophotometrically.

Part II reports spectroscopic and kinetic studies on the nature of the intermediate in the reaction of the luciferase-$FMNH_2$ complex with oxygen using the stopped flow method. Results showed that an unstable and obligatory intermediate was rapidly formed on aerobic oxidation of the luciferase-$FMNH_2$

complex. The kinetic nature of the complex formed by the reaction of this intermediate with aldehyde, as the third substrate, was examined and a sequential mechanism was proposed for the bacterial luciferase reaction *in vitro*.

Part III reports the isolation and partial characterization of native, luciferase-bound pigment(p-flavin). p-Flavin was found to be a new kind of flavin derivative, because it was more hydrophobic than other flavins, although it contained a high ratio of phosphoric acid to isoalloxazine nucleus. The chromatographic characteristics of this flavin do not correspond to those of any previously isolated flavin derivative. This flavin did not seem to be the emitter and it had an inhibitory effect on the luciferase reaction *in vitro* so its role *in vivo* requires further investigation.

PART I-A

PURIFICATION AND PROPERTIES OF NATIVE LUCIFERASE

Bacterial luciferase(native) was purified from *Photobacterium phosphoreum* and its physicochemical properties were investigated. This enzyme has a molecular weight of 82,000 and an absorption maximum at 375 nm with a shoulder at 445 nm in addition to the protein peak. The fluorescence and luminescence spectra of the enzyme and its chemiluminescent quantum yield were also investigated.

The emission of visible light from living organisms, bioluminescence, is due to the catalytic action of an enzyme called luciferase. Different species of bioluminescent organisms have their own specific luciferase with a specific action mechanism (1). A bacterial luciferase was isolated from Photobacterium fischeri by Hastings et al. (2). This enzyme requires $FMNH_2$, molecular oxygen and RCHO* as substrates for a bioluminescence activity in vitro (3). In the present work, luciferase(native) was extracted and purified from Photobacterium phosphoreum. This enzyme acts on the same substrate as that of Ph. fischeri luciferase, but differs from it in spectroscopic characteristics. This paper describes studies on the molecular weight and other physicochemical properties of this preparation and on the enzyme reaction.

MATERIALS AND METHODS

Growth and Harvesting of Cells--The bacterium was isolated from cuttle fish and was identified as Photobacterium phosphoreum. It was subcultured on solid agar medium containing 15 g of agar per liter in addition to the components of a liquid medium as described below. For preparation of the enzyme, cells were grown in the liquid medium which contained

*The abbreviations used in this paper are: RCHO, saturated, normal, long chain aliphatic aldehyde; SDS, sodium dodecyl sulfate; DTT, dithiothreitol.

the following: Beef extract 3 g, polypeptone 5 g, NaCl 30 g, glycerol 3 ml, 1 M phosphate buffer, pH 7.0 2 ml, $MgSO_4 \cdot 7H_2O$ 0.2 g, and tap water 1 liter. The medium was adjusted to pH 7.0 with 1 N NaOH. A small inoculum (1 liter) was added to 10 liters of the fresh and unsterilized medium in a large glass flask and was incubated at 20-25°C with vigorous aeration for 8-10 hr. Cells were harvested by centrifugation at 10,000 x g with a continuous flow centrifuge when light emission became maximum at a density of about 5×10^8 cells per ml. At this stage a single bacterium emitted an average of 3.6×10^4 q/sec. The yield of packed wet cells was between 5 and 8 g per liter of culture medium.

Fluorescence and Emission Spectra--The fluorescence spectrum of the luciferase solution and the emission spectrum of bacterial suspension were recorded by using a Hitachi recording spectrofluorometer, model MPF-2A. For measurement of the *in vitro* luminescence spectrum of purified luciferase, the flow system used in the enzyme assay (see below) was connected to the spectrofluorometer and I_o, the peak height of the emitted light, was measured at different wavelengths. All these spectra were represented as relative quanta per unit wavenumber width ($q/\Delta\nu$) versus wavenumber according to the method of Lippert *et al.* (4) with *m*-dimethylaminonitrobenzene, quinine sulfate and β-naphthol as standard substances.

Measurement of Absolute Light Intensity--The absolute units of light intensity of bacterial luminescence were measured by the method of Hastings and Weber (5) on a photomulti-

plier photometer apparatus combined with a micro volt-ammeter (Tōa Denpa Kogyo Co., model PM-18). The bacterial suspension (10 ml) in a glass vial was placed directly in front of the cathode of the photomultiplier, and the meter reading was converted to the absolute unit of q/sec/ml by comparison with results for the same volume of a standard solution of ^{14}C-labelled hexadecane in toluene with 2,5-diphenyl-oxazole and 2,2'-p-phenyl bis-(5-phenyl-oxazole) as scintillator (5). Appropriate corrections were made for the difference in the emission spectra and for the spectral sensitivity of the photomultiplier. The total radioactivity of the hexadecane sample was 2.1 μCi according to the supplier, the Radiochemical Center, England. The concentrations of scintillator used were the same as those described by Hastings and Weber (5). Essentially the same method was used for measuring the absolute light intensity of luminescence of luciferase *in vitro* by making appropriate spectral corrections.

Enzyme Assay--The luciferase reaction was followed in a flow apparatus (6) combined with a recording system, which consisted of a photomultiplier, a direct current amplifier and a recorder (Visigraph type FR-301 supplied by San'ei Instrument Co.). As a standard assay system, enzyme solution containing 8×10^{-4} M n-decylaldehyde, 2.4×10^{-4} M oxygen and 0.1 % Tween 80 in 0.1 M phosphate buffer, pH 7.0, was placed in one syringe of the flow system, and 4×10^{-5} M $FMNH_2$ solution in the same buffer was placed in the other syringe. The reaction was initiated by mixing these solutions in the ratio of 1 : 1, and

the light output from the reaction mixture was recorded by the stopped flow method. All the assay procedures were conducted at 20°C. The composition of the assay system was the same as that employed by Hastings et al. (2) except that 2-mercaptoethanol and bovine serum albumin were omitted. These reagents are believed to act as stabilizers for the Ph. fischeri enzyme, but the reaction of the Ph. phosphoreum enzyme was not affected by them. Furthermore, no activity loss was detected, at least, with the latter enzyme which was allowed to stand for 40 min at room temperature after dilution with n-decylaldehyde-containing buffer without these reagents. In all activity measurements, the assay mixture was used within 10 min after dilution of the enzyme stock solution.

Ultracentrifugation--The homogeneity of the luciferase preparation was tested by centrifugation in a Beckman analytical ultracentrifuge, model E.

Electrophoresis--A Tiselius-type apparatus from Hitachi Co. was used. Electrophoretic analysis was carried out at 8.5°C in 0.02 M Tris buffer containing 0.1 M NaCl.

Determination of Molecular Weight by Osmometry--The molecular weight of luciferase was determined by osmometry with the kind cooperation of Dr. Y. Hayashi of this laboratory. A Mechrolab Inc. membrane osmometer, model 503, was used for this purpose. According to van't Hoff's equation, the number average molecular weight, $\overline{Mn}$, of a solute is related to the osmotic pressure of the solution, π, by

$$Mn = RT(\pi/c)_{c=0}$$

where R is the gas constant, T, the absolute temperature and c, the concentration of the solute.

Other Measurements-- The absorption spectrum of luciferase dissolved in 0.1 M phosphate buffer, pH 7.0, was recorded with a Cary recording spectrophotometer, model 14. Enzyme concentration was determined by the Folin-Ciocalteu reaction (7) by using a calibration curve obtained on the basis of dry weight measurements of luciferase.

Reagents--The enzyme reaction is known to be strongly inhibited by riboflavin, so this was removed from commercial FMN by cellulose column chromatography according to the method of Whitby (8) with slight modifications. The purity of the resulting FMN was tested by paper chromatography. $FMNH_2$ solution was prepared in the reservoir of the flow apparatus either by photoreduction or by chemical reduction of FMN dissolved in 0.1 M phosphate buffer, pH 7.0. Photoreduction was carried out, in the presence of 2 mM EDTA, by irradiation of the FMN solution with a 20 W fluorescent lamp 10 cm apart, and chemical reduction was carried out by addition of a minimum amount of dithionite. Both methods gave the same result when used used for the luciferase assay. Fresh 10^{-2} M n-decylaldehyde solution was newly prepared before use by diluting a 1 M solution of the aldehyde (in ethanol) with distilled water containing 0.1 % Tween 80.

RESULTS

PURIFICATION OF LUCIFERASE

Extraction, Adsorption on Calcium Phosphate Gel and Fractionation by Ammonium Sulfate--Luciferase was purified by a modification of the procedure described by Hastings *et al.* (2) for the *Ph. fischeri* enzyme. One hundred grams (wet weight) of cells, whether fresh or after storage in a freezer at -20°C, was osmotically lysed and extracted with 400 ml of distilled water. The suspension was stirred for 20 min at room temperature, and the cell debris was removed by centrifugation at 13,000 x g for 15 min. All subsequent steps were carried out at 4°C. The crude extract contained a large amount of nucleic acid in addition to luciferase as evidenced by the strong absorption at 260 nm. It was mixed with 13 g (dry weight) of calcium phosphate gel to a state of thick slurry and the pH was adjusted to 5.8 with 1 N acetic acid. The slurry was stirred for 20 min. Then the calcium phosphate gel which had adsorbed most of the luciferase was collected by centrifugation at 1,500 x g for 10 min. The supernatant which contained a large amount of riboflavin and nucleic acid was discarded. The gel was washed with distilled water and then suspended in 1,200 ml of 0.5 M ammonium phosphate, pH 8.0, for extraction of the enzyme. The eluate was separated from the gel by centrifugation at 1,500 x g for 10 min, and solid ammonium sulfate was added to the supernatant to 40 % saturation. The resulting light brown precipitate was removed by centrifugation at 13,000 x g for 20 min. Solid ammonium sulfate

was added to the supernatant to 75 % saturation. The yellow precipitate thus obtained, which contained most of the luciferase activity of the lysate, was collected by centrifugation at 13,000 x g for 20 min. The resulting crude luciferase preparation was either stored in a refrigerator as a suspension in saturated ammonium sulfate solution or used immediately for further purification as described below.

Column Chromatography on DEAE Cellulose--One third of the crude enzyme from 100 g of wet cells was dissolved in 0.05 M phosphate buffer, pH 7.0, and dialyzed overnight against the same buffer in dark. Then it was centrifuged at 13,000 x g for 10 min to remove insoluble material, and charged on a column of DEAE cellulose (3 cm x 40 cm) equilibrated with 0.05 M phosphate buffer, pH 7.0. The column was exhaustively washed with 0.05 M phosphate buffer, pH 7.0, for at least 24 hr to remove the nucleic acid which remained in the dialysate. Then 0.1-0.12 M phosphate buffer, pH 7.0, was passed through the column for about 12 hr until four colored bands (I, yellow; II, brownish yellow; III, yellow; IV, brownish yellow) were clearly separated from each other, as shown diagramatically in Fig. 1. The DEAE cellulose was then removed from the glass column and band II, which contained luciferase, was carefully separated from bands I and III. Bands I and III contained unidentified flavoproteins which did not show a detectable luciferase activity.

Fig. 1

The cellulose of Band II was suspended in a minimum volume of distilled water and packed in a glass column of 3 cm diameter. Luciferase was eluted from this column with 0.3 M phosphate buffer, pH 7.0. The purified preparation could be stored in a refrigerator (in the dark) for a few days without loss of activity. The yield of luciferase from 100 g of wet packed cells was 300 mg (dry weight), which corresponded to about 4 % of the extractable protein. The activity recovery was 48 %. The purification process described above is summarized in Table I.

Table I

PROPERTIES OF LUCIFERASE

Ultracentrifugation--On ultracentrifugation of luciferase solutions at various concentrations the enzyme sedimented as a single component (Fig. 2). The sedimentation coefficient of

Fig. 2

Fig. 3

luciferase, estimated by extrapolation to zero concentration, was found to be 5.5 Svedberg units (Fig. 3).

Electrophoresis--On electrophoresis at pH 7.0 only a single peak was observed. Figure 4 shows the electrophoretic

Fig. 4

pattern. At this pH, the mobility of luciferase was calculated as 0.78×10^{-4} cm^2/V to the anode.

Gel Filtration on a Sephadex G-100 Column--The elution pattern of luciferase from a column of Sephadex G-100 is shown in Fig. 5. A single protein peak was observed in the position

Fig. 5

of the luciferase activity peak. Only a trace amount of inactive impurity was found as a slow moving protein peak. Essencially the same results were obtained by using a column of Sephadex G-150 or G-200 of the same length.

Molecular Weight Determination--The molecular weight of luciferase was determined on an enzyme preparation of known purity, which had been confirmed by the criteria as described above. The osmotic pressure of luciferase solution in 0.1 M phosphate buffer, pH 7.0, at 5.0 °C was measured at several different protein concentrations, and the values of π/c obtained were extrapolated to zero protein concentration (Fig. 6).

Fig. 6

The molecular weight of luciferase was calculated to be 82,000.

Spectra--The absorption spectrum of the purified enzyme is shown in Fig. 7. It had absorption maxima at 278 and 375

Fig. 7

nm and a shoulder at 445 nm. The absorbances of the peak at 375 nm and of the shoulder at 445 nm diminished by addition of dithionite. The fluorescence and in vitro luminescence spectra of the enzyme and the emission spectrum of the bacterial suspension are shown in Fig. 8. The fluorescence maximum of the

Fig. 8

enzyme was observed at 530 nm when excited at 380 nm whereas the maxima of the luminescence of the purified luciferase system and cell suspension were seen at 495-500 nm and 490-495 nm, respectively.

Quantum Yield Measurements--The chemiluminescent quantum yield is defined as the number of photons per reactant molecule. At the moment of initiation of the luminescent reaction, there was a rapid increase of light intensity followed by a first order decay process (Fig. 9). As with Ph. fischeri

Fig. 9

luciferase reported by Hastings and Gibson (9) no turnover of the enzyme occurred in the present assay system. When the reactants were mixed to initiate the reaction, $FMNH_2$, one of the substrates, rapidly disappeared (due to non-enzymatic oxidation by molecular oxygen) when the light emission had attained the maximum value. Thus the area under the time course curve represents the total quanta emitted from the

enzyme without turnover, and the decay curve of light emission represents the decay of an enzyme intermediate. The area increased with an increase in aldehyde concentration. With 20 mM aldehyde the enzyme emits 1.6 x 10^{15} q/mg at 18 °C and pH 7.0. Since the molecular weight of the enzyme was determined as 82,000, 1 mg of luciferase contains 7.3 x 10^{15} molecules of enzyme. Thus the quantum yield was calculated as 0.2 per enzyme molecule. The quantum yield of luciferase is dependent on the nature of the aldehyde as well as on experimental conditions such as pH and temperature, so this value is considered to be a minimum since optimal experimental conditions and the most effective aldehyde have not yet been determined.

DISCUSSION

In the present studies, a bacterial luciferase(native) was puried from Photobacterium phosphoreum in good yield (48 %). Despite many attempts to identify the emitter group of bacterial luciferase (10), no conclusive result has yet been obtained. The absorption spectrum of Ph. phosphoreum luciferase suggests that the enzyme is a flavoprotein, but in this investigation attempts to isolate flavin from the enzyme preparation by methods such as acid treatment followed by precipitation with ammonium sulfate or extraction with phenol were unsuccessful. The optical absorption spectrum of this enzyme, with a maximum at 375 nm and a shoulder at 445 nm, is different from that reported for the Ph. fischeri enzyme which had a maximum at about 400 nm (2).

This suggests that these two enzymes have different chromophores. The fact that the fluorescence spectrum of the enzyme does not coincide with the luminescence spectrum suggests that the pigment which is firmly bound to the enzyme is not the direct emitter. With the Ph. fischeri enzyme luminescence was observed when NADH and FMN were used as substrates in place of $FMNH_2$ (2). However, the purified Ph. phosphoreum luciferase used in this investigation exhibited hardly detectable luminescence when tested with these reagents under ifentical conditions to those employed for the Ph. fischeri enzyme. The value of quantum yield of Ph. phosphoreum luciferase reported in this investigation (0.2 per enzyme molecule) is comparable to that of Ph. fischeri enzyme (0.13 per enzyme molecule (2)).

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Table I. Summary of enzyme purification. The purification of luciferase from Photobacterium phosphoreum was carried out according to the procedures described in the tex with 100 g of wet packed cells as the starting material. The column A through J refer to the different measurements made on nine selected fractions obtained in the course of purification.

Fig. 1. Chromatogram of luciferase on a DEAE cellulose column at pH 7.0. For details, see to tex.

Fig. 2. Sedimentation pattern of luciferase in 0.09 M phosphate buffer, pH 7.0, at a concentration of 13.3 mg/ml. Rotor speed, 59,780 rpm; average rotor temperature, 19.9 °C. Time after rotor attained full speed, 66 min.

Fig. 3. Plot of $s_{20,w}$ against protein concentration.

Fig. 4. Electrophoretic pattern of luciferase in Tris buffer, pH 7.0, and ionic strength 0.1 at a concentration of 12.9 mg/ml. The numbers under the photographs indicate the time in min after start of the electrophoresis. Left, ascending pattern; right, descending pattern.

Fig. 5. Elution pattern of luciferase from a Sephadex G-100 column (1.7 cm x 90.5 cm). Gel filtration was performed at 4 °C with 0.1 M phosphate buffer, pH 7.0. 6.67 mg of luciferase was applied to the column and the effluent was collected in 4.1 ml fractions. The optical densities at 280 and 260 nm and I_o (at pH 7.0, 21 °C) of each fraction were measured. ○ , Optical density at 280 nm; ● , optical density at 260 nm; △ , relative value of I_o.

Fig. 6. Plot of π/c, expressed in the unit of (cm solvent)/(mg/ml), against protein concentration.

Fig. 7. Absorption spectra of luciferase in phosphate buffer, pH 7.0. The concentration of the enzyme was 10.3 mg/ml. The broken line was obtained after addition of a small amount of dithionite.

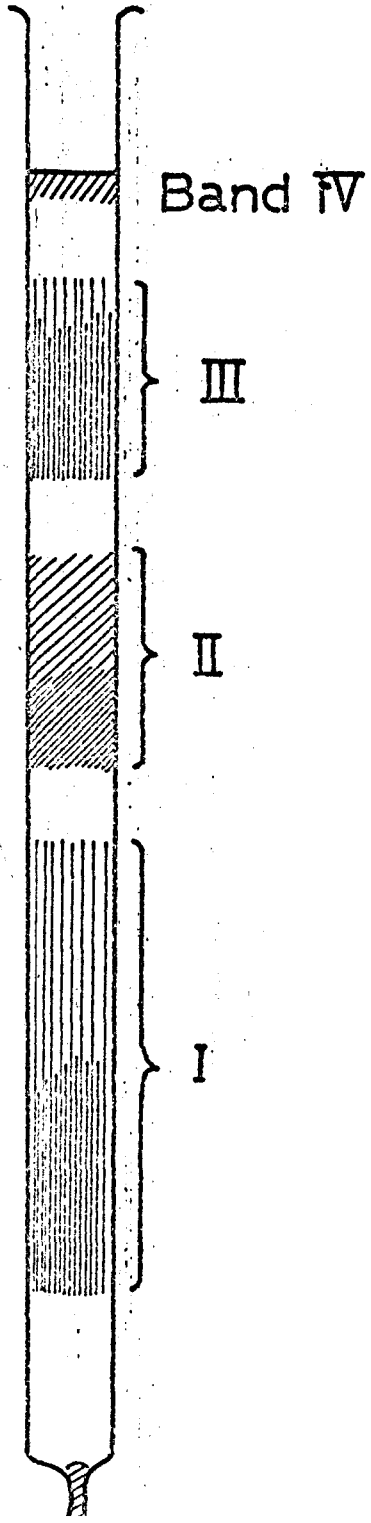
Fig. 8. Emission spectra of luciferase and living cells. ——— , Luminescence spectrum; – – – , fluorescence spectrum (excited at 380 nm) of luciferase in phosphate buffer, pH 7.0; - - - - - , bioluminescent emission spectrum of a culture of living cells.

Fig. 9. Time course of the luciferase reaction. The upper record indicates the start and stop of flow, i.e. the moment of initiation of the reaction, and the lower record shows the time course of change in light intensity.

Table I.

	A Volume (ml)	B Absorption peak (nm)	C Optical density at 278nm	D Optical density at 260nm	E C/D	F Protein (mg/ml)	G Total protein (mg)	H I_o/ml (relative)	I Total I_o	J Activity yield (%)
1. Crude extract	365	258	55	98	0.56	19.5	7,100	7.94	2,900	(100)
2. Supernatant from calcium phosphate gel	1,000	256	2.9	6.8	0.43	0.31	310	0	0	
3. Eluate from gel	1,230	258	11	18.5	0.60	3.9	4,800	16.4	2,000	69
4. Precipitated with 40 % saturation of $(NH_4)_2SO_4$ (dissolved in buffer)	54	260	60	77.5	0.77	32	1,730	2.60	140	
5. Precipitated with 40 % saturation of $(NH_4)_2SO_4$	130	260	84	138	0.61	19.5	2,500	12.0	1,560	54
6. Supernatant at 75 % saturation of $(NH_4)_2SO_4$	1,350	255	0.8	1.8	0.44	0.27	360	0	0	
7. Band I from DEAE cellulose	222	263	2.8	4.6	0.61	1.9	420	0	0	
8. Band II from DEAE cellulose	23	278	15.1	8.8	1.72	13	300	60	1,400	48
9. Band III from DEAE cellulose	78	276	5.3	3.8	1.4	7	550	0	0	

Fig. 1.



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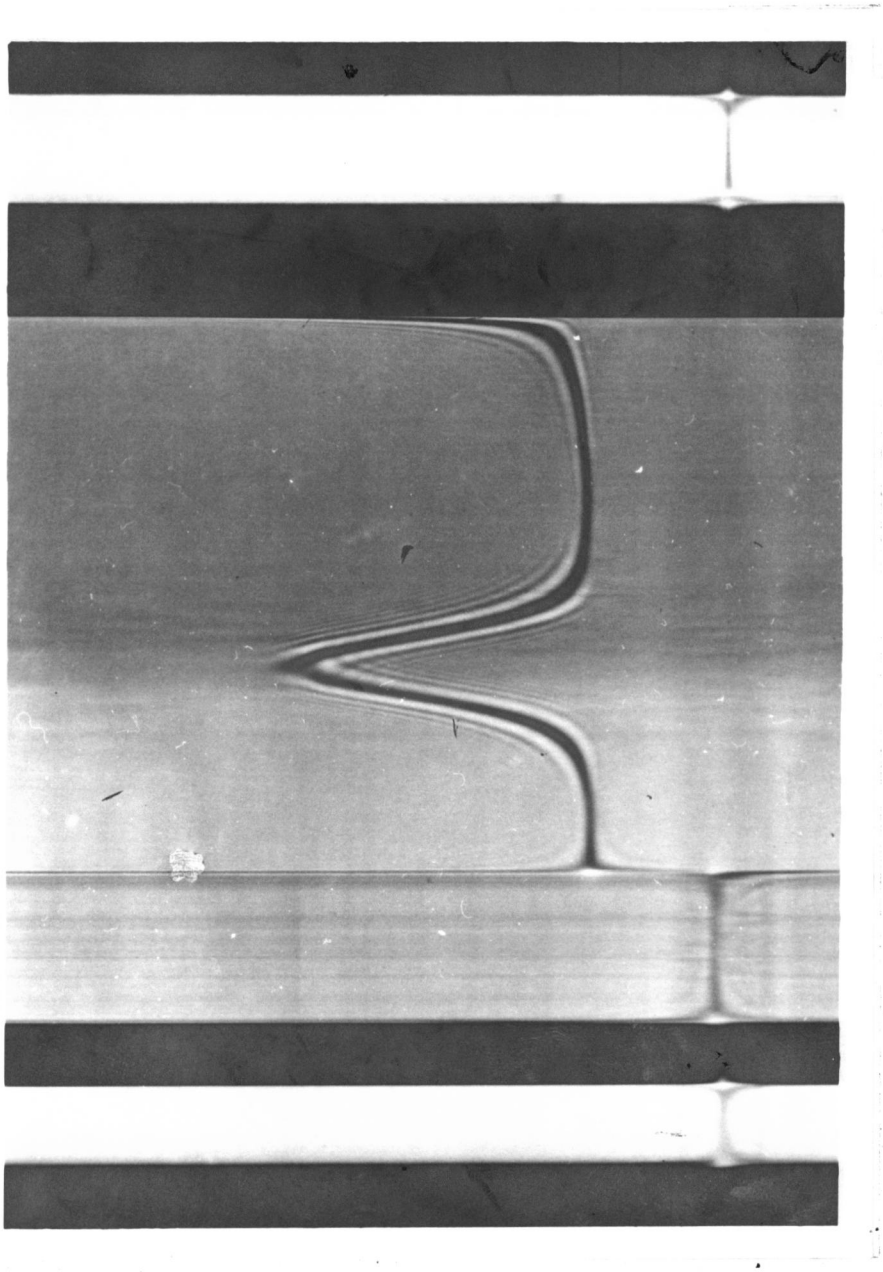


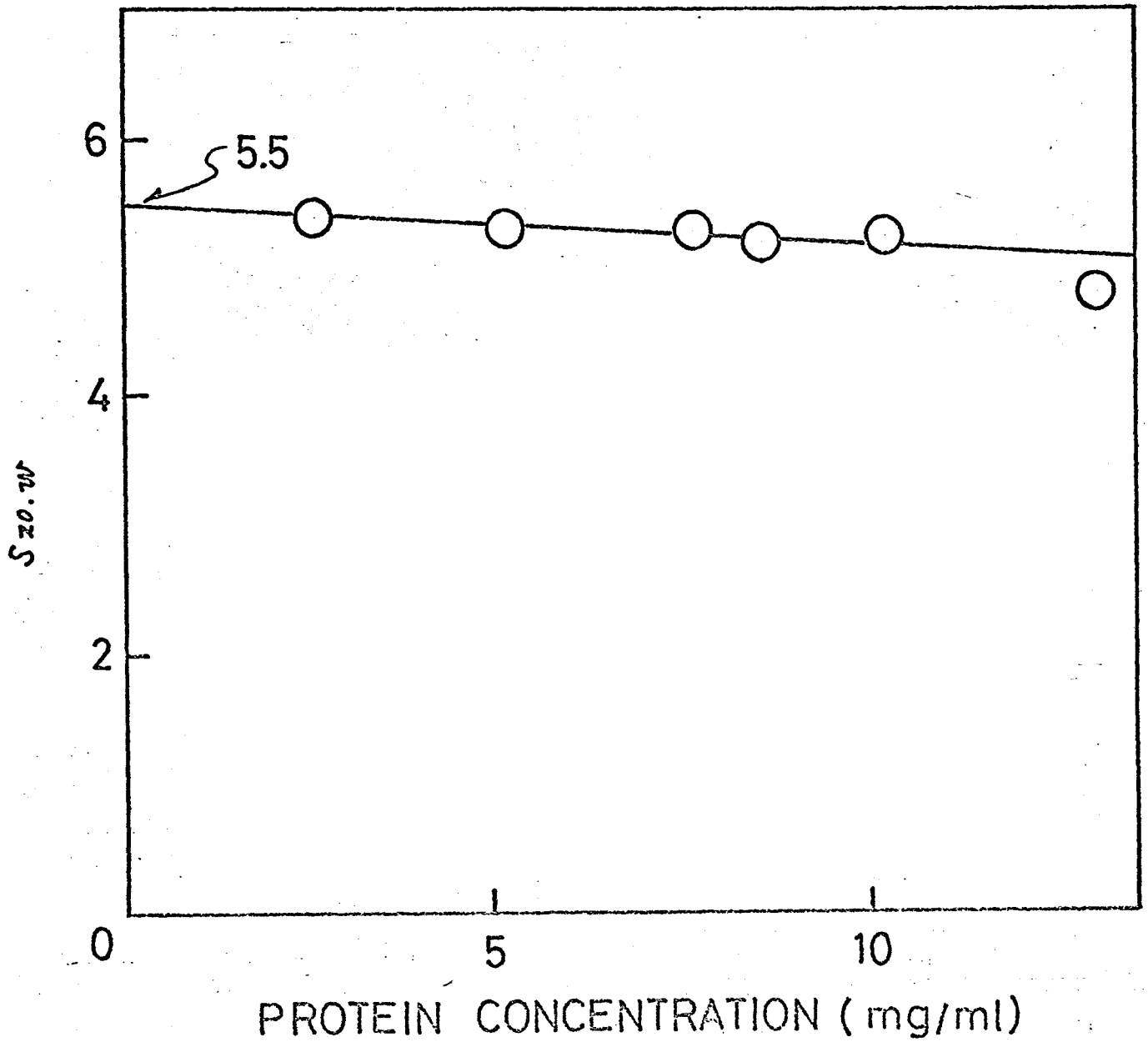
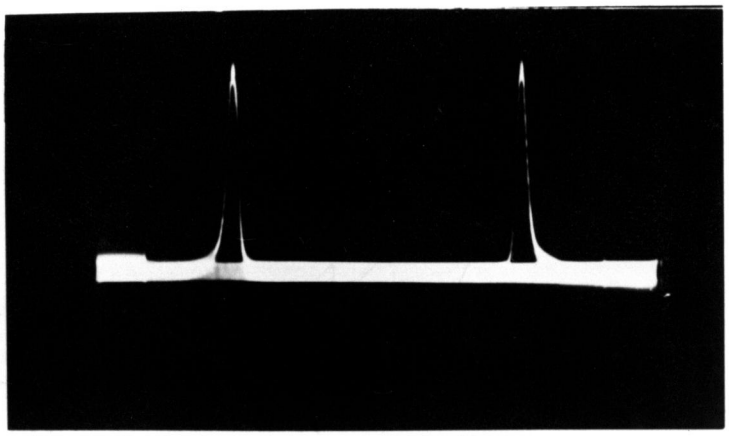


Fig. 3.

Fig. 4.



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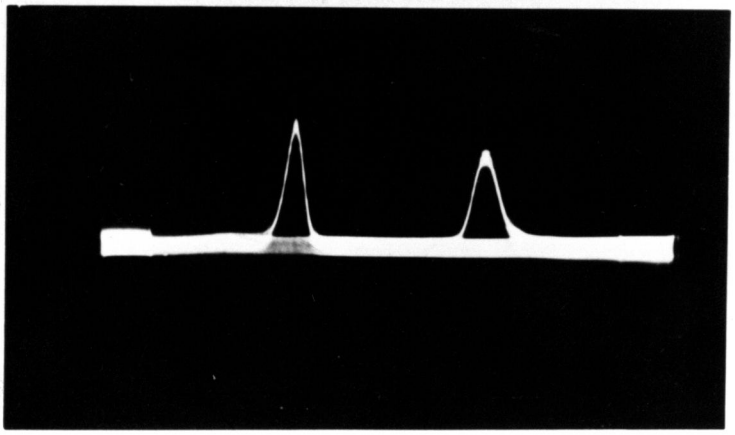

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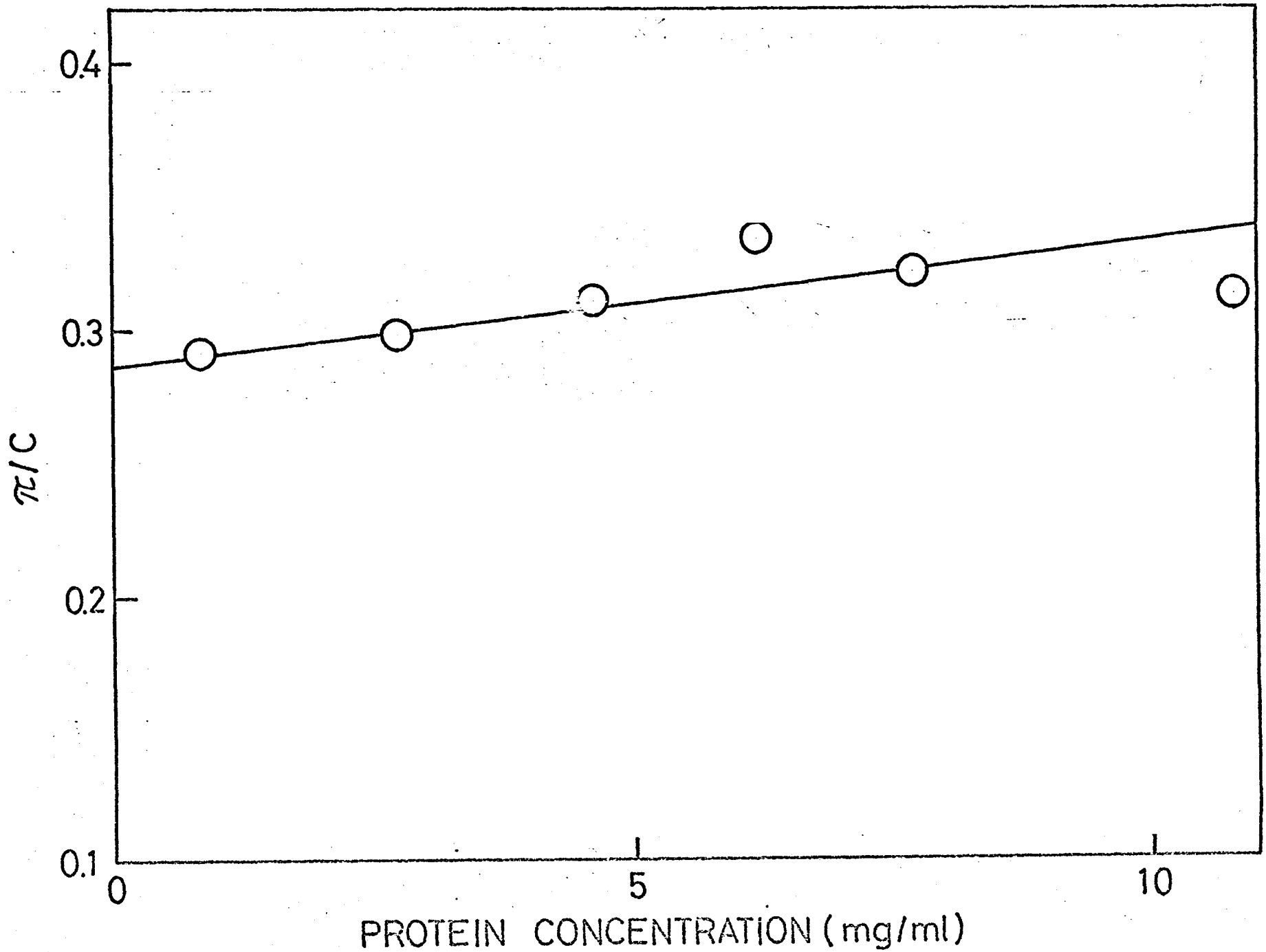
Fig. 5



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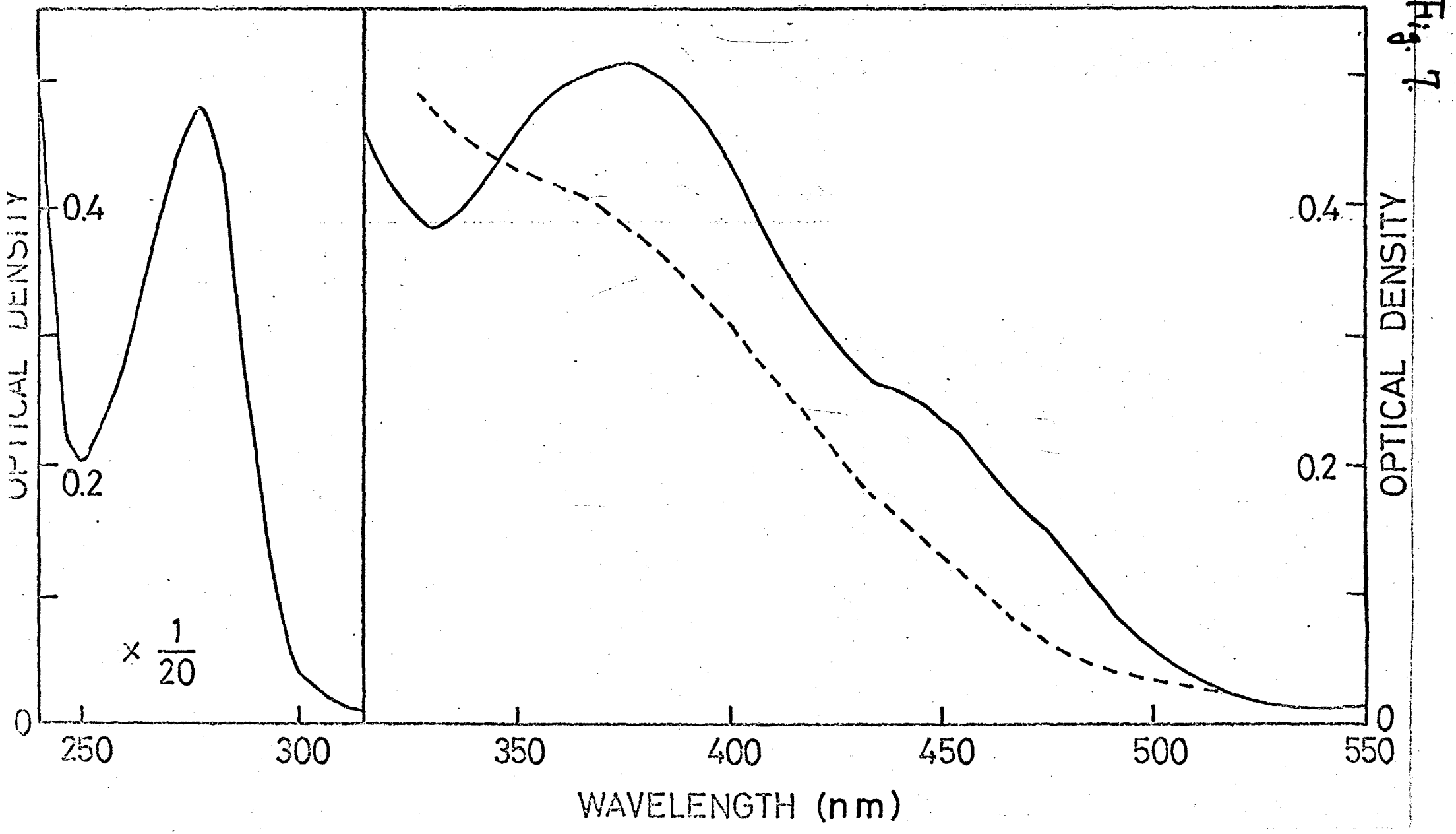
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Fig. 7

Fig. 8.

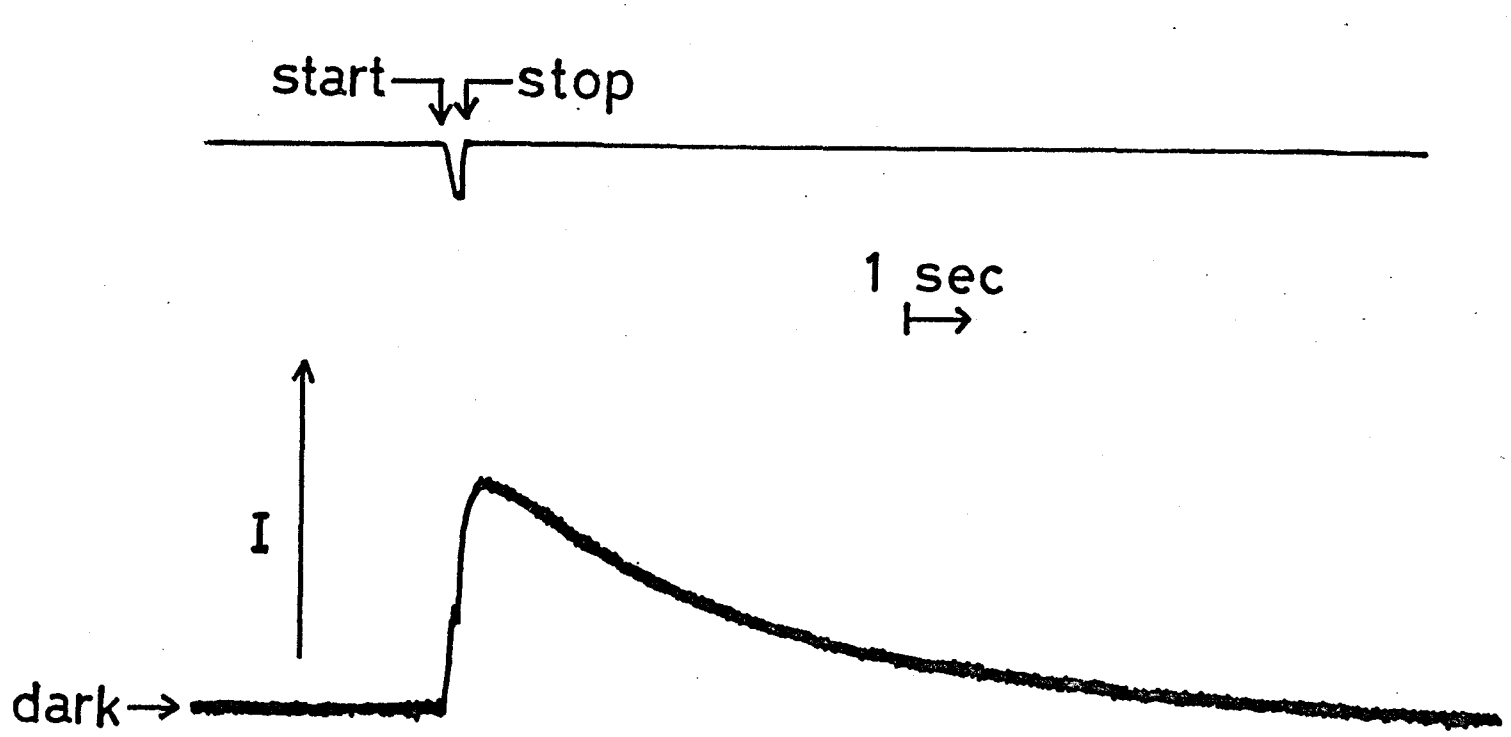

Fig. 9.

PART I-B

PREPARATION AND PROPERTIES OF STRIPPED LUCIFERASE

A luciferase preparation was obtained by reversible denaturation of native enzyme from Photobacterium phosphoreum with guanidine-HCl followed by Sephadex gel filtration. The preparation was free from p-flavin, a fluorescent flavin derivative which is bound to purified native luciferase. Luciferase was dissociated into two subunits in the presence of a high concentration of guanidine-HCl or urea. The renatured luciferase, here denoted as stripped luciferase, did not show the absorption or fluorescence in the visible region characteristic of p-flavin. The molecular weight and specific activity of the stripped luciferase and its emission spectrum were the same as those reported for the native enzyme. It was concluded that stripped luciferase catalyzes the luminescent reaction by the same mechanism as the native enzyme.

Bacterial luciferase from Photobacterium phosphoreum catalyzes a bioluminescent reaction which can be expressed as follows (1-4):

$$FMNH_2 + O_2 + RCHO \xrightarrow{\text{Luciferase}} h\nu + FMN + \text{other products},$$

where RCHO represents a long chain aliphatic aldehyde. FMN, one of the reaction products, is thought most likely to be the emitter group in the reaction (5, 6) but conclusive evidence of this has not yet been obtained and purified luciferase contains another fluorescent pigment, p-flavin, which might also act as the emitter (7). Mitchell and Hasting (8) separated "light inducible protein," which is a modified luciferase from Photobacterium fischeri, strain MAV, into a protein moiety and a fluorescent pigment (designated as B) by gel filtration on Sephadex in the presence of guanidine-HCl. They obtained an active luciferase on renaturation of the protein by dilution of the guanidine-HCl, and found that this luciferase did not require pigment B for its bioluminescent activity. This result appears to exclude the possibility that the enzyme-bound fluorescent pigment (B or p-flavin) is the emitter group. Friedland and Hastings (9) established a method for reversible denaturation of luciferase from Ph. fischeri using guanidine-HCl. However, comparative studies on the actions of native- and renatured luciferases are needed to determine whether the luciferase catalyzes the luminescent reaction by the same mechanism after removal of the bound pigment.

In the present study, a p-flavin-free luciferase, which was devoid of fluorescence in the visible region, was prepared

from Ph. phosphoreum luciferase by the method of Friedland and Hastings (9), and the characteristics of the renatured luciferase and the nature of its bioluminescent reaction were compared with those of native luciferase. It was concluded that the colorless, non-fluorescent renatured enzyme preparation catalyzed the bioluminescent reaction by the same mechanism as the native enzyme.

MATERIALS AND METHODS

Native Luciferase--Native luciferase was extracted and purified from Ph. phosphoreum by the procedures described by Nakamura and Matsuda (3).

Reagents--All the reagents used in this study were guaranteed grade reagents. Guanidine-HCl was obtained commercially and used without further purification. FMN was purified by column chromatography on DEAE cellulose (10). The procedure for preparing $FMNH_2$ solution was as described previously (3). Aldehydes were obtained commercially and used without further purification. The concentration of the aldehyde in aqueous solution was estimated on the basis of the solubility data previously reported by Watanabe and Nakamura (4).

Enzyme Assay--The luciferase reaction was measured at 20 °C in a flow apparatus fitted with a recording system, as described previously (3). The reaction was initiated by mixing one volume of a solution containing the enzyme, oxygen, aldehyde, and 0.1 % Tween 80 in 0.1 M phosphate buffer, pH 7.0, with one

volume of a solution of $FMNH_2$ in the same buffer, and the time course of light emission from the reaction mixture was recorded. Under routine assay conditions, the concentrations of O_2, tridecanal, and $FMNH_2$ after mixing were 1.2×10^{-4} M, 3.4×10^{-4}M, and 2.1×10^{-5}M, respectively.

Determination of Molecular Weight--The molecular weight of the renatured luciferase was determined by osmometry, as described previously (3). A Mechrolab Inc. membrane osmometer, model 503, was used for this purpose. For estimation of the molecular weight of luciferase in the presence of 5 M guanidine-HCl or 8 M urea, a column of Sephadex G-200 (1.5 cm x 50 cm or 1.0 cm x 95 cm, respectively) equilibrated with the same solvent was calibrated with marker proteins. Marker protein dissolved in a volume of 1 ml was charged on the column and eluted with the same solvent at room temperature, and the elution volume (Ve) was measured. The void volume (Vo) of the column was estimated using blue dextran 2000 (Pharmacia Co.), and the relative elution positions (Ve/Vo) of each protein were plotted against the logarithm of their molecular weight.

Polyacrylamide Gel Electrophoresis in SDS or Urea--Polyacrylamide gel (10 %) electrophoresis was carried out either in 0.1 % SDS using the method of Weber and Osborn (11) with slight modifications, or in 8 M urea. The electrophoretograms were scanned with a Fujiox Densitometer, FD-A IV, at a wavelength of 560 nm. The molecular weight of the enzyme subunit in the electrophoretogram was estimated using the calibration curve published by Y. Hayashi of this laboratory (12).

Emission Spectrum--The fluorescence spectrum of luciferase solution was recorded using a Hitachi recording spectrofluorometer, model MPF-2A. For measurement of the emission spectrum of the luciferase reaction, the flow system used in the enzyme assay was connected to the spectrofluorometer, and I_o, the peak intensity of the emitted light, was measured at different wavelengths. All spectra were represented as relative quanta per unit wavenumber ($q/\Delta\bar{\nu}$). The details of this procedure have been reported elsewhere (3).

Amino Acid Analysis--The amino acid composition of luciferase was determined by the method of Spackman et al. (13) using a Beckman Spinco amino acid analyzer, model 120 B, equipped with an accelerated system. For this analysis, a dry luciferase sample was hydrolyzed with distilled HCl (5.7 N) for 24 hr at 110 °C in a sealed tube. The amount of each amino acid measured was calculated as the number of amino acid residues per protein molecule, taking the molecular weight of luciferase as 82,000. Half-cystine was determined as cysteic acid after oxidation of the luciferase sample with performic acid following the method of Moore (14). The amount of tryptophan was estimated colorimetrically by the method of Spies and Chambers (15) with slight modifications. For the analysis, 1 ml of luciferase solution was added to 30 mg of p-dimethylaminobenzaldehyde in 9 ml of 21.4 N H_2SO_4 and incubated in the dark at 25 °C for 15 hr. Then 0.1 ml of 0.04 % $NaNO_2$ solution was added and the mixture allowed to stand at room temperature. After 30 min, the absorbance of the solution was measured spectrophotometrically at 600 nm.

Other Measurements--Spectrophotometric measurements were carried out either with a Cary recording spectrophotometer, model 14, or with a Hitachi spectrophotometer, model 139. Enzyme concentration was determined by the Biuret method, calibrated on the basis of dry weight measurements of purified luciferase.

RESULTS

Preparation of Stripped Luciferase--Purified native luciferase (3) was dissolved in 5 M guanidine-HCl, pH 7.0, at a protein concentration of 10-15 mg/ml, and kept at 4°C for about 20 hr. Then the mixture was submitted to gel filtration on a Sephadex G-50 column (3 cm x 20 cm), which had been equilibrated with the same solvent. A colorless protein was separated from p-flavin. The typical elution pattern using this procedure has been published previously (7). The colorless protein obtained was completely inactive. The fractions containing this colorless protein were diluted with 99 volumes of 0.1 M phosphate buffer, pH 7.0, containing 10^{-3} M DTT, and stood at 0-4°C. These conditions were essentially the same as those of Friedland and and Hastings (9), except that bovine serum albumin not added to the buffer used for dilution. After about 70 hr, the specific activity (I_o/mg protein) of luciferase in the diluted solution was found to have increased to almost that of the native enzyme (Fig. 1). This renatured luciferase was adsorbed on a DEAE

Fig. 1

cellulose column at a phosphate concentration of 0.05 M, pH 7.0, and the luciferase was eluted from the column with 0.3 M phosphate buffer, pH 7.0. The elute was concentrated using a collodion bag (Sartorius Membrane Filter, GmbH) or by ammonium sulfate precipitation, and passed through a column of Sephadex G-100 to remove a small amount of aggregated protein (Fig. 2).

Fig. 2

The fractions of effluent corresponding to the large, symmetrical protein peak with luciferase activity were collected. The final recovery of renatured protein was 10-15 % (w/w) of the initial amount of native luciferase.

Effects of Temperature, Protein Concentration, and p-Flavin on the Renaturation of Luciferase--The effect of temperature on the time course of recovery of luciferase activity is shown in Fig. 1. On replotting the data at 0 °C, it was found that the renaturation (or reactivation) process follows apparent first order kinetics. The rate of recovery was considerably slower at 0 °C than at 20 °C, but the final recovery was almost 100 % at 0 °C and only 50 % at 20 °C. Figure 3 shows the effect

Fig. 3

of protein concentrations of 4.1 to 20.8 μg/ml in the diluted mixture on the recovery of luciferase activity. In this range, the final recovery was consistently 85-95 %. As shown in

Fig. 4, the recovery of luciferase activity was inhibited by

Fig. 4

the presence of p-flavin. Recovery decreased with increase in the p-flavin concentration in the diluted mixture, and no recovery was observed when a stoichiometric amount of p-flavin was added to the enzyme.

PROPERTIES OF STRIPPED LUCIFERASE

Nature of the Renatured Luciferase--Homogeniety: As shown in Fig. 2, the renatured luciferase was homogeneous judging from its elution pattern by gel filtration on a Sephadex G-100 column. The position of the peak of activity in the elution pattern was identical with that of the native luciferase when examined on the same column under the same experimental conditions. A similar observation has been made in the case of Ph. fischeri luciferase (9).

Molecular Weight: To determine the molecular weight of the renatured luciferase, the osmotic pressure of solutions of various protein concentrations in 0.1 M phosphate buffer, pH 7.0 were measured at 5°C, and the value of π/C, the osmotic pressure divided by the protein concentration , was calculated. The values obtained were plotted and extrapolated to zero protein concentration. The molecular weight of the renatured luciferase was thus calculated as $8.4 \pm 0.2 \times 10^4$.

Amino Acid Analysis--The results of amino acid analysis

of the renatured luciferase are summarized in Table I. The

Table I

values obtained agree well with those reported for Ph. fischeri luciferase (16), except that Ph. phosphoreum luciferase contains more alanine, valine, and leucine and less threonine and glycine, than Ph. fischeri luciferase.

Absorption Spectra--As already reported (3), and shown in Fig. 5, native luciferase has a flavin-like absorption spect-

Fig. 5

rum due to p-flavin bound to the enzyme. On the other hand, the preparation of renatured luciferase had little absorption in the visible region (Fig. 5), and when excited at 380 nm its fluorescence intensity at 530 nm was less than 5 % of that of the native enzyme. Since one molecule of native luciferase contains 0.19 molecule of p-flavin (7), it was calculated that the p-flavin content of the renatured luciferase was less than 0.01 molecule per molecule.

Emission Spectra--The emission spectra of bioluminescent reactions catalyzed by the native and renatured luciferases appeared identical, as shown in Fig. 6-A. The decay rate

Fig. 6-A, B

constants (k_d (3)) of the bioluminescent reactions of the two luciferases were both 0.3 sec^{-1} at 20°C. Since the specific activity (I_o/mg) of the renatured luciferase was 85-95 % of that of the native luciferase, the quantum yield of the luminescent reaction per enzyme molecule, which is proportional to (I_o/mg)/molecular weight/k_d, was almost the same for the two luciferases. The emission spectrum of the bioluminescent reaction of the renatured luciferase was measured using aldehydes of different chain lengths. As shown in Fig. 6-B, only small differences in the peak positions of the emission spectra were observed. The emission maximum was at 500 nm when C_9 or C_{10} aldehyde was used, and at 510 nm when C_{11}, C_{12}, or C_{13} aldehyde was used.

Subunits of Luciferase--Ph. phosphoreum luciferase was dissociated into subunits in 0.1 % SDS. As shown in Fig. 7-A,

Fig. 7-A, B

the electrophoretogram of luciferase on SDS polyacrylamide gel electrophoresis showed two bands of almost equal intensity, with Rf values corresponding to molecular weights of 38,000 and 42,000, respectively. Similar results were obtained by Hastings et al. (16) for Ph. fischeri luciferase. However, the luciferases from these two organisms behaved differently on gel electrophoresis in 8 M urea: Ph. phosphoreum luciferase showed only a single band (Fig. 7-B) while under similar conditions of gel electrophoresis, Friedland and Hastings (17) observed two bands

for Ph. fischeri luciferase. They also reported that Ph. fischeri luciferase separated into two subunits by column chromatography on DEAE-cellulose in the presence of 8 M urea, but attempts to separate two subunits of Ph. phosphoreum luciferase by chromatography, both under these and other experimental conditions, were unsuccessful. However, dissociation of Ph. phosphoreum luciferase into subunits apparently does occur under these experimental conditions, as described below. Gel filtration of Ph. phosphoreum luciferase on Sephadex G-200 column was carried out in the presence of 8 M urea or 5 M guanidine-HCl, and the molecular weight of the luciferase was estimated. As shown in Fig. 8-A, B, the relative elution volume

Fig. 8-A, B

(V_e/V_o) of luciferase indicated a molecular weight of 36,000 or 39,000. This is about half that of native luciferase, and it thus appears that the Ph. phosphoreum luciferase molecule is split into subunits in 8 M urea or 5 M guanidine-HCl.

DISCUSSION

Hastings et al. (16) reported that Ph. fischeri luciferase consisted of two non-identical subunits with molecular weights of 41,000 and 38,000 which could be separated by column chromatography on DEAE-cellulose in 8 M urea (17). In the case of Ph. phosphoreum luciferase, two bands with molecular weights of 42,000 and 38,000 were observed on polyacrylamide

gel electrophoresis in 0.1 % SDS. This indicates that Ph. phosphoreum luciferase also consists of two non-identical subunits. However, these two luciferases have slightly different amino acid compositions and their subunits showed different electrophoretograms in 8 M urea. In fact, Ph. phosphoreum luciferase could not be separated to subunits by DEAE-cellulose column chromatography under the conditions used by Friedland and Hastings (17) for separation of the subunits of Ph. fischeri luciferase.

The renatured luciferase had a molecular weight of 8.4 0.2×10^4 which, within experimental error, agrees with that of the native enzyme (82,000 (3)), whereas the molecular weight of luciferase in 5 M guanidine-HCl was found to be 39,000 which is almost half this value. Friedland and Hastings (9) suggested that the rate of renaturation of luciferase might be governed by the rates of several different processes, including refolding and reassociation of the subunits. The present finding that the renaturation is apparently a first order process suggests that the rate limiting process in renaturation is not a second order reassociation process, but a refolding of the polypeptide chain of luciferase to form a structure with catalytic activity.

The renatured luciferase, here denoted as stripped luciferase, obtained free from p-flavin was found to have regained the same specific activity as that of native luciferase. The emission spectra of the native and renatured luciferase were also found to be identical. All these facts indicate that p-flavin, which is bound to purified native luciferase of Ph.

phosphoreum, is not necessary for the bioluminescent reaction. In fact, p-flavin even inhibited the renaturation process. This inhibitory effect was probably a result of the fact that p-flavin bound specifically to the active site of luciferase in competition with $FMNH_2$, one of the substrates. On the other hand, it seems that p-flavin in native luciferase (0.19 molecule /molecule) is not inhibitory and its mode of binding is different from that to renatured luciferase, since almost quantitative restoration of the original activity was observed after removal of p-flavin and renaturation of the enzyme.

In the bioluminescent reaction, renatured luciferase reacts with $FMNH_2$, O_2, and aldehyde. Of the components of the reaction, only FMN, one of the reaction products, is fluorescent in the visible region. Therefore, it seems probable that the emitter group in the bacterial luciferase reaction is FMN in a state specifically bound to the enzyme.

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Fig. 1. Effect of temperature on the time course of recovery of luciferase activity. Ordinate, % luciferase activity recovered; abscissa, time after 1 : 100 dilution of the luciferase in 5 M guanidine-HCl with 0.1 M phosphate buffer, pH 7.0, containing 10^{-3} M DTT. Incubation temperature: ○, 0 °C; ●, 20 °C.

Fig. 2. Elution pattern of renatured luciferase on a Sephadex G-100 column (2 cm x 55 cm). Gel filtration was performed at 4 °C in 0.1 M phosphate buffer, pH 7.0. 2.7 mg of protein were applied to the column and the effluent was collected in 3.4 ml fractions. The optical density at 280 nm and I_o of each fraction were measured. ○, optical density at 280 nm; ●, relative value of I_o. The void volume was 38 ml.

Fig. 3. Effect of protein concentration in the incubation mixture upon recovery of luciferase activity. Temperature, 0 °C. Protein concentration in the incubation mixture, ○, 20.8 μg/ml; □, 10.4 μg/ml; △, 4.1 μg/ml.

Fig. 4. Effect of p-flavin in the incubation mixture on the recovery of luciferase activity. Temperature, 0°C. Recovery of luciferase activity observed 80 hr after starting the incubation was plotted against the molar ratio of p-falvin added to the protein. The molarity of p-flavin was calculated from its isoalloxazine content.

Fig. 5. Absorption spectra of native and renatured luciferases in 0.1 M phosphate buffer, pH 7.0. The enzyme concentration in each case was 1.2 mg/ml. ———, native luciferase; -------, renatured luciferase.

Fig. 6. (A) Bioluminescent emission spectra of the reactions of native and renatured luciferases. C_{13} aldehyde was used. ● , native luciferase; o , renatured luciferase. (B) Emission spectra of the reaction of renatured luciferase examined with aldehydes of various chain lengths. o , C_{13}; □, C_{12}; △ , C_{11}; ■ , C_{10}; ▲, C_9 aldehyde was used.

Fig. 7. Densitometric tracings of electrophoretograms of Ph. phosphoreum luciferase in polyacrylamide gel in (A) 0.1 % SDS in phosphate buffer at pH 7.3 or (B) in 8 M urea in Tris-glycine buffer at pH 8.6. Total protein, 17 µg in each column.

Fig. 8. Estimation of the molecular weight of luciferase on a Sephadex G-200 column in the presence of denaturating reagents. (A) in 8 M urea; (B) in 5 M guanidine-HCl. The marker proteins are, GDH, L-glutamate dehydrogenase [EC 1.4.1.3]; TAA, Taka-amylase A [EC 3.2.1.1]; OA, ovalbumin; TM, Tropomyosin; TRYP, trypsin [EC 3.4.4.4]. LU represents luciferase.

Table I. Amino acid composition of Ph. phosphoreum luciferase.

Amino acid	moles/82,000g
Lysine	41.0
Histidine	22.9
Arginine	22.7
Aspartic acid	82.4
Threonine	40.7
Serine	46.0
Glutamic acid	92.0
Proline	28.3
Glycine	39.5
Alanine	63.7
Half-cystine	11.5
Valine	53.0
Methionine	18.1
Isoleucine	42.1
Leucine	65.3
Tyrosine	25.3
Phenylalanine	37.7
Tryptophan	7.0

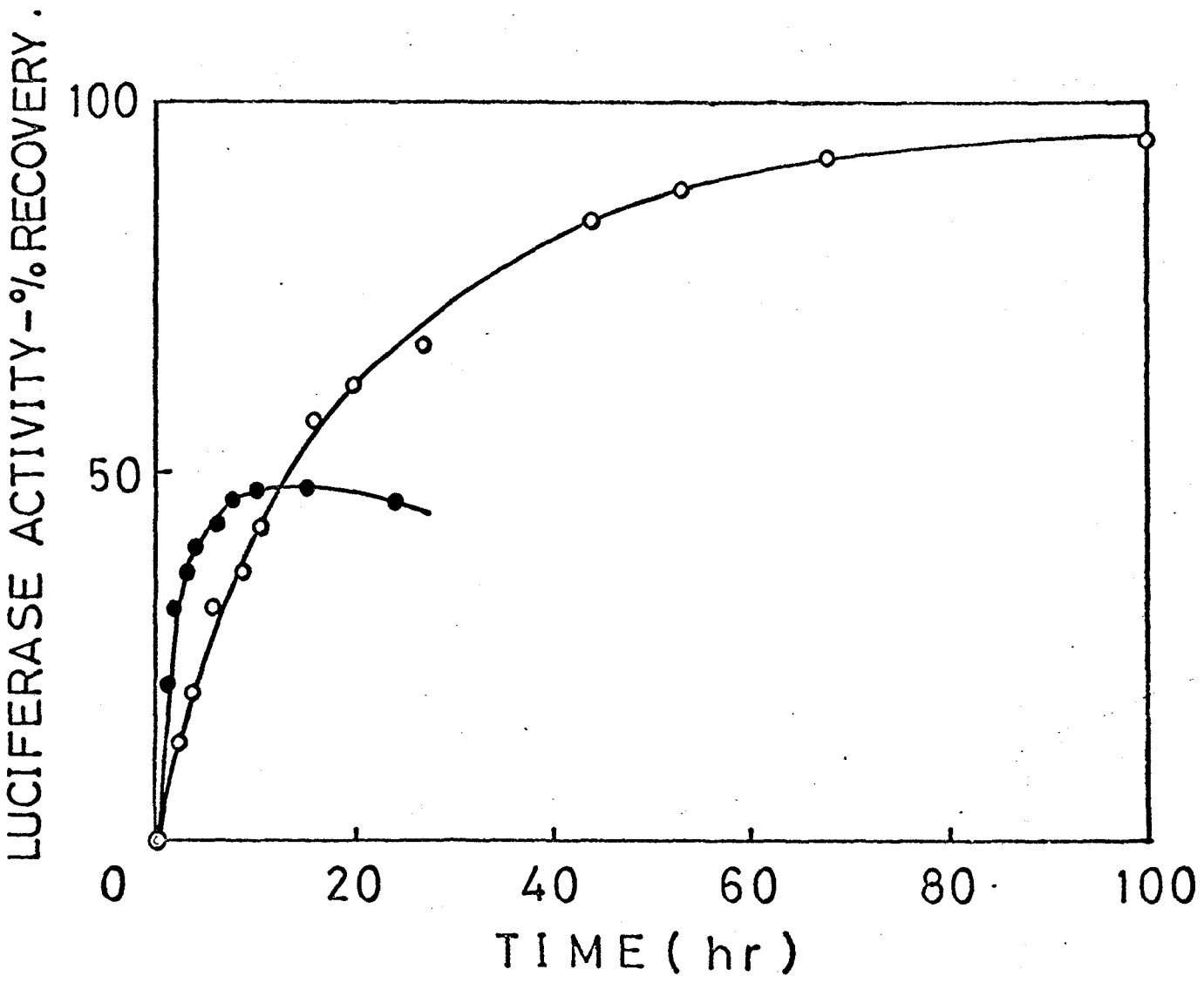
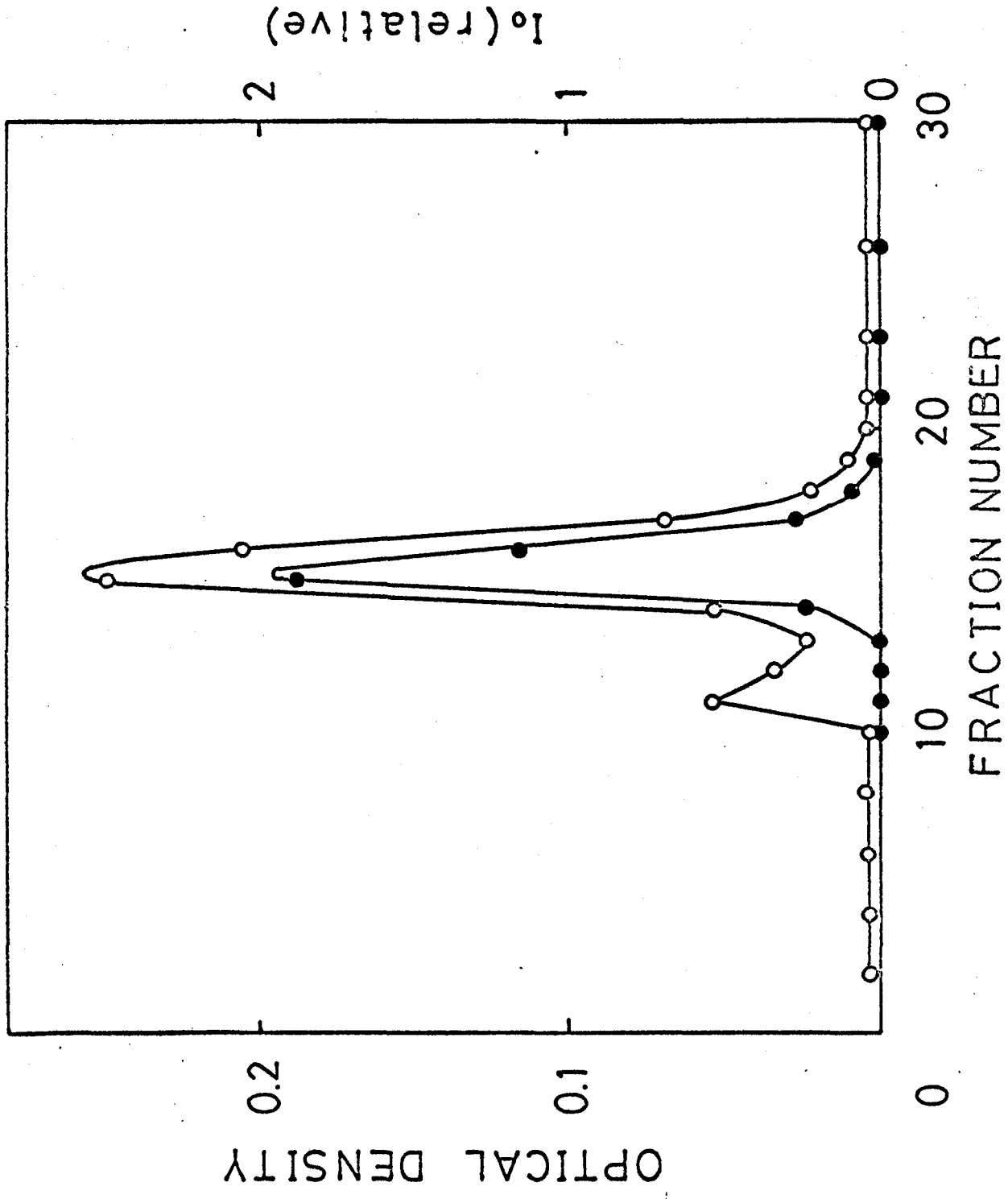


Fig. 1.

Fig. 2.


I_o (relative)
2
1
0
OPTICAL DENSITY
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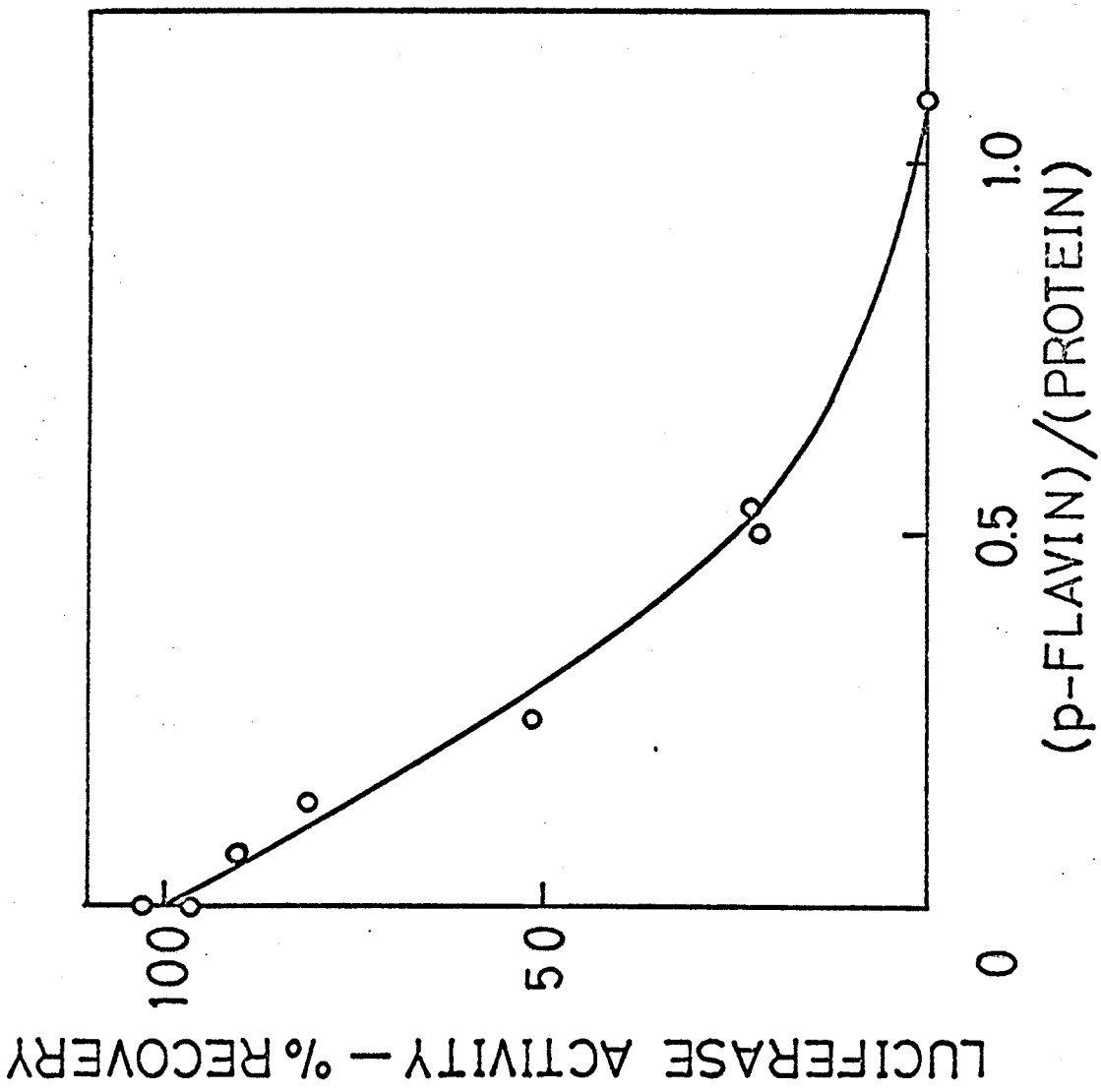
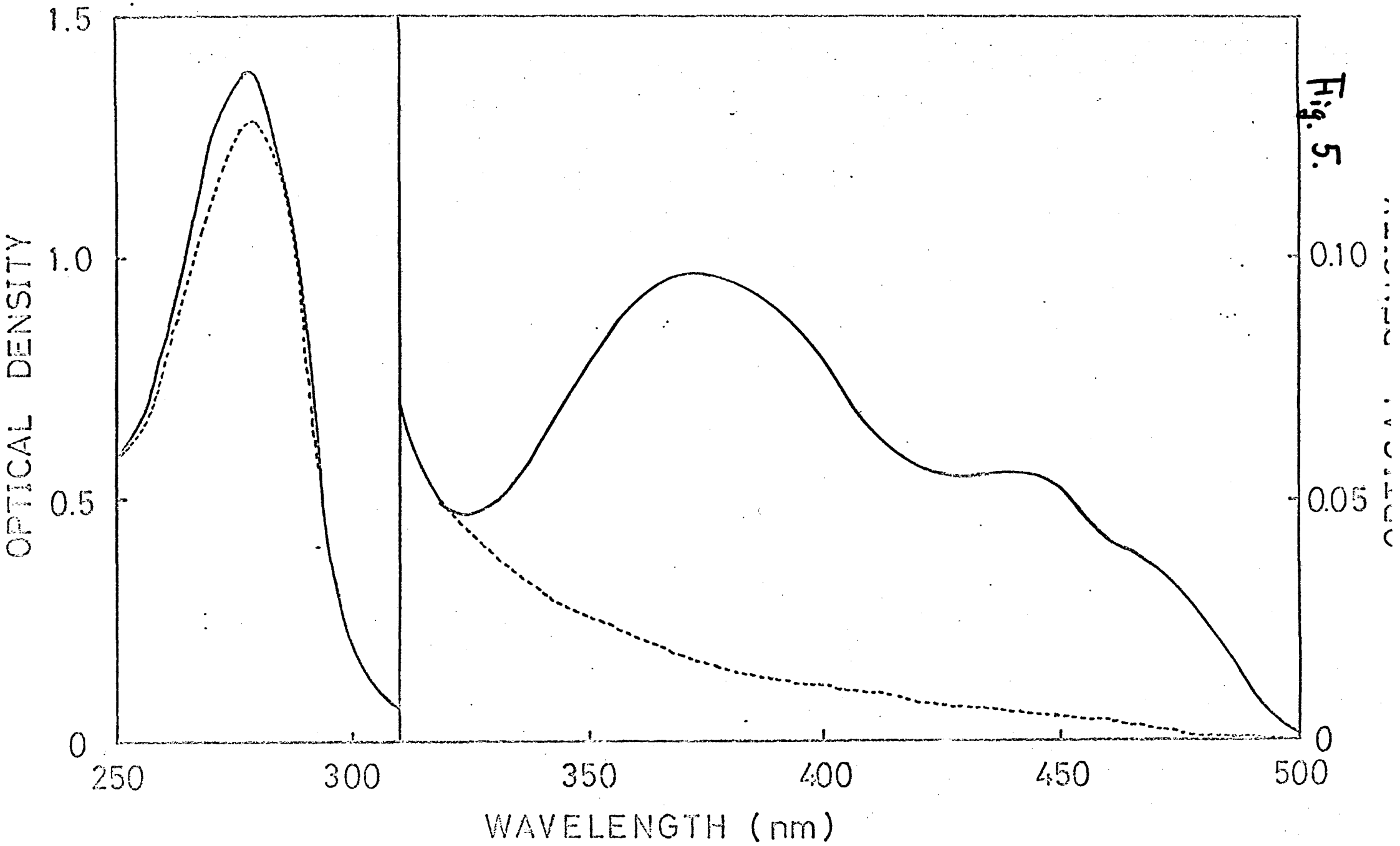
0.1
0
FRACTION NUMBER
10
20
30

Fig. 3.

Fig. 4.


LUCIFERASE ACTIVITY — % RECOVERY
100
50
0
0.5
1.0
(p-FLAVIN)/(PROTEIN)


1.5
1.0
0.5
0
OPTICAL DENSITY
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0
250
300
350
400
450
500
WAVELENGTH (nm)

Fig. 5.

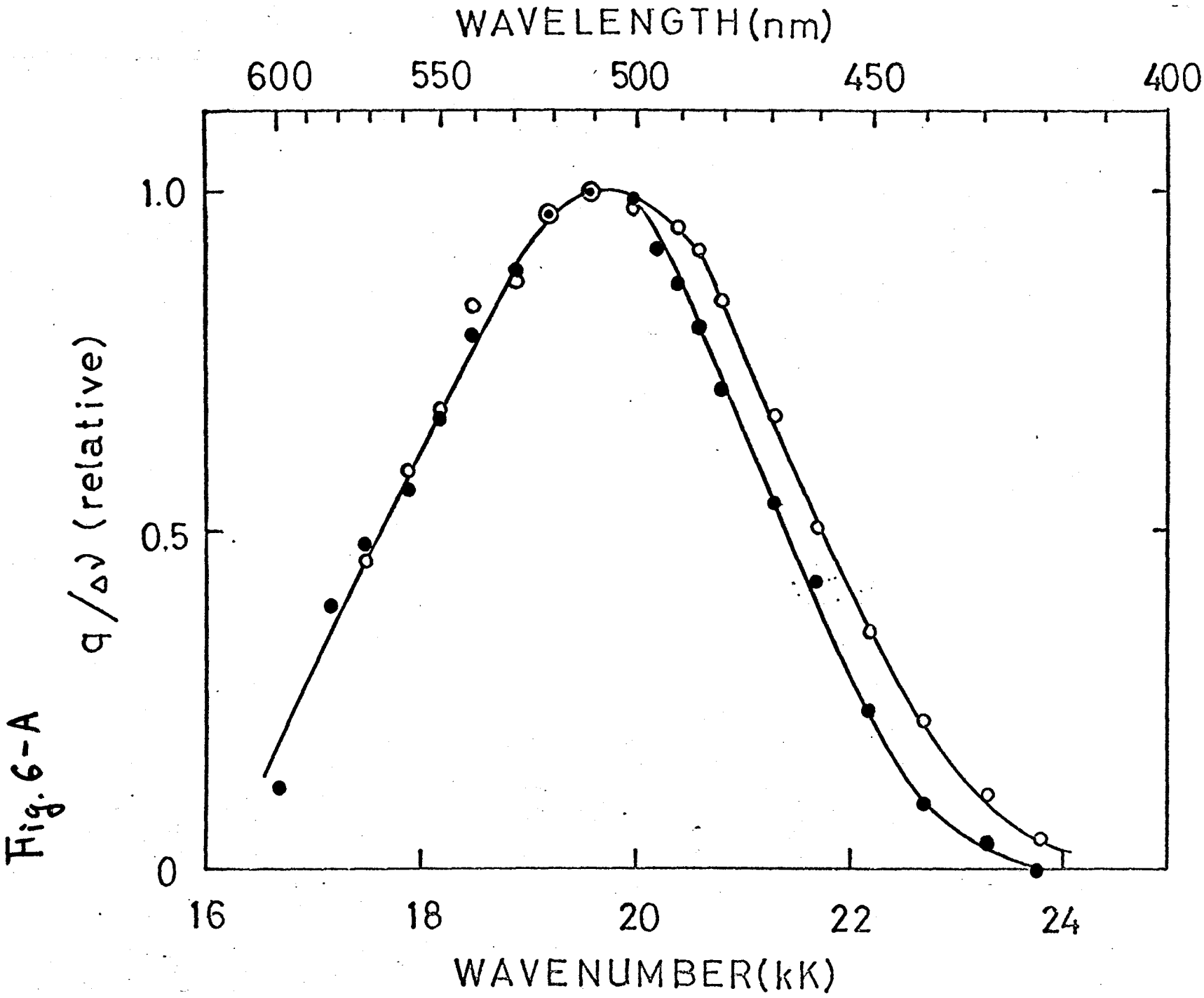
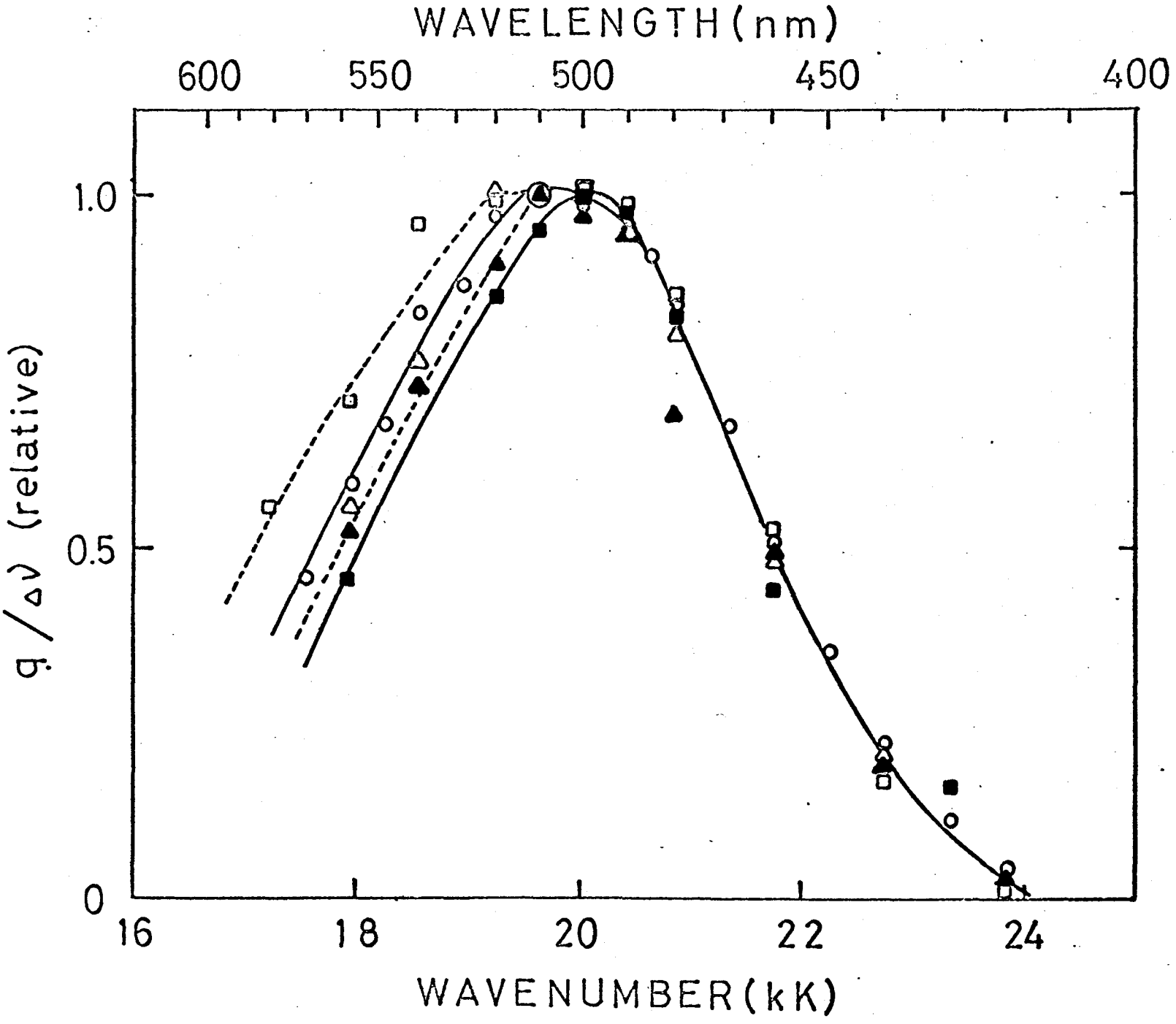

WAVELENGTH(nm)
600
550
500
450
400
1.0
0.5
0
q/Δν (relative)
16
18
20
22
24
WAVENUMBER(kK)

Fig. 6-A


WAVELENGTH (nm)
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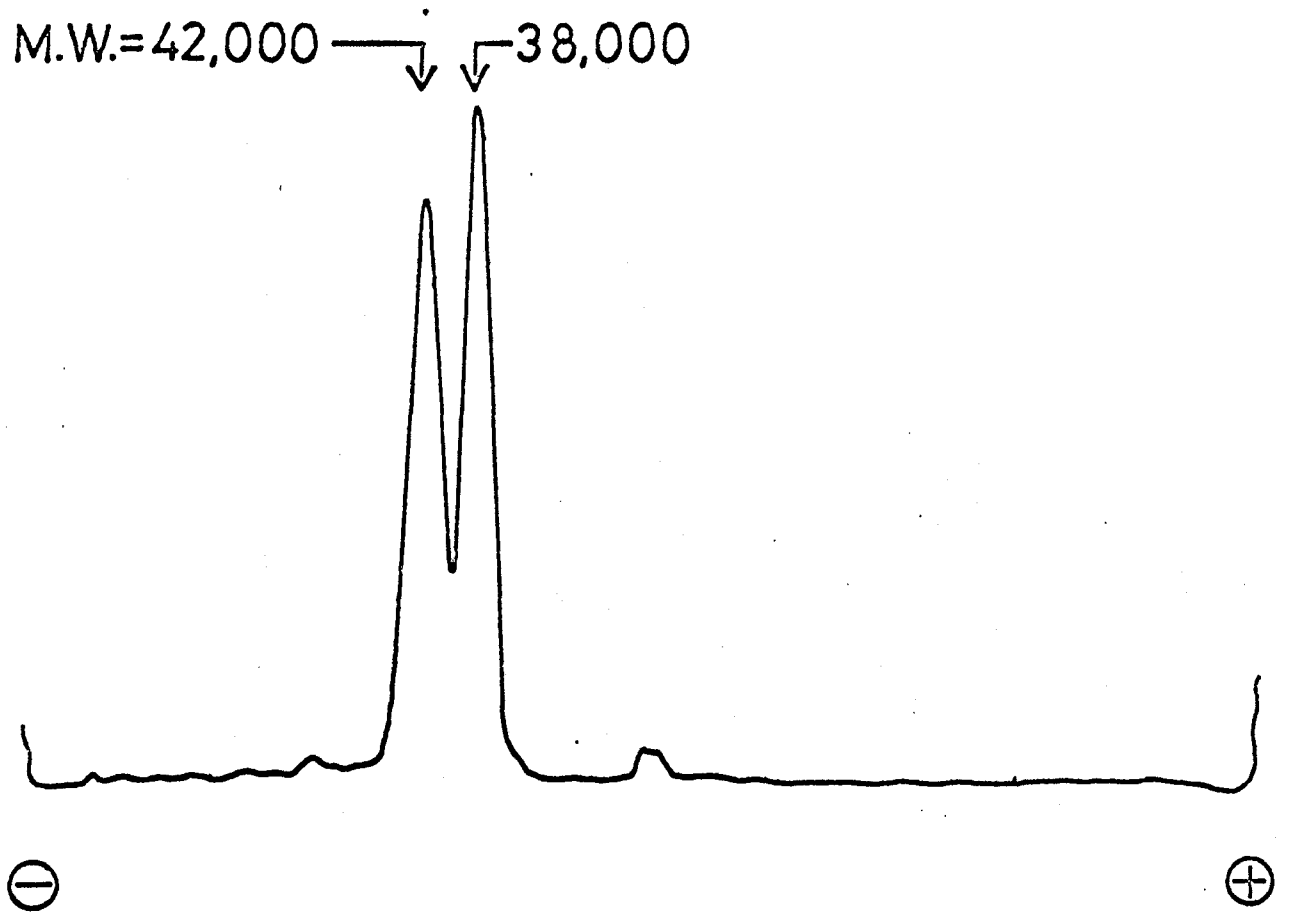
550
500
450
400
1.0
0.5
0
q / Δν (relative)
16
18
20
22
24
WAVENUMBER (kK)

Fig. 6-B

Fig. 7



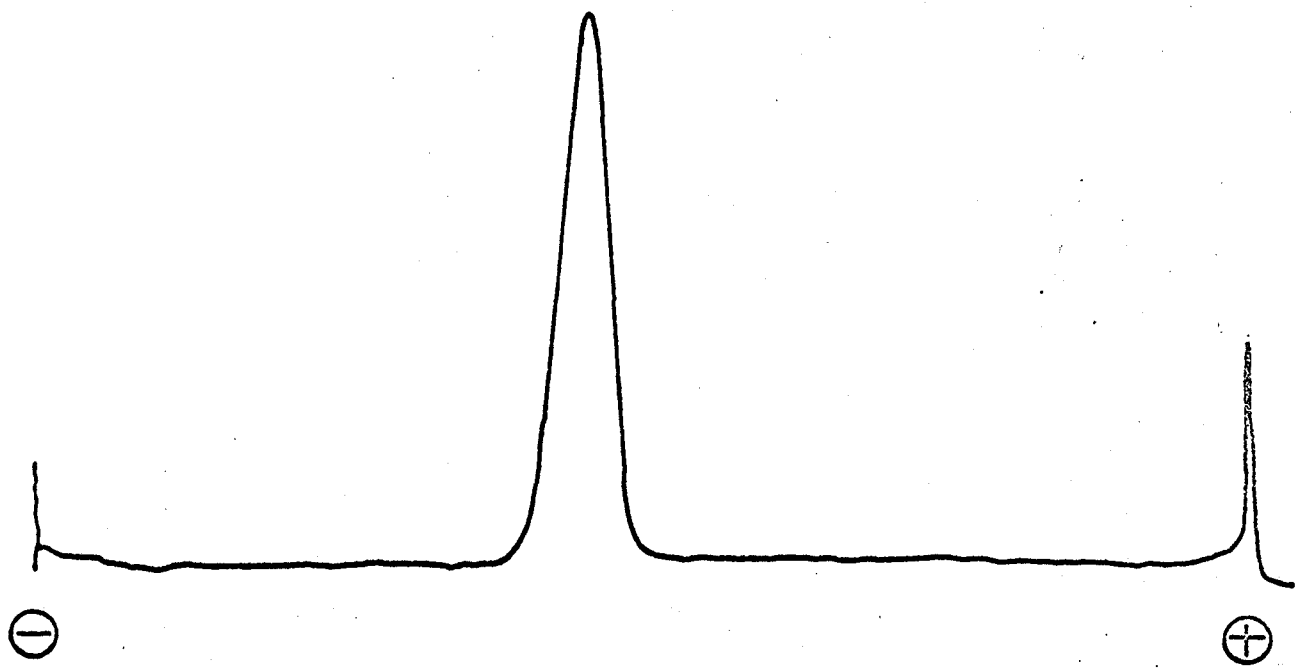


Fig. 8-A

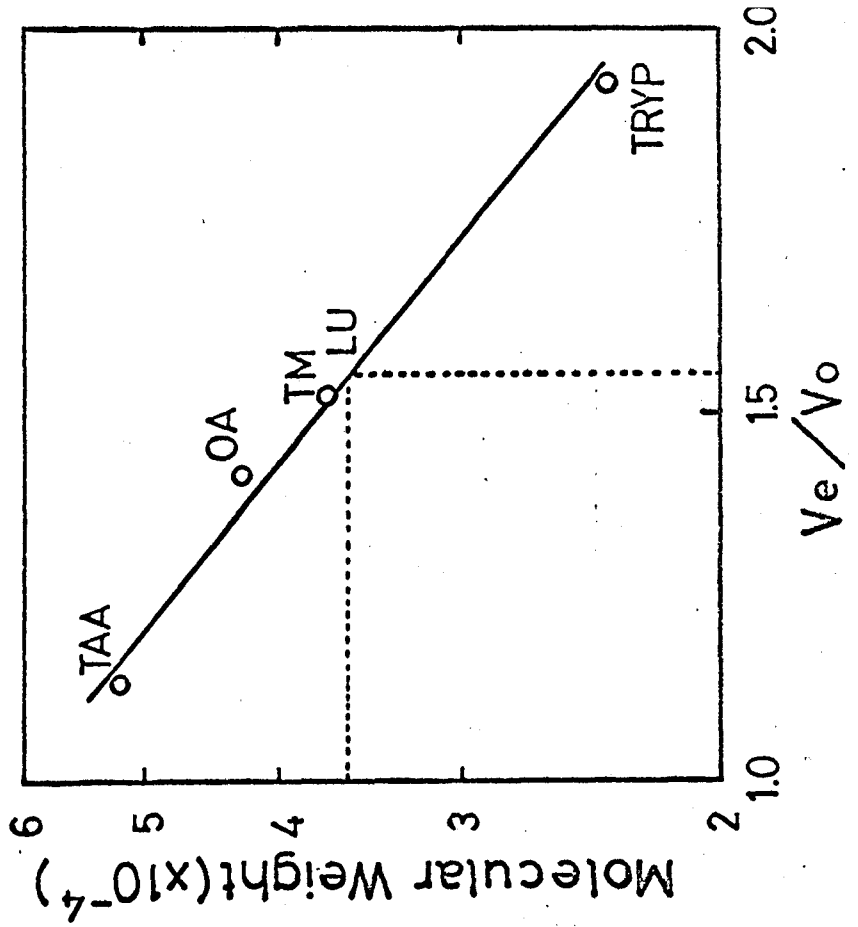
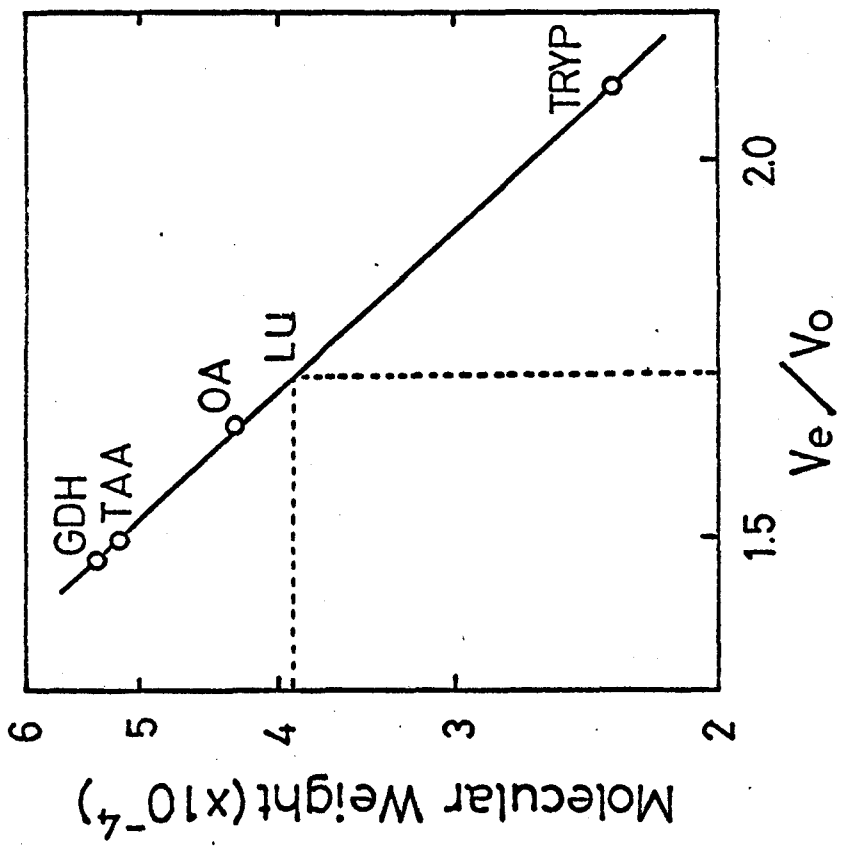


Fig. 8-B



PART II

MECHANISM OF REACTION

A luciferase-$FMNH_2$ complex was mixed with O_2 and the changes in the absorption and fluorescence spectra of the reaction mixture were followed by the stopped flow method. Rapid formation of an enzyme-FMN intermediate complex, which has a broad absorption band at 430-470 nm, was observed. The fluorescence emission maximum of the intermediate coincided with that of free FMN. The decay rate of the intermediate (0.25 sec^{-1} at 20°C) was in agreement with that of an obligatory intermediate of the luminescent reaction determined by the method of aldehyde-initiated luminescence. After forming a complex with aldehyde, the decay rate of the intermediate was dependent on the chain length of the aldehyde. The intermediate was more effectively protected by aldehydes of longer chain length against back-reduction by excess dithionite.

The bioluminescent reaction catalyzed by bacterial luciferase is described as follows (1-5)

$$FMNH_2 + O_2 + RCHO \xrightarrow{\text{luciferase}} h\nu + FMN + \text{products},$$

where RCHO denotes a long chain aliphatic aldehyde. FMN plays a key role in this reaction because it is most likely to be the emitter group of the reaction (6, 7) and aerobic oxidation of $FMNH_2$ catalyzed by this enzyme supplies, at least part of the energy required for emission of light quanta. However, detailed spectrophotometric analyses of the behavior of FMN during the reaction have been difficult since purified luciferase contains a fluorescent pigment, p-flavin (8, 9) or "B" (10), which is not essential for the light emitting process.

Recently, a stripped luciferase was prepared (9) by removal of p-flavin from the protein moiety of the enzyme. It was devoid of most of the original fluorescence and absorption in the visible region but retaining the same activity as native luciferase. In the presence of stripped luciferase, and an enzyme-bound intermediary state of FMN was discovered by rapid spectrophotometric measurements. Its decay rate was in agreement with that of an obligatory intermediate of the light emitting reaction determined by the technique of aldehyde-initiated luminescence. A direct interaction between the intermediary-bound FMN and aldehyde in the luciferase molecule was also suggested.

MATERIALS AND METHODS

Stripped Luciferase--Stripped luciferase from Photobacterium phosphoreum was prepared by the procedures described by Yoshida and Nakamura (9). Enzyme concentration was determined by the Biuret method, calibrated on the basis of dry weight measurements of purified luciferase. The molecular weight of the enzyme was taken as 82,000 (3) for calculation of the molar concentration.

Reagents--All the reagents used in this study were guaranteed grade reagents. FMN was obtained commercially and was purified further by column chromatography on DEAE cellulose (11). The procedures for preparing $FMNH_2$ were as described previously (3). Saturated, normal aliphatic aldehydes were obtained commercially and used without further purification. The method for preparing a saturated aqueous solution of an aldehyde of known concentration has been reported (4). A Thunberg tube fitted with a rubber stopper was used for preparing dithionite solution. A solution of NaOH (1/100 N) was equilibrated with N_2 gas in the tube, and then solid $Na_2S_2O_4$, which had been placed in the side arm, was dissolved in the NaOH solution under the atomosphere of nitrogen. An injection syringe was used to transfer the dithionite solution from the Thunberg tube. The molar concentration of dithionite was calibrated by spectrophotometric titration with FMN solution of known concentration under anaerobic conditions.

Transient Absorption and Fluorescence Spectrophotometries--The transient absorption change of the luciferase-

$FMNH_2$ system during aerobic oxidation was recorded by the stopped flow method as described previously (12). The fluorescence change of the same reaction system was measured using the same flow system combined with a Hitachi recording spectrofluorometer, model MPF-2A. Reactions were conducted at 20°C in 0.1 M phosphate buffer, pH 7.0.

Enzyme Assay--The bioluminescent reaction of luciferase was followed in the same flow apparatus as that described above. Detailed of particular assay methods are described in the "RESULTS".

RESULTS

Rapid Spectro(fluoro)metry of Intermediate-- $FMNH_2$ (2.7 μM) was pre-incubated with stripped luciferase in one reservoir of the flow apparatus. The total concentration of the enzyme was twice that of $FMNH_2$, and since the apparent dissociation constant of the E-$FMNH_2$ complex was reported to be 1×10^{-7} M (4), it was calculated that almost all (96 %) the $FMNH_2$ added was in the enzyme-bound state. E-$FMNH_2$ was mixed with an equal volume of O_2 solution, and the time course of change in absorption of the mixture was followed in the absence of aldehyde. As shown in Fig. 1 (a), a biphasic

Fig. 1 (a), (b)

change of the absorption, i.e. a rapid initial increase followed by a much slower increase, was observed at 460 nm after stopping

the flow. The same experiment was repeated at different wavelengths, and optical densities of the reaction mixture at intervals after the flow stop were plotted against the wavelengths (Fig. 2). It was observed that an intermediary spectrum rapidly

Fig. 2

appeared within 0.2 sec after mixing E-$FMNH_2$ with O_2. As plotted in Fig. 3, the $t_{1/2}$ value of this rapid reaction was 60 msec at 460 nm. This value was constant when examined at another wavelengths and apparently independent of the O_2 concentration with in the range tested (24-120 μM). The compound

Fig. 3

giving this intermediary spectrum had a broad absorption band at 430-470 nm and was designated as X_1. After completion of the slower reaction, which was found to be a first order process with a rate constant k=0.25 sec^{-1}, the absorption spectrum of the mixture was the same as that of free FMN, except at the shortest wavelengths examined (Fig. 2). The discrepancy at the shortest wavelengths may be due to a trace of dithionite contaminating the E-$FMNH_2$ solution. The affinity of FMN for the enzyme was reported to be at least 10^3 times less than that of $FMNH_2$ (13), so the slower process corresponds to release of FMN from X_1. In contrast to the biphasic nature of the time course of the reaction of E-$FMNH_2$ with O_2, aerobic oxi-

dation of $FMNH_2$ to FMN in the absence of luciferase followed a simple monophasic process, as exemplified in Fig. 1 (b).

Fluorometric experiments on the same reaction system were conducted in the flow apparatus, and the time course of change in fluorescence intensity of the mixture was recorded. The biphasic nature of the time course was again observed at each emission wavelength, and the results obtained are plotted in Fig. 4. The fluorescence emission spectrum of the inter-

Fig. 4

mediate X_1 thus obtained at 0.2 sec after stopping the flow was indistinguishable from that of free FMN. This is in contrast to the marked difference between the absorption spectrum of X_1 and that of free FMN. The intensity of luminescence of the reaction mixture was negligible compared with that of fluorescence under the present experimental conditions.

Life of Intermediate Measured by Aldehyde-Initiated Luminescence--E-$FMNH_2$ was mixed with O_2 in the flow apparatus, and a 0.8 ml aliquot of the resulting X_1 was directly led into a small test tube fixed in front of a photomultiplier. At a known interval after mixing, 0.2 ml of an aldehyde solution from an injection syringe was rapidly mixed with the solution of X_1, and the change in luminescence intensity of the mixture with time was recorded. The same experiment was repeated at different intervals, and results are summarized in Fig. 5.

Fig. 5 (a), (b), (c)

It can be seen that on addition of aldehyde, there was a rapid increase of light intensity over the low endogenous luminescence, followed by a first order decay process, the rate of which depended on the chain length of aldehyde (k_4=0.15, 0.12 and 0.31 sec^{-1} for C_{10}, C_{12} and C_{13} aldehyde, respectively). The same decay rate constants were obtained when the reaction was initiated by mixing $FMNH_2$ with samples of enzyme which had previously been mixed with the various aldehydes.

Luciferase undergoes a single turnover in the present experimental conditions (14, 3) since free $FMNH_2$, one of the substrates, is rapidly exhausted (oxidized) upon reaction with excess O_2. With each sample, the peak height of the light intensity (I_o) is proportional to the amount of enzyme intermediate capable of giving luminescence on reaction with aldehyde. Decrease of I_o with time after mixing E-$FMNH_2$ with O_2 was again found to be a first order process (Fig. 5) and the values of the rate constant (k_6) determined using aldehydes of different chain lengths were in good agreement (k_6=0.25, 0.24 and 0.23 sec^{-1} determined using C_{10}, C_{12} and C_{13} aldehyde, respectively). The values of k_6 obtained were again in agreement with the rate constants of the reaction $X_1 \longrightarrow E + FMN$ (0.25 sec^{-1}) observed in the preceding experiment.

Effects of Ferricyanide and Dithionite on the Time Course of the Luciferase Reaction--In the following experiments the luciferase reaction was initiated in the flow apparatus by

mixing the enzyme plus RCHO and O_2 with $FMNH_2$. A 0.8 ml aliquot of the resultant mixture was led into a small test tube in front of a photomultiplier, as in the preceding experiments. While the luminescence was decaying, 0.2 ml of ferricyanide was rapidly added to the reaction mixture from an injection syringe (final concentration of ferricyanide, 1 mM). No appreciable change in the time course of the reaction was observed (Fig. 6), demonstrating the stability of the inter-

Fig. 6

mediate to an oxidant such as ferricyanide. On the other hand, as may be seen in Fig. 7, an abrupt acceleration of the

Fig. 7

decay process was observed upon addition of dithionite when C_{10} aldehyde was used while up to 2.0 mM dithionite had much lesser effect on the time course in reaction mixture containing C_{13} aldehyde. Dithionite had an intermediary effect when the reaction mixture contained C_{12} aldehyde.

Reversible Inhibition of the Luciferase Reaction by Carboxylic Acid--As mentioned in the DISCUSSION, one of the products of the luciferase reaction is carboxylic acid formed by oxidation of the RCHO (22). In fact competitive inhibition of decanal by decanoic acid was observed (Fig. 8). Using the

Fig. 8

same technique as in the preceding section, when decanoic acid was added to a luminescent reaction mixture rapid decrease of the luminescent activity was observed (Fig. 9). On further

Fig. 9

addition of excess decanal to the mixture, recovery of the luminescent intensity to almost the original level was observed. The same results were obtained by using dodecanal-dodecanoic acid or tridecanal-tridecanoic acid system.

DISCUSSION

It has been proposed (14-17) that the sequence of the *in vitro* luminescent reaction of bacterial luciferase is,

$$E(\text{luciferase}) + FMNH_2 \rightleftharpoons E\text{-}FMNH_2$$

$$E\text{-}FMNH_2 + O_2 \longrightarrow \text{Intermediate (II)}$$

$$II + RCHO \rightleftharpoons II\text{-}RCHO \longrightarrow \text{Excited state} \longrightarrow h\nu.$$

The following facts support this scheme. (1) Luciferase has a high affinity for $FMNH_2$ (K(dissociation constant of $E\text{-}FMNH_2$) $= 1 \times 10^{-7}$ M (apparent value) (4) for *Ph. phosphoreum* luciferase and 8×10^{-7} M (5) for *Ph. fischeri* luciferase). (2) $FMNH_2$, in the free or enzyme-bound state (e.g. in old yellow enzyme), is known to be rapidly oxidized by molecular oxygen (13, 18). (3) Experiments on aldehyde-initiated bio-

luminescence, originally designed by Hastings and Gibson (14), indicated the existence of a "long-lived intermediate (II)" (14, 15, 17). This intermediate is formed when the luciferase-$FMNH_2$ complex reacts with O_2 and it emitts light upon delayed addition of aldehyde. The absorption spectrum of the intermediate (here denoted as X_1) determined in the present study by rapid mixing luciferase-$FMNH_2$ with O_2 represents that of the intermediate proposed previously since it fulfills the following requirements. (1) It is rapidly formed and the $t_{1/2}$ of its formation (60 msec) is shorter than that of the onset of the luminescent reaction (4). (2) In the absence of aldehyde, the absorption spectrum of X_1 changed to that of free FMN following a first order process, and the rate constant of this process is in agreement with the decay rate of an obligatory intermediate determined by aldehyde-initiated luminescence. A constant value was obtained for the latter irrespective of the chain length of the aldehyde used.

The biphasic nature of the absorption change at 460 nm in aerobic oxidation of $FMNH_2$ in the presence of luciferase is shown in Fig. 1 (a). A similar biphasic change has previously been reported with the luciferase from Ph. fischeri (13), but previous authors ascribed it to heterogeneity of the enzyme sample used. The rate of formation of X_1 from E-$FMNH_2$ (12 sec^{-1}) was apparently independent of the O_2 concentration and this may be a rate-determining step in the reaction, E-$FMNH_2$ + $O_2 \longrightarrow X_1$. It is suggested that this reaction proceeds in at least two steps including rapid formation of an unstable

E-$FMNH_2$-O_2 complex and then change of the complex to X_1 at a rate of 12 sec^{-1}.

The molecular structure of X_1 cannot be deduced from available information of its nature. However, since the fluorescence spectrum of X_1 was almost identical with that of FMN, it is likely that the state of FMN in X_1 is not very different from that of free FMN. The wavelength of the emission maximum of the luciferase reaction is about 30 nm shorter than that of the fluorescence of X_1 or free FMN (3, 8, 9). A shift of the emission maximum of bound FMN in X_1 by 30 nm to a shorter wavelength must occur when a molecule in the excited state is formed as a consequence of specific binding of an aldehyde molecule to X_1 in close proximity to FMN. This possibility was also supported by results on the effect of dithionite on the luciferase reaction, as will be discussed below.

The decay rate of the luminescent reaction was dependent on the chain length of the aldehyde used. The emission maximum of the luminescent reaction was also slightly dependent on the chain length of the aldehyde used (9). These facts indicate that there are differences between the structures of X_1-RCHO complexes of different chain lengths. In fact dithionite had different effects on the luminescent reaction in the presence of aldehydes of different chain length. In the presence of C_{10} aldehyde, the luminescence was rapidly extinguished when excess dithionite was added to the reaction mixture. On the other hand, dithionite caused only little decrease in luminescence intensity when C_{13} aldehyde was present in the reaction

mixture. These results indicated that X_1-RCHO could be reduced back to E-$FMNH_2$ (or E-$FMNH_2$-RCHO) by dithionite in competition with formation of the excited state molecule, and the larger hydrophobic pocket formed by binding aldehyde of longer chain length in the luciferase molecule (4) protected X_1 more effectively against the reductant. These facts indicate a rather direct interaction between FMN and RCHO in the luciferase molecule. The rate constant for the reaction X_1-RCHO(C_{10}) + dithionite $\longrightarrow$ E-$FMNH_2$(-RCHO) was estimated as 1.58 sec^{-1} at a dithionite concentration of 2 mM. It was observed further that inhibition of the luciferase reaction by carboxylic acid could be reversed by delayed addition of excess RCHO during a single turnover of the enzyme. This may suggest that the specific electronic configuration of FMN in X_1 is preserved intact in the X_1-RCHO complex until the moment of formation of the excited state molecule.

All these results may be summarized in the following reaction steps as one of the most simplified scheme possible:

$$E + FMNH_2 \underset{k_2}{\overset{K_1}{\rightleftharpoons}} E\text{-}FMNH_2, \qquad K_1' = 1 \times 10^{-7}\ M,$$

$$E\text{-}FMNH_2 + O_2 \dashrightarrow X_1, \qquad k_2 = 12\ sec^{-1}.$$

In the presence of aldehyde,

$$X_1 + RCHO \rightleftharpoons X_1\text{-}RCHO,$$

$$X_1\text{-}RCHO \xrightarrow{k_4} X_2^*(\text{excited state}),$$

$$X_2^* \xrightarrow{k_5} h\nu + \text{products}.$$

In the absence of aldehyde,

$$X_1 \xrightarrow{k_6} E + FMN, \qquad k_6 = 0.25\ sec^{-1}.$$

The values of K_m for aldehyde (4) and k_4 are, 2.8×10^{-4} M and 0.15 sec^{-1} for C_{10} aldehyde, 1.8×10^{-4} M and 0.12 sec^{-1} for

C_{12} aldehyde and 1.2×10^{-4} M and 0.31 sec^{-1} for C_{13} aldehyde, respectively, k_4 being rate limiting in the overall reaction. The factor ($0 < K < 1$) was tentatively introduced into the reaction scheme since the maximum quantum yield of the bioluminescent reaction observed was 0.2 per luciferase molecule (3). However, it is still premature to discuss the mechanism of energy dissipation in a form other than light energy.

Recently, Lee and Murphy (19) proposed a slightly different mechanism for luciferase reaction. Based on their spectroscopic observations on the reaction of luciferase-$FMNH_2$-O_2 system, they suggested that O_2 reacts in the rate limiting step in the overall reaction. However, it may be noted that ambiguities remain in their spectroscopic data since these authors employed a luciferase preparation which by itself shows flavin-like fluorescence and absorption in the visible region (20, 21).

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Fig. 1. Time course of optical density change at 460 nm upon reaction of $FMNH_2$ with O_2 in the presence or absence of luciferase followed by the stopped flow method. For (a), the total concentrations of $FMNH_2$ and O_2 were 1.35 μM and 120 μM, respectively, and a total of 1.3 μM of $FMNH_2$ was calculated to be in the enzyme-bound state. For (b), 1.3 μM $FMNH_2$ was mixed with 120 μM O_2 in the absence of luciferase. pH 7.0, 20 °C. Aldehyde was not added to the reaction mixtures.

Fig. 2. Plot of the optical density of the reaction mixture, as exemplified in Fig. 1 (a), against the wavelength at intervals after stopping the flow. The value plotted at each wavelength was the optical density change at the indicated time after stopping the flow added to the optical density of 1.35 μM $FMNH_2$. FMN and $FMNH_2$, absorption spectra of 1.35 μM FMN and $FMNH_2$, respectively.

Fig. 3. Semilogarithmic plot of time course of increase in optical density at 460 nm after mixing luciferase-$FMNH_2$ complex with oxygen. Data were replotted from Fig. 1.

Fig. 4. Plot of the fluorescence intensity change of the luciferase-$FMNH_2$ complex upon reaction with O_2, against the emission wavelength at intervals after stopping the flow. Excitation at 380 nm. Other conditions were as for Fig. 2. The broken line represents the fluorescence spectrum of free FMN (3 μM, pH 7.0, the peak intensity was normalized).

Fig. 5. Semi-logarithmic plot of the time course of the aldehyde-initiated luminescent reaction. Luciferase and O_2 were mixed with $FMNH_2$ in the flow apparatus at zero time, and C_{10} (a), C_{12} (b) or C_{13} (c) aldehyde was added to the reaction mixtures at the times indicated by arrows. ---- , Decay of peak light intensity (open circles) observed on addition of aldehyde. The first order rate constants estimated for each decay process are shown in the figure. pH 7.0, 20 °C. The concentrations of luciferase, $FMNH_2$, O_2 and aldehyde were 4.7 μM, 20 μM, 140 μM and 200 μM (C_{10}), 500 μM (C_{12}) or 360 μM (C_{13}), respectively.

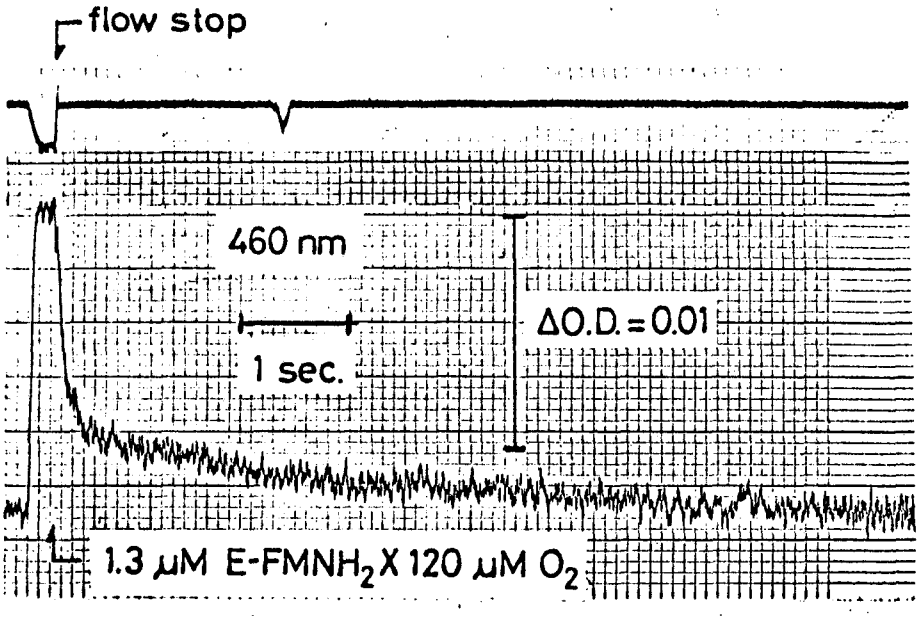
Fig. 6. Lack of effect of ferricyanide on the time course of The luciferase reaction. Ferricyanide was added at the time indicated by an arrow. C_{10} RCHO was used.

Fig. 7. Effect of dithionite on the time course of the luciferase reaction. Luciferase, O_2 and aldehyde (C_{10} in (a), C_{12} in (b) and C_{13} in (c)) were mixed with $FMNH_2$ in the flow apparatus at zero time, and dithionite was added to the reaction mixtures at the times indicated by arrows. The concentrations of dithionite added are shown near to the arrows. pH. 7.0, 23°C. The concentrations of luciferase, $FMNH_2$, O_2 and aldehyde were 3.3 μM, 30 μM, 120 μM and 400 μM (C_{10}), 200 μM (C_{12}) or 200 μM (C_{13}), respectively.

Fig. 8. Dixon plot of inhibitory effect of decanoic acid on the luciferase reaction. Concentration of C_{10} RCHO: ○, 20 μM; □, 50 μM; △, 100 μM; ●, 200 μM; ■, 400 μM. The K_i was estimated to be 200 μM.

Fig. 9. Effect of decanoic acid and its reversal by C_{10} RCHO on the time course of the luciferase reaction. The times of decanoic acid addition and the further addition of C_{10} RCHO are indicated by arrows in the figure.

Fig. 1.


flow stop
460 nm
1 sec.
ΔO.D. = 0.01
1.3 μM E-FMNH2 X 120 μM O2

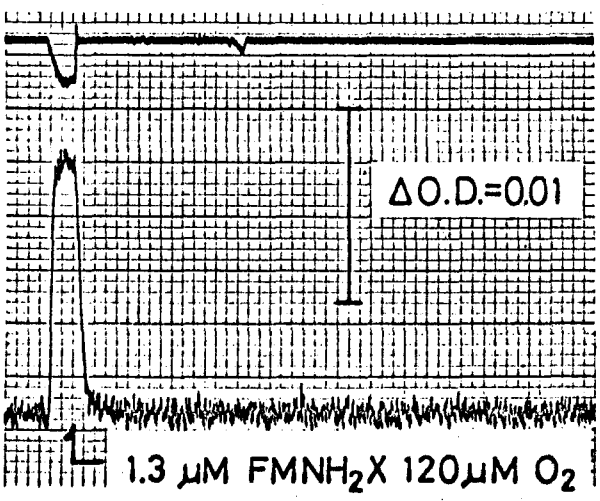
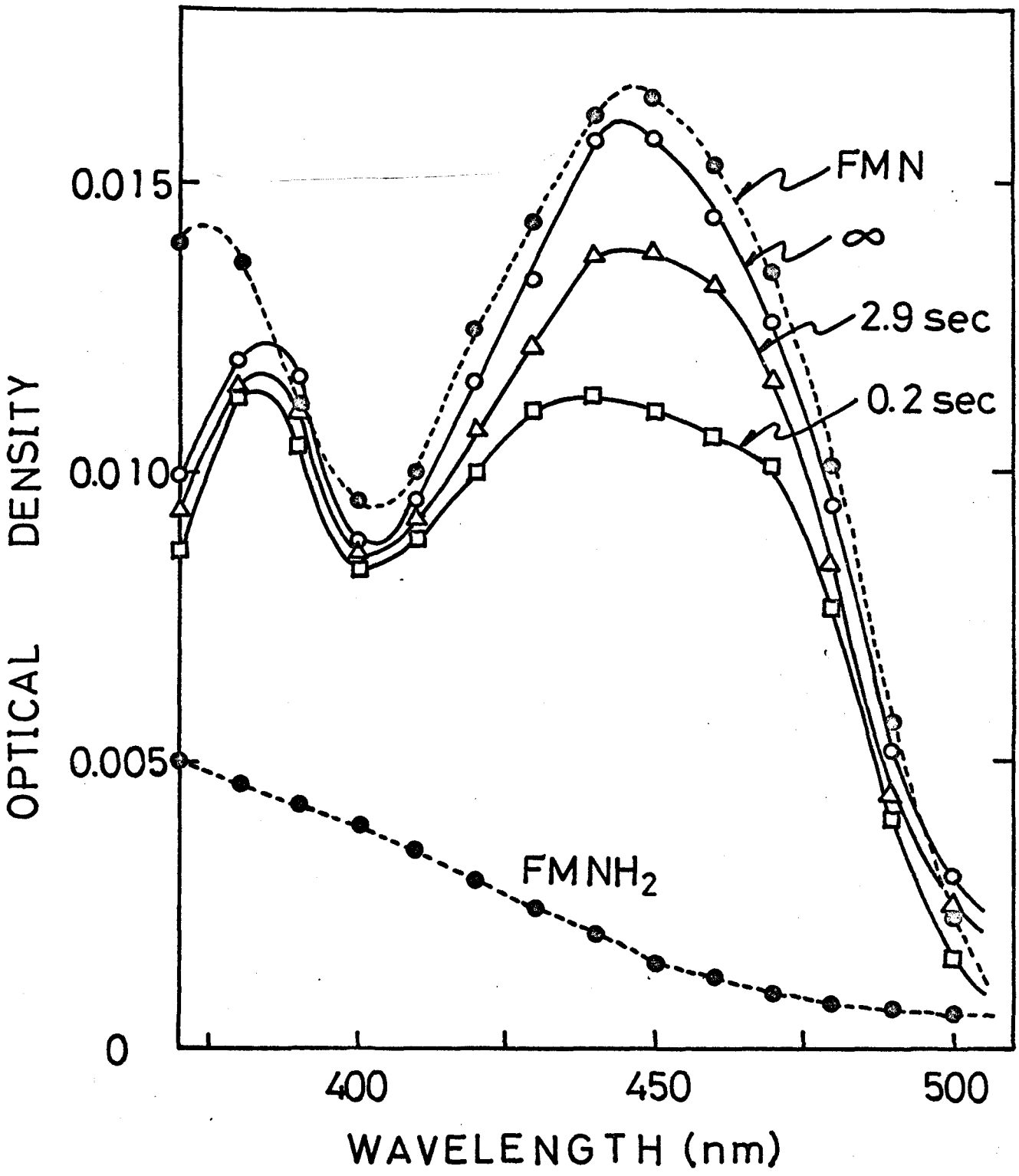

ΔO.D.=0.01
1.3 μM FMNH2 X 120 μM O2

Fig. 2


FMN
∞
2.9 sec
0.2 sec
FMNH2
0.015
0.010
0.005
0
OPTICAL DENSITY
400
450
500
WAVELENGTH (nm)

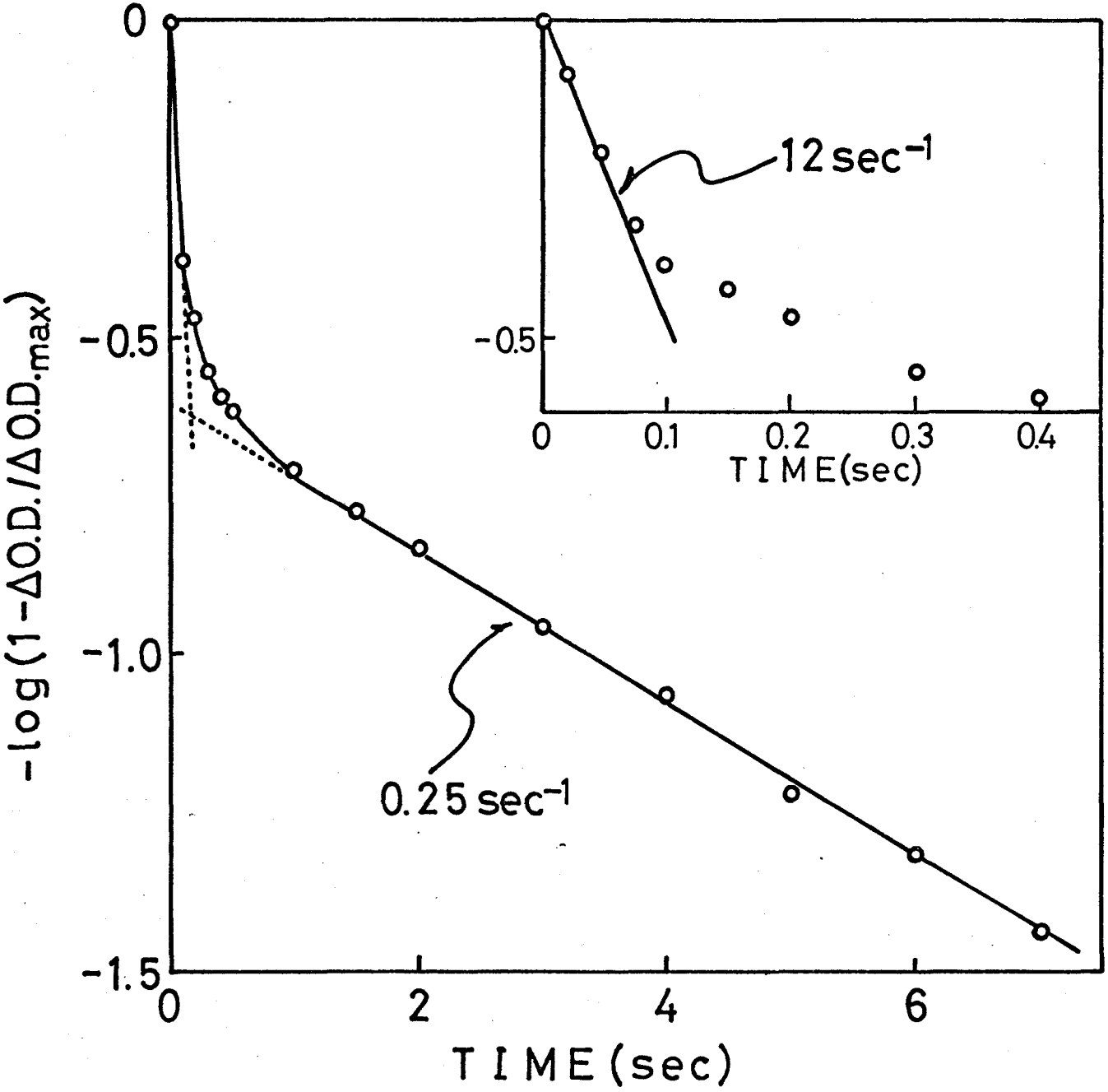

0
-0.5
-1.0
-1.5
0
2
4
6
TIME (sec)
-log (1-ΔO.D./ΔO.D.max)
0.25 sec⁻¹
12 sec⁻¹
-0.5
0
0.1
0.2
0.3
0.4
TIME(sec)

Fig. 3

Fig. 4.

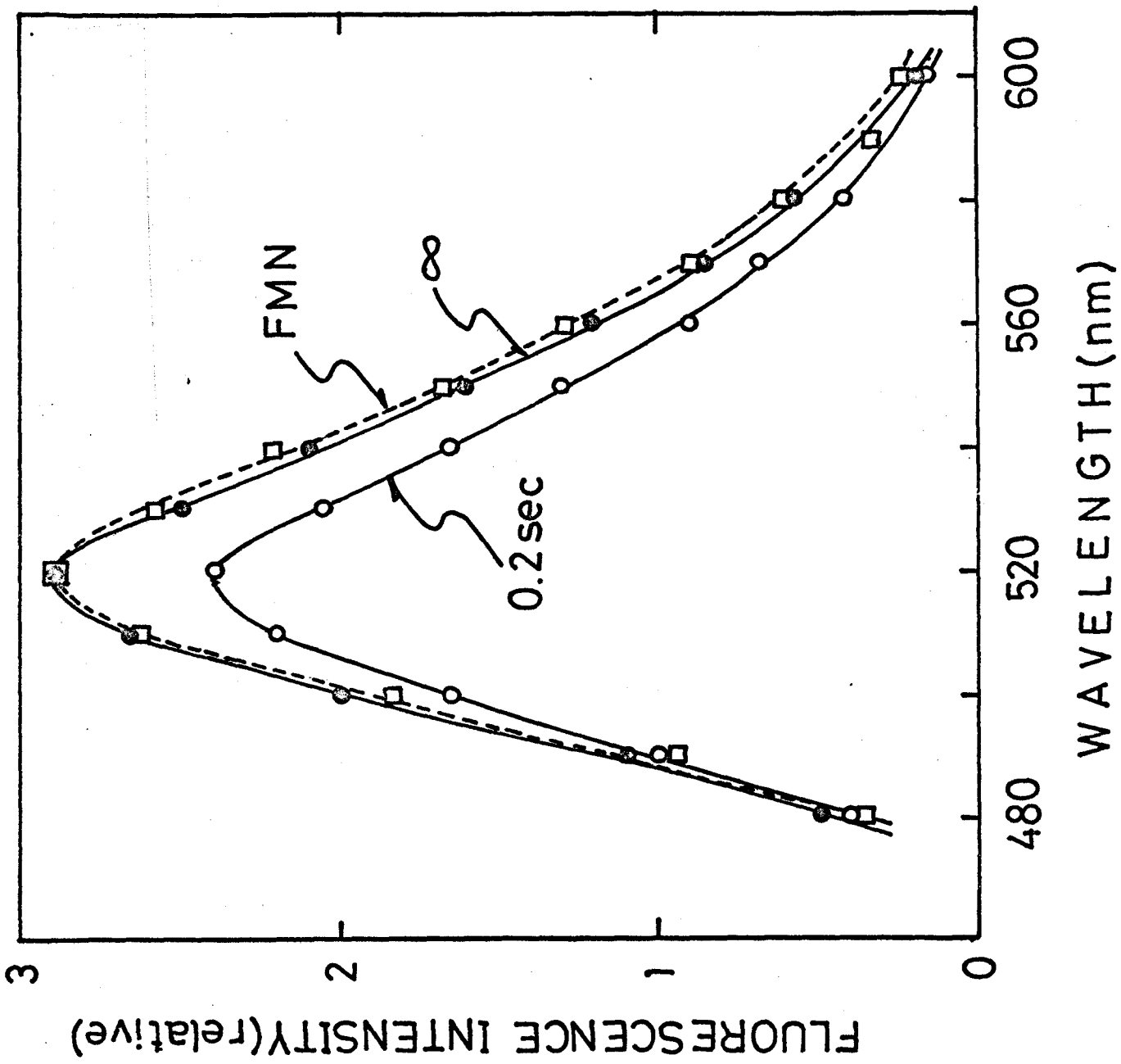
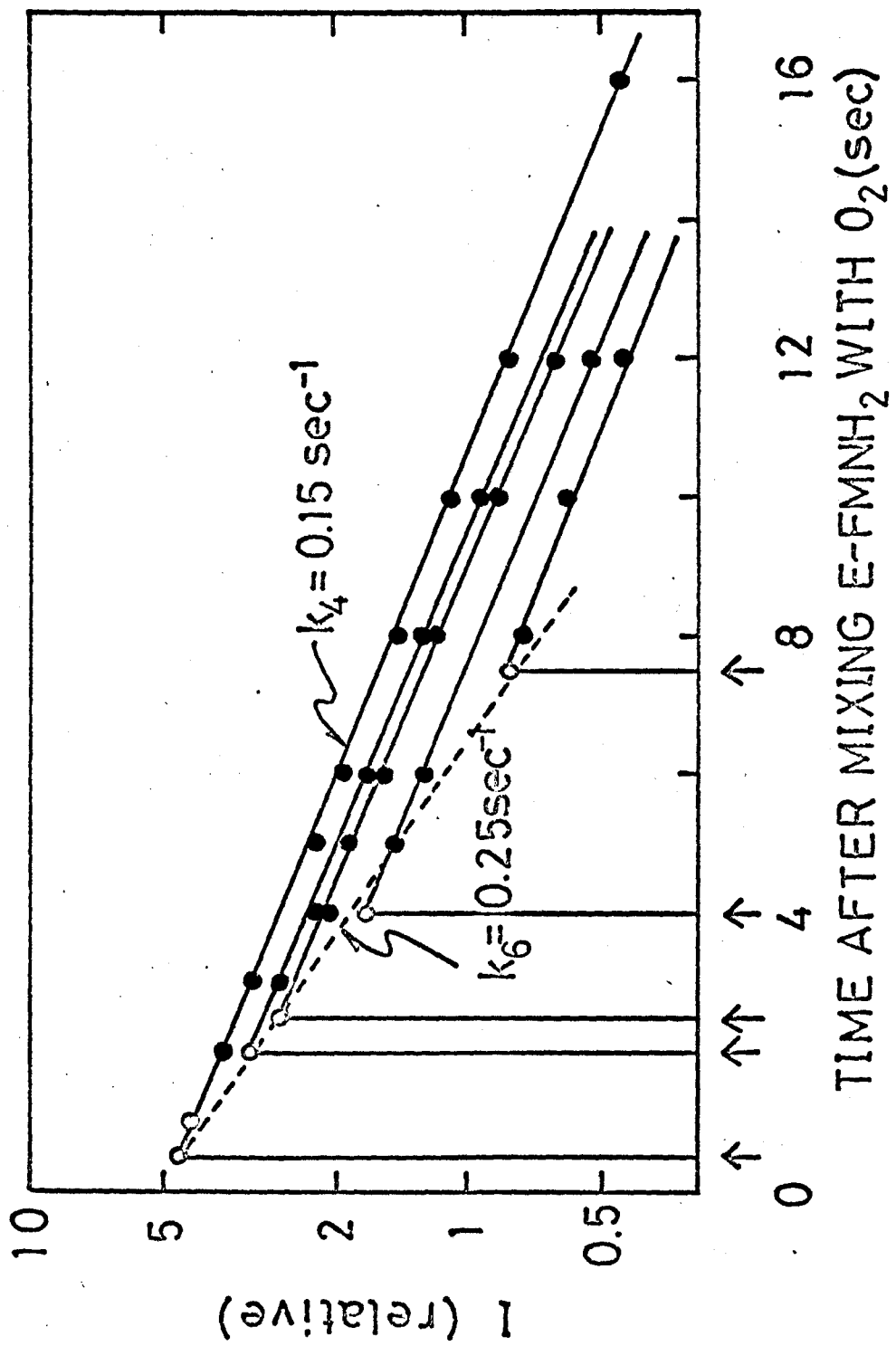

FMN
8
0.2sec
FLUORESCENCE INTENSITY(relative)
0
1
2
3
480
520
560
600
WAVELENGTH(nm)

Fig. 5-a


I (relative)
10
5
2
1
0.5
0
4
8
12
16
TIME AFTER MIXING E-FMNH2 WITH O2(sec)
k4 = 0.15 sec-1
k6 = 0.25sec-1

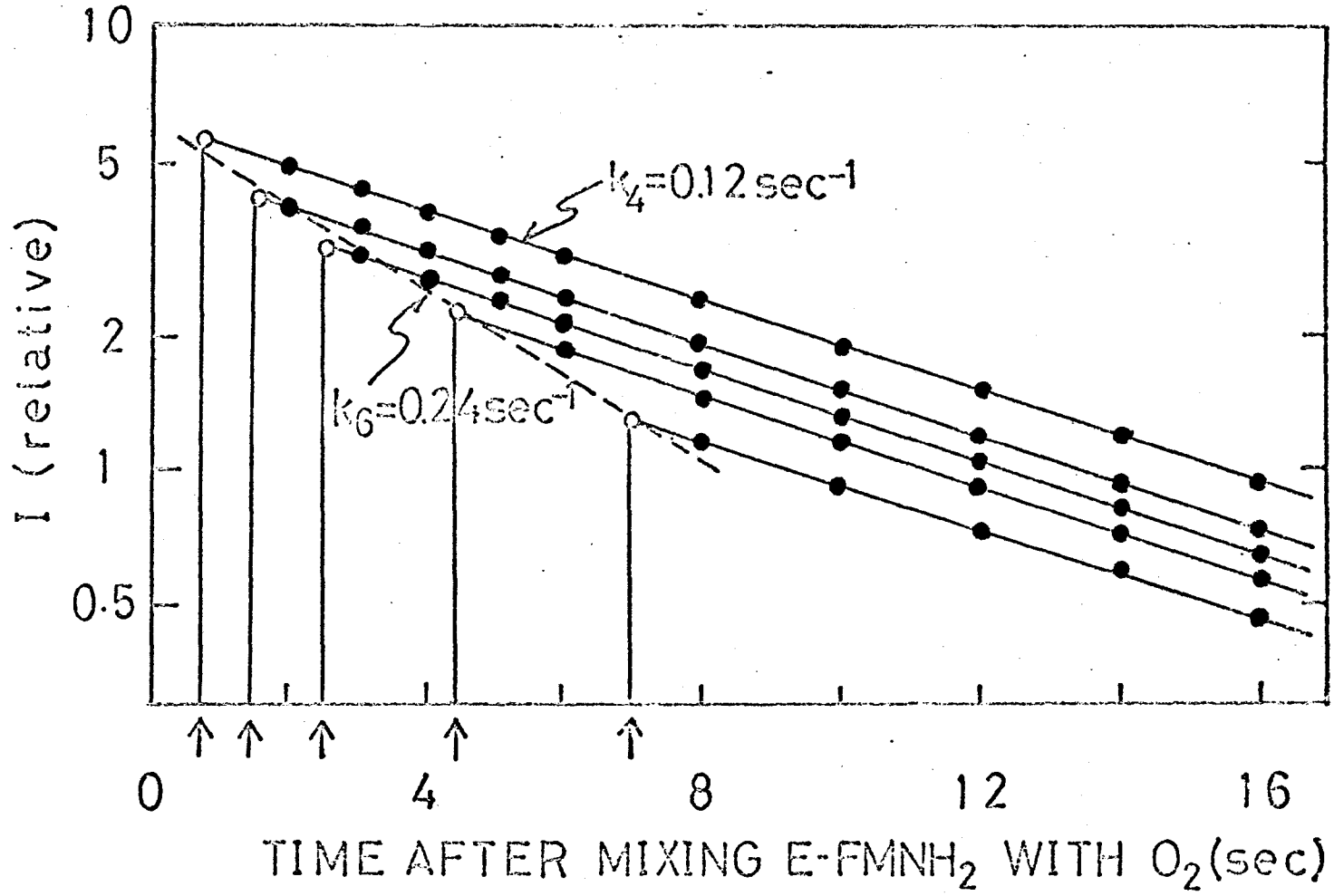
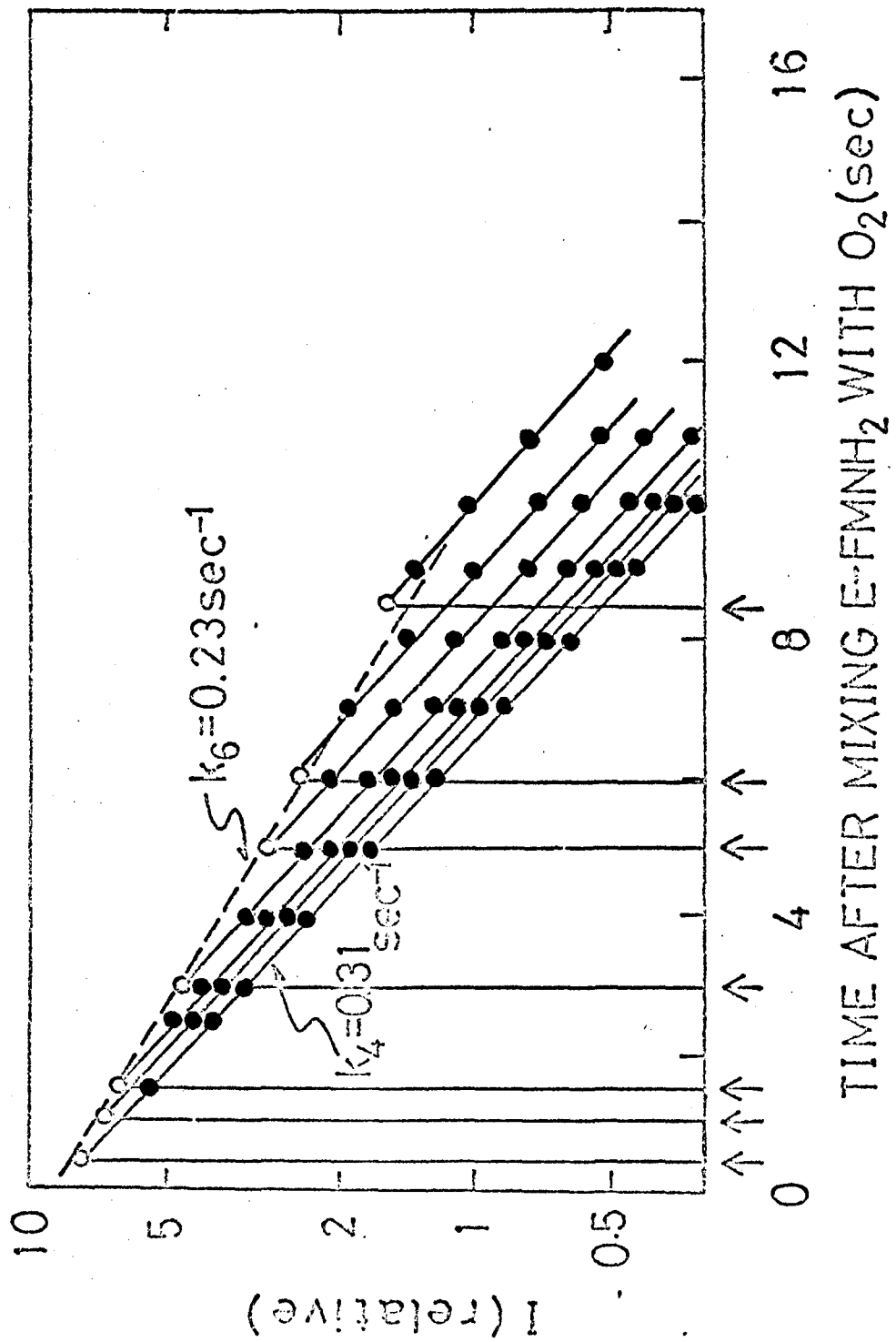

10
5
2
1
0.5
I (relative)
k_4=0.12 sec^{-1}
k_6=0.24 sec^{-1}
0
4
8
12
16
TIME AFTER MIXING E-FMNH$_2$ WITH O$_2$(sec)

Fig.5-b

Fig. 5-C


I (relative)
10
5
2
1
0.5
0
4
8
12
16
TIME AFTER MIXING E-FMNH2 WITH O2(sec)
k6=0.23sec-1
k4=0.31 sec-1

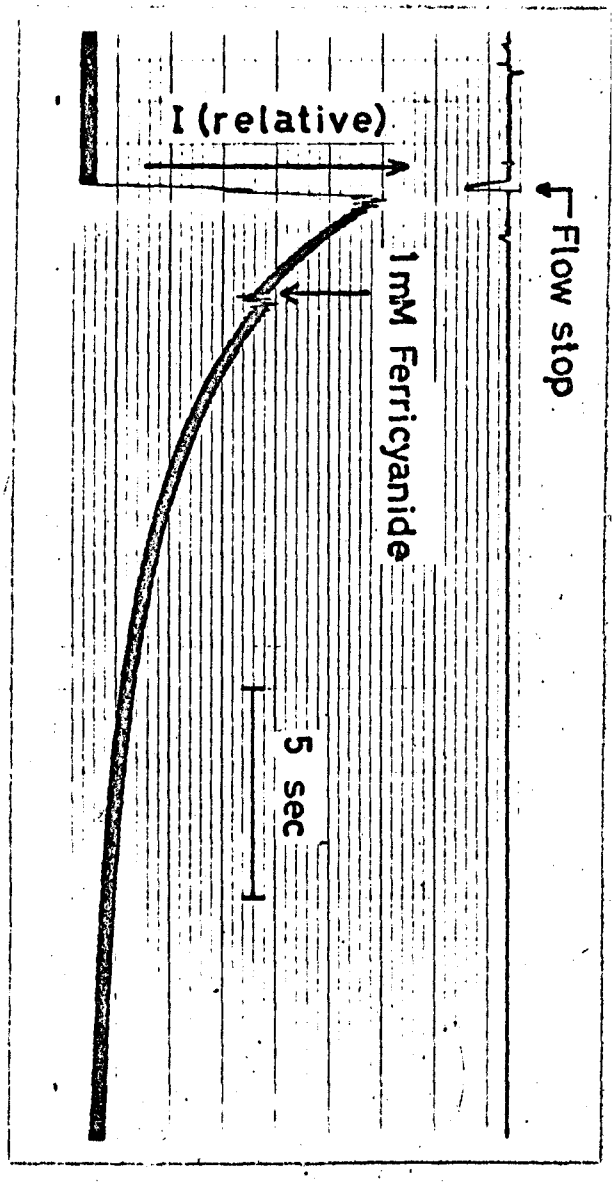
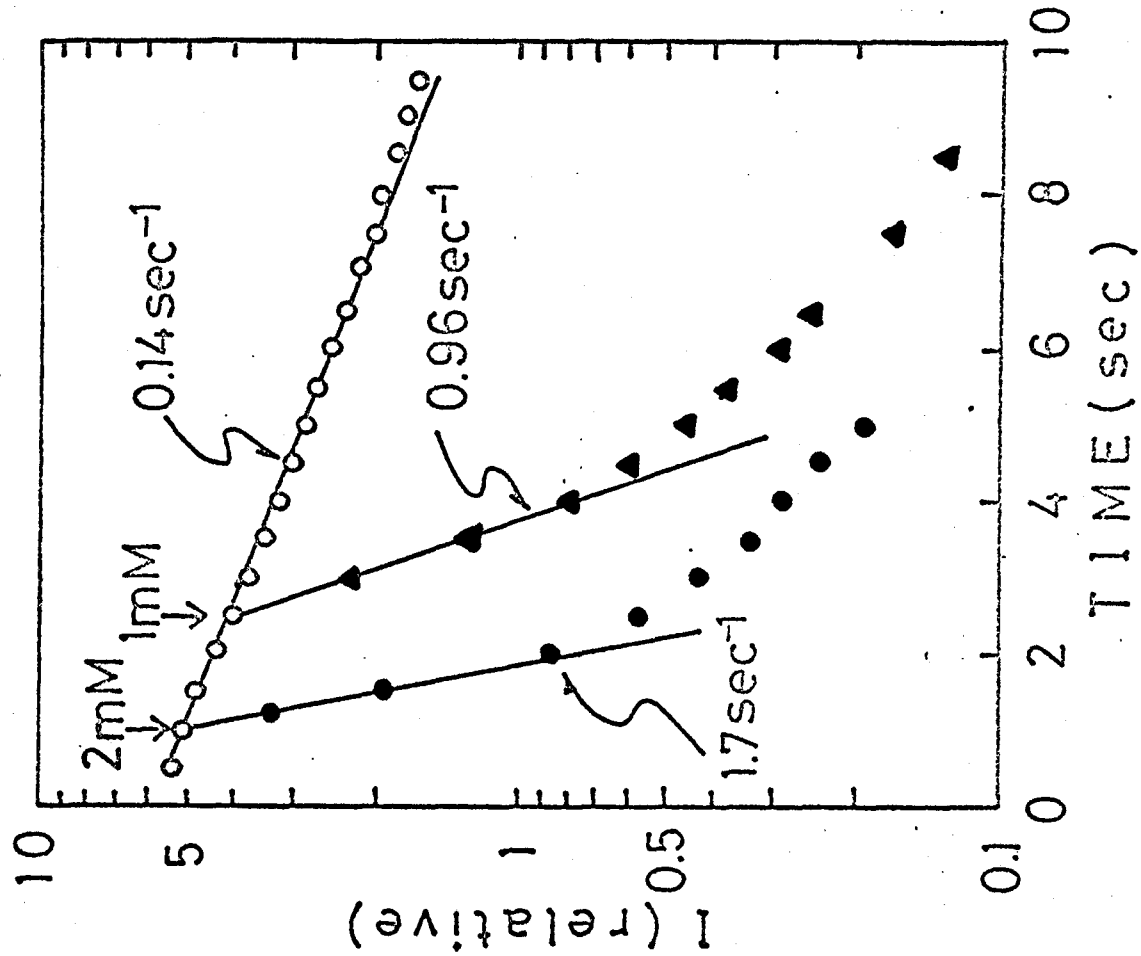


Fig. 6

Fig. 7-a


10
5
1
0.5
0.1
I (relative)
0
2
4
6
8
10
TIME (sec)
2mM
1mM
0.14 sec⁻¹
0.96 sec⁻¹
1.7 sec⁻¹

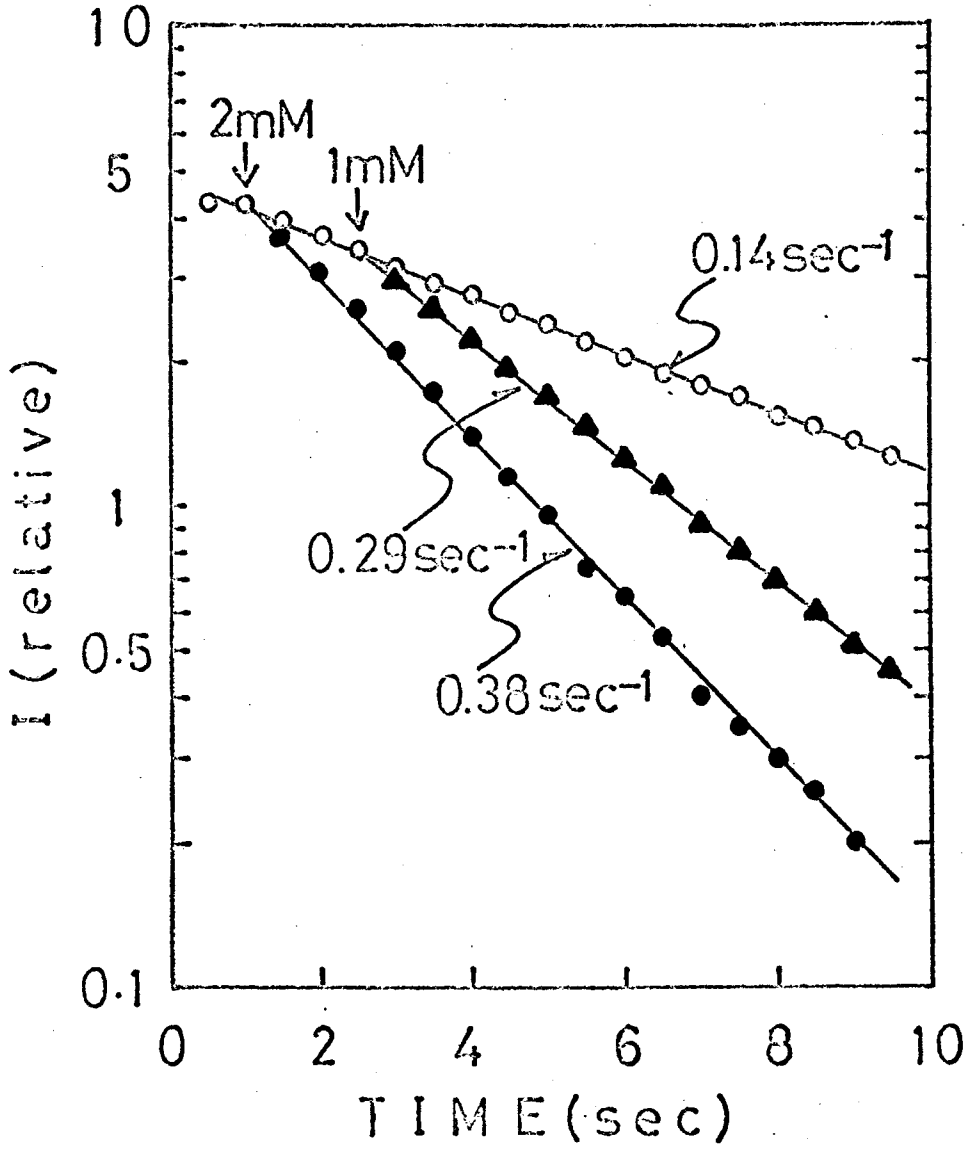

10
5
1
0.5
0.1
I (relative)
2mM
1mM
0.14sec⁻¹
0.29sec⁻¹
0.38sec⁻¹
0
2
4
6
8
10
TIME(sec)

Fig. 9-b

10
5
1
0.5
0.1
I (relative)
2mM
1mM
0.31sec⁻¹
0.39sec⁻¹
0.46sec⁻¹
0
2
4
6
8
10
TIME (sec)

Fig. 7-C

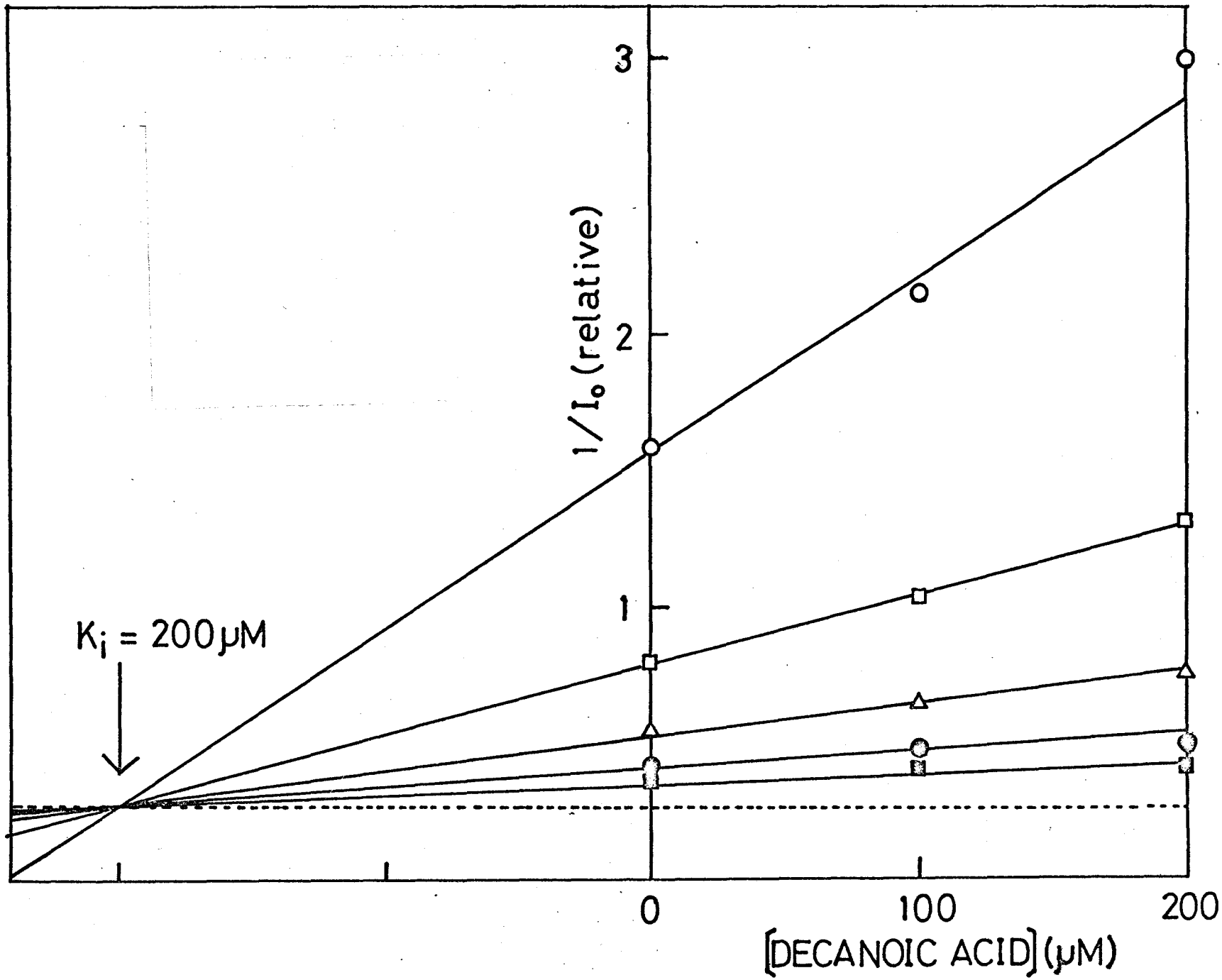


Fig. 8

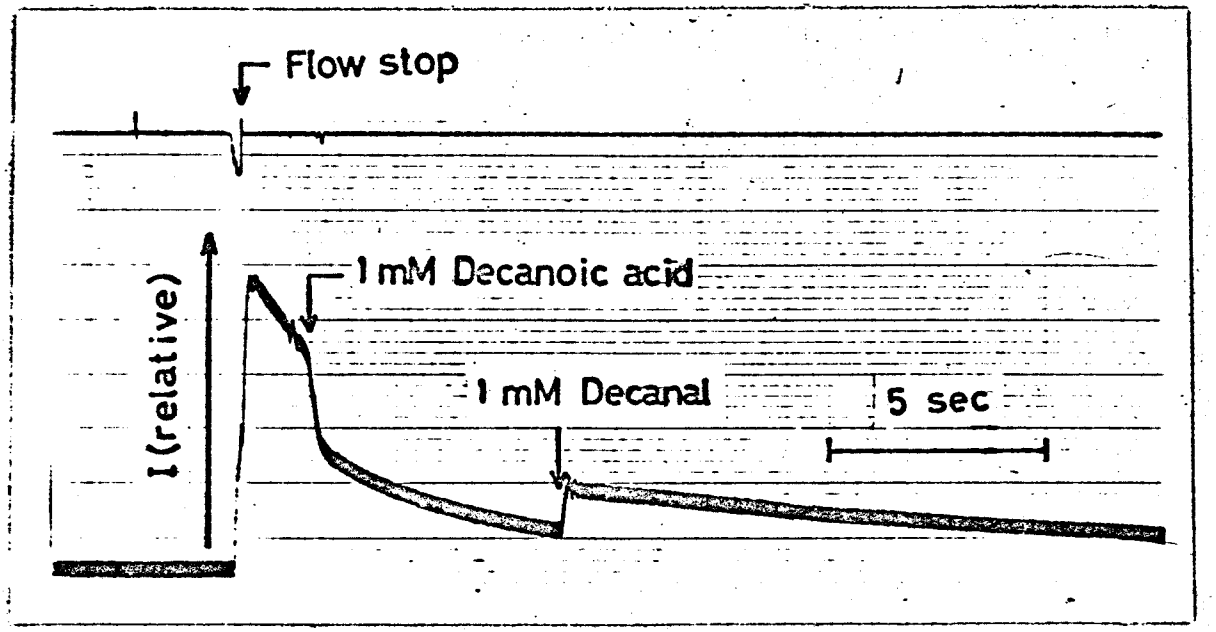


Fig. 9

PART III

ISOLATION AND PARTIAL CHARACTERIZATION OF AN ENZYME-BOUND PIGMENT

A new flavin derivative (p-flavin) was isolated from a bacterial luciferase from Photobacterium phosphoreum. It contains isoalloxazine nucleus and phosphoric acid in a ratio of 1 : 4, but is more hydrophobic than FMN or riboflavin and is soluble in chloroform. The chromatographic characteristics of this new flavin do not correspond to those of any previously known flavin derivatives. Its absorption maxima are at 224, 270, 385, and 445 nm with a shoulder at 470 nm, and its fluorescence maximum (absolute spectrum) is at 544 nm, in aqueous medium at pH 7.0. One molecule of native luciferase was found to contain an average of 0.19 molecule of this flavin, bound non-covalently.

Purified bacterial luciferase from Photobacterium phosphoreum is yellow and has an absorption maximum at 375 nm with a shoulder at 445 nm, and green fluorescence (maximum at 530 nm) (1). These characteristics suggest that a flavin-like chromophore is bound to the enzyme. The chromophore could not be isolated by methods which are commonly used to separate flavins from flavoproteins (1), and it remained firmly bound to luciferase even when the enzyme was subjected to DEAE cellulose column chromatography or gel filtration on Sephadex during the purification process.

In the present investigation, we isolated the chromophore pigment (here denoted as p-flavin), which is non-covalently bound to the enzyme, by treatment of the enzyme with either guanidine-HCl or urea followed by gel filtration on Sephadex, or by treatment with a mixture of organic solvents. p-Flavin thus obtained was bound to be a single component and was partially characterized.

MATERIALS AND METHODS

Luciferase--Luciferase was extracted and purified from Photobacterium phosphoreum as described by Nakamura and Matsuda (1). The molecular weight of the enzyme was reported to be 82,000 (1), and this value was used in calculation of the molar amount of the enzyme.

Reagents--All the reagents used in these studies were guaranteed grade reagents. Guanidine-HCl and urea were

obtained commercially and were purified further as described by Mitchell and Hastings (2). Florisil (60-100 mesh) and silica gel used for chromatography were supplied by Floridin Company and Merck, respectively.

Determination of Flavin--To determine the amount of the isoalloxazine nucleus in a test sample, Yagi's method (3) was used with slight modifications. This method involves fluorometric determination of lumiflavin derived from flavins by irradiation in an alkaline medium.

Determination of Phosphorus Content--The phosphorus content of test samples was determined by the method of Bartlett (4) after mineralization using sulfuric acid.

Spectrophotometry--Absorption spectra were recorded with a Cary recording spectrophotometer, model 14. A Hitachi recording spectrofluorometer, model MPF-2A was used to record fluorescence emission spectra. The spectrum recorded was converted to the absolute emission spectrum (relative quanta per unit wavenumber width plotted against wavenumber) by the method of Lippert et al. (5).

pH Measurement--The pH of the pigment solution was determined by a Beckman model "Expandomatic" pH-meter equipped with a combination electrode model 39142.

RESULTS

Isolation of the Pigment--A low molecular weight pigment was isolated from luciferase by denaturing the enzyme

with 5 M guanidine-HCl and then immediately submitting the mixture to gel filtration on Sephadex in the same medium (Fig. 1).

Fig. 1

It could also be isolated using 8 M urea in place of guanidine-HCl. As may be seen in the figure, a colorless protein which was devoid of green fluorescence was eluted first, and a yellow effluent with green fluorescence was obtained in later fractions separated from the protein band. The fractions with green fluorescence (fractions number 60-82 in Fig. 1) were pooled and applied to a column of Florisil equilibrated with 5 M guanidine-HCl. The pigment was adsorbed at the top of the column. The column was washed with water to remove guanidine-HCl, and with 2 % acetic acid and 0.5 % pyridine in water following the method of Dimant et al. (6). On elution with 5 % or more pyridine in water the pigment was eluted as a sharp yellow band. Fractions of eluate containing the pigment were pooled and concentrated in vacuo. In this way the pigment was obtained free from guanidine-HCl. To prepare the pigment on a larger scale, concentrated enzyme solution in water was applied on top of a column of silicic acid ("Mallinckrodt," 100 mesh), and water was allowed to evaporate at room temperature. Then the column was washed with an upper layer solution of a mixture of n-butanol : ethanol : water (3 : 1 : 3, v/v/v) and fractions of eluate containing the yellow pigment were collected. In this process, colorless denatured protein

was left adsorbed at the top of the column.

Nature of the Pigment--The sample of pigment obtained from the pure enzyme preparation gave only a single fluorescent spot on thin layer chromatography on silica gel or paper chromatography with various solvents. The R_f values of the pigment, riboflavin, FMN, and 4',5'-cyclic FMN* determined here by paper chromatography are summarized in Table I.

Table I

The R_f values of the pigment differed from those reported for known flavins. The R_f value of the pigment was larger than that of FMN using lipophilic solvents (solvents number 1-4 in Table I), and smaller than the latter using a hydrophilic solvent (solvent number 6). This suggests that the pigment is rather hydrophobic in nature. In fact, the pigment was found to be very soluble in ethanol, methanol, and pyridine, fairly soluble in chloroform, carbon tetrachloride, acetone, and benzene, and less soluble in water and dioxane. In contrast to this, FMN is known to be insoluble in chloroform, and poorly soluble in ethanol.

The absorption and fluorescence emission spectra of the pigment dissolved in phosphate buffer, pH 7.0, are shown in Fig. 2. The absorption maxima of the pigment are located at 224, 270, 385, and 445 nm, with a shoulder at 470 nm.

*A gift from Prof. K. Yagi, Nagoya University.

Fig. 2

This spectrum differs from that of FMN in that the extinction coefficient at 445 nm peak is considerably smaller than that at 385 nm. The absorption spectrum of the pigment differs slightly from that of the native enzyme (Fig 2). The absorption maxima at 385 and 445 nm of the pigment disappear upon reduction with dithionite (Fig. 2). The uncorrected fluorescence emission spectrum of the pigment has a maximum at 522 nm which is indistinguishable from that of FMN, but the maximum of the absolute emission spectrum after correction for the spectral sensitivity of the photometric system is at 544 nm (18.4 kK) which is 7 nm longer than that of FMN. Fluorescence emission spectrum of the pigment was recorded at different pHs, and no change in the fluorescence maximum (λ_{max}) was observed within the range of pH 1.4-7.2. The fluorescence intensity at 522 peak was constant within the range of pH 3.5-7.2 while it decreased in more acidic media (Fig. 3).

Fig. 3

After irradiation of the pigment in an alkaline medium a fluorescent material was extracted with acid-chloroform. The aqueous layer after chloroform extraction was colorless and non-fluorescent. This material was found to be identical

with lumiflavin produced from FMN or riboflavin by the same treatment, with respect to its fluorescence emission spectrum and R_f values on paper chromatography with the same solvents as those shown in Table I. Thus the pigment contains isoalloxazine nucleus, and can be classified as a derivative of flavin. This pigment is denoted in this paper as p-flavin. The millimolar extinction coefficients of p-flavin, determined on the basis of the amount of isoalloxazine nucleus, were 88 at 270 nm, 35 at 385 nm, and 22 at 445 nm, at pH 7.0.

p-Flavin contains an unusually large amount of phosphorus, that is 4.2 or 4.4 atoms per mole of isoalloxazine nucleus, estimated on p-flavin samples obtained from different preparations of luciferase. This high phosphorus content was not due to contaminating inorganic phosphate, since the same value was determined before and after passing the pigment, dissolved in ethanol, through a column of Sephadex LH-20 to remove a contaminant, if any, of lower molecular weight. A spot of p-flavin on filter paper was ninhydrin negative when compared with phenylalanine of 1/3 molar amount. Heat treatment of p-flavin at 100 °C for 7 min in 1 N HCl gave only an unidentified fluorescent decomposition product. This product differed from FMN on paper chromatography while the same treatment of FAD or 4',5'-cyclic FMN is known to give FMN as a decomposition product (7). The molar content of p-flavin in the native enzyme was estimated by graphical integration of the elution pattern of the p-flavin from a Sephadex column in guanidine-HCl (Fig. 1) and in two different

preparations it was found to be 0.18 or 0.19 moles per mole of enzyme.

DISCUSSION

The present work shows that bacterial luciferase(native) from Photobacterium phosphoreum contains a single flavin derivative. This is bound to the enzyme non-covalently and the details of its chemical structure is still unknown. This new flavin compound has an unusually high phosphorus content, while it is more hydrophobic than FMN or riboflavin. Possibly all of the phosphorus atoms in p-flavin are present as (hydrophilic) phosphate ester. So it seems likely that the molecule of p-flavin has a hydrophobic group, which could cancel the hydrophilicity of the phosphate groups, such as a long chain aliphatic hydrocarbon in addition to isoalloxazine and four phosphate groups, but the identity of this hydrophobic moiety of p-flavin is not known. The millimolar extinction coefficients of p-flavin at 385 nm (35 cm^{-1}) and at 445 nm (22 cm^{-1}) are considerably larger than those of FMN or riboflavin at corresponding absorption maxima (10.4 for FMN or 10.6 for riboflavin at 375 nm and 12.2 for each flavin at 450 nm (8)). Even though it is still premature to give any explanation for these differences, it seems to be suggested that another chromophore, which exhibits absorption in ultraviolet region, exists in p-flavin in addition to isoalloxazine nucleus.

Mitchell and Hastings (2) obtained two fluorescent

pigments (each with a fluorescence maximum at 525-530 nm) by guanidine-HCl treatment of the luciferase and so called "light inducible protein," a modified form of the luciferase, from Photobacterium fischeri, strain MAV. p-Flavin differs from these fluorescent pigments described by Mitchell and Hastings since the absorption spectrum of the luciferases from Photobacterium phosphoreum and from Photobacterium fischeri (9) are different, and the fluorescence maxima of p-flavin and the pigments from Photobacterium fischeri are also different.

The flavin peptides isolated from proteolytic digests of succinate dehydrogenase Succinate: (acceptor) oxidoreductase, [EC 1.3.99.1] are characterized by an anomalous pH dependency of their fluorescence intensity (10). In the case of p-flavin, such an anomaly was not observed. The fluorescence intensity of p-flavin was constant in the pH range of 3.5-7.2, and decreased in more acidic media. These characteristics are similar to those reported for FMN (10). This fact suggests that the chemical nature of p-flavin is not the type of flavin peptide. This is further supported by the observation that p-flavin is ninhydrin negative.

One molecule of Photobacterium phosphoreum luciferase contains only 0.19 molecule of p-flavin. This value is considerably lower than that expected from 1 to 1 stoichiometry of binding of p-flavin to the enzyme, but a loss of p-flavin from the enzyme in purification process is not probable, as was mentioned earlier in this report. Further-

more, the fluorescence maximum of p-flavin (544 nm in the free state and 530 nm in enzyme-bound state) is different from the emission maximum of bioluminescent reaction of the enzyme (495-500 nm (1)). Thus the role of p-flavin in the bioluminescent reaction of the luciferase still remains as a subject of investigations in future.

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Table I. R_f values of p-flavin on paper chromatography (Toyo Roshi No. 51 paper). The solvents used are (v/v/v):

1, n-Butanol : ethanol : H_2O = 3 : 1 : 3 (upper layer);

2, n-Butanol : glacial acetic acid : H_2O = 4 : 1 : 5 (upper layer);

3, n-Butanol : n-propanol : H_2O = 2 : 2 : 1;

4, Phenol : n-butanol : H_2O = 16 : 3 : 10;

5, n-Butanol : pyridine : H_2O = 5 : 3 : 2;

6, 5 % Aqueous disodium hydrogen phosphate.

Fig. 1. Elution pattern of luciferase from a Sephadex G-200 column (2.8 cm x 41.2 cm), at 25°C in the presence of 5 M guanidine-HCl and 2 mM EDTA. 3.5 mg of luciferase were applied to the column and effluent was collected in 3.25 ml fractions. The optical density at 280 nm and fluorescence of each fraction were measured. o , optical density at 280 nm; ● , relative fluorescence intensity at 520 nm (excited at 370 nm).

Fig. 2. Absorption spectrum and uncorrected fluorescence emission spectrum (excited at 380 nm) of the pigment (p-flavin). The pigment was dissolved in phosphate buffer, pH 7.0, at a concentration of 5 μM. The absorption spectrum of a solution of luciferase with the same optical density as that of the pigment at 445 nm is shown for comparison. ——— , absorption spectrum of the pigment; —·— , spectrum after reduction with dithionite; — — — , fluorescence emission spectrum of the pigment; ..... , absorption spectrum of luciferase in phosphate buffer, pH 7.0.

Fig. 3. Fluorescence intensity of the pigment (p-flavin) at 522 nm (exited at 380 nm) plotted against the pH of the medium (0.04 M phosphate buffer). p-Flavin concentration, 1.0 μM.

Table I.

Flavin	Solvent					
	1	2	3	4	5	6
p-Flavin	0.54	0.52	0.21	0.45	0.07	0.17
Riboflavin	0.37	0.35	0.23	0.85	0.42	0.30
4',5'-Cyclic FMN	0.23	0.13	0.08	0.38	0.12	0.39
FMN	0.17	0.10	0.05	0.21	0.04	0.53

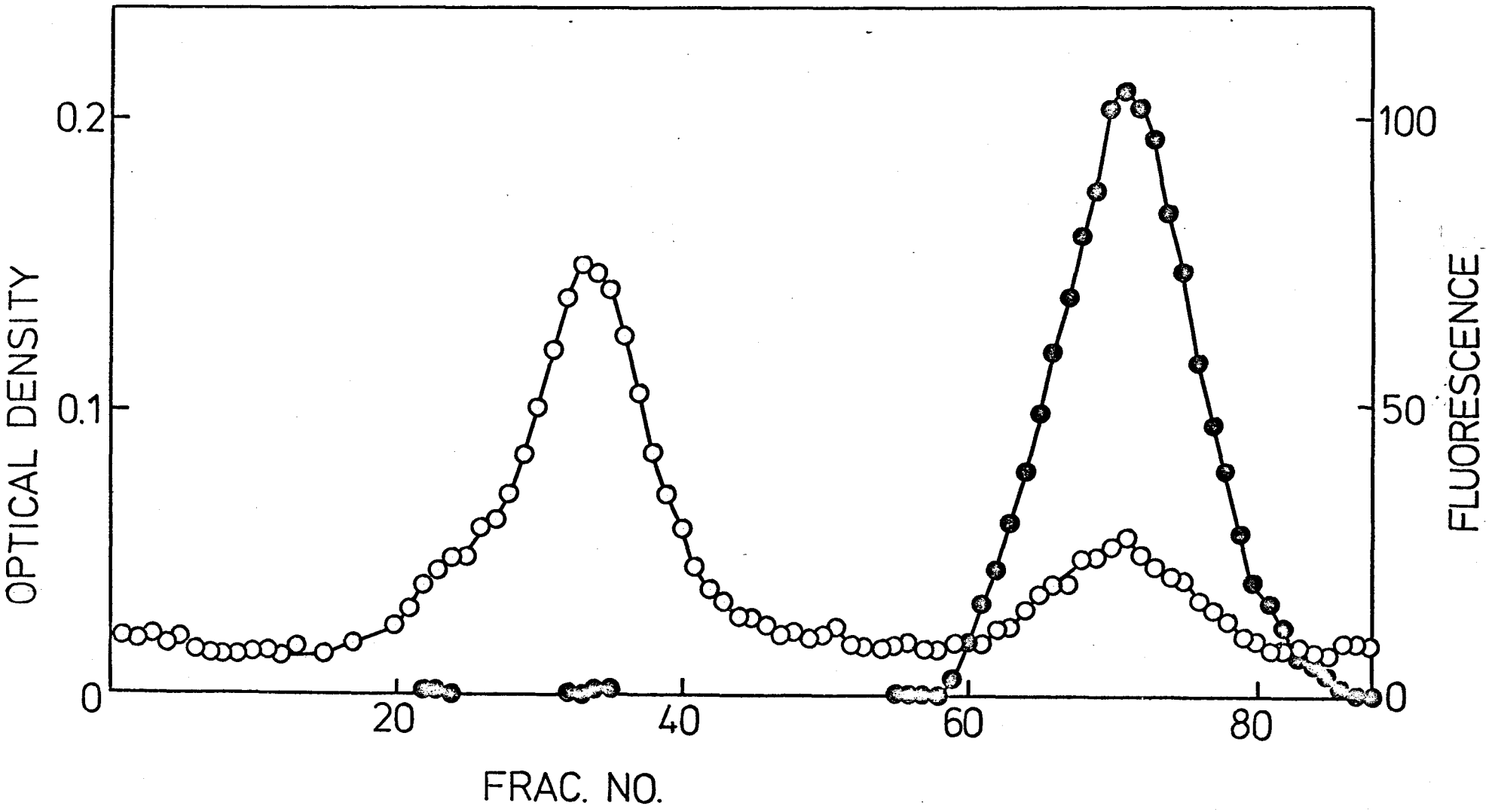
0.2
0.1
0
OPTICAL DENSITY
20
40
60
80
FRAC. NO.
100
50
0
FLUORESCENCE

Fig. 1

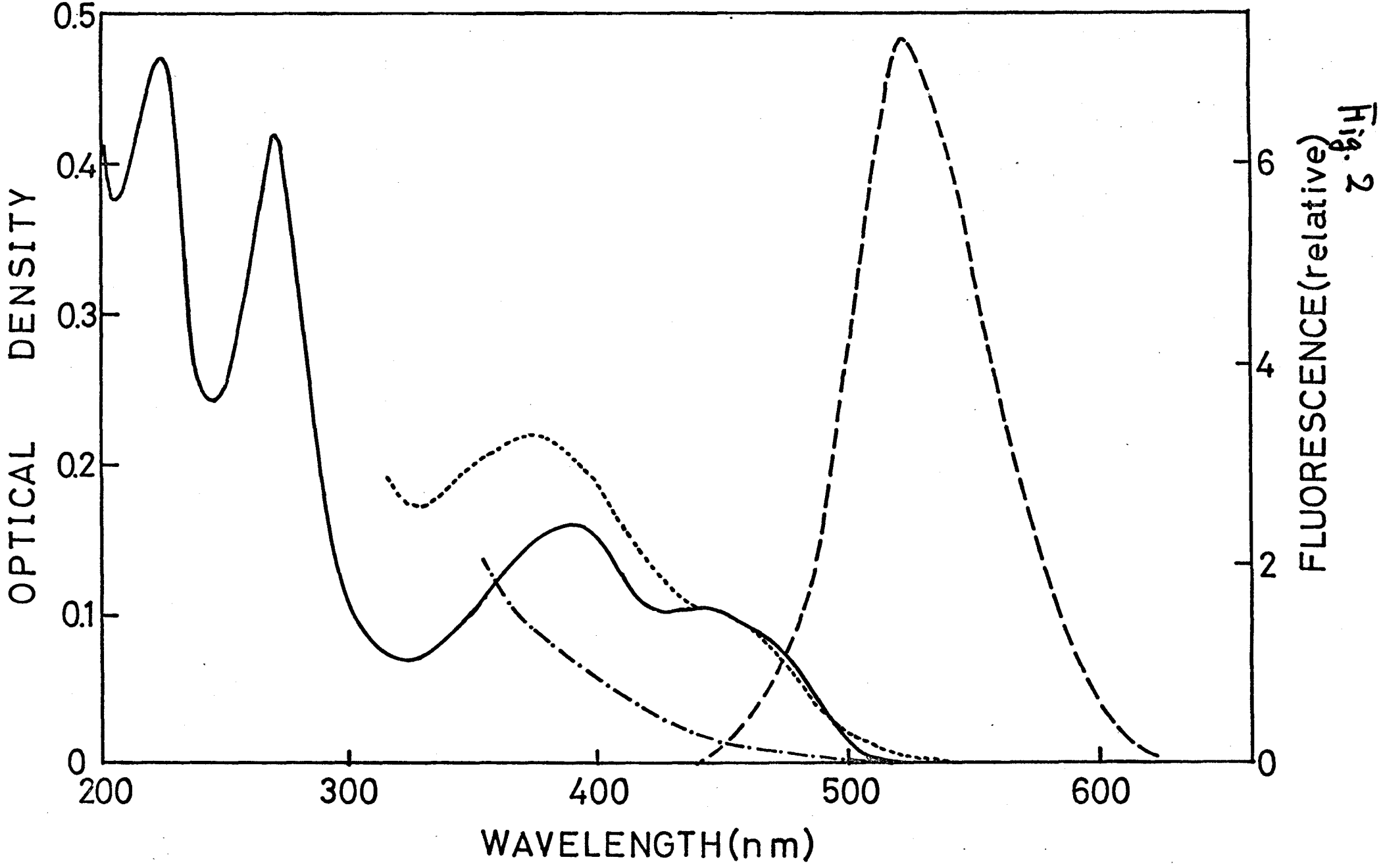
0.5
0.4
0.3
0.2
0.1
0
OPTICAL DENSITY
6
4
2
0
FLUORESCENCE(relative)
200
300
400
500
600
WAVELENGTH(nm)

Fig. 2

Fig. 3

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