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PHOTOREPAIR OF UV DAMAGE TO DNA

PURIFICATION AND PROPERTIES OF THE DNA PHOTOLYASE (the DNA-Photoreactivating Enzyme)

Progress Report

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CONTENTS

ABSTRACT

There is associated with the DNA-photolyase purified by affinity chromatography substances that have the spectral properties expected for the chromophore involved in DNA-photoreactivation. These substances can be dialyzed in part away from the enzyme when it is concentrated in a collodion membrane. The fluorescence of the dialyzate is similar to that of the enzyme except for a blue-shift in the maxima of excitation and fluorescence emission. Two yellow green materials whose spectral properties mimicked those of known pteridines were separated from the dialyzate by paper chromatography. Heating the enzyme induces this blue shift but addition of substrate, irradiated DNA, retards it. These data implicate the fluorescent substances bound to the enzyme as the actual photosensitizers in DNA photoreactivation. By assuming that the large loss in activity observed when the enzyme was purified by affinity chromatography was due to loss of the bound chromophore, we could use this enzyme preparation to bioassay for chromophore in crude yeast and algal extracts. Both were active and after each was subjected to paper chromatography two diffuse bands that assayed positively were observed. Nevertheless, these substances are designated as activators since there is not unequivocal evidence that they are actually the chromophores for DNA-photoreactivation.

INTRODUCTION

Photoreactivation is the restoration by illumination with visible light of viability to far-UV irradiated biological material. Kelner (1) discovered this process in 1949 when he reversed the UV-inactivation of Streptomyceus griseus spores by illuminating them. In subsequent years photoreactivation was observed in the microbial, animal and plant kingdoms and two early reviews of the subject were those of Dulbecco (2) and Jagger (3).

The enzymatic nature of the phenomenon was recognized about a decade later by Rupert, Goodgal and Herriott (4) and by Rupert (5). They found the enzyme in cell-free extracts of Escherichia coli and Baker's yeast by using a bioassay they developed involving transformation of Haemophilus influenzae cells.

The first serious attempt to purify the enzyme was made in J. K. Setlow's laboratory by Muhammed (6) and J. K. Setlow, who succeeded in isolating a 3000-fold purified preparation. Subsequently calculations made by Harm and Rupert (7) indicated that this material could not have contained more than a few percent active enzyme. Further purification efforts have been carried on mainly by two groups; we reported partial purification of the enzyme from the blue-green alga Anacystis nidulans (8) and extensive purification of the enzyme from Baker's yeast (9) while Sutherland and Chamberlin (10) described procedures for isolating a homogeneous enzyme preparation from E. coli.

On the molecular level the considerable efforts of the Setlows and their collaborators (11-13) have defined the substrate specificity of the enzyme while Rupert's flash photolysis technique has enabled his group to calculate several kinetic constants of initial enzyme action (14,15). Both of these groups have made numerous other significant contributions to our understanding of photoreactivation using crude enzyme preparations. Still, there are many aspects of the phenomenon that can only be studied at the molecular level with

a homogeneous enzyme preparation. This is particularly true for delineation of the protein involvement in the catalytic process. How does the enzyme recognize the substrate, cyclobutadipyrimidines on DNA? Which amino acids are involved? Is there a small molecular weight chromophore covalently or non-covalently bound to the enzyme? Is the only function of the enzyme to place in unique juxtaposition for photochemical interaction the chromophore and the substrate? Does the enzyme perform other catalytic processes besides photoreactivation? Seeking these answers in the case of a unique enzyme that requires light to perform its catalytic function, and one that may be vital to organisms exposed to sunlight, has provided us with ample justification (mission) to purify it.

All of our efforts during the past year had one ultimate objective: the development of procedures to separate and isolate a homogeneous preparation of DNA-photoreactivating enzyme. We sought to increase the yield of active enzyme by enlisting the help of a commercial laboratory to work up large batches of yeast through the first two purification steps. These efforts were quickly abandoned when we found that recovery of the enzyme was no greater than that which we could obtain in the laboratory working with smaller batches. We then concentrated on overcoming the large loss of enzyme activity that occurs during the last purification step (Table I) in the expectation that this would improve the yield of active protein. This work led to our development of a bioassay for an activator or chromophore of the photoreactivating enzyme and using this assay, we found such substances in cell-free crude yeast extracts.

We spent considerable time trying to prove that the fluorescent material associated with the photoreactivating enzyme isolated after affinity chromatography was actually involved in DNA-photoreactivation.

Without the information made available from all these studies, any attempt at final purification of the enzyme could hardly succeed. We are now poised to reach this objective.

I. Enzyme Nomenclature

Systematic enzyme nomenclature is based upon the type of reaction catalyzed and the substrates acted upon. Since this information is known with a reasonable degree of certainty in the case of the photoreactivating enzyme, the time was propitious to devise more appropriate systematic and trivial names for the enzyme. In the appended paper (8) and a research note (16) we describe reasons for designating the enzyme a deoxyribonucleate cyclobutane dipyrimidine photolyase. As a trivial name we suggest DNA-photolyase. Dr. Waldo E. Cohn has communicated to us that he prefers cyclobutadipyrimidine photolyase which we find suitable provided the prefix DNA is appended, since Dr. M. P. Gordon has reported RNA-photolyase activity in cell-free extracts of tobacco plants (17). This enzyme is distinct from DNA-photolyase because McLaren (18) was unable to photoreactivate UV-irradiated free tobacco mosaic virus ribonucleic acid with the latter.

II. Molecular Weight of DNA-Photolyase

Andrews (19) has described a procedure for estimating the molecular weights of impure proteins using chromotography on Sephadex adsorbants. A mixture of globular proteins of known molecular weights are chromatographed and their elution volumes determined. A plot of the logarithm of the elution volume versus the molecular weight is generally linear. Extrapolation of the elution volume of an unknown protein or enzyme on this linear plot provides its molecular weight. The molecular weight of the photolyase from yeast determined in this way was 53,000, but is was necessary to include 0.4 M potassium chloride in the elution buffer to obtain active enzyme. This value may be compared with that of 45,000 for the *E. coli* enzyme reported by Sutherland and Chamberlin (10). They used the technique of SDS polyacrylamide electrophoresis.

III. Is the Fluorescent Material Associated with the Partially Purified DNA-Photolyase An Obligatory Co-factor In DNA-Photoreactivation?

Figure I shows the excitation and fluorescence spectra of the photolyase preparation isolated after affinity chromatography. We proposed in the appended reprint that these spectral properties are those to be expected of the chromophore (photosensitizer of cyclobutadipyrimidine cleavage) associated with the yeast photolyase in DNA photoreactivation. Chromatography of this enzyme preparation on hydroxylapatite (Figure 2) or on Sephadex G 100 (Figure 3A) did not remove the chromophore which was present only in active fractions. In the case of the Sephadex chromatography, if the buffer did not contain a high salt concentration, partial dissociation of the chromophore from the enzyme occurred and most of the enzyme was inactivated (Figure 3 B). So far all active enzyme preparations with quantities of protein measurable by the Folin procedure exhibited the same fluorescence spectra (Figure 1).

When the photolyase preparation from the affinity chromatography step was concentrated in a collodion membrane, there appeared in the filtrate material with fluorescent properties similar to that of the enzyme, but the maxima were blue-shifted (B and D of Figure 4) to 440-445 nm in the fluorescence spectra and 350-355 nm in the excitation spectra. The corresponding values for the enzyme are 485-490 nm and 385 nm. The enzyme remaining in the collodion membrane usually lost little activity.

Fluorescent probes of protein structure such as 1-anilino-8-naphthalene sulfonate (ANS) frequently exhibit either a red or blue shift when bound to protein depending upon the polarity of the binding site (20). The blue shift of the fluorescent substances in the enzyme preparation was evidence that they were bound either to photolyase or some other protein in the enzyme preparation and were freed or became dialyzable when the enzyme was concentrated in the collodion membrane.

Thus an apparent explanation for the 50 percent loss of enzyme activity after affinity chromatography (Table I) was that some of the photoreactivation chromophore (fluorescent material observed in the filtrate of the collodion membrane) was detached from the enzyme during this purification step, and that it was non-covalently linked to the enzyme. If these notions were correct, then it might be possible to use the enzyme preparation purified by affinity chromatography to bioassay for the chromophore. Such experiments were carried out and are described in Section V.

IV Chromatographic Separation of Fluorescent Material Freed from the Enzyme

The filtrate obtained upon concentration of the photolyase in a collodion membrane was subjected to descending chromatography for 17 hours on Whatman 3 MM paper in the system n-butanol-acetic acid-water (4 : 1 : 5). The results obtained are shown in Figure 5. Three green-yellow fluorescent bands were observed, two prominent ones having R_f's at about 0.22 (B) and a lighter one at R_f 0.5 (D). The excitation and fluorescent spectra of B and D are given in Figure 4 as well as those of two compounds with similar spectra, folic acid, and 2-amino-4-hydroxypteridine. In spite of their similar fluorescence spectra, on the basis of their R_f values and response to near-ultraviolet illumination, neither B nor D could be identified with either pteridine.

V. Heat Induced Blue Shift of Photolyase Fluorescence

These experiments were carried out in collaboration with Dr. Lamola because of the paucity of photolyase available after affinity chromatography. He could obtain a fluorescence spectrum on 10 microliters of enzyme preparation. The results are shown in Figure 6A and 6B. Heating the enzyme for 10 min. at 52° blue-shifted the fluorescence (Fig. 6A) to that of the filtrate from which B and D had been isolated. These findings supported the suggestion that the fluorescent material associated with the enzyme is bound to it. Fig. 6B shows

a similar blue-shift of fluorescence when the enzyme was heated in the presence of calf thymus DNA for 45 min. at 37°. However, when this experiment was repeated with irradiated DNA, the blue-shift was markedly retarded. These findings buttress the idea elaborated upon in the appended reprint that the fluorescent material associated with the photolyase purified by affinity chromotography is the chromophore involved in DNA-photoreactivation.

VI. Evidence for an Activator of Photolyase in Crude Yeast Extracts

The data in Section III indicated that the fluorescent substances associated with the photolyase could be obtained free when the enzyme was concentrated in a collodion membrane after purification by affinity chromatography. If this were so, then the enzyme preparation at this stage of the purification should consist of active enzyme (with bound chromophore) and inactive enzyme (free of chromophore). It followed that this enzyme preparation might be useful to bioassay for free chromophore. Since in the experiments to be described we have not proved that the fraction isolated that restores activity to this enzyme preparation is actually the sought for chromophore, we refer to tne active material as an activator.

To separate the activator, Baker's yeast was autolyzed with 0.066 M dipotassium hydrogen phosphate and toluene by stirring the mixture four hours at 37°. It was centrifuged and the supernatant fraction was adjusted to pH 3.0. After the mixture was kept at room temperature for two hours, it was centrifuged and the supernatant fraction filtered through a 15Å membrane filter of a microconcentrator purchased from the Chemical Rubber Company. The filtrate was adjusted to pH 7.5 and concentrated to a small volume by rotatory evaporation.

This concentrate was assayed by mixing 0.1 ml with 0.1 ml of photolyase in 0.05 M tris buffer containing 5 percent glycerol and 0.3 ml of 0.05 M phosphate buffer, pH 7.5,containing 1 mM EDTA and 0.1% bovine serum albumin (BSA). After the mixture was held for one hour at 0°, it was diluted in phosphate-BSA buffer

The variable enhancement in activity observed favors the view that the activator is a chromophore (Table V).

We attempted to fractionate the concentrate containing the activator by paper chromatography with the same solvent system that had been used to separate B and D (Section IV). This was done with both a concentrate prepared from Baker's yeast and one prepared similarly from the blue-green alga Anacystis nidulans. The data are shown in Figures 8 and 9. In the case of the yeast extract two diffuse active bands were observed peaking at R_f's of about 0.2 and 0.45. It is no doubt coincidental that these R_f's are close to those observed for compounds B and D (Figure 5). While the results with the algal concentrate were less dramatic, activation was nevertheless observed and again, the diffuse bands exhibited maxima at R_f's of approximately 0.1 and 0.65.

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19. Andrews, P. Laboratory Practice 16, 851 (1967).
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PUBLICATIONS

"Spectral Properties of the Chromophoric Material Associated with the DNA-Photoreactivating Enzyme Isolated from Baker's Yeast" by S. Minato and H. Werbin, Biochemistry 10, 4503 (1971).

"Excitation and Fluorescence Spectra of the Chromophore Associated with the DNA-Photoreactivating Enzyme from the Blue-Green Alga Anacystis nidulans" by S. Minato and H. Werbin, Photochem. Photobiol. 15, 97 (1972).

"Separation of a Dialyzable Activator of the DNA-Photolyase from Baker's Yeast" by S. Minato and H. Werbin, Abstract Biophysical Society Meeting, February, 1972, Toronto, Canada.

TABLE I: SUMMARY OF PURIFICATION STEPS

Purification step	Total protein (mg)	Total activity (units x 10^{-3})	Specific activity (units/mg protein)	Yield %
Cell-free crude extract	760,500	6,900	9.2	100.0
Ammonium sulfate 33%-50%	97,800	5,200	53.2	76
Phosphocellulose	2,208	3,490	1,580	51
UV irradiated-DNA cellulose	0.80	574	647,000	8.3

Table I from Biochemistry 10 4503 (1971)

TABLE II. RECOVERY OF PHOTOLYASE ACTIVITY ON ADDITION OF YEAST EXTRACT* (ACTIVATOR)

Purification Step	Units of Enzyme Activity**	% Activity
Phosphocellulose	51,240	100
irr-DNA-cellulose (no activator added)	17,320	34
irr-DNA-cellulose (activator added)	40,250	79

* The preparation is discussed in the text on page 7.

** Reference 21. The assay system is discussed in the text on page 7.

TABLE III. ACTIVATION OF PHOTOLYASE BY REDUCING AGENTS AND YEAST EXTRACT* (ACTIVATOR)

Experiment 1

Additions to Photolyase**	Yeast Extract (pH 3.0)	
	-	+
	Enzyme units	
None	394	-
2-mercaptoethanol	544	902
crude yeast extract (pH 3.0)	-	706

Experiment 2

2-mercaptoethanol	1862	2638
glutathione	1503	2543
sodium bisulfite	856	1205

* The preparation is discussed in the text on page 7.

** The enzyme for the bioassay was prepared without mercaptoethanol.

TABLE IV: ASSAY OF POTENTIAL ACTIVATORS

Addition to photolyase*	Enzyme Activity (Units)
None	210
Folic acid	293
2 amino-4-hydroxy-pteridine	275
NADH	392
Pyridoxine	331
Pyridoxal-5-PO_4	277
Fraction B	318
Fraction D	397
Yeast Extract (pH 3.0)	569

* The assays were carried out in the presence of 2-mercaptoethanol. The concentrations of substances tested was 0.005 in M.

TABLE V: EFFECT OF YEAST EXTRACT* (ACTIVATOR) ON PHOTOLYASE ACTIVITY AT SEVERAL STAGES OF ITS PURIFICATION

Purification Step (Table I)	Yeast Extract - Enzyme Units B	Yeast Extract + Enzyme Units A	Enhancement factor $\frac{B}{A}$
Crude cell-free extract	930	1020	1.09
Ammonium sulfate	1329	2766	2.07
Phosphocellulose	3727	3717	1.0
irr-DNA-cellulose	7700	12,720	1.65

* The preparation and the assay are given in the text on page 7.

LEGENDS TO FIGURES

Figure 1: Excitation and fluorescence emission spectra (corrected) of DNA-photolyase in AC buffer containing 0.4 M KCl. For the excitation spectrum emission at 470 nm was recorded. The emission spectrum was excited with 380 nm radiation.

Figure 2: Chromatography on hydroxyl apatite. The photolyase fraction from affinity chromatography was applied to an hydroxyl apatite column (0.8 x 5 cm) and the column was run with a linear phosphate gradient established between 50 ml each of 0.05 M and 0.3 M phosphate buffer. pH 7.5 (-o-) enzyme activity; (-•-) absorbance at 280 nm; (-x-) fluorescence at 470 nm (excitation 380 nm).

Figure 3: Gel filtration of DNA-photolyase (purified by affinity chromatography) on a Sephadex G-100 column (1.2 x 30 cm). A: The column was equilibriated with 0.02 M phosphate buffer (pH 7.5) containing 0.4 mM EDTA, 10 mM 2-mercaptoethanol and 0.4 M KCl. The elution was performed with the same buffer and 1.5 ml fractions were collected. B: The column was equilibriated with 0.2 M 2-mercaptoethanol. The elution was performed first with KCl-free buffer then with 0.4 M KCl in the buffer, and 1.5 ml fractions were collected. (-•-), absorbance 280 nm; (-x-), DNA-photolyase activity; (-o-), fluorescence at 470 nm.

Figure 4: Excitation and fluorescence spectra (uncorrected) of substances B and D (see Figure 5) freed from photolyase, and two pteridines: folic acid and 2-amino-4-hydroxypteridine.

Figure 5: Descending Paper Chromatography. Substances were identified by near-UV illumination of the dried paper.

Figure 6 A: All the spectra are emission spectra.—filtrate obtained upon concentration of photolyase in a collodion membrane --- photolyase preparation after affinity chromatography.—•— photolyase after heating 10 min. at 52°.

Figure 6 B: —Photolyase mixed with either unirradiated or irradiated DNA and held at 5° for 1 hr. ---photolyase mixed with unirradiated DNA held at 37° for 45 min. —·—photolyase and irradiated DNA held at 37° for 45 min.

Figure 7: Activity of photolyase as a function of concentration of crude yeast extract containing activator. Calculation of enzyme units is described in (21).

Figure 8: Separation by paper chromatography of yeast fractions partially restoring activity to photolyase preparations purified through the affinity chromatography stage. The system used was n-butanol-acetic acid-water (4:1:5). Abbreviations used are Y = yellow, D = dark, B = blue, L = light. Sections of paper corresponding to R_f increments of 0.1 were cut out along the entire width of the paper, eluted with water, filtered, and assayed.

Figure 9: Separation by paper chromatography of algal fractions partially restoring activity to photolyase preparations purified through the affinity chromatography stage. The system used was n-butanol-acetic acid-water (4:1:5). Abbreviations used are Y = yellow, D = dark, B = blue, L = light. Sections of paper corresponding to R_f increments of 0.1 were cut out along the entire width of the paper, eluted with water, filtered, and assayed.

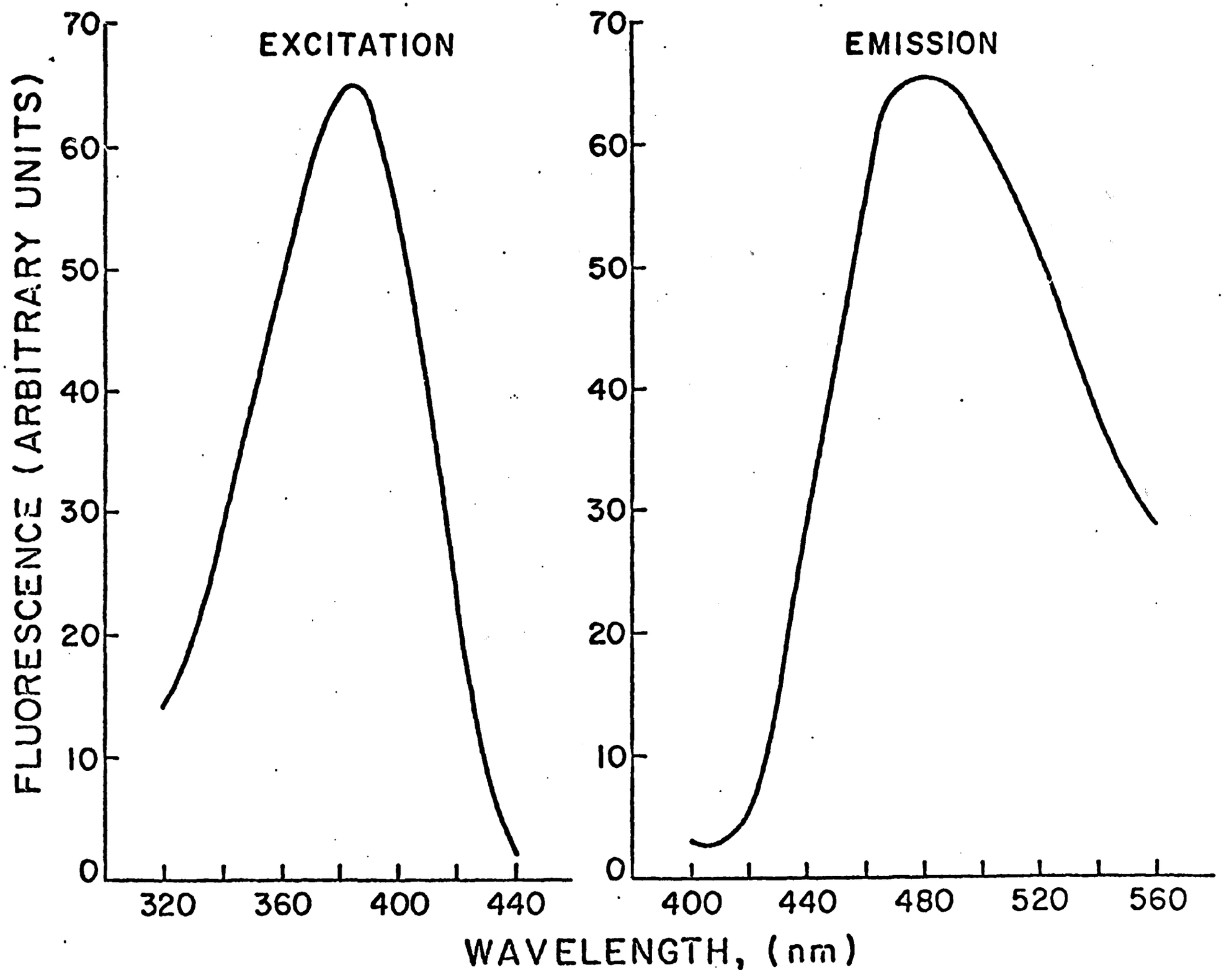
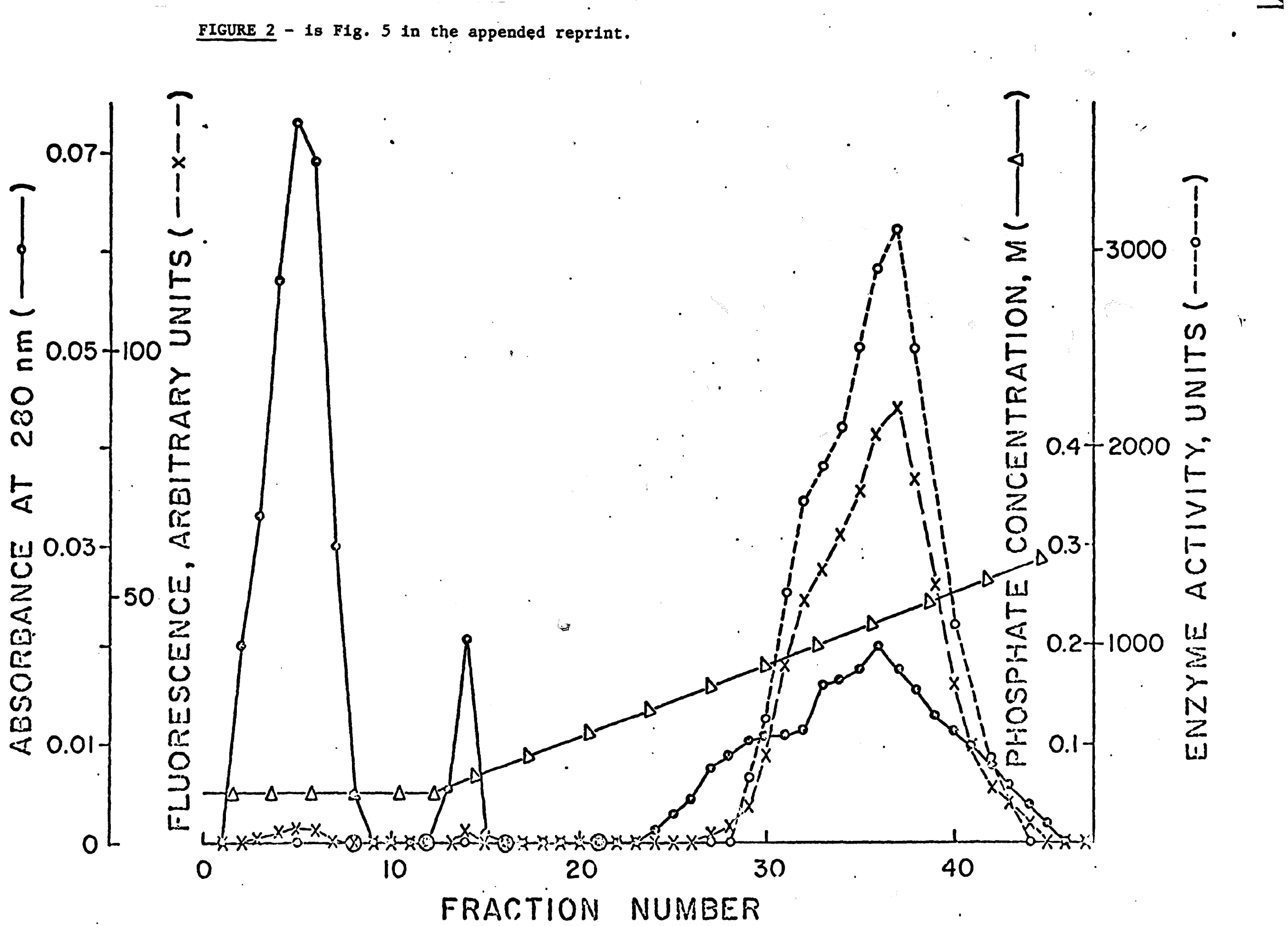


FIGURE 1 - is Fig. 7 in the appended reprint.

FIGURE 2 - is Fig. 5 in the appended reprint.



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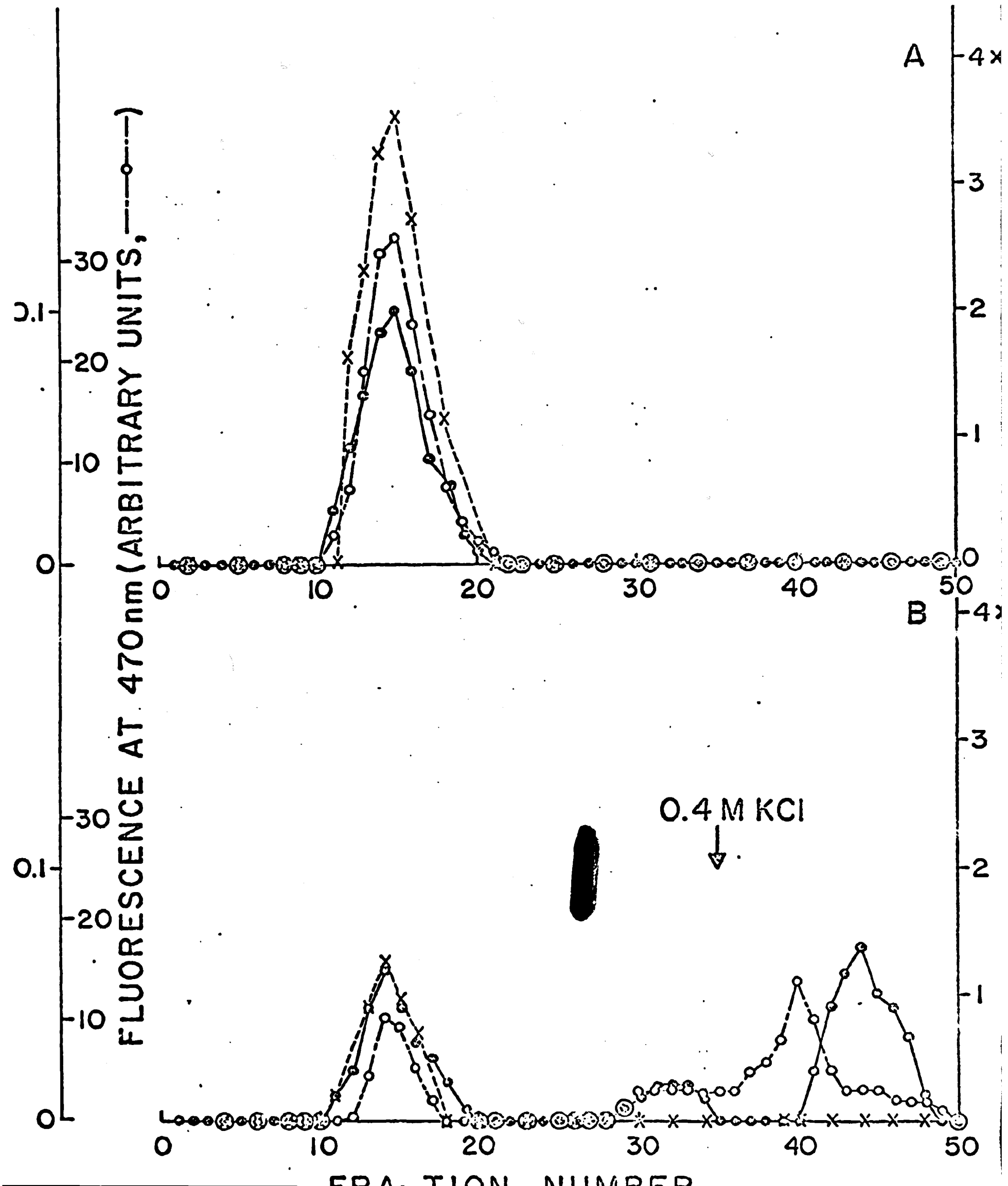
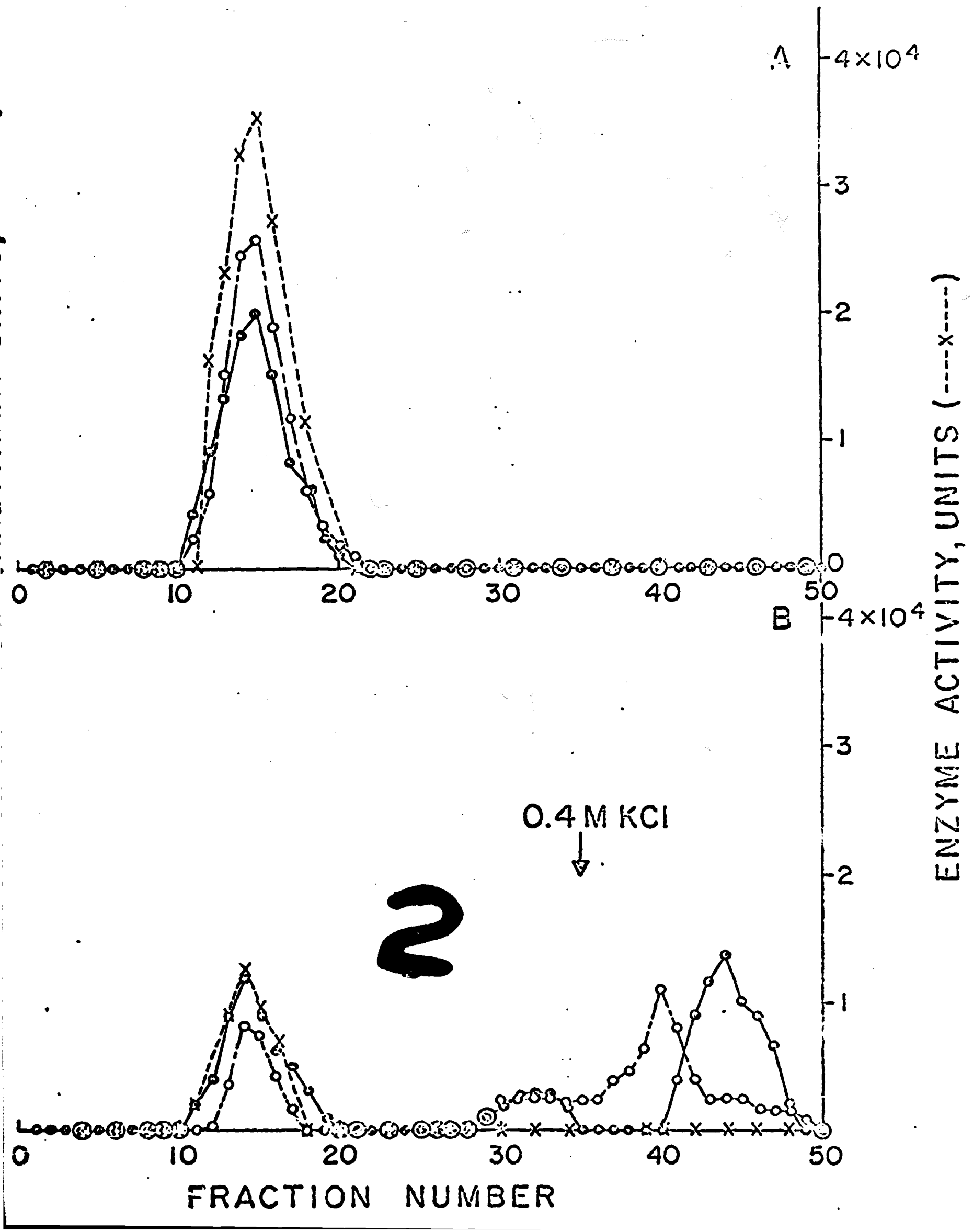


FIGURE 3 - corresponds to that in the appended reprint.



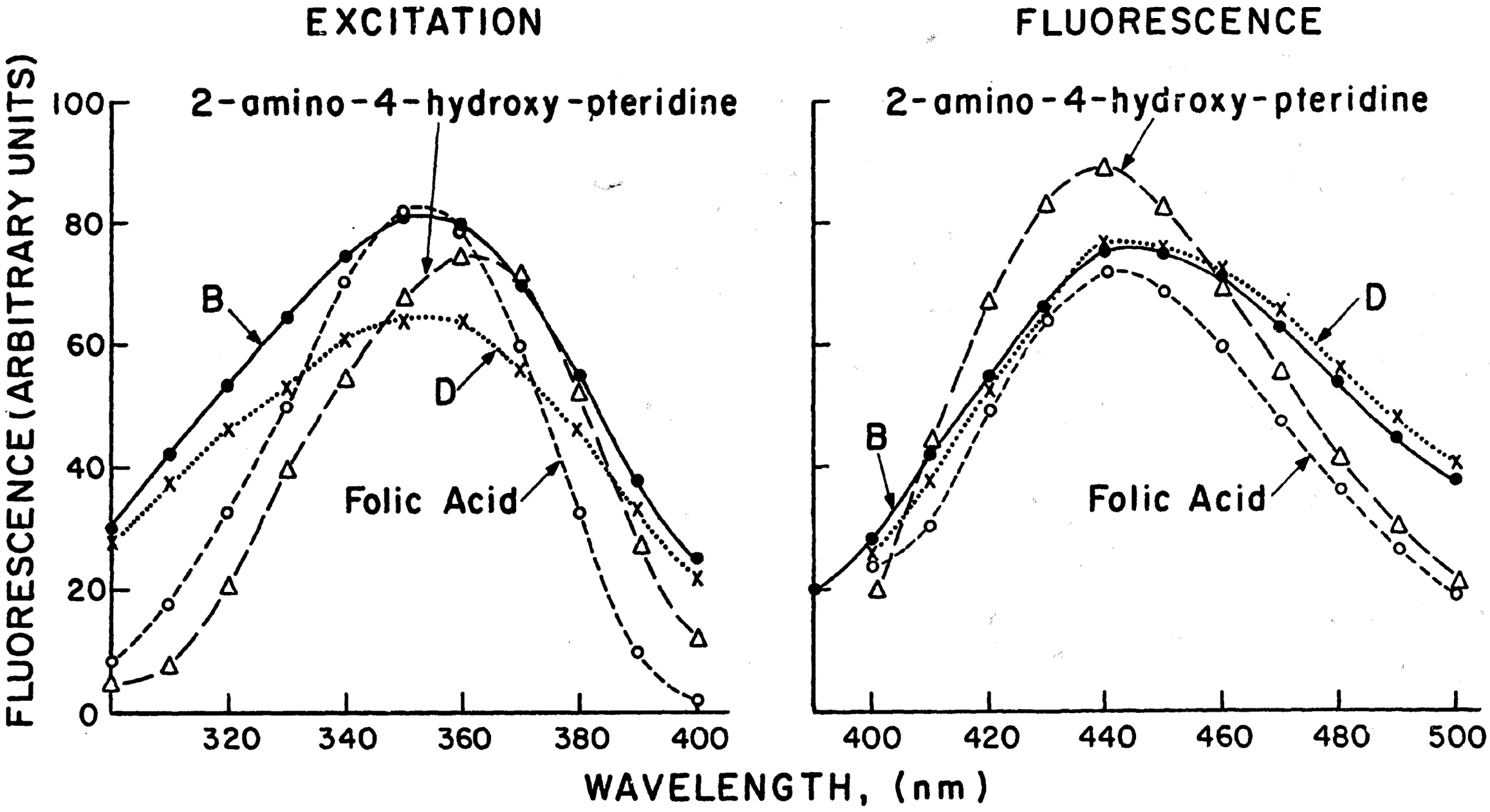

EXCITATION
FLUORESCENCE
2-amino-4-hydroxy-pteridine
2-amino-4-hydroxy-pteridine
FLUORESCENCE (ARBITRARY UNITS)
100
80
60
40
20
0
B
D
Folic Acid
B
D
Folic Acid
320
340
360
380
400
400
420
440
460
480
500
WAVELENGTH, (nm)

Figure 4

PAPER CHROMATOGRAPHY OF FILTRATE OBTAINED UPON CONCENTRATION OF PHOTOLYASE IN A COLLODION MEMBRANE

n-butanol-acetic acid-water

4 : 1 : 5

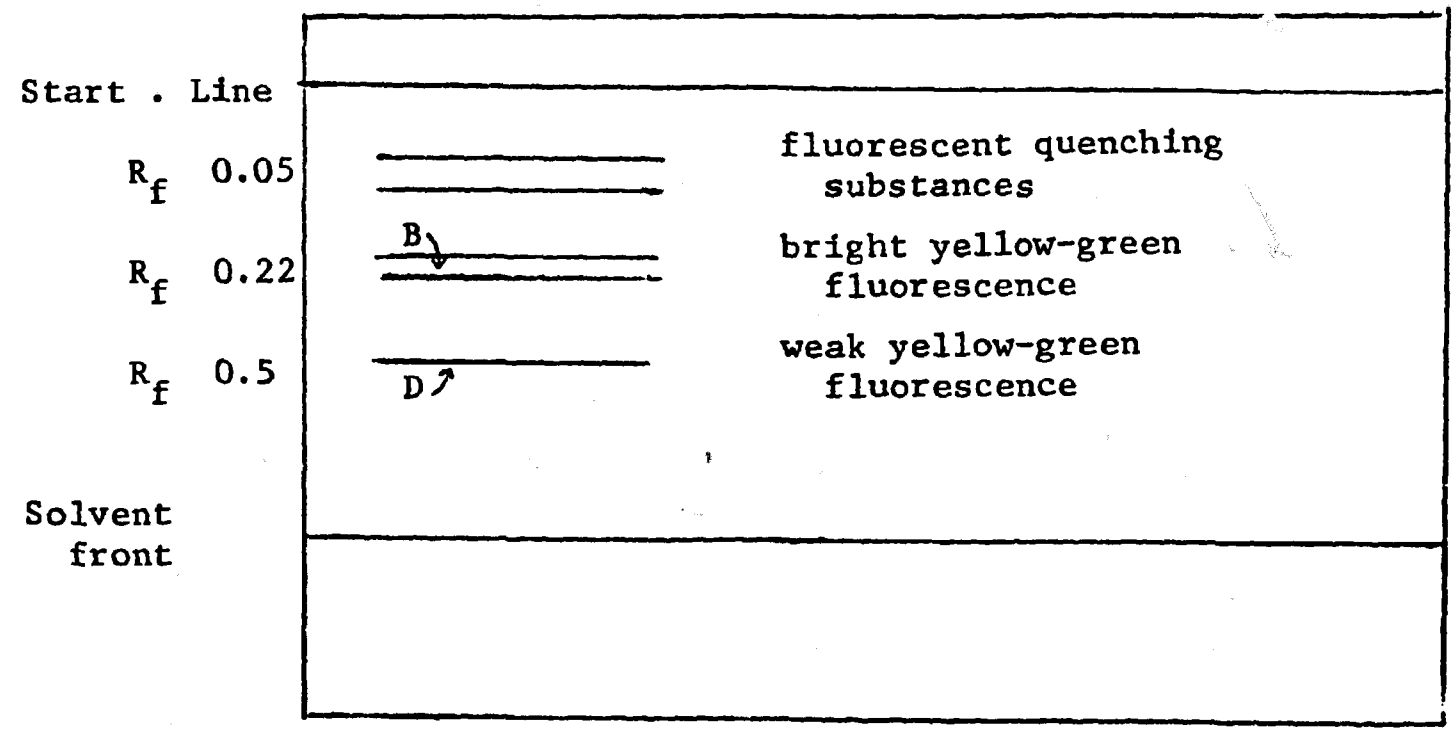


Figure 5

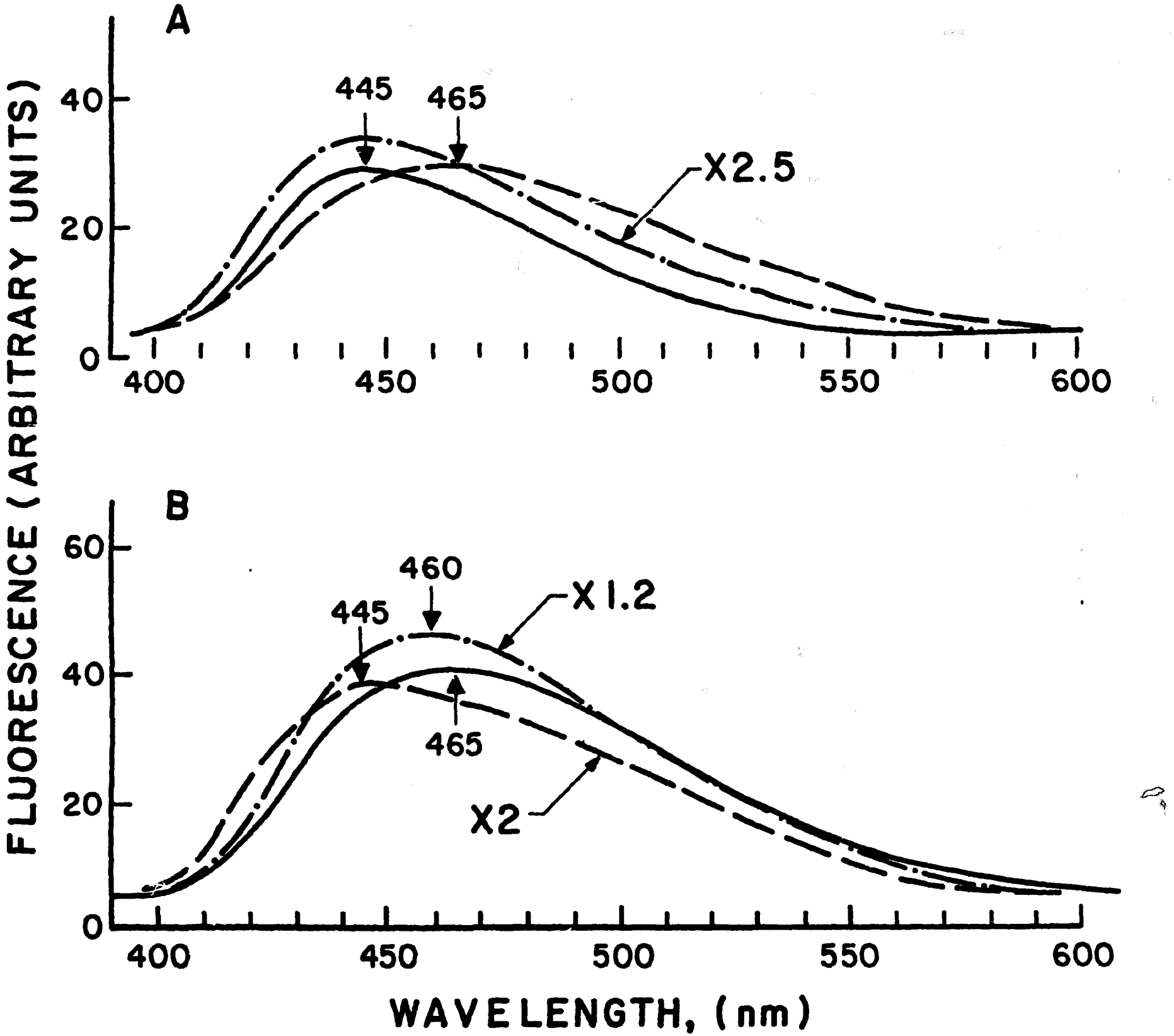

A
445
465
X2.5
40
20
0
400
450
500
550
600
B
460
445
X1.2
465
X2
60
40
20
0
400
450
500
550
600
FLUORESCENCE (ARBITRARY UNITS)
WAVELENGTH, (nm)

Figure 6

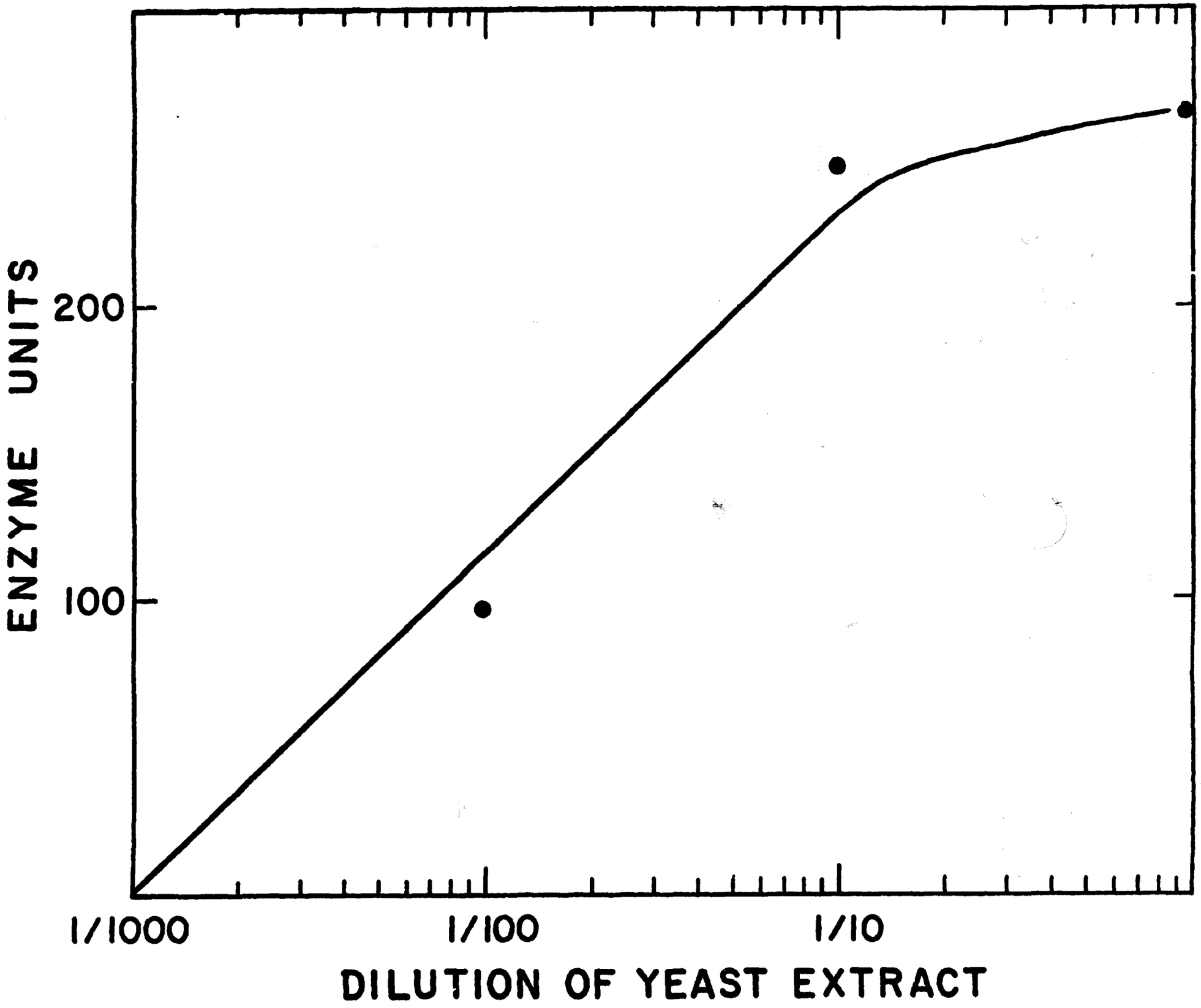

ENZYME UNITS
200
100
1/1000
1/100
1/10
DILUTION OF YEAST EXTRACT

Figure 7

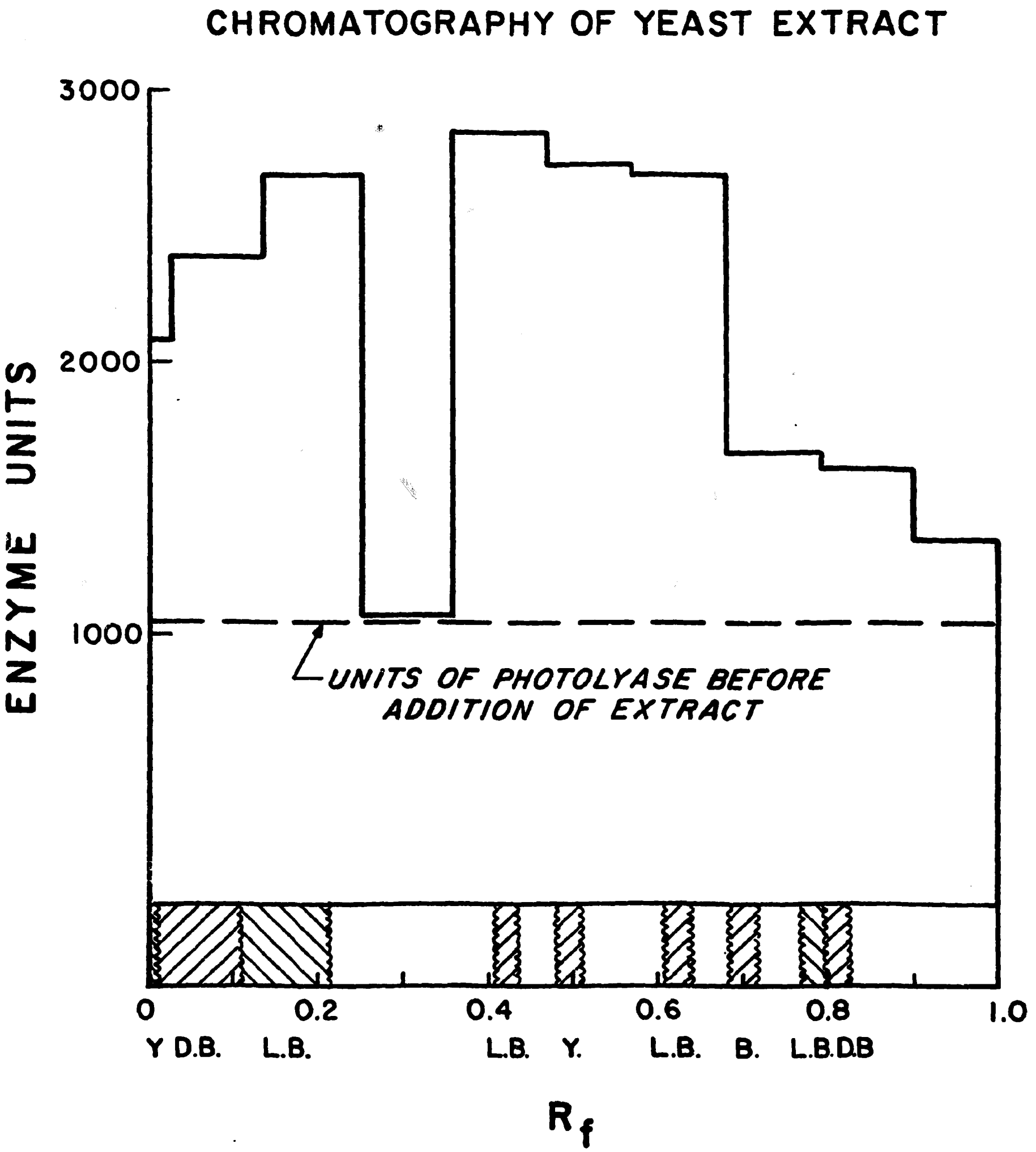

CHROMATOGRAPHY OF YEAST EXTRACT
ENZYME UNITS
3000
2000
1000
UNITS OF PHOTOLYASE BEFORE ADDITION OF EXTRACT
0
0.2
0.4
0.6
0.8
1.0
Y D.B.
L.B.
L.B.
Y.
L.B.
B.
L.B.D.B
R_f

Figure 8

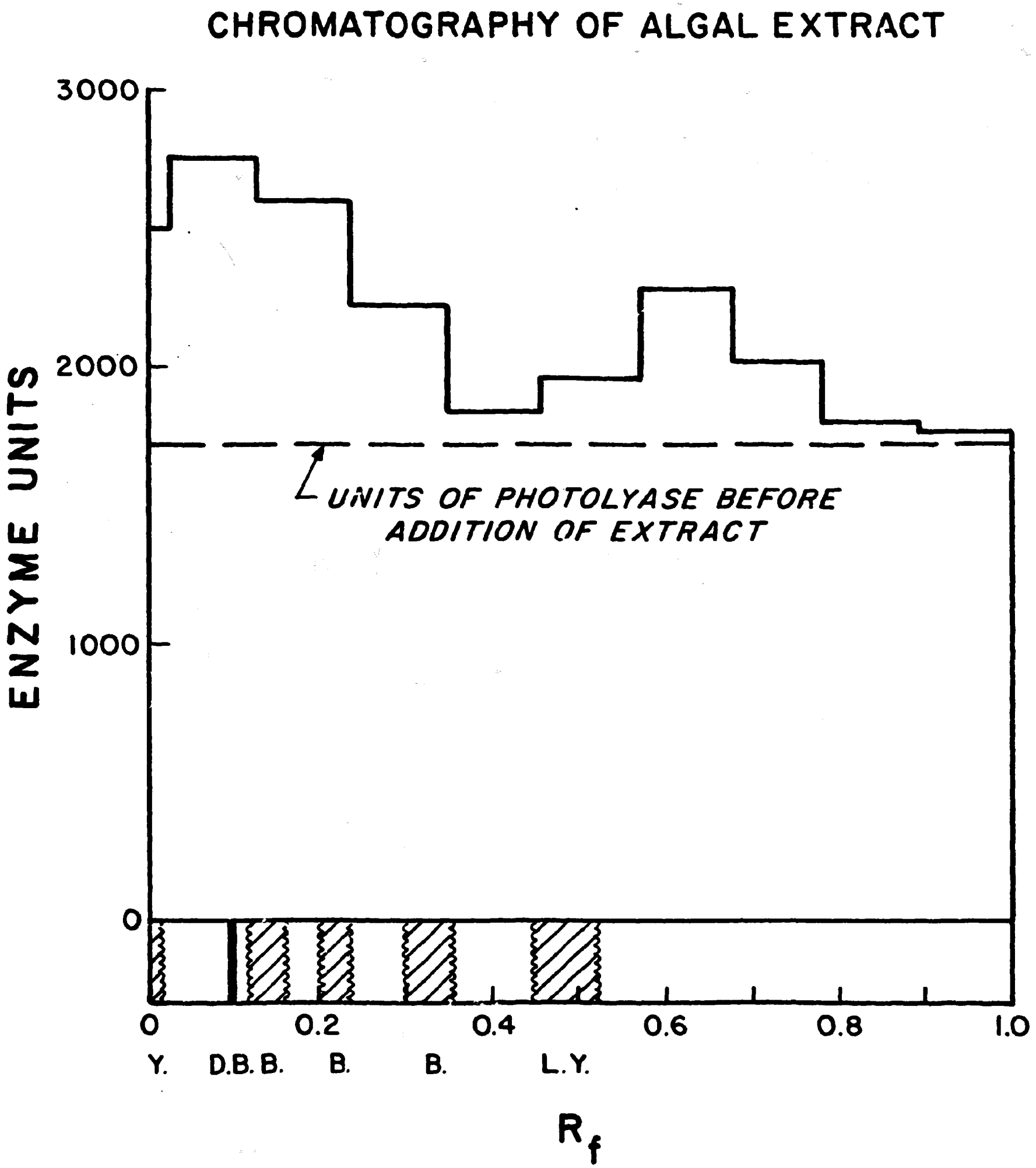

CHROMATOGRAPHY OF ALGAL EXTRACT
ENZYME UNITS
3000
2000
1000
0
UNITS OF PHOTOLYASE BEFORE ADDITION OF EXTRACT
0
0.2
0.4
0.6
0.8
1.0
Y.
D.B.
B.
B.
B.
L.Y.
R_f

Figure 9