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ELUCIDATION OF THE HYDROCARBON STRUCTURE IN AN ENZYME CATALYZED BENZO[a]PYRENE-POLY(G) COVALENT COMPLEX

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DISCLAIMER

ELUCIDATION OF THE HYDROCARBON STRUCTURE IN AN ENZYME CATALYZED BENZO[a]PYRENE-POLY(G) COVALENT COMPLEX*

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(corrected fluorescence spectra/epoxides/microsomes/benzo[a]pyrene derivatives/high pressure liquid chromatography)

Running Title: Structure of poly(G) bound benzo[a]pyrene

*This is the second paper of a series.

ABSTRACT

The carcinogen, benzo[a]pyrene, was covalently attached to poly(G) with liver microsomes from rats pre-treated with 3-methylcholanthrene. The complex was hydrolyzed with enzymes or base and products were isolated by Sephadex chromatography. Absorbance and fluorescence spectra of the products fit that of a red-shifted pyrene aromatic system and suggest that metabolism has occurred at the 7, 8, 9 and 10-positions of the hydrocarbon. Benzanthracene or chrysene fluorescence were not observed in these preparations. Benzo[a]pyrene derivatives were synthesized and purified by high pressure liquid chromatography. Dehydration of 7,8-dihydroxy-7,8,9,10-tetrahydrobenzo[a]pyrene resulted in the formation of small amounts of 7-oxo-7,8,9,10-tetrahydrobenzo[a]pyrene. A 7-keto species was also observed after similar treatment of the hydrocarbon-poly(G) hydrolysis products. Evidence of dehydration at the 9,10-positions was not observed. The hydrocarbon covalently bound to poly(G) is, therefore, a derivative of 7,8-dihydroxy-7,8,9,10-tetrahydrobenzo[a]pyrene with nucleic acid substitution at C-10 or 9.

INTRODUCTION

The relatively inert polycyclic aromatic hydrocarbon (PAH), benzo[a]-pyrene, when activated, undergoes covalent binding to nucleic acids and proteins (1-3). It has been shown that this binding can be catalyzed by liver microsomes from animals pretreated with inducers of aryl hydrocarbon hydroxylase activity (2). The structure of the complex formed between hydrocarbon and nucleic acid is unknown, in part due to low levels of binding catalyzed by the enzyme(s). Some investigators have turned to model systems since conditions are more clearly defined and yields of products are greater than with in vivo systems. These studies have approached the problem by activating carcinogens chemically or photochemically (4,5), or adding a reactive derivative of the hydrocarbon, e.g., an epoxide, directly to DNA (6). The structure of any complex produced in these model systems, however, has not yet been fully characterized.

Abbreviations: BaP, benzo[a]pyrene; 4(H)BaP, 7,8,9,10-tetrahydrobenzo[a]-pyrene; 7,8-diOH-4(H)BaP, 7,8-dihydroxy-7,8,9,10-tetrahydrobenzo[a]pyrene; 9,10-diOH-4(H)BaP, 9,10-dihydroxytetrahydro derivative of BaP; 7-OH-4(H)-BaP, 7-hydroxy-7,8,9,10-tetrahydrobenzo[a]pyrene; 9-OH-4(H)BaP and 10-OH-4(H)BaP, other monohydroxytetrahydro derivatives of BaP; 7-oxo-4(H)BaP, 7-oxo-7,8,9,10-tetrahydrobenzo[a]pyrene; 8-oxo-4(H)BaP, 9-oxo-4(H)BaP and 10-oxo-4(H)BaP, other keto tetrahydro derivatives of BaP; 7,8-2(H)-BaP, 7,8-dihydrobenzo[a]pyrene; 9,10-2(H)BaP, 9,10-dihydrobenzo[a]pyrene; 9,10-2(H)BaP 7,8-oxide, 9,10-dihydrobenzo[a]pyrene 7,8-oxide; BaP 4,5-oxide, benzo[a]pyrene 4,5-oxide; HPLC, high pressure liquid chromatography; tosic acid, p-toluene sulfonic acid.

Boyland (7) first proposed the intermediacy of epoxides in binding of PAH to nucleic acids, and subsequently epoxides have been shown to be metabolites of BaP (8,9). Borgen et al. (10) reported that 7,8-dihydroxy-7,8-dihydrobenzo[a]pyrene, when activated with microsomes, bound to DNA 10-fold greater than the parent hydrocarbon. This led Sims et al. (11) to the suggestion of a diol-epoxide intermediate in binding. Comparison of chromatographic profiles on lipophilic Sephadex between hydrolysis products of BaP-DNA formed in vivo with that of DNA treated with 7,8-dihydroxy-7,8-dihydrobenzo[a]pyrene 9,10-oxide revealed a common peak which suggested the involvement of a diol-epoxide (11,12). However, any structural designation based on R_f values is questionable. A more direct study reported the fluorescence emission spectrum of BaP-DNA formed in vivo which was consistent with that of a red-shifted pyrene nucleus, indicating that metabolism occurred at the 7,8,9,10-positions of the hydrocarbon (13). Another fluroescence study of carcinogen-nucleic acid complexes has also appeared recently (14), but definitive structural information was not obtained. In the above studies fluorescence spectra were not corrected for wavelength dependent distortions due to source monochromators and detector.

In this study, corrected fluorescence spectra were made of a BaP-poly(G) complex and compared to that of known hydrocarbon derivatives. This approach has allowed us to describe for the first time definitive evidence for the structure of BaP after enzyme catalyzed covalent binding to a nucleic acid.

MATERIALS AND METHODS

BaP-poly(G) complex: Formation, hydrolysis and isolation of hydrolysis products of a covalently linked [G-^{3}H]BaP-poly(G) complex has been described (15). Enzyme- or NaOH-hydrolyzed samples of the BaP-poly(G) complex were analyzed after isolation by LH20 column chromatography.

Spectroscopy: Fluorescence was recorded on a xenon source spectrophotofluorometer (Perkin-Elmer, model MPF-3, Norwald, Conn.). The instrument automatically records corrected excitation and emission spectra (i.e., wavelength variations due to source, monochromators and detectors are eliminated) by signal ratioing between sample and reference (rhodamine B) channels. Anthracene was used to test the correction. Spectra were recorded under the following conditions: 1) band pass 10 nm (slit width 1.29 mm) for both excitation and emission scans; 2) path lengths, excitation 10 mm and emission 3 mm; 3) absorbance <0.05 at all excitation wavelengths; 4) self-absorption effects not observed; 5) temp, 25°C; 6) samples equilibrated with air; and 7) solvents, re-distilled ethanol and/or distilled deionized water. Raman bands and Rayleigh scatter were subtracted. Bound PAH fluorescence was not observed in standard binding assays when they were carried out in the absence of either NADPH, microsomes, poly(G) or BaP.

Ultraviolet and infrared spectra were determined on a Cary model 118 (Cary/Varian, Palo Alto, CA) and Perkin-Elmer model 257 (Norwalk, Conn.) spectrophotometers, respectively. Mass spectra were recorded at 70 eV on a DuPont model 21-492-1 mass spectrometer, equipped with a model 21-094B data system.

High pressure liquid chromatography: HPLC was carried out with a Varian model 8500 liquid chromatograph (Palo Alto, CA) on a reverse phase Micropak CH-10 column (25 cm x 2 mm). The column was eluted at room temperature

with methanol-water mixtures. The flow rate was 60 ml/hr, and the effluent monitored by ultraviolet absorbance. The per cent methanol-water used was optimized for each compound. Spectroquality methanol used in these operations was obtained from Matheson Coleman and Bell (Norwood, OH).

Synthesis: 7-oxo-4(H)BaP was prepared according to Fieser and Novello (16). The following derivatives were prepared as described by Sims (17): 7,8-2(H), 9,10-2(H), cis-7,8-diOH-4(H), cis-9,10-diOH-4(H), 7-OH-4(H), 10-OH-4(H) and 9-oxo-4(H)BaP. 4(H)BaP was made by Wolff-Kishner reduction of the 7-oxo-4(H) derivative. Epoxidation of the 9,10-dihydro derivative with m-chloroperbenzoic acid in CH_2Cl_2 resulted in 7,8-epoxy-9,10-2(H)BaP. 8-oxo-4(H)BaP was a product of acid-catalyzed rearrangement of the 7,8-epoxy derivative. The reaction was carried out in benzene with p-toluene sulfonic acid (tosic acid) as catalyst. Oxidation of 10-OH-4(H)BaP with dimethyl sulfoxide-acetic anhydride (18) yielded 10-oxo-4(H)BaP. All compounds gave satisfactory ultraviolet, infrared and mass spectra.

The fluorescence spectra of 7,8-2(H) and 4(H)BaP were satisfactory and both chromatographed as a single peak by HPLC, while 9,10-2(H)BaP contained a few per cent BaP that did not interfere with fluorescence measurements. The remainder of the compounds were purified by HPLC prior to analysis.

Dehydration reactions: Monohydroxy and dihydroxy derivatives of 4(H)BaP were dehydrated by refluxing the hydrocarbon in the presence of excess tosic acid in dry ethanol for 16-20 hrs. Solvent was evaporated and samples were taken up in 0.6 ml 33% ethanol-water and applied to a LH20 column equilibrated with water. Tosic acid was eluted with water, the dehydrated BaP-poly(G) complex with ethanol and hydrocarbons with benzene. Alternatively, dehydrations were carried out in benzene, and

the tosic acid was removed by extraction with buffer. Solvents were evaporated in vacuo and samples taken up in ethanol or 50% ethanol-water for fluorescence assay.

RESULTS

Absorbance and fluorescence spectra of the BaP-poly(G) complex: Spectral analysis of the BaP-poly(G) complex indicated that a component with an absorbance and fluorescence spectra similar to pyrene was present. This is in agreement with the report by Daudel et al.(13) and indicates that metabolism of the 7,8,9 and 10-positions of the hydrocarbon occurred during enzyme activated binding. The absorbance spectrum of the BaP-poly(G) complex is shown in Fig. 1c, along with that of 4(H)BaP (Fig. 1a) and cis-7,8-diol-4(H)BaP (Fig. 1b). The fluorescence spectra of 4(H)BaP and the enzyme and NaOH hydrolyzed products of the complex are presented in Figs. 2a, 2b and 2c, respectively. The spectral relationship between the carcinogen-nucleic acid complexes and 4(H)BaP derivatives are evident, but it becomes clearer after HCl treatment of the complex to remove the ribosyl residue (15).

Acid treatment of the BaP-poly(G) complex: When a sample of the NaOH hydrolyzed product was heated with HCl in a boiling water bath, an apparent change in peak ratios and reduction of noise resulted in a more clearly defined fluorescence spectra (Fig. 3b). The spectrum now clearly shows its identity as a 4(H)BaP derivative. The same result was obtained from an enzyme hydrolyzed sample.

The emission spectrum of the complex is remarkably similar to 10-OH-4(H)BaP (Fig. 3a), which exhibits a characteristic shape different from that observed in the other 4(H) derivatives. An oxygen at C-10 of the hydrocarbon reduces the relative intensity of the first emission peak.

Dehydration of 7-OH-4(H)BaP: The fluorescence spectra of 7-OH-4(H) and 9,10-2(H)BaP are presented in Figs. 4a and 4b. Conjugation of the 7,8-double bond with the pyrene aromatic system, as expected, causes a red shift in the emission spectrum, and peaks occur at 364 and 382 nm in its excitation spectrum. The fluorescence spectrum of a sample of 7-OH-4(H)BaP which was dehydrated with tosic acid is shown in Fig. 4c. Peaks at 364 and 383 nm in the excitation spectrum, and the similarity between emission spectra of the acid-treated sample and the 2(H) derivative, indicated that elimination of water from the 7,8-positions had occurred. Similar treatment of 10-OH-4(H)BaP resulted in formation of 7,8-2(H)BaP. The fluorescence emission of 7,8-2(H)BaP is also red shifted with peaks at 399, 419 and a shoulder at 445 nm (spectrum not shown).

Dehydration of 4(H)BaP diols and HPLC analysis: The fluorescence excitation and emission spectra of cis-7,8-diol-4(H), 7-oxo-4(H) and 8-oxo-4(H)BaP are presented in Figs. 5a, 5b and 7a, respectively. After dehydration of cis-7,8-diol-4(H)BaP, small amounts (<5%) of 7-oxo-4(H)BaP were detected as evidenced by a peak at 478 nm in its emission spectrum (Fig. 5c). The same result was obtained by starting with the trans-7,8-diol (unpublished results).

HPLC of the hydration products of cis-7,8-diol-4(H)BaP resulted in 8-oxo-4(H)BaP along with a small amount (<5%) of 7-oxo isomer, as evidenced by co-chromatography and fluorescence (Fig. 6a).

Dehydration of cis-9,10-diol-4(H)BaP was carried out with tosic acid, and the products analyzed by HPLC. In this case the 9-oxo isomer was formed but the 10-ketone was not detected by HPLC retention time or fluorescence. Since dehydration of the cis-9,10-diol results in

formation of ketone only at C-9, a 9,10-diol would not be detected in the BaP-poly(G) complex, because fluorescence of 9-oxo is identical to the other 4(H)BaP derivatives.

The selective dehydration of 9,10-diol-4(H)BaP to give the 9-ketone only and of 7,8-diol to give mainly the 8-ketone (with some 7-) probably results from resonance stabilization of intermediate benzylic carbonium ions at the 7- and 10-positions. The 10-position of 4(H)BaP is adjacent to the relatively active 1-position of the pyrene residue where electrophilic substitution is known to occur. This probably results in greater resonance stabilization at 10- than at 7-, which is adjacent to an inactive position of pyrene. This accounts for dehydration of the 9,10-diol yielding exclusively the 9-ketone, while the 7,8-diol forms, in addition to 8-, detectable amounts of the 7-isomer.

Dehydration of BaP-poly(G) complex: Aliquots of the base hydrolyzed products from the BaP-poly(G) complex after dehydration and fluorescence assay revealed the presence of a 7-oxo-4(H)BaP species (Fig. 6b). The emission spectrum with peaks at 382, 400 and 418 nm are indicative of a 4(H)BaP derivative.

Fluorescence spectrum of 10-oxo-4(H)BaP: The fluorescence spectrum of 10-oxo-4(H)BaP (Fig. 7b) is easily distinguished from that of the 7-oxo or other 4(H) derivatives.

DISCUSSION

The corrected excitation and emission spectra of the enzyme and NaOH hydrolyzed BaP-poly(G) complex showed that the hydrocarbon had been metabolized to a 7,8,9,10-tetrahydro derivative. Since hydroxylated derivatives are primary and secondary products of PAH metabolism, their presence in

the BaP-poly(G) complex was investigated by dehydration of the sample with tosic acid and fluorescence assay of the products. If hydroxyls occur at the 7,8,9 or 10-positions of the hydrocarbon, dehydration would result in a number of possibilities: 1) monohydroxy at 7 or 10 would yield 9,10-2(H) or 7,8-2(H)BaP, respectively; 2) dihydroxy at either 7,8 or 9,10 would result in 8 or 9-oxo-4(H)BaP; and 3) a tri- or tetrahydroxy derivative would be expected to result in a fully aromatized BaP chromophore.

Since only pmole quantities of the BaP-poly(G) complex were available, dehydrations were carried out with excess tosic acid. The above cases were first tested on model compounds to confirm the feasibility of this approach on the BaP-poly(G) complex. Dehydration of 7-OH and 10-OH-4(H)BaP under these conditions gave the expected products, 9-10-2(H) and 7,8-2(H)BaP, respectively, as evidenced by fluorescence spectroscopy.

Dehydration of 7,8-diol-4(H)BaP has been reported to give mainly 8-oxo-4(H)BaP (17). Since fluorescence of the 7,8-diol was found to be identical to that of the 8-oxo derivative, this method followed the dehydration reaction only because detectable amounts (<5%) of the 7-oxo isomer were formed, and its spectrum is easily recognizable.

Confirmation of 7-oxo-4(H)BaP formation by 7,8-diol dehydration was shown by HPLC purification of the products. A number of prominent peaks were observed in the HPLC trace of the dehydrated sample, one of which was identified as the 7-ketone by its distinct fluorescence and co-chromatography.

Fluorescence analysis of the base hydrolyzed BaP-poly(G) complex suggested the presence of only one luminescent species. The emission spectrum of the complex was the same irrespective of the wavelength of exciting light and, conversely, the excitation spectrum did not vary with emission setting. However, the possibility that more than one hydrocarbon structure was present

with identical fluorescent spectra cannot be ruled out. Under the standard binding assay conditions used here, fluorescence measurements indicate that most of the carcinogen is intercalated in poly(G) (D. Warshawsky, personal communication). Under these conditions it is possible that the 7-, 8-, 9- and 10-positions of the hydrocarbon are the only available sites of attack by the microsomal enzyme(s), and would explain the observation that only one fluorescent product was bound to poly(G).

Dehydration of the complex resulted in formation of a 7-oxo-4(H)BaP. Since control experiments indicated that this occurred with OH at C-7 and C-8 of BaP, the hydrocarbon covalently linked to poly(G) must be a derivative of 7,8-diol-4(H)BaP, a result which further suggests that the nucleic acid is substituted at the 9- or 10-position of the carcinogen. This provides the first direct evidence on the chemical structure of enzymatically catalyzed PAH-nucleic acid complexes.

Since there is a growing body of evidence that epoxides are intermediates in binding (6,11,19), our results support the intermediacy of 7,8-diol-4(H)BaP 9,10-oxide. Opening of the oxide could leave oxygen at C-9 or C-10, but

is expected at the 9-position (cf. ref. 19, footnote 15). Thus, the covalent carbon linkage to the poly(G) would also be expected at the 10-position. Dehydration of this derivative would result in the formation of a fully aromatized BaP chromophore, but fluorescence of such a structure was not observed. Two possible explanations for these results are: (a) dehydration at 9,10- is prevented by interaction with the nucleic acid at C-10, or (b) the 9- (or 10-OH) may be involved in formation of a cyclic derivative with the nucleic acid.

PAH intercalation coupled with formation of a covalent bond to guanine, like that of N-acetylaminofluorene, probably results in a distortion of the nucleic acid tertiary structure (20,21). The biological consequence of

this would involve mutagenicity and possibly carcinogenicity. The fact that a derivative of 7,8-diol-4(H)BaP is the main product of enzyme catalyzed covalent binding of BaP to poly(G), coupled with the recent observation that the 7,8-diol 9,10-oxide is orders of magnitude more mutagenic than BaP 4,5-oxide (19), suggests that this compound may be the ultimate carcinogenic form of BaP.

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REFERENCES

1. Brookes, P. and Lawley, P. D. (1964), Nature 202, 781-784.
2. Gelboin, H. V. (1969), Cancer Res. 29, 1272-1276.
3. Wang, I. Y., Rassmussen, R. E. and Crocker, T. T. (1972), Biochem. Biophys. Res. Commun. 49, 1142-1149.
4. Umans, R. S., Lesko, S. A. and Ts'o, P.O.P. (1969), Nature 221, 763-764.
5. Ts'o, P.O.P. and Lu, P. (1964), Proc. Nat. Acad. Sci. USA 51, 272-280.
6. Blobstein, S. H., Weinstein, I. B., Grunberger, D., Weisgras, J. and Harvey, R. G. (1975), Biochem. 14, 3451-3458.
7. Boyland, E. (1950), Biochem. Soc. Symp. 5, 40-54.
8. Selkirk, J. K., Huberman, E. and Heidelberger, C. (1971), Biochem. Biophys. Res. Commun. 43, 1010-1016.
9. Selkirk, J. K., Croy, R. G. and Gelboin, H. V. (1975), Arch. Biochem. Biophys. 168, 322-326.
10. Borgen, A., Darvey, H., Castagnoli, N., Crocker, T. T., Rasmussen, R. E. and Wang, I. Y. (1973), J. Med. Chem. 16, 502-505.
11. Sims, P., Grover, P. L., Swaisland, A., Pal, K. and Hewer, A. (1974), Nature 252, 326-328.
12. Swaisland, A. J., Hewer, A., Pal, K., Keysell, G. R., Booth, J., Grover, P. L. and Sims, P. (1974), FEBS Letters 47, 34-38.
13. Daudel, P., Duquesne, M., Vigny, P., Grover, P. L. and Sims, P. (1975), FEBS Letters 57, 250-253.
14. Kodama, M. and Nagata, C. (1975), Biochem. 14, 4645-4650.

15. Meehan, T. D., Warshawsky, D. and Calvin, M. (1975), Proc. Nat. Acad. Sci. USA, in previous issue.

16. Fieser, L. F. and Novello, F. C. (1940), J. Am. Chem. Soc. 62, 1855-1859.

17. Sims, P. (1968), J. Chem. Soc.(C), 32-34.

18. Albright, J. P. and Goldman, L. (1967), J. Am. Chem. Soc. 89, 2416-2423.

19. Yagi, H., Hernandez, O. and Jerina, D. M. (1975), J. Am. Chem. Soc. 97, 6881-6883.

20. Weinstein, I. B. and Grunberger, D. (1974), in Chemical Carcinogenesis, ed. Ts'o, P.O.P. and DiPaolo, J. A. (Marcel Decker, New York), Part A, pp. 217-235.

21. Fuchs, R. and Daune, M. (1973), FEBS Letters 34, 295-298.

FIGURE LEGENDS

Fig. 1. Absorbance spectra of a) 4(H)BaP, 1.7 x 10^{-5}M, in ethanol; b) 7,8-diol-4(H)BaP, 2.95 x 10^{-5}M, in ethanol and c) enzyme hydrolyzed BaP-poly(G) products in 50% ethanol-water.

Fig. 2. Fluorescence spectra of a) 4(H)BaP, 3.40 x 10^{-7}M, in ethanol (em 3.33 x ex scale); b) enzyme hydrolyzed BaP-poly(G) products (em 10 x ex scale) and c) NaOH hydrolyzed BaP-poly(G) products (em 10 x ex scale).

Fig. 3. Fluorescence spectra of a) 10-OH-4(H)BaP, 3.24 x 10^{-7}M, in ethanol (em 10 x ex scale) and b) NaOH hydrolyzed BaP-poly(G) products, treated with HCl as described previously (15), in 50% ethanol-water (em 3.33 x ex scale).

Fig. 4. Fluorescence spectra of a) 7-OH-4(H)BaP, 3.34 x 10^{-7}M, in ethanol (em 3.33 x ex scale); b) 9,10-2(H)BaP, 5.9 x 10^{-7}M, in ethanol (em 3.33 x ex scale) and c) tosic acid dehydrated 7-OH-4(H)BaP in ethanol (em and ex scales the same), see MATERIALS AND METHODS.

Fig. 5. Fluorescence spectra of a) 7,8-diol-4(H)BaP, 1.67 x 10^{-7}M, in ethanol (em 3.33 x ex scale); b) 7-oxo-4(H)BaP, 1.85 x 10^{-6}M, in ethanol (em 10 x ex scale) and c) emission spectrum of tosic acid dehydrated 7,8-diol-4(H)BaP (-------) after LH20 chromatography, as described under MATERIALS AND METHODS.

Fig. 6. Fluorescence spectra of a) 7-oxo-4(H)BaP isolated by HPLC after dehydration of 7,8-diol-4(H)BaP (em 3.33 x ex scale) and b) NaOH hydrolyzed BaP-poly(G) complex after tosic acid dehydration (em x ex scale). Dehydration procedure is described under MATERIALS AND METHODS.

Fig. 7. Fluorescence spectra of a) 8-oxo-4(H)BaP, 1.40 x 10^{-6}M, in ethanol and b) 10-oxo-4(H)BaP, 3.61 x 10^{-6}M, in ethanol, em 3.33 x ex scale in a) and b).

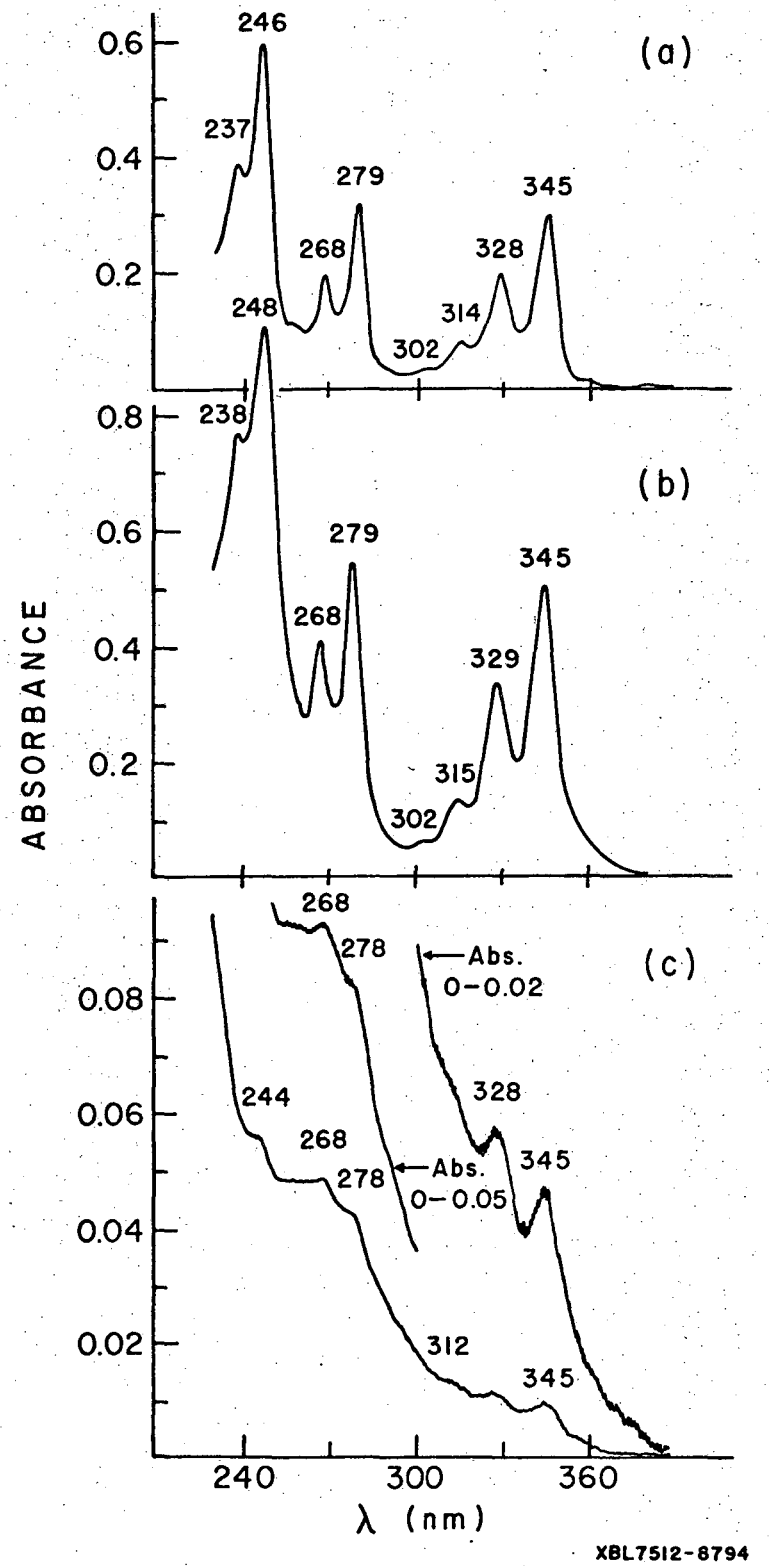
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Fig. 1

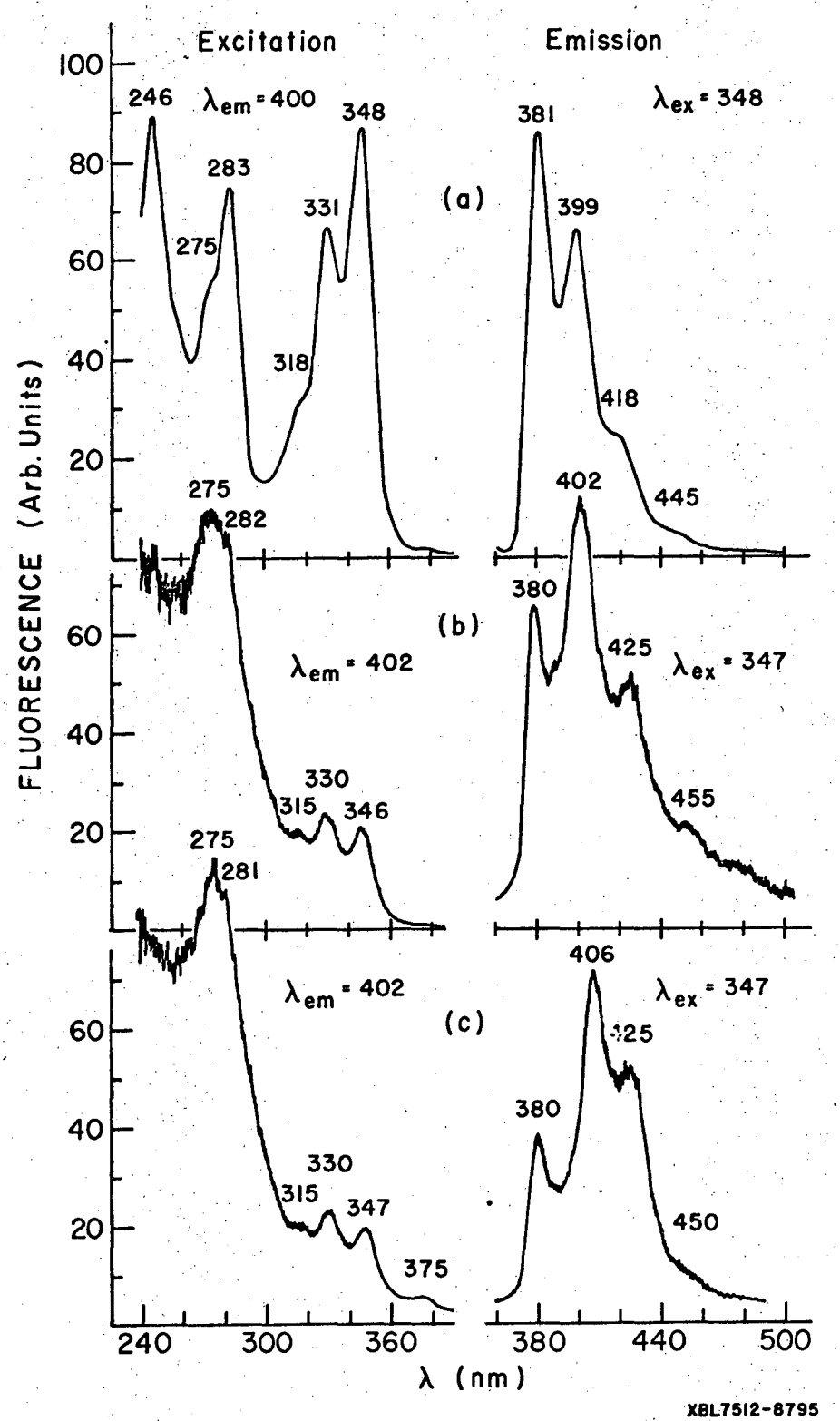

Fig. 2

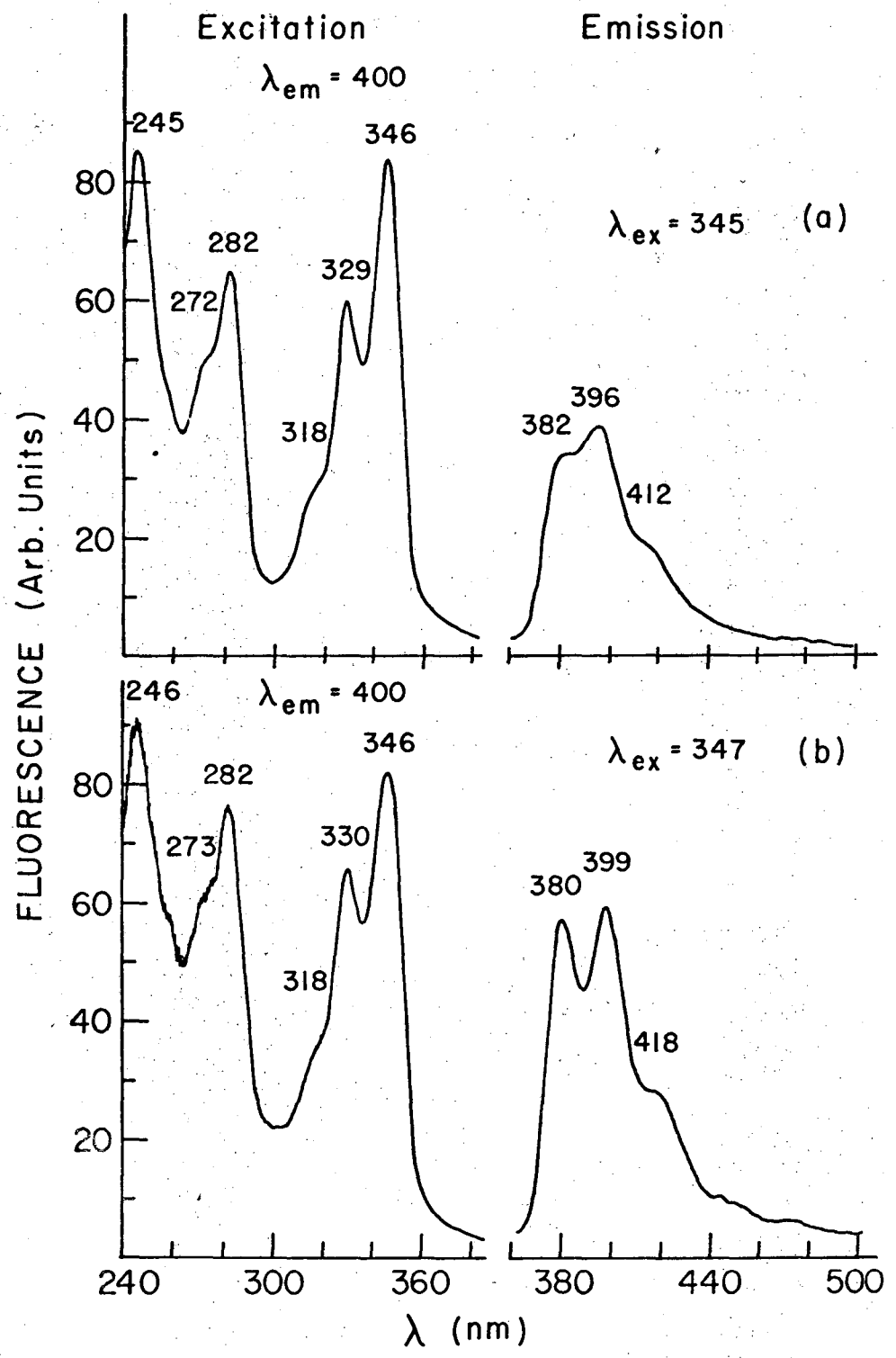
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Fig. 3

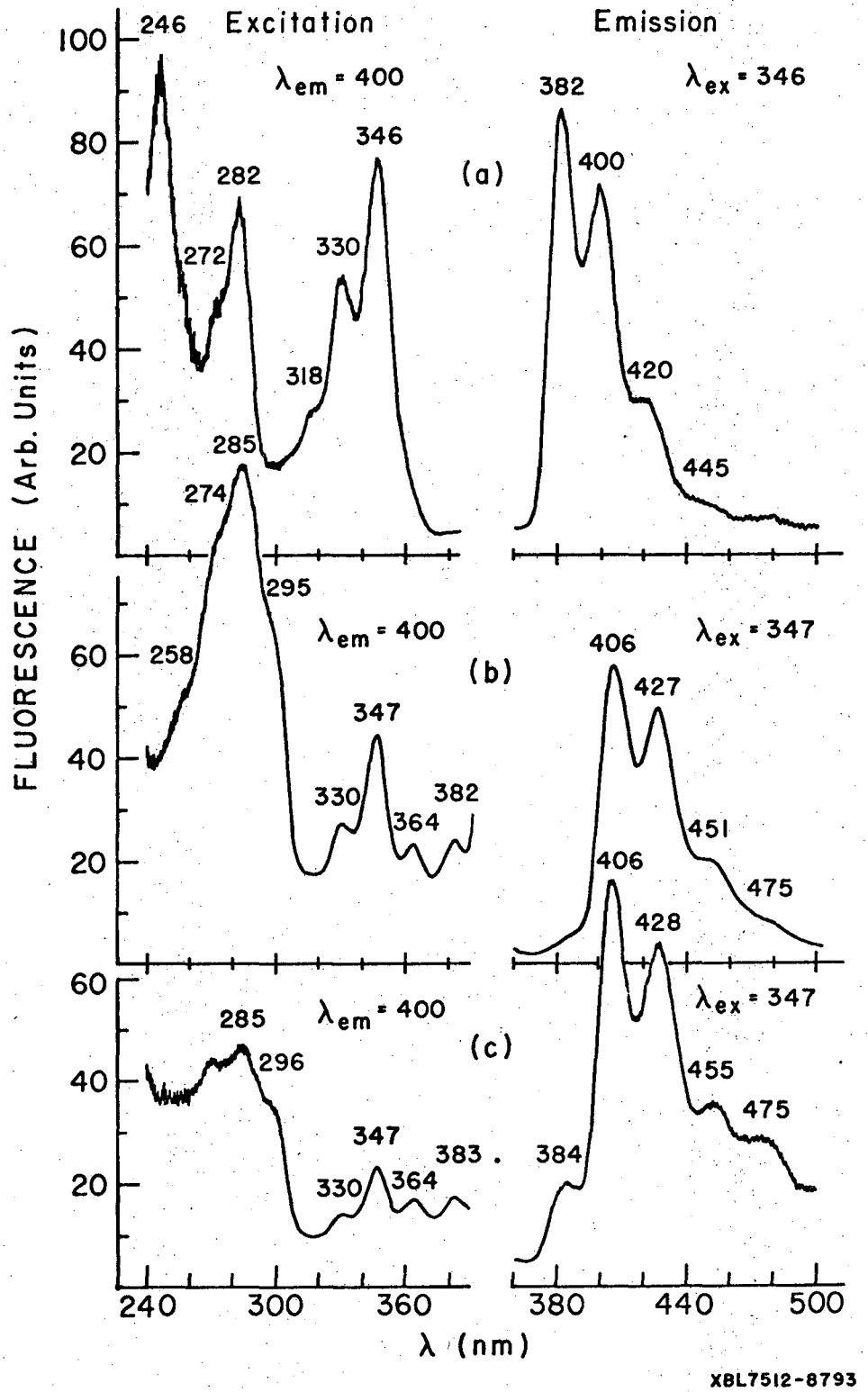
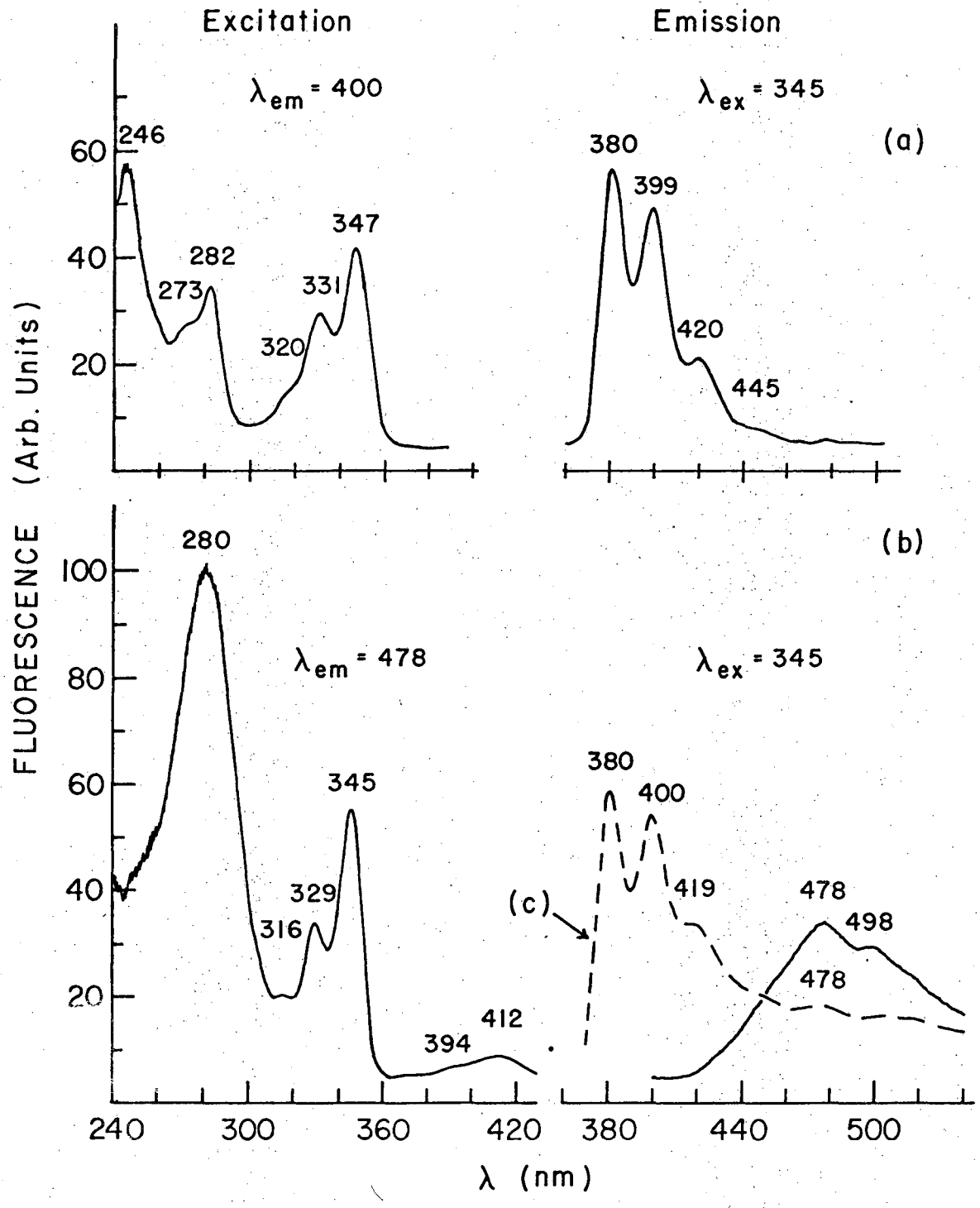


Fig. 4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λ (nm)
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Fig. 5

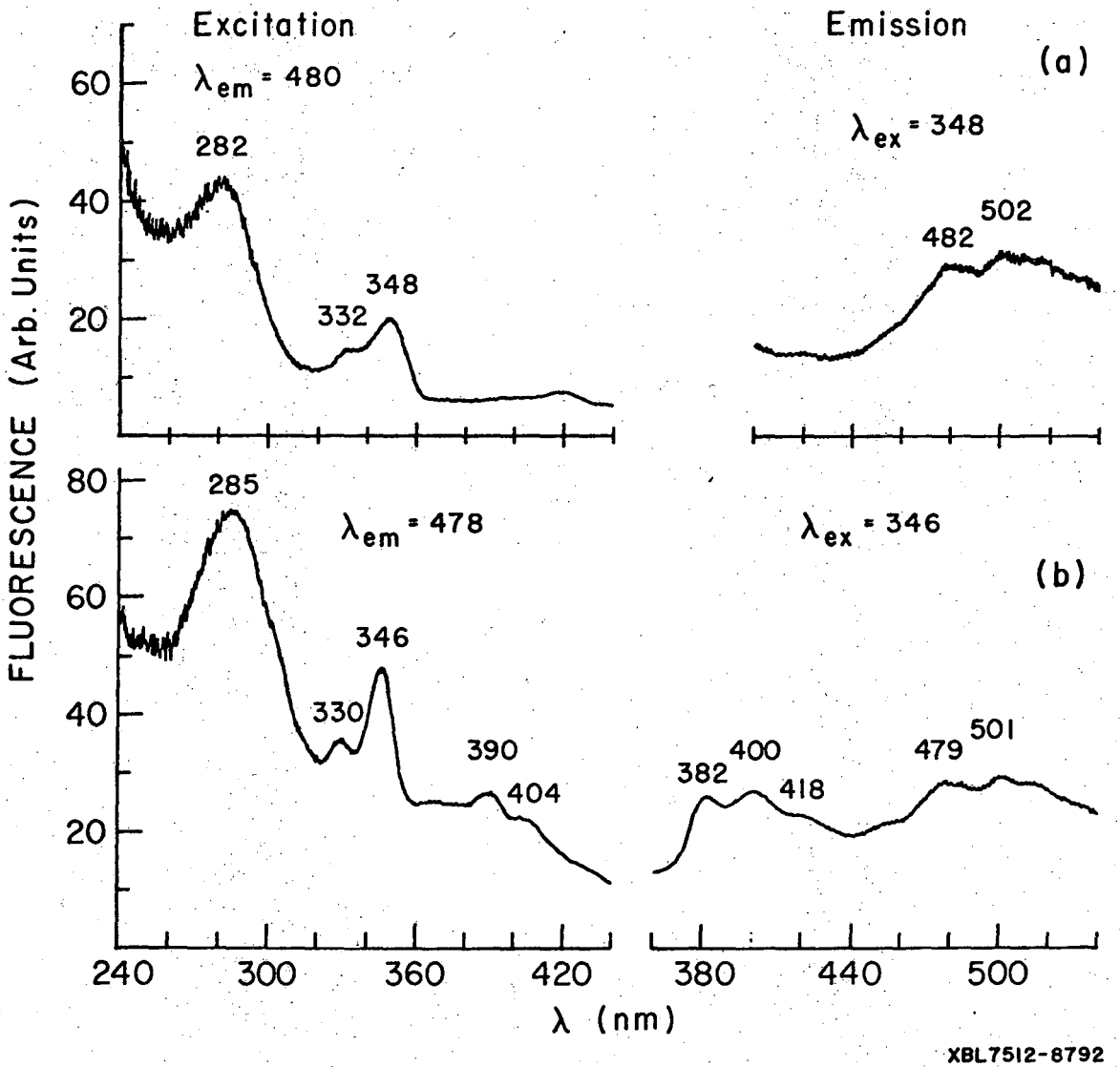
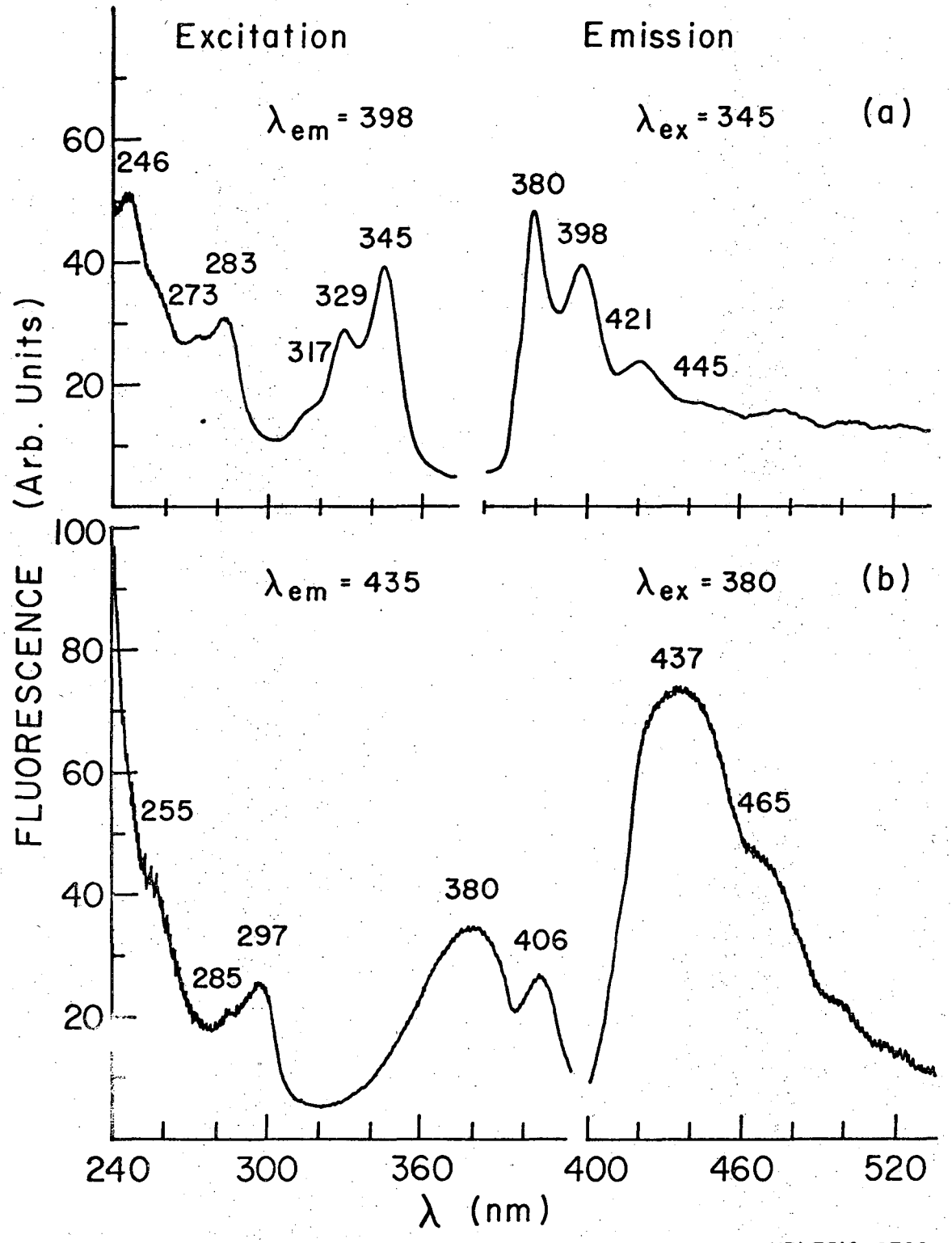


Fig. 6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(b)
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λ (nm)
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300
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400
460
520
XBL7512-8788

Fig. 7

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