## Title

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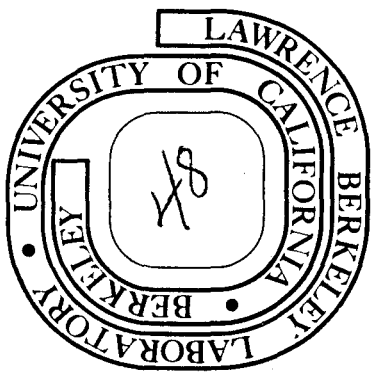

## DISCLAIMER

# THE TOXICITY AND SPECTROSCOPIC PROPERTIES OF NEUROTOXINS FROM *BUNGARUS MULTICINCTUS* VENOM*

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Running Title: Toxins from *Bungarus multicinctus* Venom

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Abstract--The venom of the taiwanese banded krait Bungarus multicinctus was fractionated into 13 components by ion exchange chromatography on CM-Sephadex. Eleven fractions were toxic. Toxicity and time to death of these toxins were studied in detail, and a correlation between time to death at high doses and the type of neurotoxicity (pre- or postsynaptic) is suggested. A tentative identification of the neurotoxins studied in this work with those described by LEE et al. (1972) is presented. Fluorescence spectra of these proteins in native and denatured states, as well as acid hydrolysates revealed the presence of tryptophan and tyrosine residues in all of them. Differences in several spectral parameters suggest that fluorescence spectra could be used to distinguish these neurotoxins among themselves, as well as to indicate denaturation.

## INTRODUCTION

The recent increase in interest in neurotoxins has been motivated by the fact that they proved to be useful tools for the study of synaptic organization and function. Purification and proper characterization of individual neurotoxins are essential prior to their use for such research. Studies of the acetylcholine receptor have focused the attention of many scientists on α-bungarotoxin (α-Bgt)* (O'BRIEN et al., 1972; SALVATERRA and MOORE, 1973; VOGEL et al., 1972; BERG et al., 1972; ETEROVIC and BENNETT, 1974). α-Bgt has been purified and studied in detail by several laboratories (CLARK et al., 1972; MEBS et al., 1972). Other toxins of Bungarus multicinctus have received very little attention, except for a few studies on β-Bgt (CHANG and LEE, 1963; LEE and CHANG, 1966; CHANG et al., 1973). Chromatograms reported by several laboratories using apparently the same fractionation procedure were different (MEBS et al., 1972; CLARK et al., 1972; BOSMANN, 1972). The identification of particular neurotoxins obtained in different laboratories was not attempted yet, except for α-Bgt and β-Bgt. During the course of this work, a paper by LEE et al. (1972) appeared describing the fractionation of B. multicinctus venom into 11 components, and some characteristics of the fractions. In the present work the venom of B. multicinctus was fractionated into 13 components. A detailed study of toxicity and the fluorescence spectra of 9 neurotoxins are presented. A tentative identification of these toxins with those described by LEE et al. (1972) is made on the basis of their toxicity characteristics.

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*Abbreviations: Bgt: bungarotoxin; DDT: dithiothreitol; Fr.: fraction.

## MATERIALS AND METHODS

Lyophilized venom of B. multicinctus was obtained from the Miami Serpentarium and was stored at -20°C until used; CM-Sephadex C-50 was obtained from Pharmacia, CM-Cellulose from Whatman, and UM-2 Diaflo membranes from Amicon. Other reagents used were analytical reagent, or electrophoretic grade obtained from usual commercial sources. All solutions were prepared in distilled water.

### Fractionation of Bungarus multicinctus venom

Lyophilized venom (355-1500 mg) was dissolved in 15 ml of 0.05 M ammonium acetate buffer, pH 5.0, and centrifuged at 39,000 x g for 15 min at 4°C. A small precipitate was discarded and the supernatant was fractionated on a CM-Sephadex C-50 column (2.5 x 85 cm). After loading the sample on the column, the column was washed with 500 ml of 0.05 M ammonium acetate buffer, pH 5.0. At the end of the wash, the absorbance at 280 nm of the effluent was negligible. The remaining material was eluted into 12 peaks with a linear gradient of ammonium acetate, 0.05 M (pH 5.0) to 1 M (pH 7.0) (LEE et al., 1968). Additional 1 M ammonium acetate, pH 7, was used after gradient solutions were exhausted to elute fractions 11 through 14.

Pure α-Bgt was obtained by rechromatography of Fraction 3 on a CM-Cellulose column of 2.5 x 24 cm. The toxin was eluted with a linear gradient of ammonium acetate, 0.05 M (pH 5.0), to 1 M (pH 6.5) (total volume 1.2 l), at a flow rate of 12 ml/hr. Desalting of fractions was achieved by ultrafiltration through UM-2 Diaflo membranes. Protein concentration was estimated by multiplying the absorbance at 280 nm by the factor 0.85 mg/absorbance unit, which had been obtained for crude venom.

This rather arbitrary conversion factor is a convenient one at present. When the absolute molar extinction coefficients are established, the toxicity values can be easily corrected as necessary. The purity of fractions was determined by electrophoresis on sodium dodecylsulfate-polyacrylamide gels. Fractions 2 and 4+5 were highly heterogeneous. All the remaining fractions had a fast migrating, low molecular weight band as their major constituent, which comprised 85-95% of the staining material.

Determination of toxicity

Toxicity of fractions after subcutaneous or intraperitoneal injections was assayed as described by CHANG and LEE (1963). Swiss (CD-1, 40 g) or BALB/cJ (25 g) male mice were used. No strain difference in toxicity/g body weight was noted. Time to death for individual mice was recorded. $LD_{50}$, $LD_{100}$ and slope functions* were calculated by the method of LITCHFIELD and WILCOXON (1949). Typically 40 to 60 mice were used for the estimation of toxicity of each fraction.

To determine the time to death at doses around $LD_{100}$, time data from mice injected with a dose of $LD_{100} \pm 20\%$ were selected. In addition, the dose intervals were so chosen that the mean dose values were within 2% of $LD_{100}$ for all fractions.

Fluorometric measurements

Fluorometric measurements were made on a Hitachi-Perkin Elmer MPF 2A recording fluorescence spectrometer. The photomultiplier was a Hamamatsu R 106 with a relatively constant response from 200 to 400 nm, and therefore the uncorrected spectra are presented. Native proteins were diluted for

*Slope function was defined by LITCHFIELD and WILCOXON (1949) as 1/2 $(LD_{84}/LD_{50} + LD_{50}/LD_{16})$; the lethal doses are obtained from semi-logarithmic plots of the data which results in straight lines.

analysis with distilled water. "Urea-treated" samples were dissolved in 8 M urea. Reduced samples were made up in 8 M urea plus 30 mM dithiothreitol (DTT).

Ultraviolet scans from 230 to 350 nm were made on a Cary 118C double beam spectrophotometer.

## RESULTS

### Fractionation of Bungarus multicinctus venom

Figure 1 illustrates the typical chromatographic pattern obtained in three runs using two different lots of lyophilized crude B. multicinctus venom. Thirteen components were separated by this single chromatography. The approximate yield of each fraction is shown in TABLE 1. About 10% of the material applied to the CM-Sephadex column was recovered in the initial buffer washing. This fraction was not identified, but it is believed that it corresponds to the guanosine peak (Peak I) of LEE et al. (1972). The designation "Fraction 4+5" was used since some evidence for separation between two different components was obtained in one of the three runs.

### Toxicity of fractions

The toxicity for the fractions obtained in run 2 (Fig. 1) are shown in TABLES 2 and 3*. Similar toxicity values ($\pm$ 20%) were obtained for the two other chromatographic separations. The slopes of mortality versus dose curves were very steep for all the fractions. This fact is reflected in the values of slope functions, which varied from 1.05 to 1.20, with Fr. 4 having the lowest and Fr. 11 the highest value. The ratios of $LD_{50}$

*We believe the toxicity of Fraction 4+5 may be in large part due to contamination from the shoulder of Fraction 3.

after intraperitoneal and subcutaneous injections varied from 0.5 to 0.9 for α-Bgt and Fractions 4 through 9, while for all the remaining fractions the values were between 0.14 and 0.2 (compare TABLES 2 and 3). These values suggest that the first group (α-Bgt and Fractions 4 through 9) has higher permeability than the second (Fractions 10 through 14).

As expected, time to death was dose dependent. To facilitate the comparison between fractions the doses were normalized to the respective $LD_{100}$. Two examples of time to death versus dose curves are shown in Figures 2a and 2b, which reveal interesting differences between the neurotoxins. At high doses, α-Bgt and Fractions 7 and 8 killed in less than 25 min. In contrast, death never occurred before 70 min for Fractions 9 through 13 in the equivalent dose range (Fig. 2 and TABLE 2, last two columns). On this basis, the first group of toxins, which included α-Bgt, was classified as α-type, and the second group, which included β-Bgt, was classified as β-type neurotoxins. The assignment of Fractions 4, 5 and 6 to α-type is less certain due to the limited amount of material isolated which precluded data for high doses. Fr. 14 behaved as β-type toxin, that is, its time to death at 14 x $LD_{100}$ was 123 min. At higher doses, however, time to death was reduced up to 15 min, probably due to the presence of an impurity.

α-Type toxins always killed within 5 hr, or the mice would recover. The symptoms were paralysis and respiratory difficulties, as was described for other α-type neurotoxins (KARLSSON et al., 1972). Fraction 9 had a very slow action, especially at low doses where death could occur as late as the third to fifth day after injection. Drying of the cornea was observed with this fraction much more often than with the others.

The remaining β-type fractions killed within 12 hr at any lethal dose. Hyperexcitability was noted approximately 1 hr after administration of β-type toxins; paralysis developed only in the final stages.

At doses below approximately 1.5 x $LD_{100}$ time to death changed very abruptly with dose, and a considerable dispersion was observed in this range. The differences in times to death at doses around $LD_{100}$ are given in TABLE 2, 4th column. Fraction 9 was significantly slower than any other fraction, followed by Fr. 14. The remaining β-toxins (Fractions 10 through 13) did not differ significantly from α-Bgt, but were slower than the α-toxins 4 through 8.

$LD_{50}$ for i.p. injections, the type of neurotoxicity, and partially the chromatographic pattern, were used to tentatively identify our fractions with those described by LEE et al. (1972) (TABLE 3). Fraction 3 (α-Bgt) was further identified by its action on frog muscle, and the similarity of amino acid composition with published values (LEE, 1972).

Time to death was successfully used for titration of botulinum and cobra toxins (BOROFF and FLECK, 1966; COOPER and REICH, 1972), since the logarithmic plot of time versus dose data resulted in a straight line. This method is also useful for the neurotoxins described here, although the range of linearity is very restricted for some of them (Fig. 3a and 3b). The dispersion shown in Fig. 2 arises from the fact that the data were obtained from three different preparations for every toxin, and during the time interval of over a year. They are presented that way to show the reproducibility of the results. For quantitative purposes, however, more precision can be obtained if the standard curves are run on the same day, as shown in Fig. 3.

Preliminary evidence was obtained for the persistence of the toxins' action *in vivo*. Most mice that survived a first injection of toxin in the range of $LD_0$ to $LD_{90}$ died after a second dose smaller than $LD_0$ was administered one day later. It was estimated from percentage mortality and time to death after the second injection, that at least 50% of the previous day's dose remained in the mouse when Fractions 3 through 11 were administered. Values for Fractions 12, 13 and 14 were less than 35%. This type of experiments was not done with Fr. 9 due to its extremely long-lasting action after a single injection.

Spectroscopic properties

The ultraviolet absorption spectra of all the neurotoxins were typical for proteins containing aromatic amino acids; the two absorption maxima were at 278-279 nm and below 240 nm, while the minimum varied from 250 to 256 nm for different fractions. The fluorescence spectra had emission maxima in the 340 nm region, which is typical for proteins containing tryptophan. The position of the maximum varied from 342 nm for α-Bgt and Fr. 7 to 335 nm for Fr. 9 (TABLE 4). In general, α-type toxins had maxima at slightly longer wavelengths than β-type, with the exception of Fractions 13 and 14. Fluorescence intensity varied greatly among the toxins, with α-Bgt having the lowest and Fr. 9 the highest value (Fig. 4, corrections made for the difference in slit width). Since the absorbancies of all solutions were similar, the variations in fluorescence intensity reflect differences in quantum yield among the toxins. Tyrosine fluorescence, which has an emission maximum at 303 nm, was not apparent in native toxins. The shoulders seen in this region for Fractions 10, 12 and 13 are artifacts due to scattered light (Raman bands),

since their position was shifted with that of the excitation band (spectra not shown). However, tyrosine presence was revealed in all the toxins by the fluorescent spectra of their acid hydrolysates. Pure tyrosine spectra were obtained, since tryptophan was destroyed during acid hydrolysis (Fig. 5). The apparent lack of tyrosine fluorescence in native toxins can be due to quenching by adjacent groups, to energy transfer, or this emission can be overshadowed by tryptophan's stronger fluorescence. If some tyrosine fluorescence is present but hidden by the tryptophan band, it will change the shape of the spectrum increasing the intensity at 303 nm (tyrosine maximum). However, tyrosine fluorescence should be absent in spectra excited at 290 nm where tryptophan absorption predominates over that of tyrosine. Therefore, the ratio of intensity at 303 nm to 340 nm should decrease when the excitation band is shifted from 265 nm to 290 nm. Such comparison was done for the neurotoxic fractions, and tyrosine fluorescence was revealed in all fractions except Fractions 7 and 8 (TABLE 5). The ratio of intensities at 380 nm and the maximum was used as a control for possible artifacts.

Partial denaturation of the toxins by 8 M urea increased the fluorescence intensity for most of the fractions, but the magnitude of the increase was not the same in two experiments. This treatment did not affect appreciably the position of the maximum or the shape of fluorescence spectra (TABLE 4 and Fig. 4). On the contrary, upon reduction of disulfide bridges by dithiothreitol in urea, a pronounced shift of the maximum towards 350 nm was observed. Shoulders in the 300-310 nm region became apparent for Fractions 8 through 14 (TABLE 4 and Fig. 4). These shoulders were not artifacts of scattered light, since their position was not affected by the displacement of the excitation band from 280 to 260 nm (spectra not shown). Since such pronounced changes in fluorescent spectra must reflect changes in protein

structure, and probably in physiological activity, we decided to measure the toxicity of the neurotoxins in urea and urea plus DTT. The results are summarized in TABLE 6. All the "urea-treated" toxins tested retained 70 to 90% of their activity, as estimated from times to death, but no toxic effects were registered even at high doses of "reduced" fractions. A shift in tryptophan maximum toward 350 nm was also observed for a dilute solution (15 µg/ml) of native Fr. 13 after a week at 6°C. A ten times more concentrated solution did not show any spectral changes under the same conditions. Since it is known that very dilute protein solutions are less stable than more concentrated solutions (this was also observed during our toxicity studies), this seems to be another example of the relationship between the position of tryptophan maximum and the integrity of these toxins.

Excitation spectra from 240 to 330 nm were monitored at the emission wavelength of 340 nm. Excitation maxima at 284 and 290 nm were seen for all fractions, corresponding to tryptophan absorption bands. No evidence was obtained in these spectra for a maximum at 278 nm, which would indicate energy transfer from tyrosine to tryptophan. Urea or urea plus DTT did not affect appreciably these spectra.

## DISCUSSION

In spite of the remarkable progress achieved in the fractionation of snake venoms, considerable confusion still exists in this field. Neurotoxins obtained in different laboratories are designated by different names and the identity can be fully established only after the laborious process of primary structure determination is completed. The venom of Bungarus multicinctus is a good example of this state of affairs. Three

laboratories have reported previously chromatographic patterns obtained after fractionation of the crude venom on CM-Sephadex by gradient elution with ammonium acetate buffers (MEBS et al., 1972; CLARK et al., 1972; BOSMANN, 1972). The chromatograms are by no means identical, which is not surprising for such a complex mixture. Minor differences in the procedure are probably responsible for the better resolution of the chromatography reported here. Description of components other than α-Bgt was reported only in the work of LEE et al. (1972) and in the present work. From toxicity and elution characteristics most of the neurotoxins isolated by LEE et al. can be related to neurotoxins described in this work (TABLE 3). Our Fr. 11 seems to be missing in the venom fractionated by LEE et al. The venoms used were from different sources, and this may be a reason for the discrepancy. Similar differences were observed for other snake venoms (KARLSSON et al., 1971).

The presence of α- and β-type neurotoxins in this venom deserve some comments. Terms α- and β-Bgt originated from the position of the respective bands after electrophoretic fractionation of the venom (CHANG and LEE, 1973). At present these terms are associated with the mechanism of toxin action. α-Type toxins decrease the sensitivity of the postsynaptic membrane to acetylcholine, without affecting the muscle fiber or the nerve membrane. The β-type toxins act presynaptically, interfering with the release of acetylcholine. To our knowledge, only two β-type toxins have been identified from other snake venoms (KARLSSON, 1973), while B. multicinctus venom contains 6 such toxins. In our work the assignment of neurotoxins to α- or β-type was not based on electrophysiological studies, but on the minimum time to death observed. Consequently, it is only assumed

that our α- and β-type neurotoxins are of the post and presynaptic type respectively. This assumption seems highly probable, since it was shown for rat phrenic nerve-diaphragm preparation that the block of neuromuscular transmission by β-Bgt required 60 min (CHANG et al., 1973). So the long time to death observed for high doses of β-toxins probably reflects this peculiarity of their mechanism of action.

A wide range of toxicity was found both among the α- and the β-type toxins. However, the most potent toxins were found among the β-type. Fraction 9 was unique in many respects. It was the least toxic among the β-toxins, differing by a factor of 11 from Fr. 13. It was also significantly slower in producing death than any other toxin at comparable doses. Fraction 9 caused drying of the cornea far more often than any other neurotoxin of this venom. By the slowness of its action, the symptoms observed in the mice, and its presumably β-type toxicity, this toxin resembles notexin, a neurotoxin from Notechis scutatus scutatus venom (KARLSSON et al., 1972). Spectroscopically, Fr. 9 had the highest fluorescence intensity per absorbance unit, and the emission maximum was shifted toward short wavelengths more than that of the other fractions. This shift probably reflects a more hydrophobic environment of tryptophan (CHEN, 1973). There is no evidence for considerable contribution of unresolved tyrosine fluorescence to this blue shift, since the same emission maximum was obtained with the excitation band at 280 or at 300 nm where only tryptophan absorbs light.

Fluorescence spectra revealed several facts about the structure of these neurotoxins. They all contain tryptophan and tyrosine, as do all the snake venom neurotoxins described until now, but the same is not true

of the cardiotoxin group (KARLSSON, 1973). Furthermore, they are all very resistant to the treatment with urea, as revealed by their toxicity and spectra. The same result was described previously for other neurotoxins (KARLSSON, 1973); the resistance to urea was attributed to relatively large numbers of disulfide bridges found in neurotoxic proteins. The same is probably true in the present case, since the reduction of disulfide bridges with dithiothreitol inactivated the toxins and modified their emission spectra. The correlation between spectral changes and toxicity suggests that fluorescence spectra can be used as a quick and convenient assay for the integrity of these proteins. Another interesting possibility is the use of fluorescence spectra for the identification of the individual neurotoxins from this venom. The present results have already shown differences in the position of the maxima, relative intensities, excitation spectra, tyrosine contribution, band widths, etc., that could be used for that purpose. Further work along this line is necessary. The only two alternative methods available at present for the identification of these neurotoxins, are the study of their toxicity characteristics, and the analysis of their amino acid composition. Both methods consume neurotoxins and are incomparably more laborious than the fluorometric method suggested here.

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TABLE 1. FRACTIONATION OF CRUDE BUNGARUS MULTICINCTUS VENOM ON CM-SEPHADEX

| Fraction | Recovery of fractions: Absorbance units (at 280 nm) recovered in each fraction | % Recovery |
|---|---|---|
| 1 | 33 | 9 |
| 2 | 4 | 1 |
| 3 | 106 | 27 |
| 4+5 | 5 | 1 |
| 6 | 10 | 3 |
| 7 | 14 | 4 |
| 8 | 23 | 6 |
| 9 | 45 | 12 |
| 10 | 48 | 12 |
| 11 | 21 | 5 |
| 12 | 23 | 6 |
| 13 | 20 | 5 |
| 14 | 9 | 2 |
| Discarded or lost | 26 | 7 |

Data from Run 2. The supernatant prepared from 355 mg of crude lyophilized venom was put onto the column; this supernatant contained 387 absorbance units at 280 nm (31 absorbance units were discarded with the small precipitate).

TABLE 2. TOXICITY OF FRACTIONS FROM BUNGARUS MULTICINCTUS VENOM

SUBCUTANEOUS INJECTIONS

| Fraction | $LD_{50}$ | $LD_{100}$ | Time to death[a] mean ± SD | Minimum time[b] to death | |
|---|---|---|---|---|---|
| | µg/g of mouse | | min | min | $\frac{\text{dose}}{LD_{100}}$ |
| 2 | >5 | | | | |
| 3 | 0.21 (0.20 - 0.22)[e] | 0.22 | 219 ± 113 | 10 | 128 |
| α-Bgt[c] | 0.14 (0.12 - 0.16) | 0.16 | | | |
| 4[d] | 1.93 (1.85 - 2.01) | 2.05 | 104 ± 53 | 42 | 3 |
| 5[d] | 2.65 (2.41 - 2.92) | 3.05 | * | 45 | 2 |
| 6 | 1.85 (1.66 - 2.05) | 2.00 | 149 ± 61 | 51 | 4 |
| 7 | 0.17 (0.15 - 0.18) | 0.19 | 138 ± 92 | 21 | 22 |
| 8 | 0.69 (0.65 - 0.72) | 0.76 | 153 ± 119 | 25 | 15 |
| 9 | 0.50 (0.46 - 0.54) | 0.60 | >500 ± 263 | 117 | 17 |
| 10 (β-Bgt) | 0.06 (0.05 - 0.08) | 0.08 | 230 ± 107 | 72 | 104 |
| 11 | 0.16 (0.14 - 0.19) | 0.21 | 267 ± 136 | 79 | 64 |
| 12 | 0.09 (0.08 - 0.11) | 0.11 | 241 ± 112 | 92 | 38 |
| 13 | 0.04 (0.03 - 0.05) | 0.05 | 215 ± 82 | 75 | 30 |
| 14 | 0.15 (0.13 - 0.17) | 0.17 | 315 ± 151 | 124 | 15 |

* Data not sufficient.

[a] Time to death for doses between 0.83 and 1.16 x $LD_{100}$.

[b] Time to death observed at the highest dose used, which is indicated in the last column. For example, for β-Bgt 104 x 0.08 = 8.32 µg/g were injected.

[c] α-Bgt (α-bungarotoxin) is obtained from Fraction 3 by rechromatography on CM-Cellulose.

[d] Fractions 4 and 5 were separated in only one of the three chromatographic runs.

[e] Figures in parentheses are intervals of confidence as calculated by the method of LITCHFIELD and WILCOXON (1949) for 19/20 probability.

TABLE 3. COMPARISON OF NEUROTOXINS DESCRIBED IN THIS WORK WITH THOSE PREPARED BY LEE ET AL. (1972)

| Fraction | $LD_{50}$ (i.p.) µg/g | Type of neurotoxicity[a] | Fraction | $LD_{50}$ (i.p.) µg/g | Type of neurotoxicity[b] |
|---|---|---|---|---|---|
| | | | | From LEE et al. (1972) | |
| α-Bgt | 0.11 (0.10 -0.12 )[c] | α | $II_2$ | 0.15 (0.10 -0.22 )[c] | α |
| 4 | 1.0 (0.7 -1.3 ) | (α) | $II_1$ | 1.10 (0.84 -1.43 ) | |
| 6 | 1.2 (1.1 -1.3 ) | (α) | $IV_1$ | 2.12 (1.40 -3.20 ) | β |
| 7 | 0.15 ( * ) | α | $III_1$ | 0.25 (0.22 -0.29 ) | α |
| 8 | 0.62 (0.57 -0.67 ) | α | $III_2$ | 0.74 (0.69 -0.80 ) | α |
| 9 | 0.4 (0.3 -0.5 ) | β | $IV_2$ | 0.28 (0.21 -0.38 ) | β |
| 10 | 0.01 (0.005-0.02 ) | β | V | 0.014(0.011-0.018) | β |
| 11 | 0.022(0.01 -0.03 ) | β | | | |
| 12 | 0.014(0.011-0.017) | β | VI | 0.053(0.039-0.072) | β |
| 13 | 0.007(0.005-0.009) | β | VII | 0.016(0.011-0.022) | β |
| 14 | 0.045(0.04 -0.05 ) | β | VIII | 0.04 - 0.05 | β |

The identification of the neurotoxins described in this work with those reported by LEE et al. (1972) was done on the basis of their $LD_{50}$ for intraperitoneal injections, their type of neurotoxicity and partially by their position on the chromatogram.

* Data not sufficient.

[a] Type of toxicity was assigned on the basis of minimum time to death (see Table 1 and "Results" for explanation).

[b] In the work of LEE et al. (1972) the type of neurotoxicity was assigned by the characteristics of the neuromuscular block.

[c] Figures in parentheses are intervals of confidence as calculated by the method of LITCHFIELD and WILCOXON (1949).

TABLE 4. EMISSION SPECTRA OF THE NEUROTOXIC FRACTIONS

| Fraction | Native | | Urea-treated | | Reduced | | |
|---|---|---|---|---|---|---|---|
| | N. | Maximum | N. | Maximum | N. | Maxima | |
| | | nm | | nm | | nm | |
| α-Bgt | 11 | 342 (340-342) | 6 | 343 (342-345) | 4 | 348 (343-351) | |
| 7 | 8 | 342 (341-343) | | | | | |
| 8 | 10 | 340 (339-341) | 6 | 341 (340-342) | 7 | 350 (348-351) | 300 |
| 9 | 10 | 335 (333-337) | 5 | 335 (334-336) | 5 | 349 (346-351) | 300 |
| 10 | 8 | 338 (337-339) | 5 | 340 (339-342) | 10 | 351 (346-355) | 308-310 |
| 11 | 7 | 339 (337-340) | 4 | 341 (340-342) | 4 | 347 (346-348) | 300-310 |
| 12 | 11 | 338 (335-339) | 7 | 340 (339-341) | 3 | 350 (347-352) | 300-310 |
| 13 | 14 | 341 (339-342) | 7 | 342 (340-346) | 7 | 351 (350-352) | 300-310 |
| 14 | 6 | 342 (340-343) | 7 | 342 (340-344) | 3 | 349 (347-352) | 300-307 |

Several solutions (in $H_2O$) from three different preparations of each neurotoxin were used for the determination of emission maximum of native neurotoxins. Urea-treated samples were dissolved in 8 M urea; reduced toxins were in 8 M urea plus 30 mM dithiothreitol. The maxima were independently determined on N different spectra. The mean and the extreme values found for each toxin are given.

TABLE 5. CONTRIBUTION OF TYROSINE FLUORESCENCE TO THE EMISSION SPECTRA OF NATIVE TOXINS

| Fraction | Excitation (nm) | | | | | |
|---|---|---|---|---|---|---|
| | 265 | 290 | %Δ | 265 | 290 | %Δ |
| | $10^3 \times I_{303}/I_{max}$ | | | $10^3 \times I_{380}/I_{max}$ | | |
| α-Bgt | 158 | 91 | 159 | 421 | 407 | 3 |
| 7 | 136 | 158 | -14 | 474 | 477 | -1 |
| 8 | 111 | 114 | -4 | 351 | 371 | -5 |
| 9 | 259 | 183 | 42 | 276 | 283 | -2 |
| 10 | 400 | 210 | 90 | 387 | 406 | -5 |
| 11 | 349 | 191 | 83 | 422 | 405 | 4 |
| 12 | 324 | 154 | 110 | 378 | 415 | -10 |
| 13 | 301 | 122 | 147 | 441 | 452 | -2 |
| 14 | 202 | 98 | 106 | 447 | 442 | 1 |

Emission spectra were obtained with the excitation band at 265 or at 290 nm. In both cases, the ratio of the intensity at 303 nm ($I_{303}$) and that at the maximum ($I_{max}$), as well as the ratio of the intensity at 380 nm ($I_{380}$) over that at the maximum, were calculated.

$$\%\Delta = \frac{\text{Ratio for excitation at 265 nm - Ratio for 290 nm}}{\text{Ratio for 290 nm}} \times 100$$

TABLE 6. COMPARISON OF TOXICITIES OF NATIVE, UREA-TREATED AND REDUCED NEUROTOXINS

| Fraction | Dose | Native | | Urea-treated | |
|---|---|---|---|---|---|
| | µg/g | % mortality | time to death (min) | mortality | time to death (min) |
| α-Bgt | 0.225 | 100 | 78 | 75 | 137 |
| 8 | 0.975 | 100 | 62 | 100 | 176 |
| 10 | 0.125 | 100 | 250 | 100 | 300 |
| 11 | 0.225 | 100 | 230 | 100 | 285 |

Fraction 10 was from run 2; the remaining fractions were from run 3. Native toxins were in distilled water; urea-treated and reduced toxins had been frozen for a month in 8 M urea and 8 M urea plus 30 mM dithiothreitol (DTT) respectively. All three forms of each toxin were diluted with saline to a concentration that represented 1.5 x $LD_{100}$ for the native toxin. They were injected subcutaneously to male CD-1, "Swiss" mice. Reduced toxins were also injected at a dose equivalent to 10 x $LD_{100}$. In all cases there were 4 mice per group. The controls injected with equivalent amount of urea or urea plus DTT survived without ill effects. All the animals injected with reduced toxins survived without symptoms of severe illness.

FIGURE LEGENDS

Fig. 1. CHROMATOGRAPHY OF THE VENOM OF BUNGARUS MULTICINCTUS.
Crude venom of Bungarus multicinctus was fractionated by gradient elution from a CM-Sephadex column (see METHODS for details). The material eluted during the washing of the column with the initial buffer was called Fraction 1 (not shown). Fraction 4+5 was so named since it was separated into two components in a previous chromatographic run. Dark zones represent the material discarded in order to minimize overlap of peaks.

Fig. 2. TIME TO DEATH VERSUS DOSE FOR α- AND β-TYPE NEUROTOXINS.
Results for α-Bgt and Fraction 12 from 3 different preparations are presented in these graphs. Dose values were normalized by $LD_{100}$. The toxins were injected subcutaneously.

Fig. 2a. TIME TO DEATH VERSUS DOSE OF α-BGT.
Fractions 7 and 8 showed very similar curves to that of α-Bgt.

Fig. 2b. TIME TO DEATH VERSUS DOSE OF FRACTION 12.
Fractions 9 through 14 belonged to this second group.

Fig. 3. TITRATION OF NEUROTOXINS BY TIMES TO DEATH.

Fig. 3a: o—o: α-BGT FROM RUN 3 INJECTED SUBCUTANEOUSLY.
●—●: FR. 8 FROM RUN 1 INJECTED SUBCUTANEOUSLY.

Fig. 3b. FR. 11 FROM RUN 3 INJECTED INTRAPERITONEALLY.

Fig. 4. FLUORESCENCE SPECTRA OF NEUROTOXIC FRACTIONS.

These spectra were obtained on a Hitachi-Perkin-Elmer MPF-2A fluorescence spectrophotometer. The excitation wavelength was 280 nm. Excitation and emission slitwidths were 6 nm for α-Bgt and Fr. 7 through 11, and 4 nm for the remaining fractions. Absorbances at 280 nm were around 0.160 for all the solutions. Note the change in intensity scale for α-Bgt and Fr. 7 through 9 as opposed to Fr. 10 through 14. Native proteins were in distilled water; "urea treated" in 8 M urea; and "reduced" in 8 M urea + 30 mM dithiothreitol.

———: Native toxins

—·— : Urea treated toxins

— — —: Reduced toxins

Fig. 5. COMPARISON OF THE FLUORESCENCE SPECTRA OF ACID HYDROLYSATE OF FR. 10 WITH THOSE OF TRYPTOPHAN AND TYROSINE.

Tryptophan solution was excited at 275 nm, tyrosine and the hydrolysate at 260 nm.

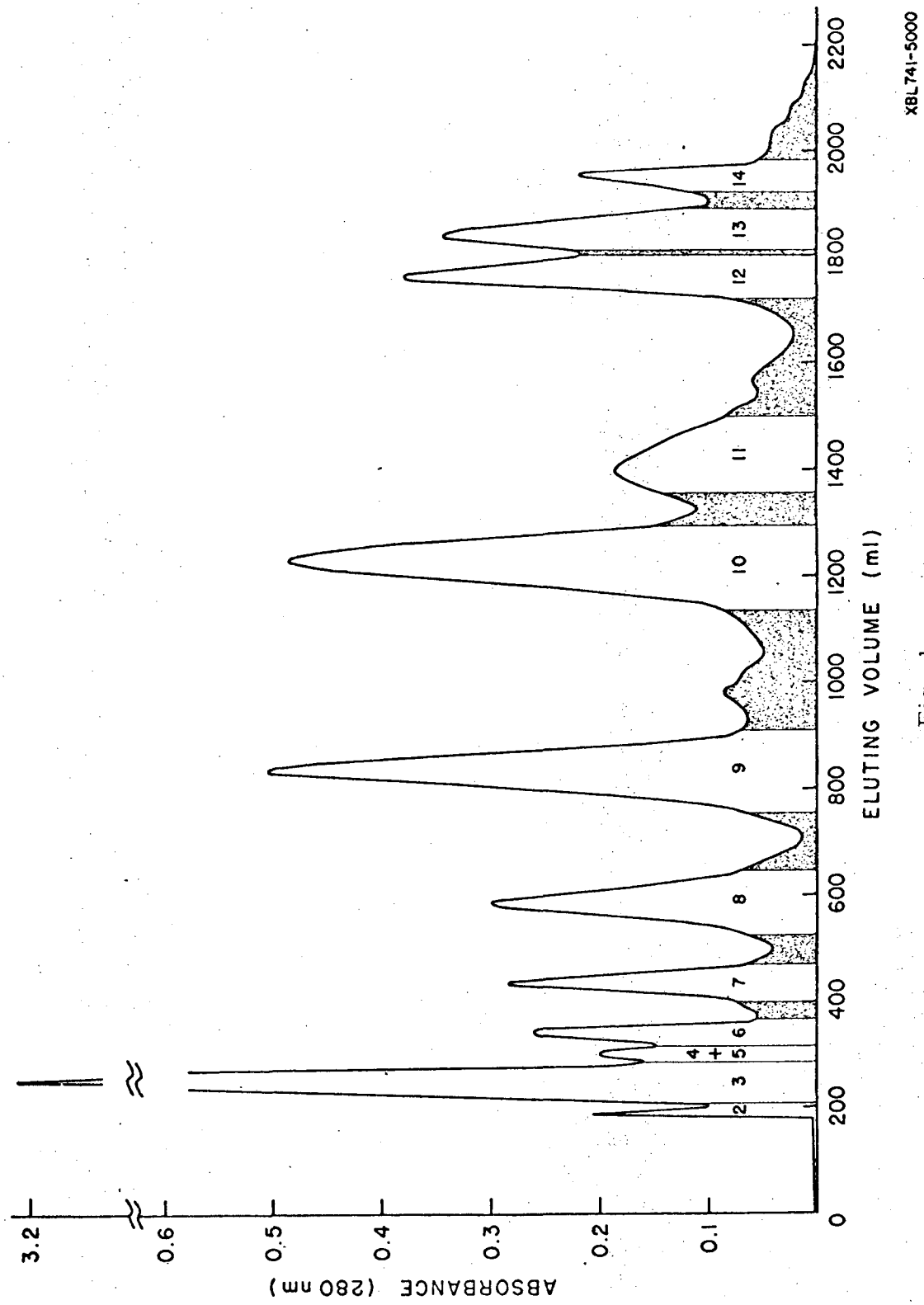

ABSORBANCE (280 nm)
3.2
0.6
0.5
0.4
0.3
0.2
0.1
0
200
400
600
800
1000
1200
1400
1600
1800
2000
2200
ELUTING VOLUME (ml)
2
3
4
+
5
6
7
8
9
10
11
12
13
14
XBL741-5000

Fig. 1.

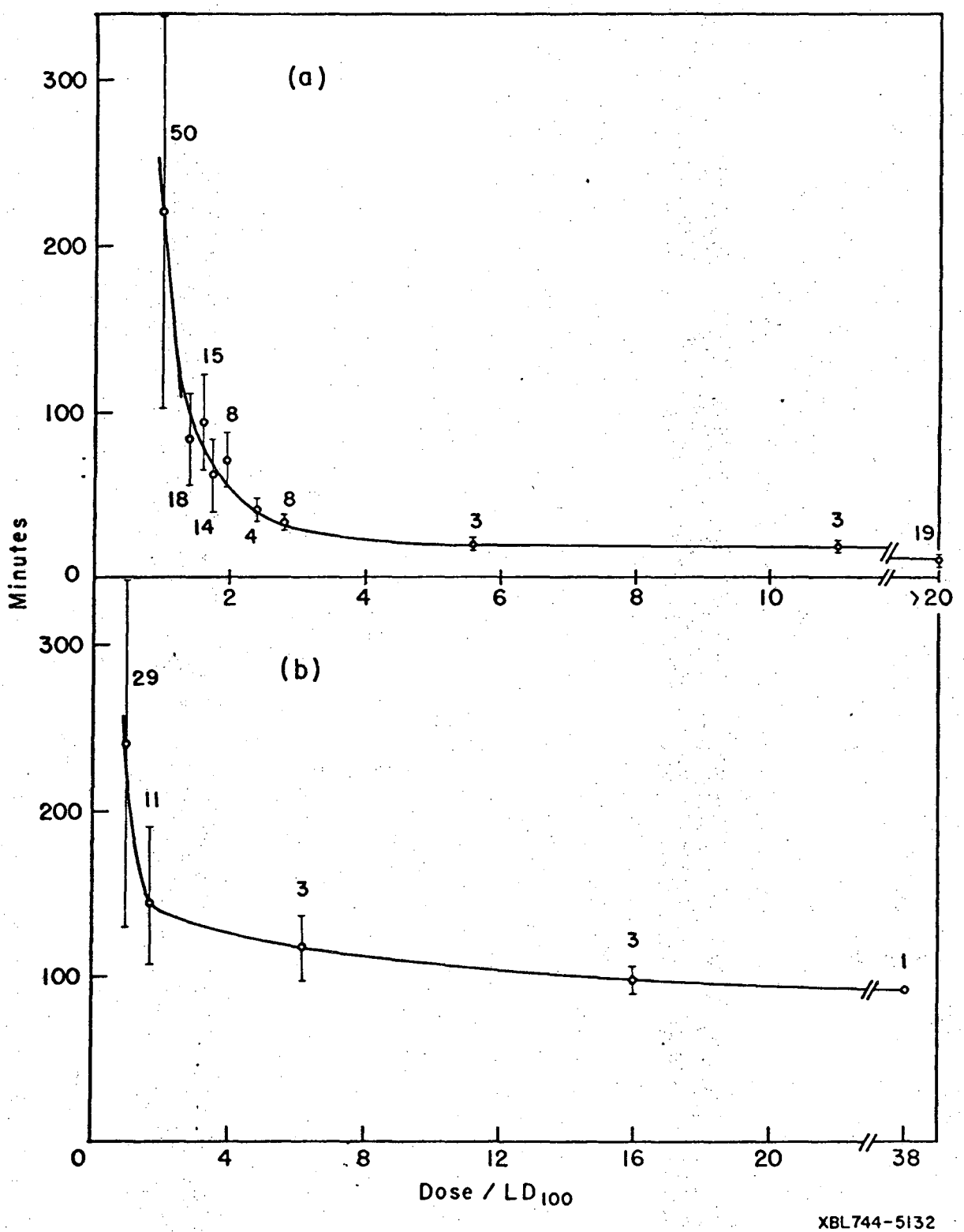

(a)
300
200
100
0
Minutes
50
15
8
18
14
4
8
3
3
19
2
4
6
8
10
>20
(b)
300
200
100
0
29
11
3
3
1
4
8
12
16
20
38
Dose / LD100
XBL744-5132

Fig. 2.

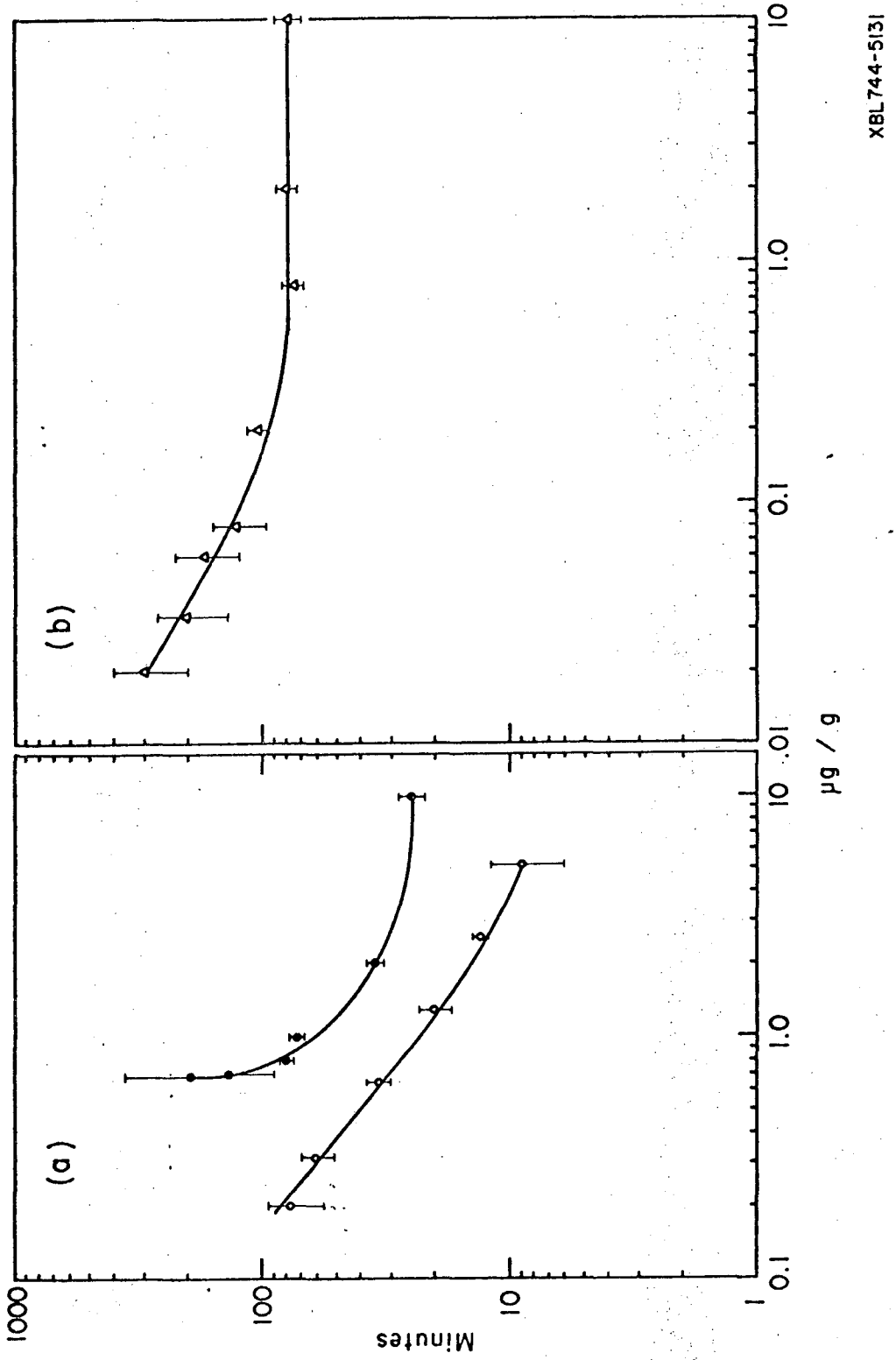



Fig. 3.

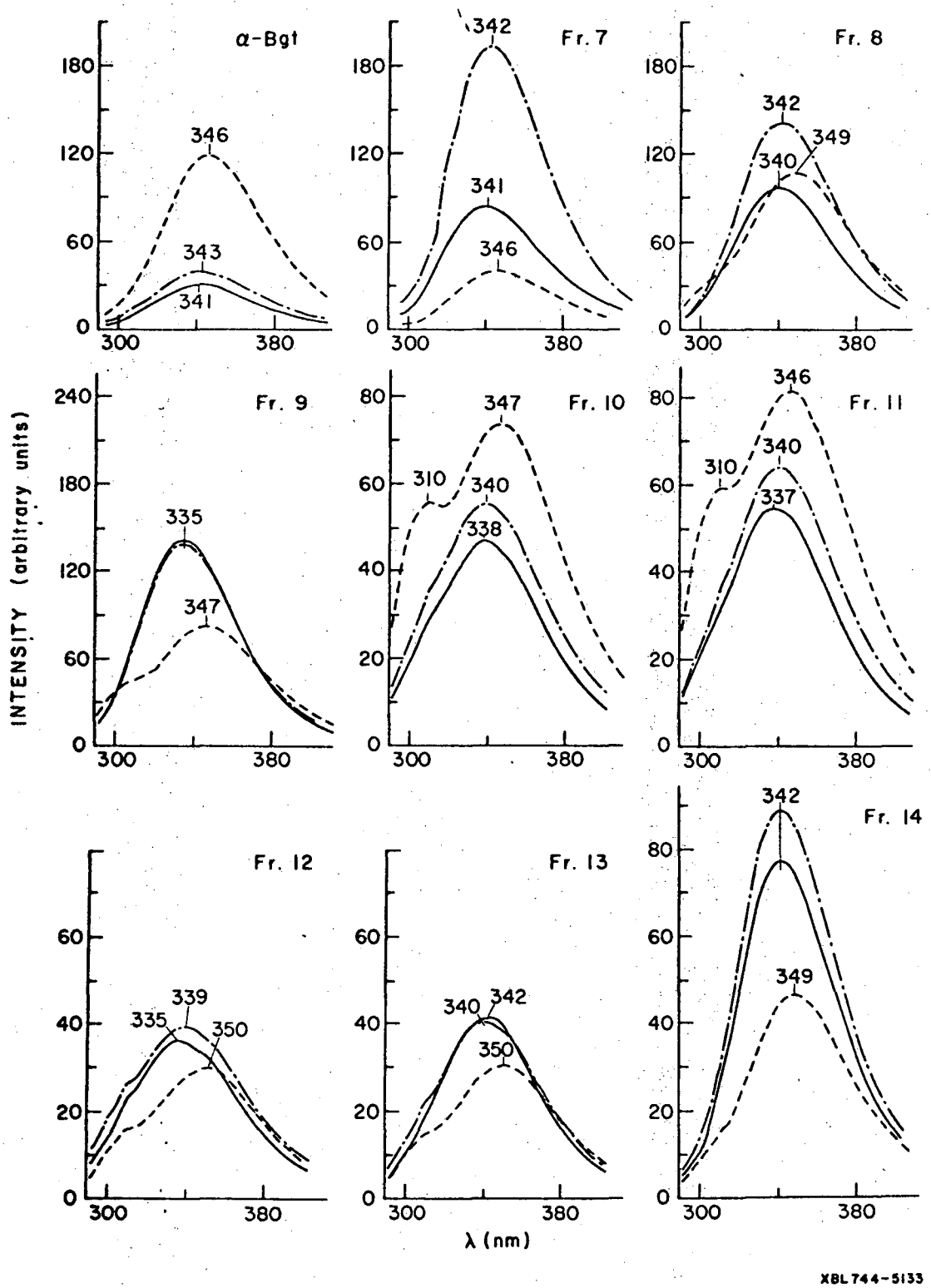
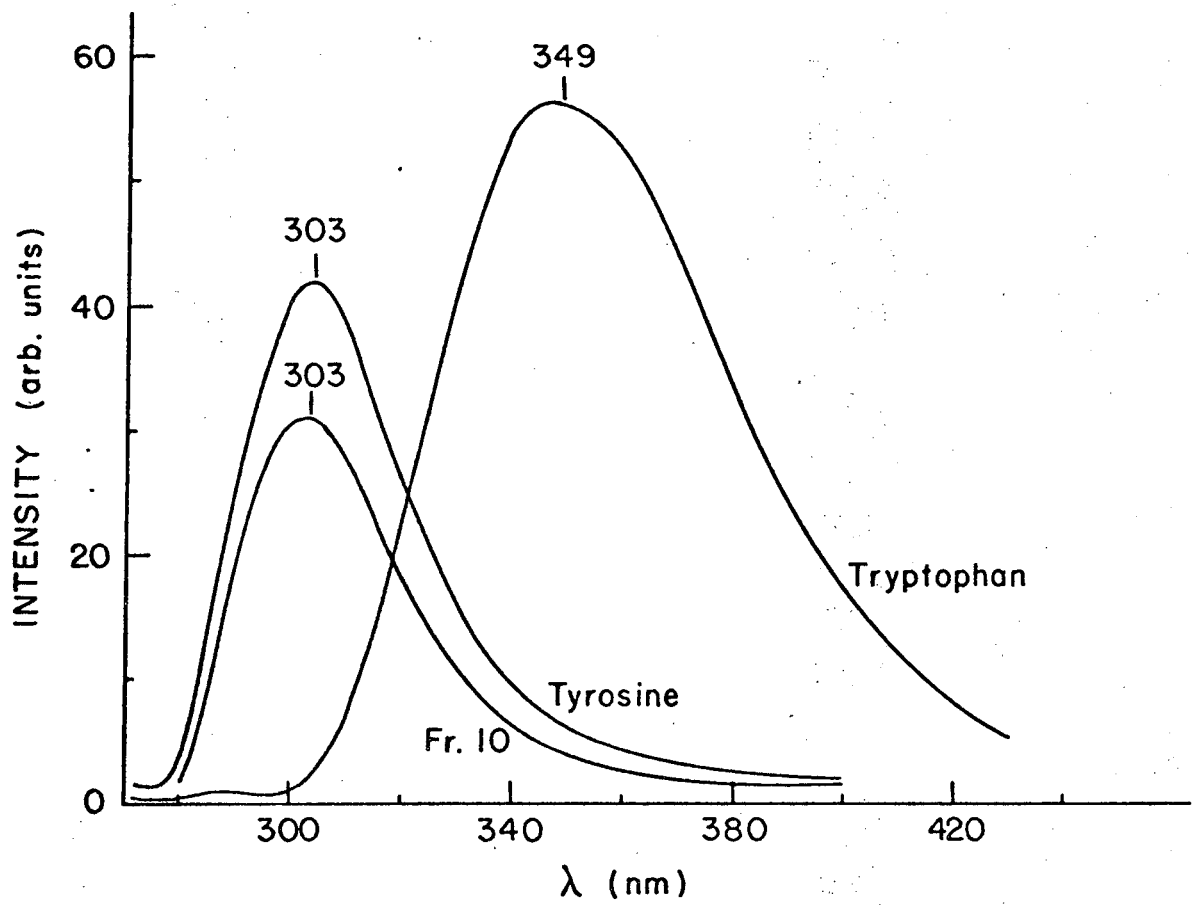

α-Bgt
346
343
341
Fr. 7
342
341
346
Fr. 8
342
349
340
Fr. 9
335
347
Fr. 10
347
310
340
338
Fr. 11
346
310
340
337
Fr. 12
339
335
350
Fr. 13
340
342
350
Fr. 14
342
349
INTENSITY (arbitrary units)
λ (nm)
XBL744-5133

Fig. 4.




XBL744-5130

Fig. 5.

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