*Biochem. J.* (1966) **99**, 624

# Studies on Sea-Snake Venoms

## CRYSTALLIZATION OF ERABUTOXINS a AND b FROM *LATICAUDA SEMIFASCIATA* VENOM

BY N. TAMIYA* AND HIROKO ARAI

*Medical School, Tokyo Medical and Dental University, Bunkyo-ku, Tokyo, Japan*

(*Received* 27 *September* 1965)

1. The toxic principles in the venom of the sea-snake *Laticauda semifasciata* were separated into two components by CM-cellulose chromatography and obtained in crystalline forms. They were named 'erabutoxins a and b'. 2. The homogeneity of each toxin was shown by rechromatography, by disk electrophoresis, by ultracentrifuging, by toxicity measurements before and after repeated crystallizations and by *N*-terminal analysis. 3. They had molecular weights of about 7000. Both of them contained 61 (or 62) amino acid residues/molecule. The only difference between erabutoxins a and b was that one of the aspartic acid (or asparagine) residues in erabutoxin a was replaced by a histidine residue in erabutoxin b. 4. Both of the toxins had $LD_{50}$ values of 0·15 $\mu$g./g. body wt. for mice and 0·07 $\mu$g./g. for rats. It was shown with frog-muscle preparations that they acted on postsynaptic membrane to block neuromuscular transmission.

There are reports that venoms of the sea-snakes *Laticauda semifasciata* and *Laticauda laticaudata* contain strongly toxic components (Tu, 1961, 1963; Homma, Okonogi & Mishima, 1964). Arai, Tamiya, Toshioka, Shinonaga & Kano (1964) reported that the toxic components in *L. semifasciata* venom were proteins. The present paper describes the crystallization and properties of the toxic proteins from *L. semifasciata* venom. They were named 'erabutoxins a and b', from the Japanese name of the snake, erabu-umihebi.

## MATERIALS

*Crude dried venom.* The sea-snakes *L. semifasciata*, captured near the Okinawa Islands, were anaesthetized with chloroform and the venom ducts from the parotid glands to the front upper teeth stopped by Kocher's forceps. The glands were taken out and the contents were squeezed out on glass and dried over NaOH pellets at −20°. The dried powder was collected and dried further *in vacuo* for 8hr. Five g. of crude dried venom was obtained from 350 individuals.

## EXPERIMENTAL AND RESULTS

*CM-cellulose column chromatography.* The crude dried venom (1·0g.) was suspended in 0·01M-phosphate buffer, pH 6·4 (5·0ml.), containing sodium chloride (0·01M) and centrifuged at 4000 rev./min. (2000*g*) for 10min. The residue was resuspended in the same buffer (5·0ml.) and centrifuged again at 4000 rev./min. (2000*g*) for 10min. The combined supernatants were dialysed against the same buffer (4·0l.) for 24hr., centrifuged at 7000 rev./min. (6000*g*) for 10min. and applied to a CM-cellulose column (1·5cm. × 46cm.) that had been equilibrated with the same buffer. The sodium chloride concentration in the eluent was raised stepwise from 0·01M to 0·02M and then to 0·5M. The results of a typical smaller-scale experiment are shown in Fig. 1(*c*). The recovery of the proteins as measured by $E_{280}$ and that of the toxicity were quantitative. Five, 50 and 45% of the toxicity were eluted with the first, second and the third protein peaks respectively. The toxic substance contained in the second peak was named erabutoxin a and that in the third peak erabutoxin b. The specific toxicities of the fractions are given in Table 1. When erabutoxin a or b was rechromatographed, it was quantitatively recovered as a reproducible single peak (Figs. 1*a* and 1*b*).

*Crystallization of erabutoxins a and b.* The combined erabutoxin a fractions (170ml.) were concentrated by freezing and dialysed against 0·85% sodium chloride solution (3·0l.) for 17hr. The procedure was repeated twice and the toxin solution was finally concentrated to 4·5ml. To the concentrated solution, saturated ammonium sulphate solution was added to make 30% saturation. After standing at 4° overnight, needle crystals were deposited (Fig. 2*a*). They were collected by centrifugation at 3000 rev./min. (1000*g*) for 10min.,

* Present address: Department of Chemistry, Faculty of Science, Tohoku University, Sendai, Japan.

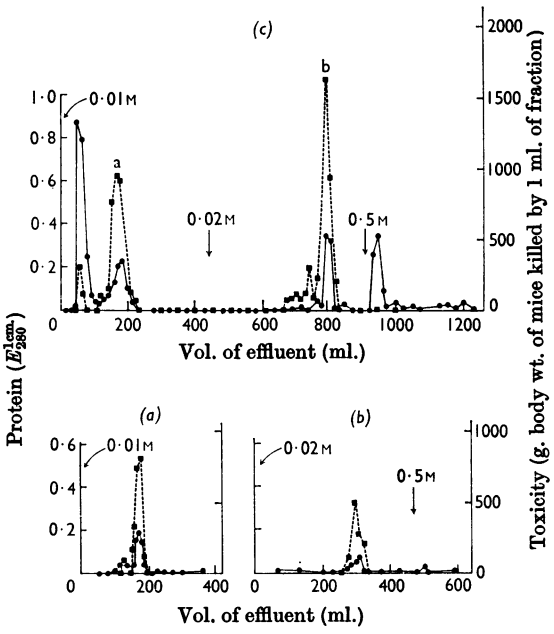


Fig. 1. CM-cellulose column chromatography of *Laticauda semifasciata* venom (*c*), and rechromatography of its components (*a* and *b*). (*c*) The crude dried venom (50mg.) was extracted with 0·01M-phosphate buffer, pH 6·4 (2.0ml.), containing NaCl (0·01M). The insoluble material was removed by centrifuging at 5000 rev./min. (3000*g*) for 10min. The supernatant was applied to a CM-cellulose column (1·5cm. × 24cm.), that had been equilibrated with the same buffer. The concentration of NaCl was raised stepwise as indicated in the Figure. The protein content of the eluate was determined by measuring $E^{1cm.}_{280}$. The toxicity of the eluate was estimated by intramuscular injection to mice as described in the text and expressed as the body wt. of mice that can be killed by 1ml. of the fraction. (*a*) and (*b*) Portions of the eluates of the toxic peaks a (*a*) and b (*b*) of Fig. 1(*c*) were concentrated, dialysed against the starting buffer and rechromatographed on the same column. ■----■, Toxicity; ●——●, protein.

dissolved in 0·85% sodium chloride solution (3·0ml.) and recrystallized by the addition of saturated ammonium sulphate solution.

The combined erabutoxin b fractions (140ml.) were concentrated to 3·4ml. by a similar procedure and treated with ammonium sulphate solution to make 50% saturation. After standing at 4° overnight, needle crystals were deposited (Fig. 2*b*). They were also recrystallized from 0·85% sodium chloride solution (3·0ml.) by the addition of ammonium sulphate.

At higher concentrations of ammonium sulphate, both toxic proteins crystallized in smaller rods. The recrystallization can be repeated several times.

The solution of each toxin showed a typical protein ultraviolet-absorption curve.

*Disk electrophoresis of erabutoxins a and b.* The recrystallized erabutoxin a was dissolved in and dialysed against 0·85% sodium chloride solution so as to contain 0·09–0·15mg. of Kjeldahl nitrogen/ml. ($E^{1cm.}_{280}$ 0·6–1·0). Then 0·1ml. of the solution was subjected to disk electrophoresis at pH 4·3 in a glass tube (0·5cm. × 7·5cm.) (Ornstein, 1964; Davis, 1964; Reisfeld, Lewis & Williams, 1962).

The recrystallized erabutoxin b and the crude dried venom were treated similarly. The results are shown in Figs. 3(*a*), 3(*b*) and 3(*c*). The recrystallized erabutoxin a or b contained no other protein components detectable in the electrophoretic system. The crude dried venom contained two main protein components, which moved 1·7 and 2·1cm. towards the cathode at pH 4·3; the former corresponded to erabutoxin a and the latter to erabutoxin b. Other protein components in the crude venom moved towards the anode in the above system (pH 4·3) and could be detected in a disk electrophoresis at pH 8·2 (Fig. 3*c'*).

*Toxicities of erabutoxins a and b.* The lethal doses of the toxins were determined as described by Arai *et al.* (1964) by injection into the hind leg muscle of mouse (average body wt. 15g.) or rat (average

Table 1. *Purification of erabutoxins a and b*

Protein was measured by total $E_{280}$. Toxicity was calculated from the body wt. of mice that can be killed by 1ml. of the fraction. For $LD_{50}$, a solution of $E^{1cm.}_{280}$ 1·0 is assumed to contain 1mg. of protein/ml.

| | Recovery (%) | | $LD_{50}$ (μg./g. body wt. of mice) |
|---|---|---|---|
| | Protein | Toxicity | |
| Crude venom extracts | 100 | 100 | 0·5 |
| Peak I (Fig. 1*c*) | 39 | 5 | 2·9 |
| Peak a (Fig. 1*c*) | 16 | 50 | 0·19 |
| Peak b (Fig. 1*c*) | 14 | 45 | 0·22 |
| Recrystallized erabutoxin a | | | 0·15 |
| Recrystallized erabutoxin b | | | 0·15 |
| Four-times recrystallized erabutoxin a | | | 0·15 |
| Four-times recrystallized erabutoxin b | | | 0·15 |

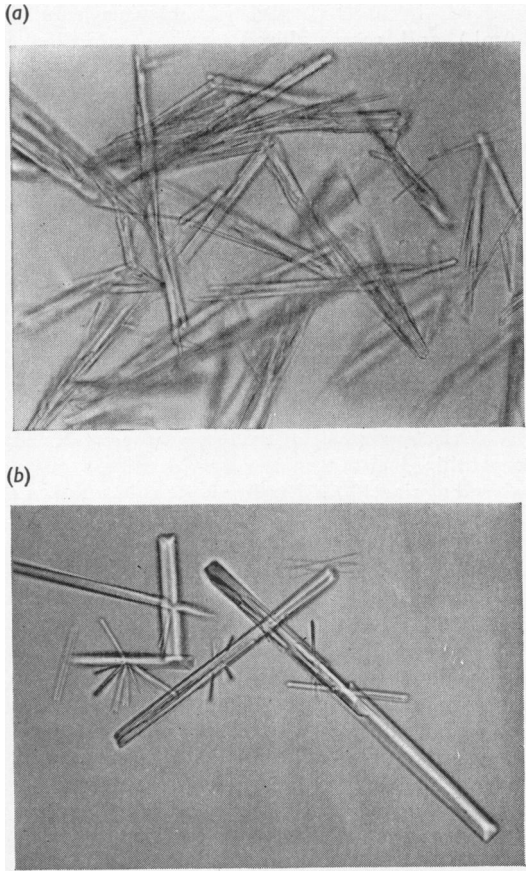


Fig. 2. Crystals of erabutoxin a (*a*) and erabutoxin b (*b*) (magnification × 250).

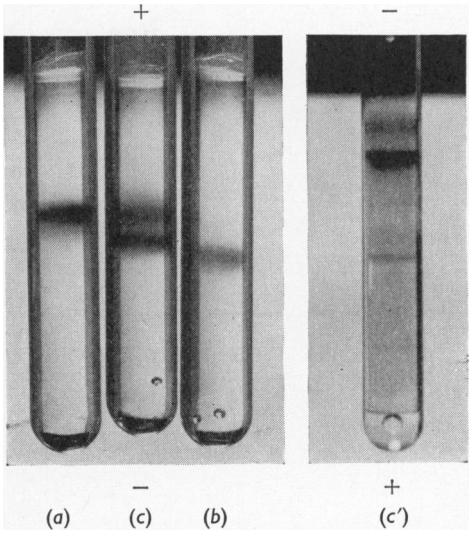


Fig. 3. Disk electrophoresis of erabutoxin a (*a*), erabutoxin b (*b*) and crude venom (*c*) at pH 4·3 in 7·5% polyacrylamide gel as described by Reisfeld *et al.* (1962) with the slight modifications that *NNN′N′*-tetramethylethylenediamine was omitted from stock solution (*b*) and that a different β-alanine–acetic acid buffer (31·2g. of β-alanine, 0·8ml. of acetic acid and water to make 1·0l., pH 5·0) was used for the trays. The concentrations of the samples are given in the text. The other proteins in the crude venom moved upwards and could be detected in disk electrophoresis at pH 8·2 (*c′*), which was carried out as described by Davis (1964).

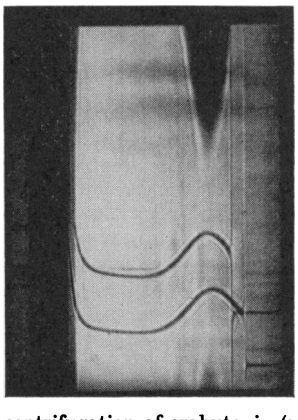

Fig. 4. Ultracentrifugation of erabutoxin (upper curve) a and erabutoxin b (lower curve). Recrystallized erabutoxins a and b were dissolved in 0·85% NaCl to their saturation and centrifuged at 345000*g* for 132min. at 20° in a Beckman–Spinco model E apparatus.

body wt. 83g.). The $LD_{50}$ values of both erabutoxins a and b were 0·15μg./g. body wt. for mice and 0·07μg./g. for rats. The toxicity remained unchanged after the fourth recrystallization as shown in Table 1. All the animals survived when half the $LD_{50}$ was given and died when twice the $LD_{50}$ was given. The $LD_{50}$ values given above were calculated assuming that a solution of $E^{1cm.}_{280}$ 1·0 contained 1mg. of protein/ml.

A solution of erabutoxin a ($E^{1cm.}_{280}$ 0·6–1·1) in 0·85% sodium chloride lost 70% of its toxicity in 5min. at 100°, and that of erabutoxin b ($E^{1cm.}_{280}$ 1·0) lost 70% in 10min. at 100°.

*Ultracentrifugation of erabutoxins a and b.* The recrystallized erabutoxins a and b were dissolved in 0·85% sodium chloride solution to their saturation (a: $E^{1cm.}_{280}$ 10·5; b: $E^{1cm.}_{280}$ 11·9) and ultracentrifuged in a Beckman–Spinco model E apparatus at 345000*g* for 132min. at 20°. The results are shown in Fig. 4. Both of the toxins are ultracentrifugally monodisperse. The difference in the sedimentation velocities of the two toxins observed in this experiment seems to be due to the difference in the

medium densities caused by the different concentrations of ammonium sulphate, which was added with the crystalline proteins.

Recrystallized erabutoxin a was dissolved in 0·85% sodium chloride solution and dialysed against a large volume of 0·85% sodium chloride to give a solution containing 0·694mg. of Kjeldahl nitrogen/ml. ($E_{280}^{1cm.}$ 4·79). Erabutoxin b was treated similarly and a solution containing 0·231mg. of Kjeldahl nitrogen/ml. ($E_{280}^{1cm.}$ 1·56) was prepared. The solutions were subjected to approach-to-equilibrium centrifugation at 31410 rev./min. for 80 min. at 20° by the Archibald method (Shacchman, 1959) in the cell described by Yphantis (1960). The partial specific volume was calculated to be 0·71 for both toxins from their amino acid contents (see below) as described by Cohn & Edsall (1943). The results are given in Table 2. The molecular weights were calculated to be 7430 for both erabutoxins a and b.

*N-Terminal amino acids of erabutoxins a and b.* The recrystallized erabutoxin a was dissolved in 0·85% sodium chloride solution and dialysed against 0·05M-tris buffer, pH 8·9 (3·0l.). The toxin solution contained 0·100mg. of Kjeldahl nitrogen/ml. ($E_{280}^{1cm.}$ 0·690). The solution (4·8ml.) was treated with 1-fluoro-2,4-dinitrobenzene (Fraenkel-Conrat, Harris & Levy, 1955). The DNP-protein was hydrolysed with 6·0N-hydrochloric acid (2·0ml.) for 15hr. at 105°. After dilution with water to give 1N acidity, the solution was extracted twice with 10ml. of ether. DNP-amino acids remained in the water layer and were adsorbed on a talc column (1·0cm. × 3·0cm.) (Sanger, 1949). The column was washed with 1·0N-hydrochloric acid (10ml.) and eluted with a mixture of N-hydrochloric acid and ethanol (1:4, v/v). The DNP-amino acid derivatives from the eluate were chromatographed on Toyoroshi no. 51 paper (Toyoroshi Corp., Tokyo, Japan) with 2-methylbutan-2-ol saturated with 0·05M-phthalate buffer, pH 6·0 (prepared from potassium hydrogen phthalate and sodium hydroxide) and developed to about 25cm. (Fraenkel-Conrat *et al.* 1955). Of four yellow spots, two were much stronger than the others. One of the strong spots was ninhydrin-negative and Sakaguchi-positive and was identified as DNP-arginine by chromatography with an authentic sample ($R_F$ 0·46). The other strong spot, which was ninhydrin-positive and Sakaguchi-negative, was ε-DNP-lysine.

A solution of recrystallized erabutoxin b (5·1ml.) containing 0·091mg. of Kjeldahl nitrogen/ml. ($E_{280}^{1cm.}$ 0·62) was prepared and treated in a similar manner. DNP-arginine was also detected.

In quantitative experiments, DNP-arginine was extracted from the paper chromatogram with aq. 1% sodium hydrogen carbonate (5·0ml.) at 55° for 15min. and determined by direct extinction measurements (Levy, 1954) and by the Sakaguchi reaction (Weber, 1930). From 0·104μmole of DNP-erabutoxin a, 0·030μmole (direct extinction measurement) or 0·038μmole (Sakaguchi reaction) of DNP-arginine was obtained. From 0·071μmole of DNP-erabutoxin b, 0·036μmole (direct extinction measurement) or 0·045μmole (Sakaguchi reaction) of DNP-arginine was obtained. The yield of DNP-arginine was 30–60% of the theoretical value in repeated experiments for both erabutoxins a and b.

Thus both erabutoxins a and b have arginine at their *N*-terminus.

*Amino acid compositions of erabutoxins a and b.* Dialysed preparations of recrystallized erabutoxins a and b containing 0·050–0·080mg. of Kjeldahl nitrogen (total $E_{280}$ 0·35–0·55) were hydrolysed with 6N-hydrochloric acid (2·0ml.) for 24, 48 or 72hr. at 110° *in vacuo* in sealed glass tubes. The amino acids in the hydrolysates were analysed by an automatic analyser (type II Mitamurariken; Tokyo, Japan). The results are given in Table 3. The tryptophan contents of the toxins were estimated by ultraviolet absorption as described by Goodwin & Morton (1946) and by the *p*-dimethylaminobenzaldehyde method of Spies & Chambers (1948).

The minimal molecular weights calculated from the amino acid contents are 6750 for erabutoxin a and 6770 for b. The values agree fairly well with those obtained from centrifugation described above. It was therefore concluded that the values in parentheses in Table 3 represent numbers of residues in one molecule of the toxins. Both toxins

Table 2. *Measurement of molecular weights of erabutoxins a and b by approach-to-equilibrium centrifuging (Yphantis, 1960)*

Molecular weights ($M$) are calculated by the equation:

$$M = \frac{1}{\bar{r}\,c_0}(\mathrm{d}c/\mathrm{d}r)_{r=\bar{r}}\frac{RT}{\omega^2(1-\bar{v}\rho)}$$

where $\bar{r}$ is the distance from the centre of rotation to the mid-point of the solution, $c_0$ is the initial concentration determined in terms of arbitrary refractive-index units by integration of the synthetic boundary trace, $(\mathrm{d}c/\mathrm{d}r)_{r=\bar{r}}$ is the refractive-index gradient at $\bar{r}$, $\omega$ is the angular velocity ($3{\cdot}29\times10^3$), $\bar{v}$ is the partial specific volume of erabutoxin a or b (0·71), $\rho$ is the density of the solution, which is assumed to be 1·0, $R$ is the gas constant and $T$ is the absolute temperature (293·2°).

| | Erabutoxin a | Erabutoxin b |
|---|---|---|
| $\bar{r}$ | 6·49 cm. | 7·15 cm. |
| $c_0$ | $3{\cdot}79\times10^{-2}$ | $1{\cdot}11\times10^{-2}$ |
| $(\mathrm{d}c/\mathrm{d}r)_{r=\bar{r}}$ | $2{\cdot}36\times10^{-1}$ | $0{\cdot}766\times10^{-1}$ |
| Mol. wt. | 7430 | 7430 |

Table 3. *Amino acid compositions of erabutoxins a and b*

| | Erabutoxin a | | Erabutoxin b | |
|---|---|---|---|---|
| | (m-moles of amino acid/g.-atom of Kjeldahl N) | (molar ratio) | (m-moles of amino acid/g.-atom of Kjeldahl N) | (molar ratio) |
| Aspartic acid | 58·5 | 5·08 (5) | 48·1 | 4·15 (4) |
| Threonine | 57·4 | 4·99 (5) | 57·4 | 4·95 (5) |
| Serine | 81·4 | 7·06 (7) | 82·0 | 7·07 (7) |
| Glutamic acid | 94·1 | 8·17 (8) | 93·8 | 8·08 (8) |
| Proline | 48·0 | 4·17 (4) | 48·1 | 4·15 (4) |
| Glycine | 58·5 | 5·09 (5) | 56·7 | 4·89 (5) |
| Alanine | 0 | (0) | 0 | (0) |
| Cystine (half) | 92·1 | 8·00 (8) | 92·1 | 7·94 (8) |
| Valine | 23·0 | 2·00 (2) | 23·1 | 1·99 (2) |
| Methionine | 0 | (0) | 0 | (0) |
| Isoleucine | 42·0 | 3·65 (4) | 44·2 | 3·81 (4) |
| Leucine | 12·2 | 1·06 (1) | 13·2 | 1·13 (1) |
| Tyrosine | 9·7 | 0·84 (1) | 10·6 | 0·92 (1) |
| Phenylalanine | 24·1 | 2·09 (2) | 23·1 | 1·99 (2) |
| Lysine | 45·1 | 3·92 (4) | 46·6 | 4·01 (4) |
| Histidine | 10·5 | 0·91 (1) | 23·1 | 1·99 (2) |
| Arginine | 34·3 | 2·98 (3) | 34·4 | 2·97 (3) |
| Tryptophan* | 15·1 | 1·31 (1 or 2) | 16·2 | 1·40 (1 or 2) |
| Ammonia | | (about 10) | | (about 10) |
| Total amino acid | | (61) | | (61) |
| Minimal mol. wt. | 6750 | | 6770 | |

* Tryptophan was measured separately as described in the text.

contain 61 amino acid residues/molecule. One more tryptophan residue/molecule may be present in both of them. It is noteworthy that the toxins contain no alanine or methionine. The amino acid compositions of the two toxins are the same except that one of the aspartic acid (or asparagine) residues in erabutoxin a is replaced by a histidine residue in erabutoxin b.

*Mode of toxic action of erabutoxins a and b.* When a lethal dose of erabutoxin a or b in 0·85% sodium chloride was injected into mice intramuscularly, the animals died, usually in 90 min. Those animals that survived for 3 hr. did not die. The animals showed symptoms of dyspnoea and paralysis of the hind legs.

When erabutoxin a (0·013 $\mu$g. of Kjeldahl nitrogen/g. body wt.) or b (the same amount as erabutoxin a) solution in 0·85% sodium chloride was injected into anaesthetized (pentobarbital, 50 mg./kg.) rats (290–310 g.) intravenously, and the respiration, blood pressure and heart rate were recorded by a polygraph, gradual decreases in respiration amplitude and in blood pressure were observed. The animals died in 40–90 min.

The contraction of an isolated sciatic-nerve–sartorius-muscle preparation of a frog (*Rana nigromaculata*) by electrical stimulation through the nerve ceased in 20–25 min. when erabutoxin a (0·11 $\mu$g. of Kjeldahl nitrogen/ml. of Ringer solution) or b (0·12 $\mu$g. of Kjeldahl nitrogen/ml.) was added to the medium, though the contraction by direct electrical stimulation of the muscle was unaffected (Fig. 5).

The contraction of the rectus abdominis muscle of a frog (*Rana nigromaculata*) by acetylcholine (0·5 $\mu$g./ml. of Ringer solution) was inhibited by erabutoxin a (0·12 $\mu$g. of Kjeldahl nitrogen/ml. of Ringer solution) or b (0·12 $\mu$g. of Kjeldahl nitrogen/ml.) as shown in Fig. 6. The inhibition was not removed by washing the muscle with the Ringer solution. In contrast, the contraction of the muscle by potassium chloride (0·02 M or 0·05 M) was not affected by erabutoxin a (0·12 $\mu$g. of Kjeldahl nitrogen/ml. of Ringer solution) (Fig. 6*c*).

## DISCUSSION

All the sea-snakes (Hydrophiidae) are venomous. *L. semifasciata*, which is the most abundant sea-snake near the coasts of Japanese southern islands (Amami and Okinawa Islands), does not bite man. There are, however, dangerous species closely related to them, e.g. *Hydrophis cyanocinctus*, *Enhydrina schistosa* and *L. laticaudata* (Reid, 1961). The present study was undertaken as part of a comparative study of sea-snake venoms.

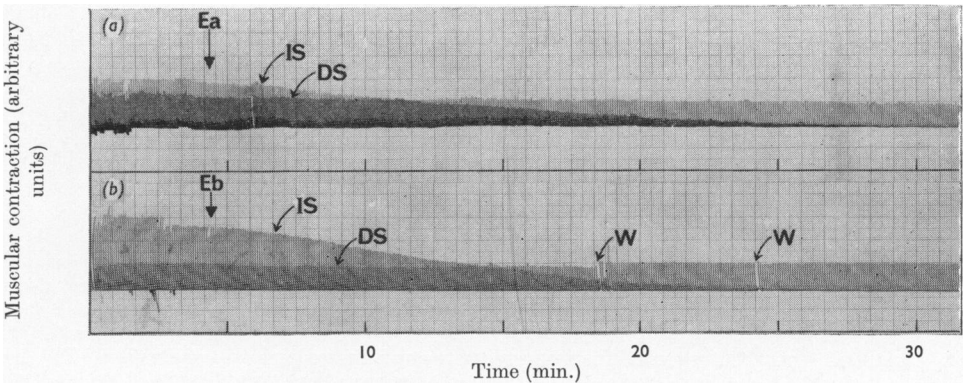


Fig. 5. Effects of erabutoxins a and b on the contraction of sciatic-nerve–sartorius-muscle preparations of frogs. An isolated sciatic-nerve–sartorius-muscle preparation of a frog (*Rana nigromaculata*) was placed in 4·3 ml. of glucose–Ringer solution (9·0 g. of NaCl, 0·42 g. of KCl, 0·24 g. of $CaCl_2$, 0·5 g. of $NaHCO_3$ and 1·0 g. of glucose in 1400 ml. of water), which was continuously bubbled with air. The electrical stimulations were given to the preparation either directly to the muscle (DS) or indirectly through the nerve (IS) every 5 sec. and the contractions were recorded by a strain-gauge transducer. The solution of recrystallized erabutoxin a (0·1 ml.) was added to the medium at the point marked Ea (final concn. 0·11 $\mu$g. of Kjeldahl nitrogen/ml. of Ringer solution) in (*a*). The recrystallized erabutoxin b solution (0·1 ml.) was added at the point marked Eb (final concn. 0·12 $\mu$g. of Kjeldahl nitrogen/ml. of Ringer solution) in (*b*). The muscle was washed with the Ringer solution at the points marked in (*b*).

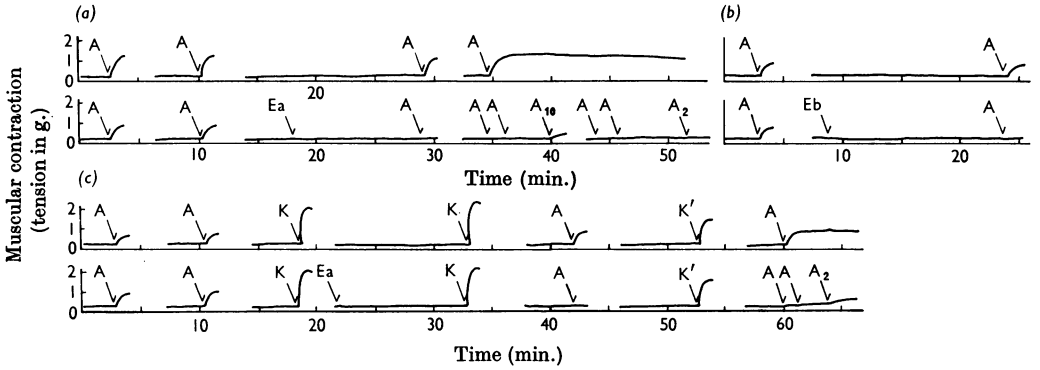


Fig. 6. Effects of erabutoxins a and b on the contraction of isolated frog rectus abdominis muscles by acetylcholine and by KCl. An isolated frog rectus abdominis muscle (*Rana nigromaculata*) was placed in 5 ml. of glucose–Ringer solution (6·43 g. of NaCl, 0·30 g. of KCl, 0·17 g. of $CaCl_2$, 0·10 g. of $NaHCO_3$ and 0·71 g. of glucose in 1000 ml. of water), which was continuously bubbled with air. The contraction of the muscle was recorded by a strain-gauge transducer. At the points marked A, $A_2$ and $A_{10}$, acetylcholine solution (0·1 ml.) was added to the medium Ringer solution to give final concentrations 0·5, 1·0 and 5·0 $\mu$g./ml. respectively. At the points marked K and K′, KCl solution (0·1 ml.) was added to the medium Ringer solution to give final concentrations 0·05 M and 0·02 M respectively. At every interval between the curves, the muscle was washed three times with 5 ml. of the Ringer solution and placed in 5 ml. of fresh Ringer solution. The right and left muscles of a single individual were used as a pair. Erabutoxin a (0·12 $\mu$g. of Kjeldahl nitrogen/ml. of Ringer solution) and erabutoxin b (the same final concentration as a) were added at the points marked Ea and Eb respectively.

The dried venoms of *L. semifasciata* and *L. laticaudata* have a very different appearance from land-snake venoms, e.g. those of 'habu' (*Trimeresurus flavoviridis*). They are obtained as white powder when dried in the air. As shown by CM-cellulose chromatography (Fig. 1*c*) about 30% of the proteins in the snake venom are the toxic proteins, half of them being erabutoxin a and the

other half erabutoxin b. It seems that these basic toxic proteins are present in the venom as their salts.

The homogeneity of each crystalline toxic protein was shown by rechromatography (Figs. 1*a* and 1*b*), by disk electrophoresis (Figs. 3*a* and 3*b*), by ultracentrifuging (Fig. 4), by toxicity measurements before and after repeated crystallizations (Table 1) and by *N*-terminal amino acid analysis.

The results of amino acid analysis and of molecular-weight measurements showed that both erabutoxins a and b consisted of 61 (or 62) amino acid residues/molecule with molecular weights of about 7000. All the Kjeldahl nitrogen of the toxins are accounted for by these amino acids, although the possibility of the existence of non-nitrogenous additional group(s) in the molecules cannot yet be eliminated.

It is noteworthy that erabutoxins a and b have the same amino acid compositions except that one aspartic acid (or asparagine) residue in erabutoxin a is replaced by a histidine residue in erabutoxin b. The differences in disk-electrophoretic mobility and in adsorption on the CM-cellulose column can be explained by this difference in amino acid composition.

Almost equal amounts of erabutoxins a and b were present in our venom preparation, which was collected from 350 individuals. The question whether a single individual has both toxins or only one of them remains to be clarified.

It is noteworthy that both erabutoxins contain no alanine or methionine among their 61 (or 62) amino acid residues. The basicity of the toxins can be explained by the contents of the basic and acidic amino acids, assuming the amide content to be about 10 (Table 3). The crystals in Fig. 2 are probably sulphate salts of these basic toxins. Carey & Wright (1960) reported that the toxin in *Enhydrina schistosa* venom is also basic. One of the three arginine residues of the toxins is located at the *N*-terminus.

The lethal doses of the toxins are the same and of the same order as that of cobrotoxin, which was obtained in crystals from Formosan-cobra (*Naja naja atra*) venom by Yang (1965). The toxins block neuromuscular transmission (Fig. 5). They inhibit the contraction of frog rectus abdominis muscle by acetylcholine (Fig. 6). The $K^+$-induced contraction of the same muscle is not affected by these toxins (Fig. 6). All these observations show that the toxins act on the postsynaptic membrane in a very similar manner to curare, like the toxins of land-living venomous snakes (Russell, Obrien & Inaba, 1961) including Crotalidae and Elapidae (Chang & Lee, 1963).

The authors thank Professor R. Kano of the Medical School for zoological advice, Dr H. Ozawa of Toyo Rayon Corp. and Professor H. Noda of the University of Tokyo for the ultracentrifuge experiments, and Professor T. Shigei of the Medical School for pharmacological examinations. They also thank Dr Y. Nagai of this Laboratory for useful advice in electrophoresis experiments. The work was partly supported by a grant from the Ministry of Education, Japanese Government.

## REFERENCES

Arai, H., Tamiya, N., Toshioka, S., Shinonaga, S. & Kano, R. (1964). *J. Biochem., Tokyo*, **56**, 568.
Carey, J. E. & Wright, E. A. (1960). *Nature, Lond.*, **185**, 103.
Chang, C. C. & Lee, C. Y. (1963). *Arch. Pharmacodyn.* **144**, 241.
Cohn, E. J. & Edsall, J. T. (1943). In *Proteins, Amino Acids and Peptides*, p. 370. Ed. by Cohn, E. J. & Edsall, J. T. New York: Reinhold Publishing Corp.
Davis, B. J. (1964). *Ann. N.Y. Acad. Sci.* **121**, 404.
Fraenkel-Conrat, H., Harris, J. I. & Levy, A. L. (1955). *Meth. biochem. Analy.* **2**, 359.
Goodwin, T. W. & Morton, R. A. (1946). *Biochem. J.* **40**, 628.
Homma, M., Okonogi, T. & Mishima, S. (1964). *Gumma J. med. Sci.* **13**, 283.
Levy, A. L. (1954). *Nature, Lond.*, **174**, 126.
Ornstein, L. (1964). *Ann. N.Y. Acad. Sci.* **121**, 321.
Reid, H. A. (1961). *Brit. med. J.* p. 1284.
Reisfeld, R. A., Lewis, U. J. & Williams, D. E. (1962). *Nature, Lond.*, **195**, 281.
Russell, F. E., Obrien, B. A. & Inaba, D. I. (1961). *Proc. 10th Pacific Science Congress, Honolulu*, p. 455.
Sanger, F. (1949). *Biochem. J.* **45**, 563.
Schachman, H. K. (1959). *Ultracentrifugation in Biochemistry*, p. 181. New York: Academic Press Inc.
Spies, J. R. & Chambers, D. C. (1948). *Analyt. Chem.* **20**, 30.
Tu, T. (1961). *Biochem. Pharmacol.* **8**, 75.
Tu, T. (1963). *J. Formosan med. Ass.* **62**, 87.
Weber, C. J. (1930). *J. biol. Chem.* **86**, 217.
Yang, C. C. (1965). *J. biol. Chem.* **240**, 1616.
Yphantis, D. A. (1960). *Ann. N.Y. Acad. Sci.* **88**, 586.