248, 2107.
Palmiter, R. D., and Smith, L. (1973), *Mol. Biol. Rep. 1,* 129.
Palmiter, R. D., and Wrenn, J. T. (1971), *J. Cell Biol. 50,* 598.
Rhoads, R. E., McKnight, G. S., and Schimke, R. T. (1971), *J. Biol. Chem. 246,* 7407.
Rhoads, R. E., McKnight, G. S., and Schimke, R. T. (1973), *J. Biol. Chem. 248,* 2031.
Roberts, B. E., and Paterson, B. M. (1973), *Proc. Natl. Acad. Sci. U.S.A. 70,* 2330.
Rosen, J. M., Woo, S., Holder, J. W., Means, A. R., and O'Malley, B. W. (1975), *Biochemistry 14,* 69.
Rosenfeld, G. C., Comstock, J. P., Means, A. R., and O'Malley, B. W. (1972), *Biochem. Biophys. Res. Commun. 46,* 1695.
Ross, J., Ikawa, Y., and Leder, P. (1972), *Proc. Natl. Acad. Sci. U.S.A. 69,* 3620.
Socher, S. H., and O'Malley, B. W. (1973), *Dev. Biol. 30,* 411.
Sullivan, D., Palacios, R., Stavnezer, J., Taylor, J. M., Faras, A. J., Kiely, M. L., Summers, N. M. Bishop, J. M., and Schimke, R. T. (1973), *J. Biol. Chem. 248,* 7530.
Tompkins, G. M., Gelehrter, T. D., Granner, D., Martin, D., Jr., Samuels, H. H., and Thompson, E. B. (1969), *Science 166,* 1474.
Woo, S. L. C., Rosen, J. M., Liarakos, C. D., Robberson, D., Choi, Y. C., Busch, H., Means, A. R., and O'Malley, B. W. (1975), in preparation.
Yu, J.Y.-L., Campbell, L. D., and Marquardt, R. R. (1971), *Can. J. Biochem. 49,* 348.

Structure–Function Relationship in the Binding of Snake Neurotoxins to the Torpedo Membrane Receptor†

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ABSTRACT: The Cys_{30}–Cys_{34} bridge present in all long neurotoxins (71–74 amino acids, 5 disulfide bridges), but not in short toxins (60–63 amino acids, 4 disulfide bridges), is exposed at the surface since it can be reduced rapidly and selectively by sodium borohydride. Reduction and alkylation of the Cys_{30}–Cys_{34} bridge of *Naja haje* neurotoxin III hardly alter the conformational properties of this model long toxin. Although alkylation by iodoacetic acid of the -SH groups liberated by reduction abolishes the toxicity, alkylation by iodoacetamide or ethylenimine does not affect the curarizing efficacy of the toxin. The Cys_{30}–Cys_{34} bridge is not very important for the toxic activity of long neurotoxins. Reduction of the Cys_{30}–Cys_{34} bridge followed by alkylation with radioactive iodoacetamide gave a labeled and active toxin which is a convenient derivative for binding experiments to the toxin receptor in membranes of the Torpedo electric organ. The binding capacity of these membranes is 1200 pmol of toxin/mg of membrane protein. The dissociation constant of the modified toxin–receptor complex at pH 7.4, 20° is 10^{-8} *M*. Reduction with carboxamidomethylation of the Cys_{30}–Cys_{34} bridge decreases the affinity of the native *Naja haje* toxin only by a factor of 15. Carboxymethylation after reduction prevents binding to the membrane receptor. The binding properties of the derivative obtained by reduction and aminoethylation of Cys_{30}–Cys_{34} are very similar to those of native neurotoxin III; the affinity is decreased only by a factor of 5. Binding properties to Torpedo membranes of long neurotoxins (*Naja haje* neurotoxin III) and short neurotoxins (*Naja haje* toxin I and *Naja mossambica* toxin I) have been compared. Dissociation constants of receptor–long neurotoxin and receptor–short neurotoxin complexes are very similar ($5.7–8.2 \times 10^{-10}$ *M* at pH 7.4, 20°). However, the kinetics of complex formation and complex dissociation are quite different. Short neurotoxins associate 6–7 times faster with the toxin receptor and dissociate about 5–9 times faster than long neurotoxins. Acetylation and dansylation of Lys_{53} and Lys_{27} decrease the affinity of long and short toxins for their receptor by a factor of about 200 at pH 7.4, 20°, mainly because of the slower rate of association with the receptor.

During the last decade considerable work has been carried out on snake venoms, and more than 50 toxins with neurotoxic activity have been isolated in a pure state from elapid venoms (cobra, krait, tiger snakes, mambas) as well as from hydrophid venoms (sea-snakes).

These neurotoxins form two different groups—the 60–63 amino acids group and the 71–74 amino acids group. Neurotoxins with a chain length of 60–63 amino acid residues in a single polypeptide chain are cross-linked by four disulfide bridges; they are called "short neurotoxins." Neurotoxins with a chain length of 71–74 amino acid residues are cross-linked by five disulfide bridges; they are called "long neurotoxins." Both short and long neurotoxins are postsynaptic or curariform toxins which combine firmly with the acetylcholine receptor on the motor end plate and produce a nondepolarizing block of neuromuscular transmission, just like *d*-tubocurarine (Lee, 1970).

Both short and long neurotoxins have the same overall arrangement of four disulfide bridges (Figure 1). The two

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	1	5	10	15	20
1. *N. haje haje*	(toxinα) Leu Glu Cys His Asn Gln Gln Ser Ser Gln Pro Pro Thr Thr Lys Thr Cys Pro -				Gly Glu Thr
2. *N. nigricollis mossambica*	(toxin I) Leu Glu Cys His Asn Gln Gln Ser Ser Glu Pro Pro Thr Thr Thr Arg Cys Ser				Gly Gly Glu Thr
3. *N. haje*	(toxin I) Leu Glu Cys His Asn Gln Gln Ser Ser Gln Pro Pro Thr Thr Lys Thr Cys Pro -				Gly Glu Thr
4. *N. nivea*	(toxin α) Ile Arg Cys Phe - - -	Ile Thr Pro Asp Val Thr Ser Glu Ala Cys Pro Asp			Gly - His
5. *N. haje*	(toxin III)Ile Arg Cys Phe - - -	Ile Thr Pro Asp Val Thr Ser Gln Ala Cys Pro Asp			Gly Gln Asn

	25	30	35	40	45
1. *N. haje haje*	Asn Cys Tyr Lys Lys Arg Trp Arg Asp His - - -	-	Arg Gly Ser Ile Thr Glu Arg Gly Cys Gly - -		Cys
2. *N. nigricollis mossambica*	Asn Cys Tyr Lys Lys Arg Trp Arg Asp His - - -	-	Arg Gly Tyr Arg Thr Glu Arg Gly Cys Gly - -		X
3. *N. haje*	Asn Cys Tyr Lys Lys Arg Trp Arg Asp His - - -	-	Arg Gly Ser Ile Thr Glu.....		
4. *N. nivea*	Val Cys Tyr Thr Lys Met Trp Cys Asp Asn Phe Cys Gly Met Arg Gly Lys Arg Val Asp Leu Gly Cys Ala Ala Thr Cys				
5. *N. haje*	Ile Cys Tyr Thr Lys Thr Trp Cys Asp Asn Phe Cys Gly Met Arg Gly.....				

	50	55	60	65	70	75
1. *N. haje haje*	Pro Ser Val Lys Lys Gly	Ile Glu Ile Asn Cys Cys Thr Thr Asp Lys Cys Asn Asn				
2. *N. nigricollis mossambica*	Pro Thr Val.....			Cys Asn Asn		
3. *N. haje*	.....			Cys Asn Asn		
4. *N. nivea*	Pro Lys Val Lys Pro Gly Val Asn Ile Lys Cys Cys Ser Arg Asp Asn Cys Asn Pro Phe Pro Thr - Arg Lys Arg Ser					
5. *N. haje*	.....			Lys Arg Ser		

FIGURE 1: Comparison of partial amino acid sequences of *Naja haje* toxins I and III (Kopeyan et al., 1973) and of *Naja nigricollis mossambica* toxin I (Rochat et al., 1974) with sequences of *Naja haje haje* toxin α (Botes and Strydom, 1969) and *Naja nivea* toxin α (Botes et al., 1971a,b). The amino acids are numbered according to Yang (1974). Determination of complete sequences of *Naja haje* and *Naja nigricollis mossambica* toxins used in this work is under way. (—) Deletion; (···) or (X) not determined.

nonhomologous half-cystine residues in long toxins form an "extra" disulfide bridge that pinches off a short pentapeptide sequence in a large loop which is present in both short and long neurotoxins (Botes, 1971). Sequence comparisons to study the phylogenetic relationships of snake toxins (Lee, 1972; Yang, 1974) suggest that the more primitive toxins are the short toxins. This speculation is strengthened by the fact that long neurotoxins are not present in sea-snake venoms, but appear in venoms from the elapid family, the more highly evolved species. We describe in this paper kinetic and thermodynamic differences in binding properties between short and long neurotoxins. We also study the structural and functional role of the extra disulfide bridge present only in the long neurotoxins.

Several groups have chemically modified snake neurotoxins to try to identify the amino acid side chains involved in the active site area of the toxins. The results, reviewed by Yang (1974), have indicated that two amino acids with positively charged side chains are of great importance for the neurotoxicity; they are lysine-53 and arginine-37.[1] Chemical modifications of these two residues drastically decrease neurotoxicity measured as LD_{50}. We analyze in this paper the effect of chemical modification of Lys_{53} in short and long neurotoxins upon the binding properties of the toxins to the toxin receptor of the electric organ membranes of Torpedo.

Materials and Methods

Neurotoxins and Chemicals. Neurotoxins I and III from the venom of *Naja haje* and neurotoxin I of *Naja nigricollis mossambica* were prepared as described by Miranda et al. (1970a) and Rochat et al. (1974). These proteins were homogeneous by equilibrium chromatography on Amberlite CG-50 or Biorex-70, gel electrophoresis, and amino acid composition.

Toxicity was determined according to Miranda et al. (1970a) by subcutaneous injection of the neurotoxins into mice. LD_{50} for neurotoxins I and III of *Naja haje* and neurotoxin I of *Naja nigricollis* are 1.1, 1.3, and 0.8 μg/20 g of body weight, respectively.

Iodoacetic acid, iodoacetamide, ethylenimine, and dansyl chloride were Sigma products. [^{14}C]Iodoacetic acid and [^{14}C]iodoacetamide were obtained from the Radiochemical Centre, Amersham. Cyanogen bromide was obtained from Pierce Chemical Company.

Optical Rotatory Dispersion (ORD) and Spectrophotometric Measurements. Optical rotatory dispersion measurements were carried out in a Fica Spectropol I spectropolarimeter with a cell thermostated between 5 and 90 ± 0.2°. Melting curves of the various neurotoxins were obtained as previously described (Chicheportiche et al., 1972).

Concentrations of the neurotoxins were estimated by optical density measurements at 280 nm with $E_{1cm}(1\%)$ 11.48 for neurotoxin III of *Naja haje* venom (mol wt 7806), $E_{1cm}(1\%)$ 13.49, for neurotoxin I of *Naja haje* venom (mol wt 6843) (Miranda et al., 1970a), and $E_{1cm}(1\%)$ 13.70 for neurotoxin I of *Naja nigricollis mossambica* (mol wt 7176) (Rochat et al., 1974).

Chemical Modifications. Reduction of disulfide bridges of *Naja haje* neurotoxin III (1 mg/ml) by 0.1 *M* sodium borohydride was carried out at pH 9.0 at 20° and at 0° under nitrogen. The kinetics were followed by taking aliquots at different times. The aliquots were immediately acidified to pH 2 for at least 15 min at 0° to destroy excess borohydride. Newly formed -SH groups were then titrated both by the Ellman technique (Ellman, 1959) and by alkylation with [^{14}C]iodoacetic acid (50 m*M*, pH 8.0, 30 min,

[1] The numbering used for the amino acid residues is that of Yang (1974) (see Figure 1).

and 20°) or [^{14}C]iodoacetamide (50 mM, pH 8.0, 30 min, and 20°). Excess labeled iodoacetic acid or iodoacetamide were separated from alkylated neurotoxin III by Sephadex G-25 chromatography on a column (2 × 22 cm) equilibrated and eluted with ammonium bicarbonate (50 mM, pH 7.8) or with 1 mM HCl with the same results.

Acetylation of the 5 amino groups of neurotoxin III and selective dansylation of the ϵ-amino groups of lysine-27 and lysine-53 of neurotoxin I from *Naja haje* venom were performed as previously described (Chicheportiche et al., 1972).

Location in the Sequence of the Selectivity Reducible Disulfide Bridge. As will be seen in the Results section, one disulfide bridge in *Naja haje* neurotoxin III can be selectively reduced by borohydride.

For cyanogen bromide cleavage as described by Inglis and Edman (1970), 0.46 μmol of neurotoxin was incubated at 30° for 15 hr with a 2000-fold molar excess of cyanogen bromide.

Automatic Edman degradation was performed in a Sequenceur PS 100 (Socosi 94 100, St Maur, France) using a program for the degradation cycle prescribed by Edman and Begg (1967). Identification of phenyl isothiocyanate derivatives was made by thin-layer chromatography (Edman and Begg, 1967). The average yield at each step was 93% for the nine first steps.

Samples for amino acid analysis were hydrolyzed in 6 N HCl in sealed evacuated tubes at 110° for 20 hr. Amino acid analyses were carried out in a Spinco automatic amino acid analyzer, Model 120 C, according to Spackman et al. (1958).

Preparation of Membrane Fragments from Torpedo marmorata Electrical Tissue. Membrane fragments containing the cholinergic receptor protein were prepared from Mediterranean *Torpedo marmorata* electrical tissue according to Cohen et al. (1972). The protein content of the membrane preparation was estimated by the method of Hartree (1972) using bovine serum albumin as the standard protein. The receptor concentration was calculated from the maximal binding capacity of membranes (1200 pmol of toxin/mg of membrane protein, see Figure 5) assuming a 1:1 stoichiometry for the interaction between the toxin and its receptor.

Neurotoxin–Receptor Association Kinetics. To determine complex formation, Torpedo membranes (final receptor concentration, 65 nM) were equilibrated at 20° in a 10 mM sodium phosphate buffer (pH 7.4) containing 0.25 M NaCl, 5 mM KCl, 2 mM $MgCl_2$, and 0.1 M sucrose. The association reaction was started by adding [^{14}C]RCAM-★TIII[2] to give a final concentration of 103 nM. Aliquots (1 ml) of the incubation mixture were taken at different times and filtered under reduced pressure through Millipore cellotate filters[3] (EGWP 02500, 0.2 μ). The filters were then washed twice with 5 ml of cold phosphate buffer and finally shaken in 10 ml of Bray's solution for 20 min. The amount of labeled toxin bound to Torpedo membranes was determined by liquid scintillation counting in a Packard TriCarb spectrometer, Model 3375.

Kinetics of association of the membrane toxin receptor with nonradioactive neurotoxins were followed by competition with [^{14}C]RCAM★TIII. [^{14}C]RCAM★TIII (0.26 μM) and the nonradioactive toxin (20 nM to 4 μM) were incubated together at 20° and pH 7.4 in the phosphate buffer already described. The association reaction was started by adding membranes (receptor concentration, 20 nM) to the neurotoxins. The amount of radioactive toxin bound to Torpedo membranes was evaluated after a 10-min incubation by the filtration technique described above.

Dissociation Kinetics of the RCAM★TIII–Receptor Complex. The complex was first prepared by incubating membranes (final receptor concentration, 50 nM) and [^{14}C]RCAM★TIII (final concentration, 103 mM) at 20°, pH 7.4, for 2 hr in the usual phosphate buffer. As will be seen in the Results section, 2-hr incubation is long enough to reach equilibrium under the experimental conditions described. Displacement of [^{14}C]RCAM★TIII from the complex was started by adding a 100-fold molar excess of native toxin III. Aliquots were taken at different times to measure the amount of [^{14}C]RCAM★TIII which remained bound to the receptor, using the filtration technique described above.

Equilibrium Binding Experiments. Direct evaluation of the dissociation constant of the RCAM★TIII–receptor complex was readily made from the titration curve of the receptor with radioactive toxin. Dissociation constants of the complexes formed between the toxin receptor and unlabeled neurotoxins were determined from the equilibrium positions obtained in competition experiments involving the receptor, [^{14}C]RCAM★TIII, and the nonradioactive neurotoxin. [^{14}C]RCAM★TIII (50 nM to 0.5 μM) was incubated with the Torpedo membranes (receptor concentration, 25 nM) at 20°, pH 7.4, in the usual phosphate buffer. After 2 hr, when the association between both partners was maximal, [^{14}C]RCAM★TIII was competitively displaced from the receptor-[^{14}C]RCAM★TIII complex by the nonradioactive toxin (40 nM to 2 μM). The nonradioactive toxin was also displaced from its complex with the toxin receptor by [^{14}C]RCAM★TIII under the same experimental conditions. Competition between the nonradioactive toxin and [^{14}C]RCAM★TIII for the toxin receptor was followed by measurement of the amount of membrane-bound radioactive RCAM★TIII until equilibrium was attained. The membrane-bound radioactivity at equilibrium with the known dissociation constant for the toxin receptor–RCAM-★TIII complex allowed an estimation of the dissociation constant for each one of the toxin receptor–nonradioactive toxin complexes.

Results

Selective Reduction of One Disulfide Bridge in Neurotoxin III. Figure 2 shows the kinetics of reduction of disulfide bridges of neurotoxin III by 0.1 M borohydride. It indicates that reduction rapidly generated two sulfhydryl groups at 20°. The fast reduction process lasted about 6 min and was followed by a slower reduction. The fast reduction process followed pseudo-first-order kinetics (inset of Figure 2). The corresponding second-order rate constant was $8.2 \times 10^{-2}\ M^{-1}\ \text{sec}^{-1}$ at 20°. Exactly the same type of

[2] Abbreviations used are: RCAM★TIII, *Naja haje* neurotoxin III selectively reduced and carboxamidomethylated at the disulfide bridge Cys_{30}–Cys_{34}; RCOM★TIII, neurotoxin III selectively reduced and carboxymethylated at Cys_{30}–Cys_{34}; RAE★TIII, neurotoxin III selectively reduced and aminoethylated at Cys_{30}–Cys_{34}; dansyl toxin I, *Naja haje* neurotoxin I selectively dansylated on the ϵ-amino groups of Lys_{27} and Lys_{53}; acetyl toxin III, *Naja haje* neurotoxin III fully acetylated on its five amino groups (1 α-amino and 4 ϵ-amino).

[3] Millipore cellotate filters are routinely kept before use in the phosphate buffer previously described containing 1% bovine serum albumin at 0°. This pretreatment considerably decreases nonspecific binding of labeled neurotoxin to the filters themselves.

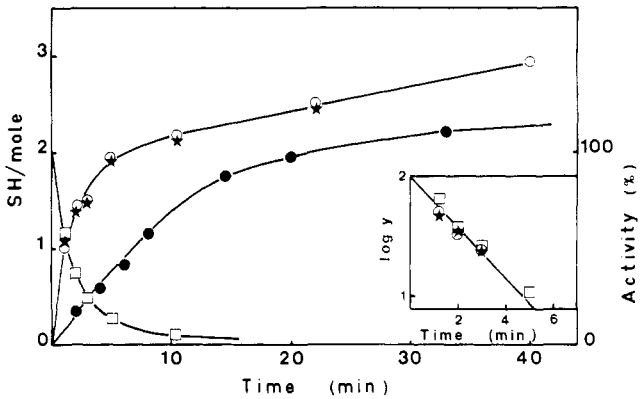

FIGURE 2: Kinetics of reduction of the disulfide bridges of *Naja haje* neurotoxin III by 0.1 *M* sodium borohydride. The time course of the reduction at 20° was followed by measuring the number of newly formed -SH groups by titration with the Ellman reagent (○) or by alkylation with [^{14}C]iodoacetic acid (★). Alkylation with [^{14}C]iodoacetamide gave results identical with those obtained with [^{14}C]iodoacetic acid. The loss of activity after reduction and alkylation with [^{14}C]iodoacetic acid was followed by LD_{50} measurements (□) (the activity is expressed as percent from the ratio: LD_{50} of the native toxin/LD_{50} of an aliquot taken at time t of the reduction). The inset shows a pseudo-first-order representation of the reduction of the most reactive disulfide bridge followed both by titration of the -SH groups liberated and by the loss of toxicity. y represents either the percent of toxicity during reduction followed by carboxymethylation of the -SH groups or the percent of the rapidly reducible disulfide bridge which remained unaltered. Symbols used in the inset correspond to symbols used in the main part of the figure. The time course of the reduction was also followed at 0° (●) by titration of the -SH groups with the Ellman reagent.

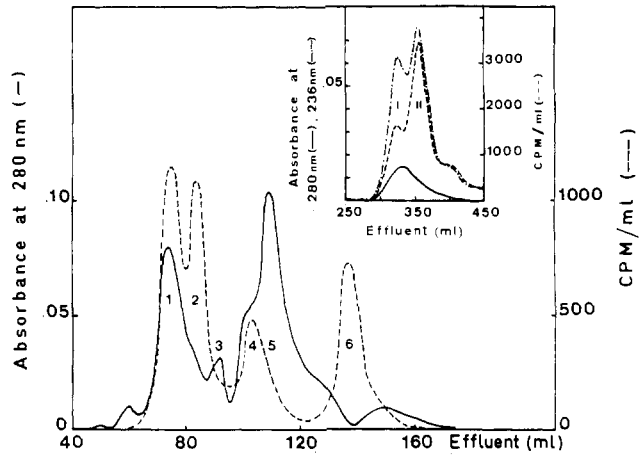

FIGURE 3: Gel filtration on Sephadex G-50 of peptide obtained after BrCN cleavage. The elution profile of the cyanogen bromide peptide separated on a Sephadex G-50 column (2 × 50 cm) equilibrated and eluted with 1 *M* acetic acid with a flow rate of 12.5 ml/hr. The inset presents the rechromatography of fraction 2 on a Sephadex G-50 column (2 × 150 cm) eluted with 1 *M* acetic acid with a flow rate of 25 ml/hr.

profile was obtained at 0°; in that case the second-order rate constant for the fast appearance of two -SH groups was $1.7 \times 10^{-2}\ M^{-1}\ \text{sec}^{-1}$.

Figure 2 also shows that the loss of toxicity was parallel to the reduction when the -SH groups were alkylated by iodoacetic acid. Toxicity was decreased by more than 90% when two -SH groups had been alkylated.

A comparison of the reduction kinetics of neurotoxin I and neurotoxin III of *Naja haje* was carried out at 0°. At 33 min when 2.25 -SH groups were liberated by borohydride reduction of neurotoxin III, only 0.25 group was liberated from neurotoxin I. Since the four disulfide bridges of neurotoxin I are in homologous positions in neurotoxin III (Figure 1), it is likely that the disulfide bridge, which does not exist in neurotoxin I, is particularly vulnerable to borohydride reduction.

Sequence work has been carried out to demonstrate this particular point. Neurotoxin III was reduced with borohydride and carboxymethylated with [^{14}C]iodoacetic acid as shown in Figure 2 to obtain a derivative with 1.8 ^{14}C-labeled carboxymethyl group per mol of protein. The modified toxin was then diluted six times with native neurotoxin III. Intact disulfide bridges in the modified neurotoxin III and in the native toxin added for dilution were then completely reduced at 20° by 70 m*M* dithioerythritol in 5 *M* guanidine hydrochloride at pH 6.7 (phosphate buffer 0.5 *M*). Carboxymethylation of the -SH groups liberated by this complete reduction was carried out with cold iodoacetic acid (0.25 *M*) for 1 hr at 20°. The modified protein was separated from excess reagents by filtration on a Sephadex G-25 column (2 × 42 cm) equilibrated and eluted with 1 m*M* HCl. If one of the five disulfide bridges of neurotoxin III is selectively reducible as we believe, then, this procedure should produce a completely reduced and alkylated toxin with the particularly sensitive S-S bond radioactively labeled. The fully carboxymethylated toxin III was then subjected to cyanogen bromide treatment. Although neurotoxin III has only one methionine residue in its sequence, cyanogen bromide cleavage gave six different peptide fractions which were separated by filtration on Sephadex G-50 (Figure 3). The amino acid composition and specific radioactivity of four of the fractions are presented in Table I. Identification of the peptides in the sequence was made from the known partial sequence of neurotoxin III (Kopeyan et al., 1973) and from the amino acid composition. The results in Figure 3 and in Table I indicate that cyanogen bromide treatment was not complete since some of the material was not cleaved at all (30%). Moreover it was not selective for the methioninyl bond. A subsidiary cleavage occurred under our conditions at the peptide bond Trp_{29}-Cys_{30}. Three of the four peptides of Table I were highly radioactive and had similar specific radioactivities; they were the uncleaved neurotoxin (fraction 1) and peptides Cys_{30}-Ser_{76} (fraction 2) and Cys_{30}-Met_{36} (fraction 6). Conversely peptide Ile_1-Trp_{29} (fraction 5) had a specific radioactivity about 16–17% that of the other peptides. All these results taken together indicate that the fast reduction observed in Figure 2 occurred selectively on disulfide bridge Cys_{30}-Cys_{34}.

Automatic sequencing confirmed this interpretation of the cyanogen bromide cleavage results. Toxin III selectively alkylated on the rapidly reduced disulfide bridge with [^{14}C]iodoacetic acid (1.8 ^{14}C-labeled carboxymethyl group per mole of protein) was further reduced on other disulfide bridges by dithioerythritol and S-methylated by ICH_3 on the new -SH groups formed. Automatic sequencing from the N-terminal end was carried out with 0.47 μmol of toxin. The technique for alkylation of the -SH groups by ICH_3 before sequencing and the automatic sequence procedure have been previously described (Kopeyan et al., 1973). The sulfhydryl groups alkylated with [^{14}C]iodoacetic acid were located by radioactivity measurements on phenylthiohydantoin amino acids at each step of the automatic Edman degradation. Automatic sequencing was carried out for the first 32 amino acids (Figure 4). The amount of radioactivity incorporated into each half-cystine was calculated from Fig-

Table I: Amino Acid Composition of Peptides Obtained by Cyanogen Bromide Cleavage.

Amino Acid	Fraction 1	Fraction II	Fraction 5[a]	Fraction 6
Carboxymethyl-cysteine	10.0 (10)[b]	6.4 (7)	3.0 (3)	1.8 (2)
Aspartic acid	9.6 (10)	6.2 (6)	3.6 (3)	2.1 (2)
Threonine	7.3 (7)	4.0 (4)	3.6 (4)	0.2 (0)
Serine	2.8 (3)	1.8 (1)	1.1 (1)	0.1 (0)
Glutamic acid	3.3 (3)	1.3 (1)	2.0 (2)	(0)
Proline	5.6 (6)	4.3 (4)	2.1 (2)	(0)
Glycine	5.1 (5)	3.6 (4)	1.1 (1)	1.2 (1)
Alanine	3.1 (3)	2.2 (2)	1.2 (1)	(0)
Valine	3.9 (4)	3.2 (3)	1.0 (1)	(0)
Methionine	0.8 (1)	0.1 (1)	(0)	(0)
Isoleucine	3.6 (4)	1.1 (1)	2.8 (3)	0.1 (0)
Leucine	1.0 (1)	1.1 (1)	(0)	(0)
Tyrosine	0 (1)	(0)	(1)	(0)
Phenylalanine	2.9 (3)	1.7 (2)	1.0 (1)	1.0 (1)
Lysine	4.0 (4)	3.1 (3)	1.1 (1)	0.2 (0)
Arginine	4.6 (5)	4.1 (4)	1.0 (1)	(0)
Tryptophan	(1)	(0)	(1)	(0)
Corresponding peptide	Ile_1–Ser_{76}	Cys_{30}–Ser_{76}	Ile_1–Trp_{29}	Cys_{30}–Met_{36}[c]
Specific radioactivity[d] (cpm/μmol)	8.2×10^4	8.7×10^4	1.4×10^4	8.4×10^4

[a] Figure 2 shows that fraction 5 is partially contaminated by about 20% of radioactivity from fraction 4. [b] Figures in parentheses represent the expected number of each amino acid evaluated from the amino acid composition and the knowledge of the partial sequence of *Naja haje* neurotoxin III. [c] Cyanogen bromide treatment transforms Met_{36} into an homoserine residue. [d] Specific radioactivity was calculated from the radioactivity measured with 1 ml of the top fraction of each peak (Figure 2) and from the corresponding quantity of peptide determined by amino acid analysis.

ure 4, correcting for background activity and allowing for an average yield of 89% at each step of Edman degradation. Cys_{30} contained 42.4% and Cys_{34}, 43.3% of the total radioactivity of the [^{14}C]RCOM★TIII (10^6 cpm). Thus 86% of the initial radioactivity was present on the two half-cystines constituting the single disulfide bridge 30–34, showing selective reduction at this position.

At 0°, pH 9.0, the Cys_{30}–Cys_{34} bridge in neurotoxin III is reduced by sodium borohydride ($k = 1.7 \times 10^{-2}$ M^{-1} sec^{-1}) 8–30 times faster than models such as oxytocin, cystine, or glutathione ($k = 0.5$–2×10^{-3} M^{-1} sec^{-1} (Light et al., 1969)) and nearly as rapidly as the Cys_{14}–Cys_{38} bridge of the pancreatic trypsin inhibitor ($k = 3.0 \times 10^{-2}$ M^{-1} sec^{-1} (unpublished result from this laboratory)) which is known from X-ray crystallographic data to be in a very exposed position at the protein inhibitor surface (Huber et al., 1971).

After selective reduction of the Cys_{30}–Cys_{34} disulfide bridge, alkylation of the two -SH groups can also be carried out with iodoacetamide or ethylenimine to obtain RCAM-★TIII and RAE★TIII. After carboxamidomethylation or aminoethylation of two -SH groups per mole of neurotoxin III the LD_{50} value was little changed, 1.7–1.8 μg/20 g of body weight of mice as compared to 1.3 μg/20 g of body weight with the native neurotoxin III. Neither carboxamidomethylation nor aminoethylation suppresses the neurotoxicity.

Conformational Properties of RCAM★TIII, RCOM-★TIII, RAE★TIII, and Native Neurotoxin III. The conformational properties of all these species of neurotoxin III have been compared using the ORD technique to follow thermal denaturation. Neurotoxin III is extremely resistant to thermal denaturation since, even at pH 2.1, denaturation did not start before 50° and temperatures over 80° were necessary for complete unfolding. This high stability is most

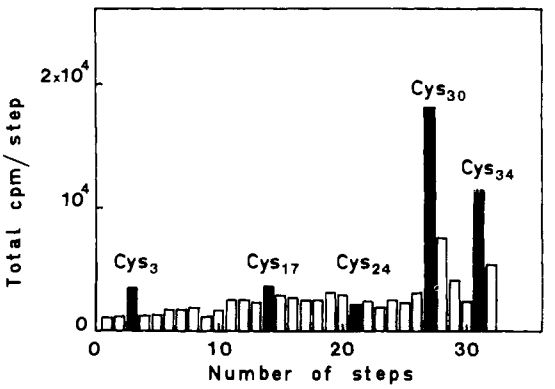

FIGURE 4: Histogram of automatic sequencing of [^{14}C]RCOM★TIII up to residue Gly_{35}; 0.47 μmol of [^{14}C]RCOM★TIII with 1.8 [^{14}C]carboxymethyl groups (total radioactivity 10^6 cpm) was submitted to the automatic Edman degradation for 32 steps. The phenylthiohydantoins obtained in the organic phase were dissolved in 100 μl of ethylene chloride and their radioactivity was measured.

probably due to the large number of disulfide cross-links (Chicheportiche et al., 1972; Tu, 1973). However, reduction and alkylation of the Cys_{30}–Cys_{34} bridge in RCAM-★TIII, RCOM★TIII, and RAE★TIII did not cause important changes in the thermal denaturation profile; melting curves for native and modified toxins were nearly superimposable. Transition temperatures (temperature for 50% denaturation) at pH 2.1 were: 68.5° and 67.5°, for native neurotoxin III and RCOM★TIII, 66° for RCAM★TIII and RAE★TIII. Reduction and alkylation of the Cys_{30}–Cys_{34} bridge in neurotoxin III hardly affected the conformational properties of the protein, even when charged groups are introduced.

Titration of the Toxin Receptor in Torpedo Membrane by [^{14}C]RCAM★TIII. Since selective reduction of the

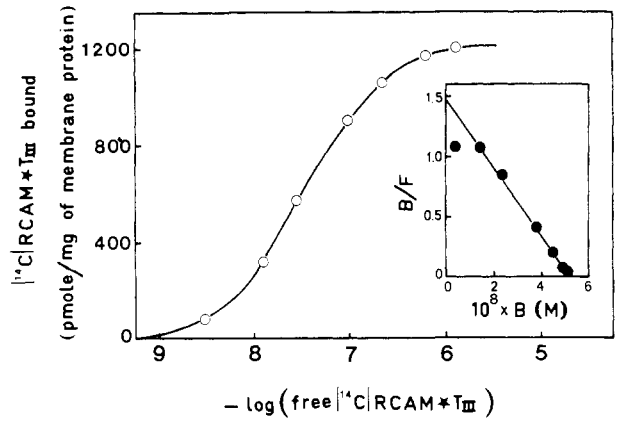

FIGURE 5: Titration of the neurotoxin receptor in Torpedo membranes with [^{14}C]RCAM★TIII. Torpedo membranes (41.4 μg of protein/ml) were incubated at 20°, pH 7.4 with increasing concentrations of [^{14}C]RCAM★TIII (54 Ci/mol). After a reaction time of 2 hr, aliquots (1 ml) were filtered on Millipore as described under Materials and Methods. (○) Toxin binding to Torpedo membranes; (●) Scatchard plots of the same experimental data. B refers to the concentration of [^{14}C]RCAM★TIII which was bound to Torpedo membrane after an incubation time of 2 hr; F is the concentration of [^{14}C]RCAM★TIII which remained free.

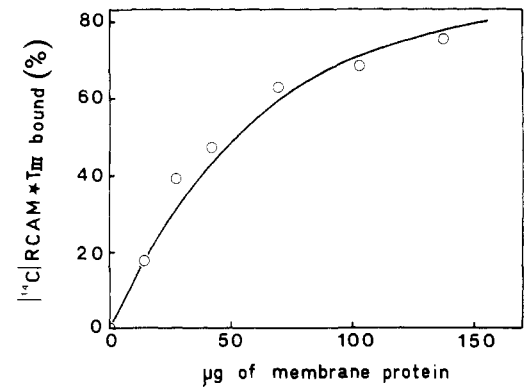

FIGURE 6: Titration of [^{14}C]RCAM★TIII by the neurotoxin receptor of Torpedo membranes. [^{14}C]RCAM★TIII (52 nM) was incubated (20°, pH 7.4) in the usual phosphate buffer with increasing concentration of Torpedo membranes. The reaction was terminated after 2 hr by filtration on Millipore filters. (○) Percent of the total [^{14}C]RCAM-★TIII concentration which is bound to Torpedo membranes. The solid line is a calculated curve using the value of the dissociation constant of the toxin receptor–[^{14}C]RCAM★TIII complex obtained from the data in Figure 5, i.e., $K_d = 3.4 \times 10^{-8}$ M. Saturation of the labeled toxin could not be reached because of an experimental limitation; when the membrane concentration exceeded 150 μg of membrane protein/ml, the incubation medium could no longer be filtered through the Millipore filters.

Cys_{30}–Cys_{34} bridge followed by alkylation with iodoacetamide has little effect on toxicity of neurotoxin III, [^{14}C]RCAM★TIII is a useful tool for titration of the receptor in membranes containing the acetylcholine receptor. Membranes from the electric organ of the Torpedo fish were chosen for this study because they contain large amounts of the cholinergic receptor protein (Cohen et al., 1972).

We have seen that reduction of the Cys_{30}–Cys_{34} disulfide bridge by borohydride in *Naja haje* neurotoxin III is much faster than that of the four other disulfide bridges. However, when the reduction of the toxin was stopped after liberation of two -SH groups per mole of toxin, the preparation obtained after alkylation by iodoacetamide consisted of about 95% of toxin III with the Cys_{30}–Cys_{34} bridge selectively reduced and alkylated, about 5% of native neurotoxin III, and traces of toxin III with more than one disulfide bridge reduced and alkylated. In contrast to native neurotoxin III and RCAM★TIII both of which have high toxicity, derivatives of toxin III with more than one disulfide bridge reduced and carboxamidomethylated have lost neurotoxicity. Reduction and alkylation of neurotoxin III to incorporate 2.0 carboxamidomethyl group per mole of toxin was used for conformational studies. For binding experiments to the toxin receptor, the RCAM★TIII preparation must be essentially free of native neurotoxin III. Native toxin could easily displace [^{14}C]RCAM★TIII bound to Torpedo membranes if it binds to the toxin receptor with a higher affinity than the modified toxin. To eliminate traces of native neurotoxin in the RCAM★TIII preparation, we made a borohydride reduction followed by alkylation with [^{14}C]iodoacetamide to reach an incorporation of 2.7 carboxamidomethyl groups incorporated per mole of neurotoxin. The concentration of *active* [^{14}C]RCAM★TIII (one disulfide bond reduced) in this sample was 65% of the total concentration of toxin and its specific radioactivity was 54 Ci/mol. All concentration and specific radioactivity calculations have been made using these latter data.

Figure 5 presents a binding curve obtained at 20°, pH 7.4, by adding increasing concentrations of [^{14}C]RCAM-★TIII to a constant concentration of receptor. Concentrations of RCAM★TIII higher than 10^{-6} M are saturating. The maximal binding capacity of membrane fragments containing the receptor is 1200 pmol of toxin/mg of membrane protein. The Scatchard plot in the inset of Figure 5 is linear and gives a value of the dissociation constant of the RCAM★TIII receptor complex of 3.4×10^{-8} M. The linearity of the Scatchard plot indicates that there is no heterogeneity among the toxin receptor binding sites and that no cooperativity (positive or negative) is involved in the binding process.

Figure 6 shows the results of experiments in which a constant concentration of [^{14}C]RCAM★TIII was associated with increasing amounts of Torpedo membranes. The experimental points agreed well with a curve calculated by assuming a value of the dissociation constant identical with that determined in Figure 5, 3.4×10^{-8} M.

The [^{14}C]RCAM★TIII-Receptor Complex: Kinetics of Formation and Dissociation of the Complex. Figures 5 and 6 have shown that the association between the toxin receptor and each one of the snake neurotoxins used in this work may be represented simply by the following scheme:

$$R + T \underset{k_d}{\overset{k_a}{\rightleftharpoons}} RT$$

where R is the toxin receptor, T, the neurotoxin, and k_a and k_d are the rate constants for association and dissociation, respectively.

The rate of association is expressed by

$$v = k_a[R][T] - k_d[RT] \quad (1)$$

Figure 7 shows the kinetics of association of the toxin receptor with [^{14}C]RCAM★TIII at 20°, pH 7.4. In this representation, the amount of labeled toxin bound at equilibrium is 735 pmol/mg of membrane protein. Since the maximal binding capacity of the membranes is 1200 pmol/mg of membrane protein (Figure 5) only a part of the receptor sites (60%) was saturated by the toxin. At the beginning of the association reaction, the complex concentration, [RT],

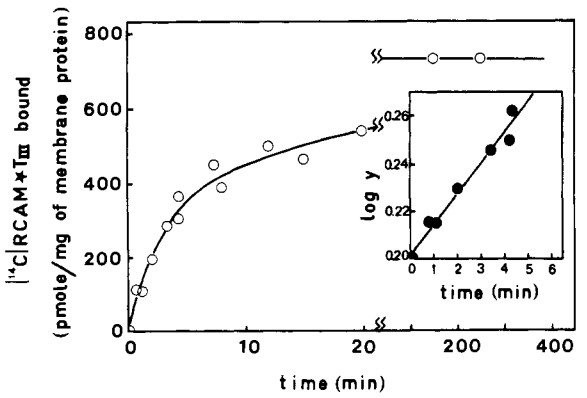

FIGURE 7: Kinetics of association of the toxin receptor from Torpedo membranes with [^{14}C]RCAM★TIII. Kinetics of association were evaluated by following the increase in membrane-bound [^{14}C]RCAM-★TIII. (○) Association between the toxin receptor (65 nM) and [^{14}C]RCAM★TIII (103 nM); (●) linear plot demonstrating second-order kinetics during the first 5 min of association. y refers to the ratio $([T_0] - [RT])/([R_0] - [RT])$ (eq 3). After 5 min, the association ceases to follow pure second-order kinetics because dissociation of the complex already formed is no longer negligible (see eq 1 in the text). k_a was calculated from the slope ($k_a([T_0] - [R_0])/2.3$) of the log y vs. time plot. An identical value of k_a was obtained when 25 nM receptor was associated with 51.5 nM [^{14}C]RCAM★TIII, other experimental conditions being the same: 20°, pH 7.4.

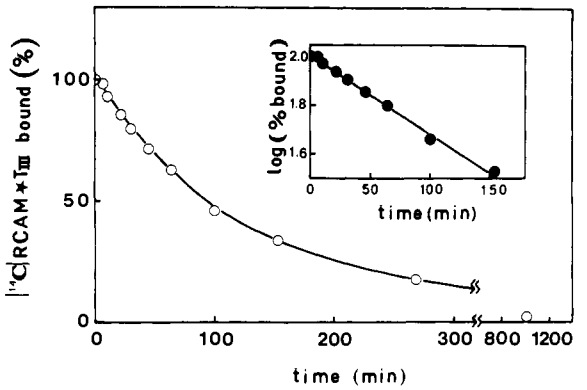

FIGURE 8: Dissociation kinetics of the complex formed between the toxin receptor of Torpedo membrane and [^{14}C]RCAM★TIII. The receptor-[^{14}C]RCAM★TIII complex was first formed by incubating Torpedo membranes (receptor concentration 50 nM) with [^{14}C]RCAM★TIII (103 nM) for 2 hr (sufficient for maximal association). Dissociation was started by adding a 100-fold molar excess of native neurotoxin III (10 μM). Replacement of [^{14}C]RCAM★TIII by native toxin III in the complex was followed by measuring the decrease of [^{14}C]RCAM★TIII bound to Torpedo membranes. (○) Time course of the dissociation; (●) pseudo-first-order plot of the data; 20°, pH 7.4.

is low as compared to concentrations of free receptor, [R], and free toxin, [T]. Moreover, as will be seen later, the value of the dissociation rate constant, k_d, is also very low. In consequence, eq 1 simplifies to eq 2 in the first few minutes of the association

$$v = k_a[R][T] \quad (2)$$

Classical integration of eq 2 gives

$$\log \frac{[T_0] - [RT]}{[R_0] - [RT]} = ([T_0] - [R_0])k_a t + \log \frac{[T_0]}{[R_0]} \quad (3)$$

As expected, the variation of $\log ([T_0] - [RT])/([R_0] - [RT])$ is linear with time during the first 5 min of association (inset of Figure 7). The value of k_a calculated from the slope is $1.2 \times 10^4\ M^{-1}\ \text{sec}^{-1}$. This value of k_a has been confirmed from plots similar to those of Figure 7, but in which different initial concentrations $[R_0]$ and $[T_0]$ were used.

Radioactive RCAM★TIII can be displaced from its association with the toxin receptor by native neurotoxin III of *Naja haje*. The dissociation process can be schematized as follows:

$$RT^* \underset{k_a^*}{\overset{k_d^*}{\rightleftharpoons}} T^* + R \xrightarrow[+T]{k_a} RT$$

T* and T are the labeled RCAM★TIII and the native neurotoxin, respectively. k_a* and k_d* refer to the complex formed with the radioactive toxin. k_a is the second-order rate constant for the association of the native toxin with its receptor. The displacement of T* from RT* is followed by measuring the decrease of labeled toxin bound to Torpedo membranes. Figure 8 presents a typical dissociation experiment. On addition of native toxin III in a 100-fold molar excess to the RT* complex, the system evolved toward an equilibrium position in which the initially complexed RCAM★TIII was completely liberated from its association with the receptor and replaced in the complex by the native toxin. The half-life of the displacement was 96 min. The association of the receptor with native toxin III being much faster than the displacement observed in Figure 8, the rate of this displacement gives an evaluation of the first-order rate constant, k_d*, for the dissociation of the receptor-RCAM★TIII complex (inset of Figure 8); $k_d^* = 1.2 \times 10^{-4}\ \text{sec}^{-1}$ at pH 7.4 and 20°. The same displacement profile and, in consequence, the same first-order rate constant of dissociation was observed when RCAM★TIII was displaced from the RT complex by native neurotoxin I of *Naja haje* (100-fold molar excess) instead of neurotoxin III.

The dissociation constant of the toxin receptor-RCAM-★TIII complex which was already evaluated from Figures 5 and 6 can also be calculated from kinetic data, using the equation: $K_d = k_d/k_a$.

The rate constants of association and dissociation previously evaluated give a dissociation constant of 10^{-8} M at pH 7.4 and 20°, in good agreement with that determined by equilibrium measurements.

Complexes Formed between the Toxin Receptor and Other Neurotoxins Either Native or Chemically Modified. The analysis of the kinetic and thermodynamic properties of the RCAM★TIII-receptor association permits measurements, by competition, of K_d, k_a, and k_d values for the binding of other nonlabeled neurotoxins to the toxin receptor in Torpedo membranes.

A typical example of such a competition between RCAM★TIII and native toxin III is presented in Figure 9. As expected, the displacement of neurotoxin III from the receptor-neurotoxin III complex by a fivefold molar excess of [^{14}C]RCAM★TIII gives the same equilibrium position than that obtained when [^{14}C]RCAM★TIII is displaced from its complex with the toxin receptor by native toxin III under the same conditions. The equilibrium position corresponds to the displacement of about 50% of [^{14}C]RCAM-★TIII initially bound to the toxin receptor. The experimental data in Figure 9 allow the determination of the ratio K_d^*/K_d where K_d* and K_d represent dissociation constants of the receptor -[^{14}C]RCAM★TIII and the receptor-native toxin III complexes, respectively.

$$\frac{K_d^*}{K_d} = \frac{[T^*]}{[RT^*]} \frac{[R_0] - [RT^*](1 + K_d^*[T^*])}{[T_0] - [R_0] + [RT^*](1 + K_d^*[T^*])} \quad (4)$$

Table II: Kinetic and Thermodynamic Characteristics of the Interaction between Various Native Neurotoxins or Chemically Modified Neurotoxins and the Toxin Receptor of Torpedo Membrane at 20°, pH 7.4.[a]

Toxin	k_a (M^{-1} sec^{-1})	k_d (sec^{-1})	$t_{1/2}$ (min)	K_d (M)	ΔC°_a (kcal mol^{-1})
Naja haje toxin III (71aa, 5S-S)	4.8×10^4	3.2×10^{-5}	360	6.7×10^{-10}	−12.4
RCAM*Toxin III	1.2×10^4	1.2×10^{-4}	96	10^{-8}	−10.8
RAE*Toxin III	1.4×10^4	4.8×10^{-5}	240	3.4×10^{-9}	−11.4
RCOM*Toxin III	0				
Acetyl toxin III	8.6×10^2	1.2×10^{-4}	96	1.4×10^{-7}	−9.3
Naja haje toxin I (61aa, 4S-S)	3.4×10^5	2.8×10^{-4}	41	8.2×10^{-10}	−12.3
Dansyl toxin I	2.4×10^3	3.2×10^{-4}	36	1.3×10^{-7}	−9.3
Naja mossambica toxin I (62aa, 4S-S)	3.0×10^5	1.7×10^{-4}	68	5.7×10^{-10}	−12.5

[a] K_d values for complexes formed between the toxin receptor and native or chemically modified neurotoxins were obtained from competition experiments similar to those described in Figure 9. Concentration ratios between [^{14}C]RCAM*TIII and each one of the unlabeled toxins were as follow: three concentration ratios 1:1, 5:1 (Figure 9), and 10:1 were used in competitions involving [^{14}C]RCAM*TIII on one hand and native *Naja Haje* neurotoxin I or native *Naja Haje* neurotoxin III or native *Naja Mossambica* neurotoxin I on the other hand; two concentration ratios, 1:1 and 1:10, were used for competitions involving [^{14}C]RCAM*TIII and RAE*TIII; two different concentration ratios, 1:2 and 1:20, were used with dansyl toxin I as the unlabeled toxin; these ratios were 1:4 and 1:40 with acetyl toxin III as the unlabeled toxin. The data for K_d presented in the table correspond to a mean value of K_d data obtained from the different concentration ratios. The precision of K_d is 20%.

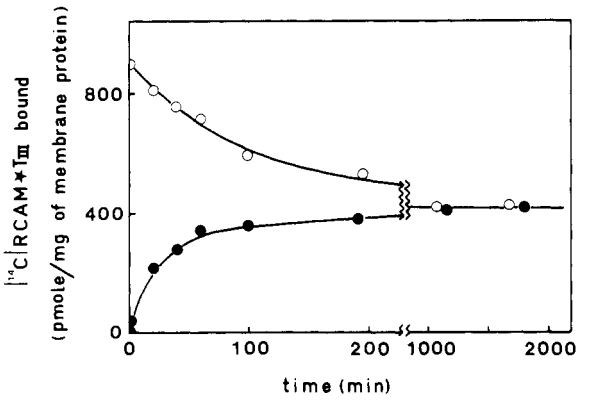

FIGURE 9: Competition experiments for the evaluation of the dissociation constant of the toxin receptor–native toxin III complex. Both initial complexes involving the toxin receptor and either [^{14}C]RCAM-★TIII or native toxin III were first formed by incubating the receptor (25 nM) with either the native toxin III (46 nM) or [^{14}C]RCAM-★TIII (258 nM) at 20°, pH 7.4, for 2 hr. Displacements were then carried out as follows. (●) Native toxin III was partially displaced from the receptor–native toxin III complex by [^{14}C]RCAM★TIII (258 nM); (○) [^{14}C]RCAM★TIII was partially displaced from the receptor –[^{14}C]RCAM★TIII complex by native toxin III (46 nM). As shown in the figure, equilibrium was attained after 1000 min.

[R_0] is the total concentration of receptor, [T_0] is the total concentration of native neurotoxin III, [RT*] is the concentration of the toxin receptor–RCAM★TIII complex at equilibrium, and [T*] is the concentration of free RCAM-★TIII at equilibrium ([T*] = [T_0*] − [RT*] where [T_0*] is the total concentration of RCAM★TIII).

Results in Figure 9 give a K_d^*/K_d ratio of 15. Taking a value of $K_d^* = 10^{-8}$ M calculated from the kinetic results for the association of RCAM★TIII with the toxin receptor (see above), K_d, the dissociation constant of the receptor–neurotoxin III complex, is found to be 6.7×10^{-10} M. The same ratio of K_d^*/K_d has been obtained in competition experiments involving different molar ratio of labeled RCAM★TIII and native toxin III ([T_0*]/[T_0] = 1 or 5 (see Figure 9) or 10.

The same type of approach described in Figure 9 permitted the determination of K_d values for complexes formed between the toxin receptor and *Naja haje* toxin I, *Naja nigricollis mossambica* toxin I, RCOM★TIII, RAE★TIII, acetyl toxin III, and dansyl toxin I (Table II).

Association kinetics between nonlabeled neurotoxins and the toxin receptor were also followed by competition experiments involving the Torpedo membranes, [^{14}C]RCAM-★TIII, and each one of the nonlabeled toxins. The competition technique was previously used and described in detail for the determination of the association kinetics between pseudotrypsin (Vincent and Lazdunski, 1972) or anhydrotrypsin (Vincent et al., 1974) and pancreatic trypsin inhibitors. The competition can be described as follows:

$$\begin{array}{rcl} & + \underset{(a-x)}{T^*} \xrightarrow{k_a^*} \underset{(x)}{RT^*} \\ \underset{(c-x-y)}{R} & & \\ & + \underset{(b-y)}{T} \xrightarrow{k_a} \underset{(y)}{RT} \end{array}$$

abbreviations are: R for the toxin receptor, T* for [^{14}C]RCAM★TIII, T for the nonlabeled toxin. a, b, and c are the initial concentrations of [^{14}C]RCAM★TIII, of the nonlabeled toxin and of the receptor, respectively. x and y are the concentrations of toxin–receptor complexes formed from RCAM★TIII or from nonlabeled toxin at a given time of the association. k_a^* and k_a are the second-order rate constants of association of the receptor with RCAM-★TIII and with nonradioactive toxin, respectively. It has been previously shown that the competition system used obeys the following relationship (Vincent and Lazdunski, 1972):

$$\frac{k_a^*}{k_a} = \frac{\log [a/(a-x)]}{\log [b/(b-y)]} \qquad (5)$$

Concentrations of R and RCAM★TIII in these experiments were chosen so that, if the time of incubation were long enough to reach equilibrium, the receptor would be nearly saturated by RCAM★TIII (more than 95%). The time of incubation of the ternary mixture receptor-[^{14}C]RCAM★TIII-nonlabeled toxin for determination of k_a from eq 5 should be long enough for complex formation of all receptor molecules with either [^{14}C]RCAM★TIII or nonradioactive toxin, but not long enough for significant displacement of [^{14}C]RCAM★TIII in the receptor-RCAM★TIII complex by nonradioactive toxin; 10 min fitted both conditions. The half-life of the RT* complex has already been shown to be 96 min. Different values of half-lives have been found with nonlabeled toxin; as will be seen later in the paper (Table II), they range between 36 and 360 min. x was evaluated from measurement of [^{14}C]RCAM★TIII bound to the receptor after 10 min. Knowing a, b, c, and x, the value of y can be calculated from $y = c - x$.

Figure 10 shows typical examples of log $[a/(a - x)]$ vs. log $[b/(b - y)]$ plots. The non-labeled toxins in these cases were native *Naja haje* toxin I and toxin III. The graphs are linear as expected from eq 5; they allow an evaluation of the ratio k_a^*/k_a. k_a can then be calculated since k_a^* is already known. Typical values taken from Figure 10 indicate that toxin III associates four times faster than RCAM★TIII with the toxin receptor whereas association of toxin I is 28 times faster than that of RCAM★TIII. Values of k_a corresponding to each one of the other nonradioactive toxins tested are compiled in Table II.

Since both K_d and k_a have been evaluated, the first-order rate constants of dissociation k_d were calculated ($k_d = k_a K_d$) and are given in Table II.

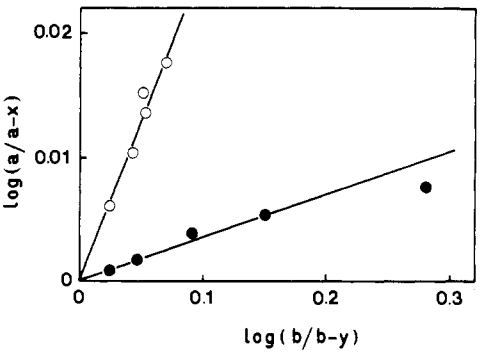

FIGURE 10: Analysis of the association of the toxin receptor with native toxins I and III. (●) Competition for the toxin receptor between [^{14}C]RCAM★TIII and native *Naja haje* toxin I; (○) competition for the toxin receptor between [^{14}C]RCAM★TIII and native *Naja haje* toxin III. Incubation time was 10 min for both competitions. Data were treated according to equation 5; 20°, pH 7.4.

Discussion

It has been shown in this paper that the Cys_{30}-Cys_{34} disulfide bridge, which is present in the long neurotoxins but absent in the short neurotoxins, can be selectively reduced in neurotoxin III of *Naja haje*, our model of long neurotoxins. No superreactive disulfide bridge is present in *Naja haje* neurotoxin I.

Reduction and alkylation of the Cys_{30}-Cys_{34} bridge hardly affect the main conformational properties of the long neurotoxin; this bridge does not play an important role for the maintenance of the overall structure of the long neurotoxin.

A useful application of the selective reduction of the Cys_{30}-Cys_{34} disulfide bridge is the preparation of a radioactive-labeled neurotoxin, [^{14}C]RCAM★TIII. This labeled toxin permits a facile analysis of the interaction of snake neurotoxins with excitable membranes containing the cholinergic receptor. Other types of chemical modifications have been used with success by other workers to label snake neurotoxins used for studies of the acetylcholine receptor in membranes of different origins: electric tissues of *Electrophorus electricus* and *Torpedo marmorata* and muscle fibers of frog sartorius (Miledi and Potter, 1971; Raftery et al., 1971; Menez et al., 1971; Fulpius et al., 1972; Karlson et al., 1972a; Cooper and Reich, 1972; Franklin and Potter, 1972; Chang et al., 1973; Weber and Changeux, 1974). In all these cases the toxin was found to bind to membranes with a high affinity. However, in most instances the location of the radioactive label in the neurotoxin sequence was not known.

The stoichiometry of the reaction of toxin with receptor in Torpedo membranes found by titration with [^{14}C]RCAM★TIII, 1200 pmol/mg of protein, is similar to that found by Weber and Changeux (1974), 1100 ± 600 pmol/mg of protein, with a [^{3}H]toxin from *Naja nigricollis* in which the radioactive label was incorporated on two histidines residues (Menez et al., 1971).

The possible functional importance of the Cys_{30}-Cys_{34} disulfide bridge was first evaluated by comparison of native neurotoxin III of *Naja haje* with reduced and alkylated derivatives of this toxin: RCAM★TIII, RCOM★TIII, and RAE★TIII. Reduction and alkylation with iodoacetamide decrease the affinity of the toxin for its receptor by a factor of 15. The increase in dissociation constant K_d is due both to a decrease of the rate of association (k_a) and to an increase of the rate of dissociation (k_d). Aminoethylation after reduction of the Cys_{30}-Cys_{34} bridge introduces 2 new positive charges in the toxin molecule without decreasing the affinity of neurotoxin III for its receptor by more than a factor of 5 (Table II). The changes in affinities observed with RCAM★TIII and RAE★TIII are not accompanied by similar changes in LD_{50} values. Native neurotoxin III, RCAM★TIII, and RAE★TIII all have LD_{50} values ranging between 1.3 and 2.0 μg/20 g of body weight. Alkylation with iodoacetic acid after reduction of the Cys_{30}-Cys_{34} bridge introduces two negative charges in the neurotoxin molecule. This treatment abolishes both binding and neurotoxicity measured as LD_{50}.

Disulfide interchange between one of the disulfide bridge of snake neurotoxins and the super reactive disulfide bridge of the acetylcholine receptor (Karlin and Winnik, 1968) has been suggested as a possible explanation for the tight binding of the toxins to their receptor in excitable membranes (Bartels and Rosenberry, 1971; Yang, 1974; Vincent and Lazdunski, 1972). The Cys_{30}-Cys_{34} bridge in long neurotoxins would have been an excellent candidate for such an interchange: it is at the surface of the toxin molecule in a very exposed position and it is in a region of the sequence including residues Tyr_{25}, Trp_{29}, and Arg_{37} which are believed to have some role for the structure and function of the toxin active site. The binding properties of RAE★TIII and RCAM★TIII are not drastically different from those of the native *Naja haje* toxin III. In consequence it is improbable that a disulfide interchange occurs between the long toxin and its receptor. If it occurs, it certainly does not contribute much to the stability of the interaction.

Comparison of the binding properties of the long neuro-

toxin, neurotoxin III of *Naja haje*, with those of the short neurotoxins, neurotoxin I of *Naja haje* and of *Naja nigricollis mossambica*, reveals an interesting phenomenon. Although the dissociation constants (i.e., the free energies of binding) of the receptor–long or –short neurotoxin complexes are nearly identical and although the LD_{50} values are also identical for both families of neurotoxins, there are large differences in the kinetic characteristics of the binding. The short neurotoxins associate with the toxin receptor 6–7 times faster and dissociate from the neurotoxin–receptor complex 5–9 times faster than the corresponding long neurotoxin.

The very slow dissociation of the long neurotoxin–receptor complex ($t_{1/2}$ = 360 min) probably explains the pharmacological observations of Lee et al. (1972). These authors studied the neuromuscular blocking activity of several neurotoxins on the sciatic-nerve sartorius muscle preparation of *Rana tigrina* and on the rat phrenic-nerve diaphragm preparation. They have observed that neuromuscular blockage by short toxins such as erabutoxin b or cobrotoxin (62 amino acids) was completely reversible after 1–2 hr depending upon the preparation and the toxin used. Conversely long neurotoxins such as α-bungarotoxin (74 amino acids) and *Naja haje* toxin A (71 amino acids) display a blocking activity which appears to be irreversible or nearly irreversible by repeated washing even after 2 hr. Electrophysiological experiments with the nerve-sartorius preparation of the frog have given results for the rates of association of snake neurotoxins with their receptor which are also qualitatively very similar to those found in this work in binding experiments to Torpedo membranes. Banks et al. (1974) have shown that short neurotoxins from *Dendroaspis viridis* venom associate faster than long neurotoxins from the same venom. The slow dissociation of the receptor–long neurotoxin complex as compared to the complex with short neurotoxins cannot be due to the presence of the Cys_{30}–Cys_{34} bridge in the long toxins. It has been demonstrated in this paper that the rates of dissociation of *Naja haje* toxin III and of the reduced and aminoethylated derivative from the toxin–receptor complex are nearly identical. The few amino acids common to both short and long toxins are probably mainly responsible for the common mode of action, i.e., the curarizing action. There are other amino acids which are invariant in the sequence of *either* all short *or* all long neurotoxins, but which are not necessarily present in the other class of toxin (Yang, 1974). They are Asn_5, Ser_8, Thr_{13}, Lys_{27}, Arg_{43}, Gly_{46}, Val_{52}, Gly_{55}, Val/Ile_{56}, and Asn_{67} in short toxins and Gly_{20}, Cys_{30}, Cys_{34}, Lys_{39}, Val_{41}, Leu_{43}, the sequence Ala_{46}-Ala_{47}-Thr_{48}, and Ser_{62} in long toxins. This difference in the overall arrangement of the sequence may be responsible for the difference in the kinetics of action between the two classes of toxin. Differences in kinetics might also be due to the C-terminal sequence which is present only in long toxins and which is rich in proline, arginine, and lysine residues.

There are now more than 30 snake toxins with known amino acid sequences. Alignment of these sequences (Yang, 1974) shows that besides the invariant distribution of the eight half-cystine residues common to all short and long toxins, seven other amino acid residues are also present in all sequences known to date and are therefore good candidates for a structural and/or a functional role Tyr_{25}, Trp_{29}, Asp_{31}, Arg_{37}, Gly_{38}, Gly_{44}, and Pro_{50}. In fact analysis for side-chain similarity indicates that four additional residues should be added to the list, they are Ser/Thr_9, Asp/Glu_{42}, Lys/Arg_{53}, and $Ile/Leu/Val_{58}$.

Physicochemical experiments and chemical modification of several neurotoxins have given valuable information concerning the role of Tyr_{25}, Trp_{29}, and Arg_{37} (Chang and Hayashi, 1969; Seto et al., 1970; Tu et al., 1971; Tu and Toom, 1971; Chang et al., 1971a; Chicheportiche et al., 1972; Karlson and Eaker, 1972; Yang et al., 1974).

Chemical modification of lysine residues in snake neurotoxins indicated that two lysines, Lys_{27} and Lys_{53}, are particularly exposed in the molecule and more reactive than other lysine residues (Chicheportiche et al., 1972, Chang et al., 1971b). Lys_{53} is one of the few residues common to all toxins sequenced. Lys_{27} is present in all short neurotoxins and in most long neurotoxins. It is present in the sequence of *Naja haje* neurotoxin III (Kopeyan et al., 1973). Selective dansylation of Lys_{27} and Lys_{53} in *Naja haje* neurotoxin I decreased the toxic activity to less that 7% of the normal value of the native toxin. Acetylation or maleylation of the lysine residues in the same toxin decreased the toxicity to less than 5% of the normal value (Chicheportiche et al., 1972). Selective trinitrobenzylation of both Lys_{27} and Lys_{53} in cobrotoxin completely supresses toxicity (Chang et al., 1971b). Although Lys_{27} is particularly exposed in snake neurotoxin molecules and although it is near the invariant Tyr_{25} and Trp_{29} it seems much less important for the neurotoxic activity than Lys_{53}. In two of the long neurotoxins sequenced to date, *O. hannah* toxin (Joubert, 1973) and *Dendroaspis viridis* toxin (Banks et al., 1974), a glutamic acid residue replaces Lys_{27}. Moreover pentacarbamylation of lysines, including Lys_{27} in a toxin which has an arginine residue in place of Lys_{53}, decreases the toxic activity only to 20% (Karlson et al., 1972b).

The ε-ammonium of Lys_{53} could participate in the formation of an ion pair with a negatively charged partner in the toxin receptor. A chemical modification which preserves the positive charge of the Lys_{35} side chain such as a guanidination with *O*-methylisourea left the toxicity unaltered (Chang et al., 1971b; Tu et al., 1971; Karlson et al., 1972b).

The data presented in this paper show that chemical modification of Lys_{27} and Lys_{53} in *Naja haje* neurotoxins by acetylation or dansylation significantly decreased, but did not suppress the binding to the toxin receptor in Torpedo membranes. The affinity after modification of the lysine residues was decreased by a factor of about 200. This ratio corresponds to a difference in free energy of binding of about 3 kcal mol^{-1}. Differences in affinities between the native toxin and the toxin blocked on lysine residues are essentially due to differences in the second-order rates of association k_a. k_a was decreased by a factor of 140 on dansylation of Lys_{27} and Lys_{53} in *Naja haje* toxin I and by a factor of 56 by acetylation of the lysines in neurotoxin III. Chemical modification of Lys_{27} and Lys_{53} did not greatly affect the rates of dissociation, k_d, of the toxin–receptor complexes.

Lys_{53} (and possibly also Lys_{27}) is an important residue for the recognition of the toxin by its receptor, but it is not essential. The interaction of the snake neurotoxin with its receptor probably involves an array of noncovalent interactions. The suppression of only one of these interactions does not suffice to prevent binding of the neurotoxin to target membranes. The stereochemical details and the thermodynamics of a model interaction of a mini-protein (56 amino acids) with its receptor are now well known; it is the pancreatic trypsin inhibitor–trypsin interaction (Vincent and Lazdunski, 1972). This complex is associated by 200 van der

Waals contacts and 7 hydrogen bonds (Blow et al., 1972; Rühlmann et al., 1973). The primary interaction is the formation of a salt bridge between the lysine (Lys_{15}) in the active site of the protein inhibitor and the carboxylate side chain of the aspartic acid residue at the specificity site of the trypsin partner. In this case, too, chemical modification of the active site lysine strongly alters the stability of the association but does not suppress complex formation (Vincent and Lazdunski, 1972).

The ability of the Cys_{30}-Cys_{34} bridge in long neurotoxins to be selectively reduced may be useful in at least two ways: to attach an active modified toxin to a solid phase for affinity chromatography of the toxin receptor and to incorporate into the toxin molecule fluorescent probes that can serve either as reporter groups for physicochemical studies of the toxin receptor embedded in the excitable membrane or for cytological purposes.

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References

Banks, B. E. C., Miledi, R., and Shipolini, R. A. (1974), *Eur. J. Biochem. 45,* 457–468.

Bartels, E., and Rosenberry, T. L. (1971), *Science 174,* 1236–1237.

Blow, D. M., Wright, C. S., Kukla, D., Rühlmann, A., Steigemann, W., and Huber, R. (1972), *J. Mol. Biol. 69,* 137–144.

Botes, D. P. (1971), *J. Biol. Chem. 26,* 7383–7391.

Botes, D. P., and Strydom, D. J. (1969), *J. Biol. Chem. 244,* 4147–4157.

Botes, D. P., Strydom, D. J., Anderson, C. G., and Christensen, P. A. (1971a), *J. Biol. Chem. 246,* 3132–3139.

Botes, D. P., Strydom, D. J., Strydom, A. J. C., Joubert, F. J., Christensen, P. A., and Anderson, C. G. (1971b), in Toxins of Animal and Plant Origin, Vol. 1, De Vries, A., and Kochva, E., Ed., London, Gordon and Breach, p 281.

Chang, C. C., Chen, T. F., and Chuang, S. T. (1973), *Br. J. Pharmacol. 47,* 147–155.

Chang, C. C., and Hayashi, K. (1969), *Biochem. Biophys. Res. Commun. 37,* 841–846.

Chang, C. C., Yang, C. C., Hamaguchi, K., Nakai, K., and Hayashi, K. (1971a), *Biochim. Biophys. Acta 236,* 164–173.

Chang, C. C., Yang, C. C., Nakai, K., and Hayashi, K. (1971b), *Biochim. Biophys. Acta. 251,* 334–344.

Chicheportiche, R., Rochat, C., Sampieri, F., and Lazdunski, M. (1972), *Biochemistry 11,* 1681–1691.

Cohen, J. B. Weber, M., Huchet, M., and Changeux, J. P. (1972), *FEBS Lett. 26,* 43–47.

Cooper, D., and Reich, E. (1972), *J. Biol. Chem. 247,* 3008–3013.

Edman, P., and Begg, G. (1967), *Eur. J. Biochem. 1,* 80–91.

Ellman, G. L. (1959), *Arch. Biochem. Biophys. 82,* 70–78.

Franklin, G. I., and Potter, L. T. (1972), *FEBS Lett. 28,* 101–106.

Fulpius, B., Cha, S., Klett, R., and Reich, E. (1972), *FEBS Lett. 24,* 323–326.

Hartree, E. F. (1972), *Anal. Biochem. 48,* 422–427.

Huber, R., Kukla, D., Rühlmann, A., and Steigemann, W. (1971), *Proc. Int. Res. Conf. Proteinase Inhibitors, 1st, 1970,* 56.

Inglis, A. S., and Edman, P. (1970), *Anal. Biochem. 37,* 73–80.

Joubert, F. J. (1973), *Biochim. Biophys. Acta 317,* 85–98.

Karlin, A., and Winnik, M. (1968), *Proc. Natl. Acad. Sci. U.S.A. 60,* 668–674.

Karlson, E., and Eaker, D. (1972), *Tai-wan I Hsueh Hui Tsa Chih 71,* 358–364.

Karlson, E., Eaker, D., and Ponterius, G. (1972b), *Biochim. Biophys. Acta 257,* 235–248.

Karlson, E., Heilbronn, E., and Widlund, L. (1972a), *FEBS Lett. 28,* 107–111.

Kopeyan, C., van Rietschoten, J., Martinez, G., Rochat, H., Miranda, F., and Lissitzky, S. (1973), *Eur. J. Biochem. 35,* 244–250.

Lee, C. Y. (1970), *Clin. Toxicol. 3,* 457–472.

Lee, C. Y. (1972), *Annu. Rev. Pharmacol. 12,* 265–286.

Lee, C. Y., Chang, C. C., and Chen, Y. M. (1972), *T'ai-wan I Hsueh Hui Tsa Chih 71,* 344–349.

Light, A., Hardwick, B. C., Hatfield, L. M., and Sondack, D. L. (1969), *J. Biol. Chem. 244,* 6289–6296.

Menez, A., Morgat, J. L., Fromageot, P., Ronseray, A. M., Boquet, P., and Changeux, J. P. (1971), *FEBS Lett. 17,* 333–335.

Miledi, R., and Potter, L. T. (1971), *Nature (London) 233,* 599–604.

Miranda, F., Kopeyan, C., Rochat, H., Rochat, C., and Lissitzky, S. (1970a), *Eur. J. Biochem. 17,* 477–484.

Miranda, F., Kopeyan, C., Rochat, H., Rochat, C., and Lissitzky, S. (1970b), *Eur. J. Biochem. 16,* 514–523.

Raftery, M. A., Schmidt, J., Clark, D. G., and Wolcott, R. G. (1971), *Biochem. Biophys. Res. Commun. 45,* 1622–1629.

Rochat, H., Gregoire, J., Martin-Moutot, N., Menashe, M., Kopeyan, C., and Miranda, F. (1974), *FEBS Lett. 42,* 335–339.

Rühlmann, A., Kukla, D., Schwager, P., Bartels, K., and Huber, R. (1973), *J. Mol. Biol. 77,* 417–436.

Seto, A., Sato, S., and Tamiya, N. (1970), *Biochim. Biophys. Acta 214,* 483–489.

Spackman, D. H., Stein, W. H., and Moore, S. (1958), *Anal. Chem. 30,* 1190–1197.

Tu, A. T. (1973), *Annu. Rev. Biochem. 42,* 235–258.

Tu, A. T., Hong, B. S., and Solie, T. N. (1971), *Biochemistry 10,* 1295–1304.

Tu, A. T., and Toom, P. M. (1971), *J. Biol. Chem. 246,* 1012–1016.

Vincent, J. P., and Lazdunski, M. (1972), *Biochemistry 11,* 2967–2977.

Vincent, J. P., Peron-Renner, M., Pudles, J., and Lazdunski, M. (1974), *Biochemistry 13,* 4205–4213.

Yang, C. C. (1974), *Toxicon 12,* 1–43.

Yang, C. C., Chang, C. C., and Liou, I. F. (1974), *Biochim. Biophys. Acta 365,* 1–14.

Weber, M., and Changeux, J. P. (1974), *Mol. Pharmacol. 10,* 1–14.