## Discussion

Recently we have found that CT increases serum glucose concentration in fed and fasted rats.[6,7] This effect of CT does not result from epinephrine release from the adrenal glands in rats.[10] In the present work, it was examined whether CT could increase serum glucose independently of insulin secretion.

The administration of CT caused a significant increase in serum glucose, while it did not significantly alter serum insulin. Also, CT had no significant effect on serum glucose and insulin levels increased by a glucose load in fasted rats. These results suggest that CT does not inhibit insulin secretion in rats. In rats treated with somatostatin, furthermore, CT produced a significant increase of serum glucose. Since somatostatin inhibits glucagon and insulin releases in intact rats,[11] this result suggests that the hyperglycemic effect of CT is not related to insulin and glucagon releases.

On the other hand, the increase in serum glucose caused by CT administration was significantly inhibited by insulin treatment, indicating that CT does not prevent the action of insulin to decrease serum glucose in rats. This suggests that the hyperglycemic effect of CT does not result from an inhibition of insulin action on serum glucose in rats.

Thus, in the present investigation, it was demonstrated that CT increases serum glucose independently of insulin secretion in rats.

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# The Primary Structure of Toxin C from the Venom of the Indian Cobra (*Naja naja*)

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The primary structure of toxin C, a neurotoxin isolated from Indian cobra (*Naja naja*) venom, was determined. Toxin C is a highly toxic polypeptide consisting of 71 amino acid

residues (molecular weight 7800) crosslinked by five disulfide bridges. A high degree of homology is observed among the long-chain neurotoxins. Toxin C differs from toxin A only in having -Ala-28 and -Lys-49 in place of -Gly-28 and -Arg-49, and from toxin B in having -Ala-28, -Ile-32 and -Lys-49 in place of -Gly-28, -Ser-32 and -Arg-49 residues. The amino acid sequence of toxin C is the same as that of *Naja naja siamensis* neurotoxin 3.

**Keywords**——primary structure; neurotoxin; Edman degradation; snake venom; amino acid composition; Indian cobra venom

Venoms of many snakes belonging to the families *Elapidae* and *Hydrophiidae* are highly toxic, and produce flaccid paralysis and respiratory failure in animals. These effects have been attributed to so-called "neurotoxins" contained in the venoms. The mechanism of neuromuscular junction at the muscular endplate has been studied extensively by using snake neurotoxins. The Indian cobra (*Naja naja*) venom contains toxins A[2] and B[3] as major neurotoxins and toxins C and D as minor components.

Studies on the chemical properties of toxin C revealed that the amino acid composition was very similar to those of toxins A, B and D and moreover the composition was the same as that of neurotoxin 3, a major neurotoxin in the venom of *Naja naja siamensis*.[4] Because the amino acid composition suggested a high degree of structural homology among these toxins, we felt that the primary structure determination of these neurotoxins might help to identify the essential amino acid residues necessary for the maintenance of active conformation.

This paper presents the complete amino acid sequence of toxin C from the venom of Indian cobra (*Naja naja*).

## Experimental

**Materials**——The crude lyophilized Indian cobra (*Naja naja*) venom was purchased from Sigma Chemical Co., Ltd., USA. Bovine trypsin (2×crystallized) and bovine α-chymotrypsin (3×crystallized) were products of Worthington Biochemical Corp., USA. Acid carboxypeptidase produced by *Penicillium janthinellum* was provided by Dr. S. Yokoyama of Takara Shuzo Co., Ltd., Otsu, Japan. Toxin C was purified from the venom of Indian cobra by gel filtration on a column of Sephadex G-50 then on a CM-cellulose column as described previously.[2]

**Methods**——Polyacrylamide disc gel electrophoresis was carried out by the method of Williams.[5] The isoelectric point was determined by the method of Vesterbërg and Svensson[6] using 0.5% ampholytes, pH 3—10, and a sucrose gradient at 4° for 20 hr. The $LD_{50}$ in mice (NIH strain) by subcutaneous injection was determined according to the method of Lichfield and Wilcoxon.[7] The reduction and S-carboxymethylation (RMC) of toxin C were performed as described by Crestfield *et al.*[8] Amino acid analyses of the toxin and the tryptic and chymotryptic peptides were carried out by the standard method using a Hitachi KLA 3B automatic amino acid analyzer.

**Separation of the Peptide**——The peptides obtained by treatment with proteolytic enzymes were separated by a combination of gel filtration on a column of Sephadex G-15 using 0.2% acetic acid as the eluent, high voltage paper electrophoresis using a buffer, pH 3.6, of pyridine: acetic acid: water=1: 10: 289, by volume, and descending paper chromatography using a solvent system of *n*-butanol: acetic acid: pyridine: water=15: 3: 10: 12, by volume.

**Sequence Analysis**——The N-terminal sequence of the toxin and the peptides were determined by Edman's phenylthiohydantoin (PTH) procedure.[9] The PTH amino acids were identified by thin-layer chromatography on Kiesel gel $F_{254}$ plate (E. Merck, West Germany) using solvent systems IV and V described by Jeppsson and Sjöquist[10] and on polyamide sheets (Carle Place, New York, USA) using a solvent system of toluene: *n*-pentane: acetic acid=60: 30: 35, by volume, containing 250 mg of 2-(4′-butylphenyl)-5-(4′-biphenyl)-1,3,4-oxadiazole per liter.[11] The aqueous phase containing PTH arginine was evaporated to dryness, the residue was dissolved in 0.1 ml of 70% ethanol, and an aliquot was spotted on filter paper and tested for arginine with Sakaguchi's reagent. The carboxyl-terminus of toxin C was determined by the use of *Penicillium janthinellum*[12] and by hydrazinolysis.[13]

## Results and Discussion

Gel filtration of the venom of *Naja naja* on Sephadex G-50 followed by CM-cellulose chromatography yielded several kinds of neurotoxins which were homogenous on disc gel

electrophoresis. Toxin C was isolated in a yield of 1.0% by weight from the crude venom and the $LD_{50}$ of the toxin in mice after subcutaneous injection was estimated to be 0.1—0.15 μg/g body weight. The isoelectric point was about 9.0 as determined by isoelectric focusing in polyacrylamide gel.[14] The molecular weight was estimated by gel filtration to be about 7000.

The amino acid composition of toxin C is shown in Table I. The Edman degradation (32 cycles) of RCM-toxin C revealed the amino-terminal sequence to be $H_2N$–Ile–Arg–Cys–

Table I. Amino Acid Compositions of RCM-toxin C and Tryptic Peptides Derived from RCM–Toxin C

| Amino acid | RCM-toxin C | T-1 | T-2 | T-3 | T-4 | T-5 | T-6 | T-7 | T-8 |
|---|---|---|---|---|---|---|---|---|---|
| Carboxymethylcysteine | 8.24 | | 1.00 | 2.01 | 1.60 | | 2.15 | 3.04 | |
| Aspartic acid | 9.20 | | 1.25 | 2.00 | 0.93 | | 1.31 | 4.41 | |
| Threonine | 8.74 | | 1.78 | 1.21 | 1.05 | | 2.00 | 3.22 | |
| Serine | 3.05 | | 1.21 | | 1.28 | | | 1.10 | |
| Glutamic acid | 1.30 | | | | | | | 1.32 | |
| Proline | 5.78 | | 1.10 | 1.05 | | | 0.95 | 2.22 | 1.00 |
| Glycine | 4.25 | | | 1.00 | | 0.96 | 1.00 | 1.31 | |
| Alanine | 2.90 | | | | 0.97 | | 1.52 | | |
| Valine | 3.95 | | | 0.83 | | | 1.90 | 1.01 | |
| Isoleucine | 4.30 | 0.99 | 1.62 | | 0.94 | | | 0.90 | |
| Leucine | 1.00 | | | | | | 0.75 | | |
| Tyrosine | 1.30 | | | 0.60 | | | | | |
| Phenylalanine | 2.70 | | 0.89 | | 0.93 | | | 0.80 | |
| Tryptophan | +[a] | | | | + | | | | |
| Lysine | 4.36 | | 1.00 | 1.00 | | 1.00 | 1.00 | | 1.00 |
| Histidine | 0.79 | | | 0.60 | | | | | |
| Arginine | 4.62 | 1.00[b] | | | 1.00 | 1.03 | | 1.00 | 1.06 |
| Yield (%) | | 51 | 20 | 15 | 18 | 47 | 18 | 10 | 56 |

*a*) Tryptophan was determined spectrophotometrically.
*b*) The value of the underlined amino acid was taken as 1.00.

Table II. Amino Acid Compositions of Chymotryptic Peptides derived from RCM–Toxin C

| Amino acid | C-I | C-II | C-III | C-IV | C-V | C-VI |
|---|---|---|---|---|---|---|
| Carboxymethylcysteine | 1.00[a] (1)[b] | 2.00(2) | | 1.00(1) | 5.72(6) | |
| Aspartic acid | | 3.07(3) | | 0.93(1) | 4.79(5) | |
| Threonine | | 2.01(2) | 1.72(2) | | 4.41(5) | |
| Serine | | 1.03(1) | | | 2.28(2) | |
| Glutamic acid | | | | | 1.00(1) | |
| Proline | | 2.20(2) | | | 3.32(3) | 1.30(1) |
| Glycine | | 1.00(1) | | | 2.83(3) | |
| Alanine | | | | 0.97(1) | 1.95(2) | |
| Valine | | 0.87(1) | | | 2.26(3) | |
| Isoleucine | 0.99(1) | 1.67(2) | | | 1.76(2) | |
| Leucine | | | | | 0.93(1) | |
| Tyrosine | | 0.75(1) | | | | |
| Phenylalanine | 1.00(1) | | | 0.93(1) | 0.90(1) | |
| Tryptophan | | | +[c] (1) | | | |
| Lysine | | 0.93(1) | 1.00(1) | | 2.00(2) | 1.00(1) |
| Histidine | | 0.92(1) | | | | |
| Arginine | 0.89(1) | | | | 1.87(2) | 2.30(2) |
| Yield (%) | 18 | 20 | 18 | 27 | 23 | 14 |

*a*) The value of the underlined amino acid was taken as 1.00.
*b*) The numbers in parentheses represent the values from the amino acid sequence.
*c*) Tryptophan was determined spectrophotometrically.

Phe–Ile–Thr–Pro–Asp–Ile–Thr–Ser–Lys–Asp–Cys–Pro–Asn–Gly–His–Val–Cys–Tyr–Thr–Lys–Thr–Trp–Cys–Asp–Ala–Phe–Cys–Ser–Ile–. The carboxyl-terminal sequence of toxin C was determined to be –Arg–Lys–Arg–Pro–OH by the use of acid carboxypeptidase isolated from the culture fluid of *Penicillium janthinellum*[12] and by hydrazinolysis.[13]

To determine the amino acid sequence of the central part of toxin C, RCM-toxin C was digested with chymotrypsin or trypsin. The resulting peptides were separated by gel filtration on a Sephadex G-15 column with monitoring of the eluate at 226 and 280 nm. Further purification of heterogenous peptides was carried out by high voltage paper electrophoresis or descending paper chromatography. The amino acid compositions and the yields of the tryptic and chymotryptic peptides are given in Tables I and II. The peptides derived by the trypsin or chymotrypsin digestion of RCM-toxin C are indicated by the symbol T- or C-, respectively. The peptide numbers refer to their location in the sequence starting from the amino-terminal end.

Except for peptides T-4, T-6, C-IV and C-V in Tables I and II, the amino acid compositions of all peptides derived from the digestion of trypsin or chymotrypsin were the same as the results obtained in the studies of the amino acid sequence of toxin B isolated from the venom of *Naja naja*.[3] From the amino acid composition of the peptides and the amino-terminal sequence of RCM-toxin C, the presence of –Ala–Phe–Cys–Ser–Ile– in place of –Gly–Phe–Cys–Ser–Ser– at positions 28—32 in the amino acid sequence of toxin B was deduced. This was supported by the sequence study on peptide T-4 having the amino acid sequence –Thr–Trp–Cys–Asp–Ala–Phe–Cys–Ser–Ile–Arg. The carboxyl-terminal of peptide T-6 was determined to be lysine from the tryptic specificity.

On the basis of the above results, the complete amino acid sequence of toxin C was determined as shown in Fig. 1. There was a remarkable similarity among the amino acid sequences

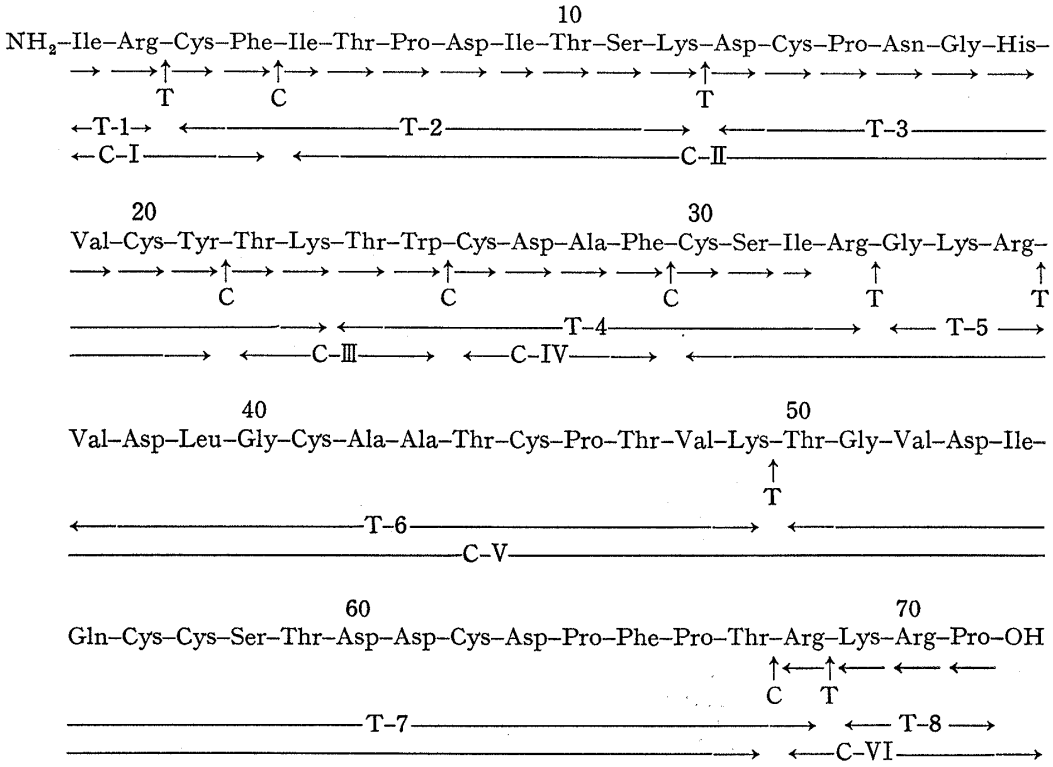


Fig. 1. Amino Acid Sequence of Toxin C from Indian Cobra (*Naja naja*) Venom

Horizontal arrows below amino acid residues denote the sequences of tryptic and chymotryptic peptides. Right- and left-handed arrows show that the sequence was elucidated by Edman degradation, or by hydrazinolysis, respectively. T and C represent the peptides obtained by hydrolysis with trypsin or chymotrypsin, respectively.

of toxins A, B and C. Toxin C differs from toxin B only in having –Ala–28, –Ile–32, and –Lys–49 in place of –Gly–28, –Ser–32, and –Arg–49 residues. Also, toxin C differs from toxin A in having –Ala–28 and –Lys–49 in place of –Gly–28 and –Arg–49.

The primary structure of toxin C thus elucidated was the same as that of *Naja naja siamensis* neurotoxin 3, which is a major neurotoxin in the venom.[4] At present, it is not known whether the presence of toxin C in the venom of *Naja naja* is due to contamination of the venom of *Naja naja siamensis* during the milking or whether the venom of *Naja naja* originally contains toxin C as one of the neurotoxin components.

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# A Model Independent Approach to describe the Blood Disappearance Profile of intravenously Administered Drugs

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A model independent approach is proposed to describe the blood disappearance profile of intravenously administered drugs. This approach requires a new definition of the total volume of distribution as a function of time. The total volume of distribution is regarded as the sum of $V_1$ and $V_2$. $V_1$ represents the initial volume of distribution after the intravenous administration of drugs. $V_2$ represents the additional distribution volume where the drug is distributed after the initial rapid distribution has ceased. To characterize $V_2$, two parameters, the maximum value of $V_2$, $(V_2)_{max}$, and the distribution constant, $K_d$, which is equal to the time when $V_2$ is equal to one-half its maximum, are adopted. It has been suggested by a simulation study that these two parameters, $(V_2)_{max}$, and $K_d$, are the major determinants of the rapid initial disappearance (*i.e.* distribution