sufficiently scattered over the length of the I-Aβ chain to account for the extreme variations that were noted in the tryptic peptide maps. However, the fact that most of the differences are confined to the β_1 domain suggests that mouse I-A and human HLA-DR-like β-chain genes have evolved along similar lines. Specifically, the Aβ chain of one individual is 5 to 10 percent different from that of another individual, and most of these differences are found in the β_1 domain. This clustering of variation in the NH_2-terminal extracellular domain of this polypeptide may be related to the function of Ia molecules as targets for T-cell recognition and cell-to-cell interactions.

The comparison of the amino acid sequences of the I-Aβ chain alleles suggests that the evolution of these genes has not involved gross insertions or deletions in the exons. By contrast, restriction map analysis of the sequences flanking the I-Aβ chain exons (*7, 17*) suggests that there have been large insertions and deletions in these regions. In particular, the intron between β_1 and β_2 differs in size (by as much as 0.9 kilobase) in all three of the haplotypes we examined (Fig. 1). Also striking is the complete sequence divergence among the three haplotypes just 3′ of the TM exon (Fig. 2). From the data presented here we cannot determine whether the large differences between the introns of these alleles are a result of the same mutational events that altered the exons or whether the introns have been affected by an independent series of mutational events. However, our data do allow us to exclude certain models for the genetic basis of I-A polymorphism. Earlier data on I-A polymorphism suggested that models involving mechanisms analogous to the mechanism by which trypanosomes vary their coat protein genes were plausible (*18*). Such mechanisms, involving the insertion and deletion of large stretches of DNA, are not consistent with the data presented here. The pattern of sequence differences presented in Fig. 3 is more consistent with multiple separate events that affect one or a few nucleotides. How these relatively small changes in amino acid sequence can have such a profound effect on immune responsiveness remains to be determined. Future studies on the structure of mutationally altered genes detected in laboratory strains of mice may determine the precise nature and frequency of the mutational events. Eventually these studies should determine whether the chromosomal region containing these genes is hypermutable or whether a selective advantage conferred by multiple forms of these genes leads to the maintenance of a large number of polymorphic alleles.

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21. We thank Terri Broderick for her editorial assistance, R. Robinson for the use of the pI-Aβ-1 cDNA clone, and P. Peterson for the HLA-DR-like β-chain cDNA clone. Supported by NIH grant AI18436, American Cancer Society grant JFRA66 (to J.G.S.), Helen Hay Whitney award (to E.C.), and American Cancer Society awards (to R.N.G. and K.M.).

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Peptide Cotransmitter at a Neuromuscular Junction

Abstract. *The neuropeptide proctolin (H-Arg-Tyr-Leu-Pro-Thr-OH) is present in the nerve terminals of an identified slow skeletal motoneuron in the cockroach. Proctolin is released onto the target muscle, a coxal depressor, by neuron stimulation and by depolarization with potassium. The physiological action of the motoneuron suggests that proctolin acts as a cotransmitter. Proctolin and neural stimulation produce delayed and sustained contractile effects without muscle depolarization.*

Evidence that cotransmitters may act with classical transmitters at skeletal nerve-muscle junctions in both vertebrates and invertebrates is accumulating. Thus, although the vertebrate motor end plate has long been regarded as exclusively cholinergic (*1*), synthetic enzymes for neuroactive substances other than acetylcholine have now been found in mammalian motoneurons (*2*). Similarly, excitatory motoneurons in insects are generally regarded as L-glutaminergic (*3*), but we have shown that the insect gut neuropeptide proctolin (H-Arg-Tyr-Leu-Pro-Thr-OH) (*4*) is associated with some excitatory motoneurons in the cockroach *Periplaneta americana* (*5*). In considering the role of this peptide at the insect neuromuscular junction, we studied the individually identified, proctolin-containing, slow coxal depressor or Ds motoneuron (*5, 6*) in the cockroach. We chromatographically characterized the proctolin-like immunoreactivity (PLI) in Ds nerve terminals and showed a calcium-dependent release of proctolin from Ds. The Ds motoneuron produces transient effects consistent with the recognized effects of L-glutamate in addition to sustained, delayed effects consistent with the action of proctolin. Our findings provide direct evidence for a peptide

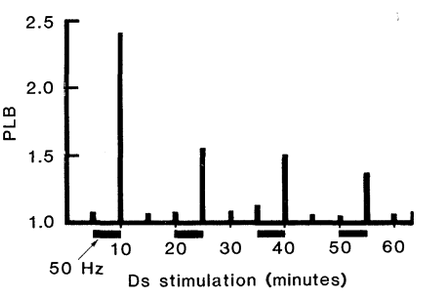

Fig. 1. The release of PLB from a single Ds coxal depressor muscle preparation (*7, 10*) by electrical stimulation of the Ds axon at 50 Hz for 5 minutes on four successive occasions (black bars). Bioassay of the perfusate (*10*) showed significant elevation of PLB after each stimulus.

acting as a skeletal neuromuscular transmitter and challenge the idea that actions of skeletal motoneurons in insects are mediated by L-glutamate alone. The physiological findings suggest possible functions for the additional nonacetylcholine neuroactive substances now suspected in vertebrate nerve-muscle junctions (*2*).

The cell body, axon, and nerve terminals of the Ds motoneuron are immunoreactive to a polyclonal rabbit antiserum specific to proctolin (*5*). The coxal depressor muscle (muscle 177, d and d′) is innervated by Ds and at least four other motoneurons (*6*). The only one containing PLI is Ds (*5*). To characterize further the PLI in Ds terminals, we purified extracts of muscle 177 using reverse-phase (C_{18}) high-performance liquid chromatography (HPLC) combined with a sensitive and specific bioassay of proctolin-like bioactivity (PLB) (*7*). Muscles were homogenized in a mixture of methanol, water, and acetic acid (90:9:1). The soluble fraction was dried and again dissolved, either in a volatile liquid-phase buffer for HPLC fractionation or in physiological saline for bioassay preceding chromatography (*8*). Chromatographed fractions were dried, dissolved in saline, and bioassayed. Nonchromatographed extracts contain PLB equivalent to a mean ± standard deviation of 31 ± 16.8 femtomoles of proctolin per muscle (N = 10). All PLB recovered from muscle 177 is chromatographically and biologically indistinguishable from authentic proctolin. Extracts made from muscle in which Ds terminals had degenerated after denervation showed a reduction in PLB of 90 percent (89.6 ± 5.8; N = 5) (*9*). This is evidence that the PLB and PLI in the motor terminals of Ds are due to the presence of authentic proctolin.

Release of proctolin from Ds nerve terminals was achieved by eliciting action potentials in the Ds axon or by bathing nerve-muscle preparations in high potassium saline. Figure 1 shows PLB in the muscle perfusate after each 5-minute stimulus (50 Hz) applied to the Ds axon (*10*). Assays for PLB gave basal values immediately after saline wash, but PLB reappeared after each additional stimulus. Depolarization of several muscles simultaneously by bathing them in saline with an elevated potassium concentration yielded sufficient released PLB to make possible (i) determination of calcium dependence of release, (ii) purification by HPLC of the released PLB, and (iii) an estimate of the amount of proctolin released relative to the total amount present in the nerve muscle preparations.

Up to 15 coxal depressor muscles were perfused with physiological saline and saline with altered ionic composition (*11*). The different perfusates were drawn over the muscles by a peristaltic pump and through a small cartridge (Sep-Pak) containing C_{18} reverse-phase packing (Fig. 2A). The C_{18} adsorbs and accumulates released hydrophobic peptides from the aqueous perfusates. For each

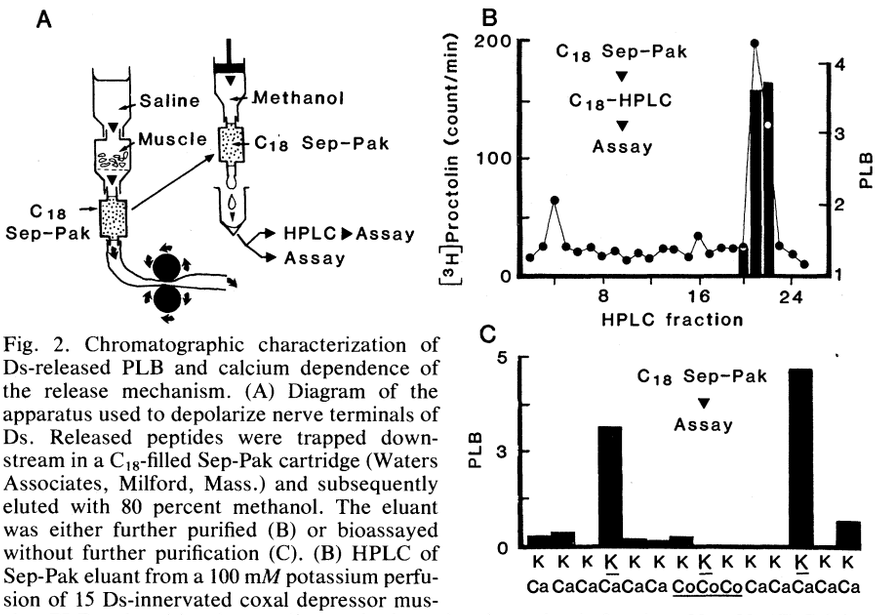

Fig. 2. Chromatographic characterization of Ds-released PLB and calcium dependence of the release mechanism. (A) Diagram of the apparatus used to depolarize nerve terminals of Ds. Released peptides were trapped downstream in a C_{18}-filled Sep-Pak cartridge (Waters Associates, Milford, Mass.) and subsequently eluted with 80 percent methanol. The eluant was either further purified (B) or bioassayed without further purification (C). (B) HPLC of Sep-Pak eluant from a 100 m*M* potassium perfusion of 15 Ds-innervated coxal depressor muscles. PLB and a tritiated proctolin standard are eluted together in fractions 20 to 23. (C) Calcium dependence of proctolin release is shown by replacing calcium with 5 m*M* cobalt. The cobalt inhibition of release is reversible on reintroduction of calcium. Each black bar represents the PLB eluted from a different Sep-Pak in a sequence of 10-minute exposures to 20 ml of saline. Normal physiological saline is represented by a small K and Ca. The altered saline solutions are underlined. Elevated potassium is represented by a large K and cobalt by Co.

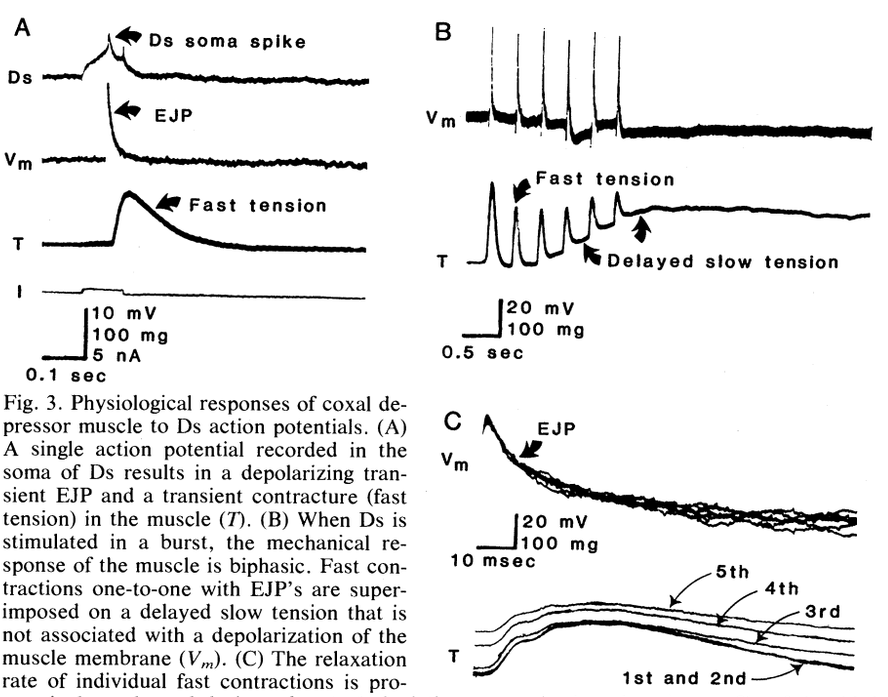

Fig. 3. Physiological responses of coxal depressor muscle to Ds action potentials. (A) A single action potential recorded in the soma of Ds results in a depolarizing transient EJP and a transient contracture (fast tension) in the muscle (*T*). (B) When Ds is stimulated in a burst, the mechanical response of the muscle is biphasic. Fast contractions one-to-one with EJP's are superimposed on a delayed slow tension that is not associated with a depolarization of the muscle membrane (V_m). (C) The relaxation rate of individual fast contractions is progressively prolonged during a burst. A deviation occurs in the relaxation profile of the third response in a burst compared to the first and second. The change in the rate of relaxation is not associated with a change in the shape of the EJP (upper trace). *Ds*, intracellular soma recording from Ds; V_m, membrane potential of coxal depressor muscle recorded intracellularly; *T*, tension (force) transduction of muscle's mechanical response; *I*, current injected into Ds soma.

saline condition, we changed the cartridge and subsequently eluted the trapped compounds with methanol. In some experiments, we further purified the released and C_{18}-retained material using an analytical HPLC C_{18} column. Eluted samples were assayed for PLB after being dried and dissolved in physiological saline.

High potassium saline caused release of PLB (Fig. 2, B and C). Purification of released PLB on HPLC against a tritiated proctolin standard (Fig. 2B) showed that PLB and proctolin elute into identical fractions. Replacement of calcium with equimolar cobalt reversibly blocked proctolin release (Fig. 2C). We conclude that proctolin is released from Ds nerve terminals by a calcium-dependent process. The mean amount of proctolin released by high potassium treatment is approximately 0.9 percent of the total proctolin extractable from the Ds nerve terminals (*12*).

Contractile and electrophysiological responses of muscle to stimulation of the Ds motoneuron were examined for evidence of an action of released proctolin (*13*). Intracellular recordings and tension measurements from the Ds-innervated coxal depressor muscle (177d) show a transient excitatory junctional potential (EJP) followed by a single transient contractile response when a single action potential is activated in the Ds neuron (Fig. 3A). This response to Ds is typical of the glutamate-mediated action of insect motoneurons and has been described in numerous accounts of insect neuromuscular transmission (*3*). When Ds is stimulated in a short train, however, the contractile response becomes biphasic (Fig. 3B). There is a delayed and persistent rise in tension that is not associated with EJP's or with a membrane depolarization. In addition, although the shape of individual EJP's remains unaltered, the relaxation phase of transient contractions becomes progressively prolonged (Fig. 3C). The temporal separation of these effects from the usual transient responses and the absence of an associated muscle depolarization suggest that a non-glutamate-mediated mechanism is responsible. These effects are specifically associated with Ds and with no other motoneuron innervating the same muscle.

Can the delayed effects, not associated with the EJP, be attributed to the proctolin released from Ds terminals? The following evidence suggests that they can. Proctolin applied in low concentration to muscle innervated by Ds produces the effects observed when Ds is stimulated in a train (Fig. 4). Thus, proctolin causes a slow and persistent rise in tension without a membrane depolarization (Fig. 4A). The proctolin-induced contraction is related to the dose, with a threshold of about $5 \times 10^{-10} M$. Furthermore, when applied during Ds stimulation at nanomolar concentrations, proctolin prolongs the relaxation phase of the transient Ds-initiated contraction (Fig. 4B). Proctolin therefore can account for the additional effects seen when Ds is stimulated in a brief burst, but cannot account for the classical EJP and transient twitch contraction. We propose therefore that released proctolin acts as a cotransmitter that has both a direct action causing slow contraction and a modulatory action in altering relaxation of twitch contractions. The site of action of proctolin is postsynaptic on the muscle because proctolin induces contraction of denervated muscle on which all motor terminals have degenerated.

We have demonstrated the presence, release, and action of a neuropeptide at skeletal neuromuscular junctions on the coxal depressor muscle 177d. We do not yet fully understand the functional significance of this activity, but it may be related to the special requirement of certain slow muscle fibers to develop and sustain tensions over extended periods (*14*). Coxal depressor muscles 178 and 179, which are adjacent to 177d (*6*), receive no proctolin innervation, are not sensitive to proctolin, and produce twitches but do not produce slow, sustained contraction on nerve stimulation. This apparent association of a cotransmitter with a muscle capable of both transient and sustained contracture may provide clues to the possible roles of additional transmitters now suspected in vertebrate motoneurons and end plates (*2*).

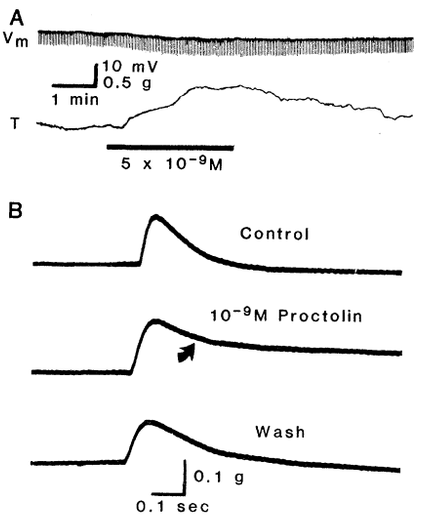

Fig. 4. Action of proctolin on the coxal depressor muscle. (A) Bath application of $5 \times 10^{-9} M$ proctolin causes a tonic contracture (T) not associated with a muscle depolarization (V_m). Membrane response to injected current pulses (downward deflections of V_m) suggests a small (20 percent) increase in input resistance associated with bath-applied proctolin. (B) Proctolin ($10^{-9} M$) prolongs the relaxation (arrow) of fast contractions evoked by single action potentials in the Ds neuron. The proctolin also caused a small tonic contraction. The three tension records shown are parts of a continuous recording. The Ds neuron was stimulated at 0.1 Hz.

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7. The bioassay [M. O'Shea and M. E. Adams, *Science* **213**, 567 (1980); M. O'Shea and C. A. Bishop, *J. Neurosci.* **2**, 1242 (1982)] consists of a myogenic bundle of muscle fibers that form part of the main extensor muscle of the locust (*Schistocerca nitens*) hind leg. PLB was quantified by dividing the intercontraction interval before sample application by the interval at the peak of the response. Since proctolin increases the rhythm frequency (threshold from 10^{-11} to $10^{-10} M$), the PLB ratio is a number greater than 1 (see scale for PLB in the figures).
8. Extracts were chromatographed on a 10-μm μBondapak C_{18} reverse-phase column (Alltech). A volatile liquid phase (50 m*M* ammonium acetate, 12 percent acetonitrile, *p*H 4.5) was pumped at 1.3 ml/min, 1800 pounds per square inch, and 50-drop fractions were collected. Elution time in this system was determined by chromatography of [^{3}H-Tyr^2]proctolin. This was prepared by catalytic reduction of an I_2-Tyr^2 analog of proctolin (synthesized by E. T. Kaiser and W. F. DeGrado, Department of Chemistry, University of Chicago). Chromatographed fractions were bioassayed after the liquid phase was evaporated under reduced pressure and dissolved in isotonic physiological saline containing (in millimolar concentration): NaCl, 140; KCl, 5; $CaCl_2$, 5; $MgCl_2$, 1; $NaHCO_3$, 4; trehalose, 5; trimethylaminoethane sulfonic acid (TES), 5; and sucrose, 100; *p*H 7.2
9. For denervation experiments, the nerve (N5) to the coxal depressor muscle was severed unilaterally in the third thoracic segment with the contralateral muscle serving as control. Extracts were made 7 days after the operation and contained either two denervated or two control muscles.
10. An individual coxal depressor muscle 177, d and d′, with its innervating nerve (N5) was isolated and bathed in a 7-μl drop of physiological saline (*8*). Portions (4 μl) were removed from the bath and bioassayed for PLB. The innervating nerve root was stimulated with a suction electrode

with 0.5-msec square pulses at 50 Hz for 5 minutes. Portions were assayed at the end of each successive control or stimulus period.

11. High potassium saline was identical to that described above, except that KCl was elevated to 100 m*M* and NaCl lowered to 40 m*M*. High potassium cobalt saline contained 5 m*M* $CoCl_2$ replacing $CaCl_2$.
12. The mean value was determined from four such release experiments; the range was 0.5 to 1.6 percent. Mean PLB extracted from those muscles was equivalent to 30 fmole of proctolin per muscle. Amounts of PLB recovered from the Sep-Pak before and after HPLC purification did not differ significantly.
13. The third thoracic ganglion containing the Ds motoneuron and the coxal depressor muscle 177d were removed intact from the animal and perfused in vitro with physiological saline. Ds was stimulated either intracellularly with a microelectrode placed in the cell body or extracellularly with a suction electrode placed on the nerve root. Muscle membrane potentials were recorded intracellularly with flexibly mounted microelectrodes, and the contractile response was measured with a force transducer (RCA 5734).
14. A "catch" property of coxal depressor muscle 177d described by M. Chesler and C. R. Fourtner [*J. Neurobiol.* **12**, 391 (1981)] may be related to the sustained tension we describe in this report.
15. Supported by NIH grant NS-16298 and NSF grant BNS-8202515, by the University of Chicago, and by the Zoecon Corporation. We thank K. G. Pearson for the RCA transducer.
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Synthesis and Secretion of the Plasmid-Coded Heat-Labile Enterotoxin of *Escherichia coli* in *Vibrio cholerae*

Abstract. *Both cholera toxin and heat-labile enterotoxin were made and secreted into culture supernatants by* Vibrio cholerae *containing the enterotoxin plasmid pCG86. Several regulatory mutations in* V. cholerae *that increased or decreased the synthesis of cholera toxin did not affect production of heat-labile enterotoxin. In contrast, a mutation in* V. cholerae *that interfered with the secretion of cholera toxin also decreased the secretion of heat-labile enterotoxin, indicating that they are processed by a common secretory pathway.* Vibrio cholerae *should be useful as a model system for analyzing the secretion of true extracellular proteins by Gram-negative bacteria.*

Vibrio cholerae and enterotoxigenic strains of *Escherichia coli* can cause diarrhea by producing enterotoxins that stimulate secretion of fluid and electrolytes in the small bowel (*1*). Cholera toxin (CT) and *E. coli* heat-labile enterotoxin (LT) are closely related in mode of action, subunit structure, and immunochemistry (*2–4*); the structural genes for CT and LT show strong homology although they are not identical in sequence (*5*), and they are organized into similar operons (*6, 7*). Nevertheless, several differences in the genetic control of these heat-labile enterotoxins have been reported. Most of the CT produced by *V. cholerae* is secreted (*8*), but most of the LT synthesized by *E. coli* remains cell-associated (*4, 9, 10*). The A and B subunits of LT are synthesized as separate polypeptides (*6, 11*), but one preliminary study suggests that the subunits of CT might be derived from processing of a larger precursor polypeptide (*12*). The structural genes for CT appear to be chromosomal in *V. cholerae* (*7*), but the genes for LT are encoded on plasmids in *E. coli* (*5, 13*).

We constructed bacterial strains that produced both CT and LT by transferring the enterotoxin plasmid pCG86 (*14*) from *E. coli* into *V. cholerae*. By comparing the synthesis and secretion of CT and LT in isogenic strains of *V. cholerae* with wild-type or mutant alleles at loci that regulate toxinogenesis, we evaluated the roles of specific host functions on the expression of the homologous structural genes of CT and LT. We used specific competitive-binding radioimmunoassays to measure each enterotoxin antigen in the presence of the other toxin (Fig. 1).

Our analyses of the synthesis and secretion of CT and LT in wild-type and mutant strains of *V. cholerae* containing plasmid pCG86 are summarized in Table 1. All strains of *V. cholerae* containing plasmid pCG86 produced detectable amounts of LT antigen, whereas none of the control strains lacking pCG86 produced the antigen. Therefore, the structural genes for LT can be expressed in *V. cholerae*. In cultures of wild-type *V. cholerae* 569B (*8*) and of 569B containing plasmid pCG86 [569B(pCG86)], almost all of the CT was in the culture supernatant. Approximately 95 percent of the LT produced by 569B(pCG86) was also in the culture supernatant. Extracellular protein presented less than 4 percent of the total protein in these cultures. Therefore, both CT and LT are secreted by *V. cholerae*. In contrast, studies of the expression of cloned CT structural genes in *E. coli* showed that CT (*7*), like LT (*4, 9, 10*), remained cell-associated in *E. coli*.

Synthesis of CT is controlled by regulatory genes in at least two different loci on the genetic map of *V. cholerae*, and different alleles at these loci are associated with hypertoxinogenic or severely hypotoxinogenic phenotypes (*15, 16*). When plasmid pCG86 was present in strain M13, a severely hypotoxinogenic mutant of 569B (*8*), LT antigen was produced and secreted in amounts comparable to those observed with 569B(pCG86). Strain CA401 is a wild-type, virulent strain of *V. cholerae* that produces much less CT than 569B does (*17*). HV131 is a highly toxinogenic mutant of CA401 that produces CT in yields comparable to those produced by 569B; HV133 is a hypotoxinogenic mutant of CA401. Both HV131 and HV133 were isolated from survivors of nitrosoguanidine mutagenesis (*18*) of CA401 that exhibited altered toxin production in a toxin-dependent plate hemolysis assay (*19*). Although the total amount of CT produced by HV131 was approximately 100 times that produced by CA401 or

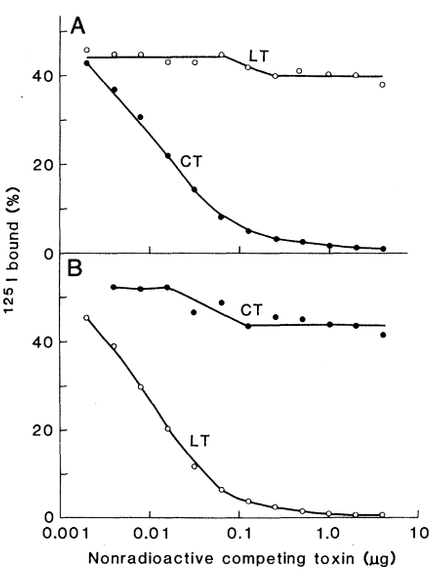

Fig. 1. Specific radioimmunoassays for cholera enterotoxin (A) and *E. coli* heat-labile enterotoxin (B). Purified cholera enterotoxin (*CT*) from *V. cholerae* 569B (*22*) and purified heat-labile enterotoxin (*LT*) from *E. coli* strain HE12 (*4, 10*) were labeled with $Na^{125}I$ (*23*) and used in competitive-binding radioimmunoassays (*24*). Each reaction mixture contained ^{125}I-labeled toxin, rabbit antiserum to the homologous purified toxin, and unlabeled CT (●) or LT (○) as the competing antigen; immune complexes were absorbed on protein A–bearing *Staphylococcus aureus*, and the amount of ^{125}I bound was determined. Most of the antibodies in each immune serum were directed against the unique determinants of the homologous toxin antigen. In the presence of a large excess of heterologous toxin antigen, there was only a slight decrease in the amount of ^{125}I bound in the assay for CT (A) or for LT (B).