rats. These data imply that the opiate-induced hypotension occurred only in the hypertensive animals and only in response to fasting. While the possibility that endogenous opiates also contribute to the fasting-induced reductions in systolic blood pressure in WKY rats cannot be dismissed, such an effect was not evident in WKY rats under the same conditions in which it was observed in SH rats.

If the actions of endogenous opiates in fasting SH rats (Fig. 2) include the reduction in SNS activity (Fig. 1), then the pressor effect of naltrexone in these animals should be associated with activation of the SNS. To examine this possibility, we measured the turnover of norepinephrine in SH rat hearts after 4 days of food deprivation. Half the rats received naltrexone after the administration of tritiated norepinephrine and the other half received saline. The rates of disappearance of the tracer from heart tissue did not differ between the two groups; the calculated norepinephrine turnover rates were 13.1 ± 4.1 ng per hour for naltrexone-treated animals and 18.5 ± 5.7 ng per hour for saline-treated animals (95 percent confidence intervals). Thus SNS activity, as measured by cardiac norepinephrine turnover, was not increased by naltrexone in unfed SH rats. While the pressor effect of naltrexone could be associated with SNS stimulation in a noncardiac tissue, preliminary experiments suggest that the blood pressure increase in food-deprived SH rats given naltrexone occurs even in the presence of alpha-adrenergic blockade (*10*). Thus the endogenous opiate mechanism activated by fasting in the SH rat does not appear to mediate the SNS suppression.

Previous attempts to explain the hypotensive effect of fasting focused on weight loss per se or a limitation in dietary sodium intake. Recent findings have tended to diminish the importance of these factors in the lowering of blood pressure induced by fasting in human subjects and SH rats (*1–4*), and the present results support two other mechanisms. The 4-day fast decreased SNS activity in the heart and did so to the same extent in both hypertensive and normotensive rats. Although these studies provide no evidence of a causal relation between SNS withdrawal and blood pressure reduction during fasting, such a connection is a reasonable presumption because of the intimate involvement of the SNS in blood pressure regulation. Fasting also elicited an opiate-mediated vasodepressor response, but one observed exclusively in the hypertensive animals. Since opiate antagonism in food-deprived SH rats only partially restored blood pressure to the levels measured before fasting, the additive effects of SNS suppression and opiate stimulation appear to have accounted for the greater hypotensive response to fasting in SH rats compared to WKY rats. Although similar changes in blood pressure and in biochemical indices of SNS activity appear to occur in dieting humans (*2*), no information is available to indicate whether opiate mechanisms are also operative.

Evidence suggests that the combination of SNS suppression with opiate activation is not unique to fasting SH rats (*7, 8*). Diminished SNS tone and increased opiate activity have been demonstrated in animals given 2-deoxyglucose, a nonmetabolizable glucose analog (*11*), and in genetically obese rats (Zucker *fa/fa*) and mice (*ob/ob*) (*12*). In traumatic injury, SNS activity decreases rapidly (*13*), a situation analogous to forms of experimentally induced hypotension (hemorrhagic or endotoxin shock) in which a role for endogenous opiates in blood pressure regulation has been inferred (*14*). Moreover, the frequently antagonistic effects of opiates and the SNS on blood pressure and metabolic rate suggest that reciprocal changes in the activity of both systems may be important in homeostatic regulation.

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Events in the Evolution of Pre-Proinsulin

Abstract. *An extensive computer-assisted analysis of known pre-proinsulin coding sequences has shown correlations that can be interpreted as evidence for an intron-mediated juxtaposition of exons in the evolution of these genes. The evidence includes the discovery that the regions of the pre-proinsulin genes that code for the signal peptide consist of nearly tandem repeating units of nine base pairs. This pattern reappears in the C region of the genes after a large intron that occurs in three of the four genes analyzed. A model is proposed in which primordial insulin was coded for by two separate minigenes arising from a gene duplication, each with identical or nearly identical signal peptide coding regions. The minigenes fused into one transcriptional unit mediated by the large intron, and the signal peptide coding region of one of the putative minigenes evolved into the latter portion of the C peptide coding region.*

The genomic structure of a number of pre-proinsulin genes have been elucidated. These include the human (*1*), the two rat variants—rat 1 and rat 2 (*2*)—and the chicken (*3*). These genes all contain a short intron in the 5′ noncoding region, and all except the rat 1 gene contain a long intron interrupting the region coding for the connecting peptide.

Gilbert (*4*) postulated that one function of introns is to bring together various exons to form new structural genes. The

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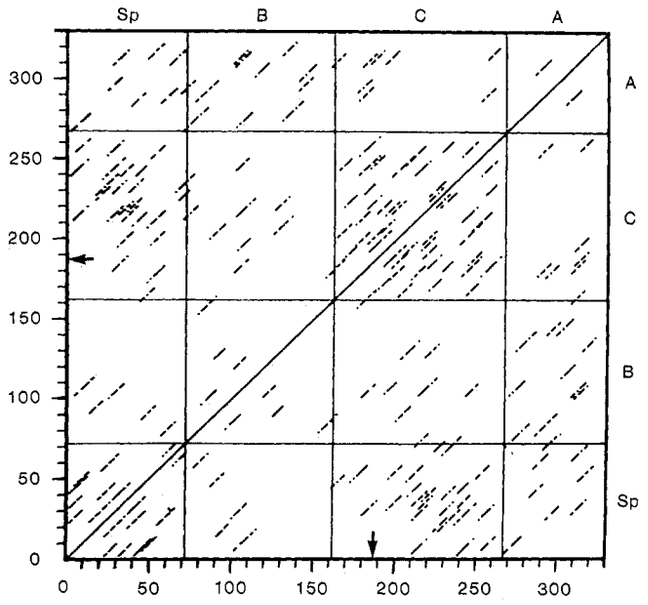


Fig. 1. Match matrix for human pre-proinsulin. Contiguous runs with six or more matches per nine bases, parallel to the diagonal, are shown. The arrows show the position of the large intron. Densities, calculated as 100 times the number of base matches divided by the area of the domain, were: (Sp,Sp), 2.93; (C,C), 3.00; (Sp,C), 1.77. Densities of all the other domains were between 1.48 (B,A) and 0.45 (A,A). Domain densities for other coding sequences were similar. Rat 2: (Sp,Sp), 3.87; (C,C), 2.12; (Sp,C), 1.77; all other values were between 1.48 (B,A) and 0.40 (A,A). Syrian hamster: (Sp,Sp), 2.28; (C,C), 2.12; (Sp,C), 1.23; all other values were between 1.09 (B,A) and 0.54 (C,A). Chicken: (Sp,Sp), 1.77; (C,C), 0.77; and (Sp,C), 0.44; all other values were between 1.19 (Sp,B) and 0.00 (A,A). All areas were calculated from the position of the large intron except (C,C). An intron was assumed at base 187 in Syrian hamster.

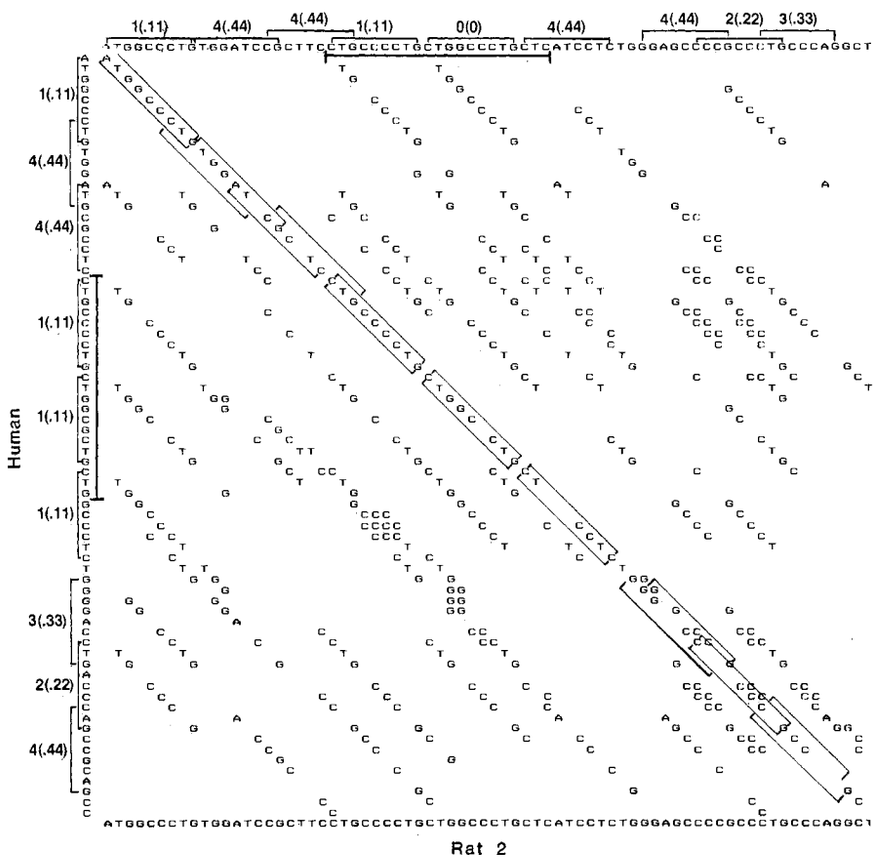


Fig. 2. Comparisons of human and rat 2 Sp regions. Contiguous off-diagonal sequences (direct repeats) are shown with a minimum match of six out of nine bases except on the main diagonal where all matches are shown. Tandem sequences having six or more matches per nine bases are projected onto the diagonal. The E.D. from the consensus sequence CTGGCCCTG is given as E.D.(K), where E.D. is the evolutionary distance and K is the E.D. normalized to the length of the region. For these short sequences, the value $(1 - K) \times 100$ is the percentage of match to the consensus sequence except for the last sequence CTGCCAG of rat 2, which must be gapped to give a minimum E.D. of 3. The underlined segments code for the hydrophobic core Leu-Pro-Leu-Leu-Ala-Leu-Leu, which is invariant in the four sequences analyzed.

existence of both the large and small intron in the pre-proinsulin genes may reflect the structure of an ancient gene; the loss of the large intron in rat 1 occurred after the mammalian radiation (85 million years ago). Since the large intron does not seem to play a functional role in gene expression, it could be a remnant of some ancient evolutionary process.

Computer programs, based primarily on the algorithms of Sellers (*5*), were written to analyze the relatedness of DNA sequences. The sequences are compared by the construction of a matrix in which nonzero elements represent base matches. The program finds the best alignment of two sequences by a process that is equivalent to maximizing the number of base matches by sliding and gapping when a manual comparison is made. For a particular alignment, a parameter called the evolutionary distance (E.D.), which is an index of the degree of relatedness between the two sequences, can be calculated. The best alignments of the sequences, including gaps, are those of minimum E.D.

For a given alignment, represented by the diagonal of the matrix, off-diagonal runs of base matches that are parallel to the main diagonal represent homologous regions in domains outside that alignment. For two identical sequences, these off-diagonal runs represent direct repeats.

An analysis of the off-diagonal runs of human, rat 2, chicken, and Syrian hamster (*6*) genes revealed a striking nonuniform distribution of direct repeats having six or more matches per nine bases (Fig. 1). For all four sequences, the greatest density of direct repeats was usually found in the signal peptide domain (Sp,Sp) of the matrix. For human, rat 2, and Syrian hamster genes the C peptide domain (C,C), also has a high density of repeats. Comparisons of the Sp and C regions [the (Sp,C) domain of the matrix] also show a high density of repeats after the large intron in the rat 2 and human genes, as well as the hamster gene, in which an intron is assumed to exist at base position 187 by analogy with the rat 2 and human genes. For the chicken gene, the density of repeats in the (C,C) and (Sp,C) domains is relatively low.

Programs were also written to examine sets of nucleotide strings and to construct consensus sequences if they occur. When these programs were applied to the pre-proinsulin genes, consensus sequences consisting of nine base pairs could be derived from the direct repeats in the Sp regions of all four coding sequences. These sequences are

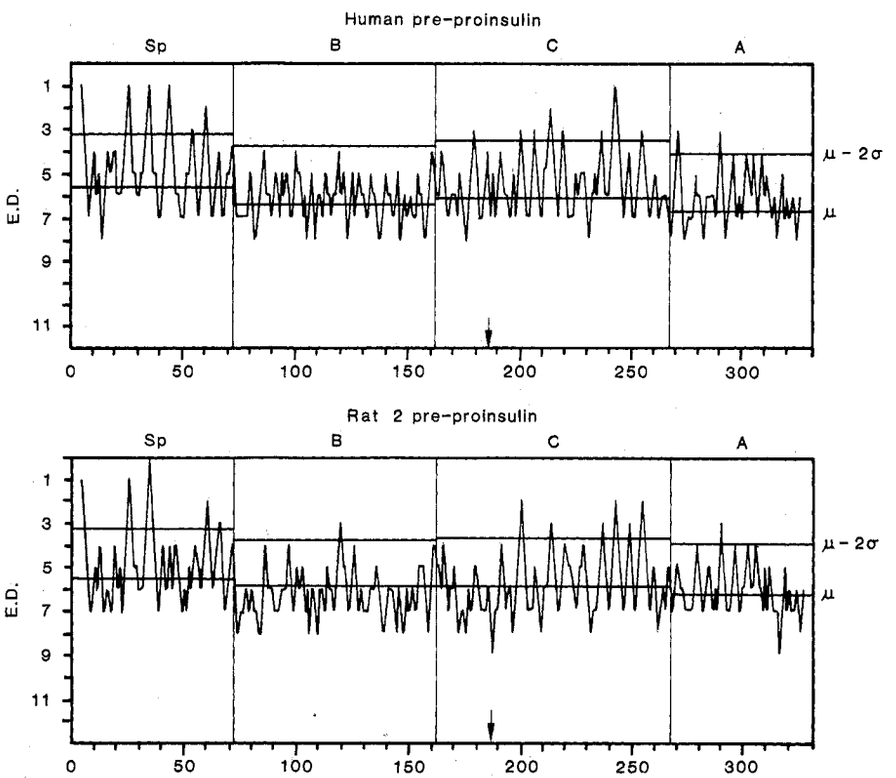
CTGGCCCTG for the human and rat 2, TGNCCCTGT for the hamster, and CTGGCTCTT for the chicken (C, cytosine; T, thymine; G, guanine; N, any base). The maximum E.D. between any two of these three consensus sequences is 3, which suggests a common evolutionary origin. For these small sequences, the E.D. represents the number of base mismatches in the best alignment without gapping. Only permutations of one candidate consensus sequence could be obtained from each Sp region. Furthermore, these sequences are arranged in an almost tandem fashion covering the entire Sp regions (see Fig. 2 for human and rat 2). Consensus sequences that are not as distinctive, or are not tandemly arranged, but that are closely related to the Sp repeating sequences, were also derivable from the regions with a high density of repeats in the C peptide coding region of the human, rat 2, and hamster genes, but not from the chicken gene. Proper consensus sequences could not be constructed from the other coding regions of any of the four sequences.

The methods used in this analysis were tested with computer-generated random chains of appropriate size and base composition. Although direct repeats of significant length infrequently appear, in no test case could a proper repeating consensus sequence be obtained.

The amino acid sequence Leu-Pro-Leu-Leu-Ala-Leu-Leu (Leu, leucine; Pro, proline; Ala, alanine) is preserved in the Sp regions of all four coding sequences. Across this region the nine–base pair repeats appear in perfect tandem arrangement with minimal E.D. from the derived consensus sequences. This region is underlined in the human and rat 2 sequences displayed in Fig. 2. This "hydrophobic core" is presumably associated with the function of the signal peptide.

The results of the base sequence analysis strongly suggest that the evolutionary precursor of the signal peptides of human, rat 2, Syrian hamster, and chicken pre-proinsulin was constructed of identical or nearly identical, small, tandemly repeating subunits. Furthermore, the nonuniform distribution of direct repeats and the finding of consensus sequences related to the Sp repeating units in DNA sequences past the large, or assumed large, intron in the human, rat 2, and hamster C regions suggests an evolutionary relation between the Sp and C regions of these genes. The shortening of the C region in chicken caused by deletions coupled with point mutations may have obliterated any remnant of a relation between the Sp and C regions in this particular gene.

The nine-base consensus repeating sequences of the Sp region were compared to the entire coding regions of the appropriate pre-proinsulin genes in one-base increments, and values of E.D. were calculated (shown in Fig. 3 for human and rat 2 genes). A similar analysis was made for Syrian hamster pre-proinsulin (not shown). Very long pseudorandom sequences with the same base compositions as the various coding regions were also generated. The means and standard deviations for each of the consensus repeating sequences were calculated and are shown in the legend to Fig. 3. The expected values for each E.D. were computed by summing the number of times an E.D. $\leqq (\mu - 2\sigma)$ appeared, where μ is the mean and σ is the standard deviation, and dividing the result by the total number of data points. For these three pre-proinsulin coding sequences, the number of points in the Sp and C regions more than two standard deviations (2σ) from the mean (μ) was at



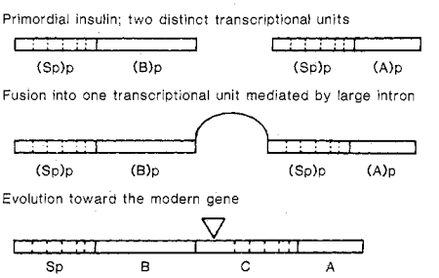


Fig. 3 (left). Evolutionary distance (E.D.) plotted against base position for the Sp consensus repeating sequences of rat 2 and human genes. The Sp consensus repeating sequences were compared to the total coding regions in one-base increments, and values of E.D. were calculated and plotted by computer. The position of the large intron is indicated by the arrows. Values of μ and $\mu - 2\sigma$ were calculated by use of pseudorandom chains of the same base composition and at least 100 times the length of each region. The consensus sequence for human and rat 2 genes is CTGGCCCTG; for the Syrian hamster gene, TGNCCCTGT; and for the chicken, CTGGCTCTT. The observed frequencies and the expected frequencies (in parentheses) of E.D. $\leqq (\mu - 2\sigma)$ were the following. Human: Sp, 6 (0.50); B, 3 (7.02); C, 11 (2.57); and A, 1 (0.73). Rat 2: Sp, 12 (3.25); B, 1 (1.53); C, 9 (2.4); and A, 1 (0.82). Syrian hamster: Sp, 9 (0.85); B, 4 (4.95); C, 10 (3.28); and A, 2 (4.08). Chicken: Sp, 10 (2.98); B, 3 (4.27); C, 5 (4.34); and A, 1 (3.86). Similar results were obtained with pseudorandom chains 100 times the length of each region and with regional dinucleotide frequencies preserved. Human: Sp, 6 (3.04); B, 0 (2.60); C, 11 (2.57); and A, 1 (0.73). Rat 2: Sp 7 (2.37); B, 1 (2.05); C, 9 (3.65); and A, 1 (2.30). Pseudorandom chains 100 times the length and maintaining the base composition of the entire pre-proinsulin genes gave the following values of μ and of $\mu - 2\sigma$ (in parentheses): human, 5.96 (3.62); rat 2, 5.99 (3.65); Syrian hamster, 5.51 (3.24); and chicken, 6.08 (3.81). The observed and expected (in parentheses) frequencies of E.D. $\leqq (\mu - 2\sigma)$ were human, 26 (8.13); rat 2, 23 (6.73); hamster, 31 (14.2); and chicken, 14 (5.22). Fig. 4 (right). Model of the events in the evolution of pre-proinsulin; (Sp)p, (B)p, and (A)p indicate primordial regions. The arrowhead indicates the position of the large intron.

least three times the expected values. In the B and A regions of the genes, actual values were much closer to expected values. Pseudorandom sequences maintaining the dinucleotide frequencies of each region were also generated. Calculation of μ and σ and expectation values were made as above (see legend to Fig. 3) and led to the same conclusions as those obtained with regional chains generated with single nucleotide frequencies maintained.

In addition, pseudorandom chains of the same base composition as the entire genes were generated and also compared with appropriated consensus sequences (see legend to Fig. 3). If a Gaussian distribution is assumed, E.D. values $\leqq (\mu - 2\sigma)$ have a maximum probability of occurrence of $\sim .023$ (*7*).

The relation between the Sp and the latter part of the C region is therefore significant and not a fortuitous result of similar base composition or dinucleotide frequency. For the chicken, no significant regions of E.D $\leqq (\mu - 2\sigma)$ calculated for all three types of pseudorandom chains were found outside of the Sp region from which the consensus sequence was derived.

We speculate that the pre-proinsulin gene evolved from two separate minigenes, each with identical or nearly identical Sp regions, but separate functional regions corresponding to the B and A regions of the modern gene. Primitive pre-proinsulin may have arisen through selection pressures favoring the juxtaposition of these two minigene products so that proper disulfide bridges could form more easily in the maturation of the hormone. The functional genomic units, mediated by the large intron, evolved into a single transcriptional unit. The nucleotide sequence coding for the Sp region of the putative A minigene became the latter portion of the C region. Evolution into a functional C peptide coding region probably occurred at a very rapid rate because of the great advantage of such a unit for proper folding and disulfide bridge formation. This model is depicted in Fig. 4.

The evolutionary relationship between the Sp regions of the four pre-proinsulins examined in this study implies a common origin. In the context of the foregoing speculation, at least the signal peptide coding portion of the putative minigenes must have arisen from a common ancestral source, probably by gene duplication.

An alternative interpretation of our findings is that an ancient pre-proinsulin precursor was made up entirely of tandem repeats. Divergent evolution with strong selection pressures for particular amino acids in the functional B and A regions could have obliterated any identifiable remnant of the primordial base repeats, or for that matter, of any other homologous relation. This alternative model does not account for the existence and possible evolutionary role of the large intron.

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Neurotoxin-Specific Immunoglobulins Accelerate Dissociation of the Neurotoxin–Acetylcholine Receptor Complex

Abstract. *Toxin isolated from cobra venom and labeled with tritium was incubated with membranes rich in acetylcholine receptors. The amount of toxin bound to the receptors was determined and the kinetics of dissociation of the receptor-toxin complex was followed. Addition of an excess of horse antiserum to the venom resulted in a significant acceleration of the dissociation reaction. Similarly, a monoclonal antibody against the toxin accelerated dissociation of the receptor-toxin complex. The results indicate that specific antibody binding destabilizes the toxin-receptor complex.*

Injection of cobra venom into mammals produces a flaccid muscle paralysis, and death occurs by respiratory deficiency (*1*). The lethal effect is due to the presence in the venom of neurotoxic proteins (molecular weight, 6000 to 7000), which bind tightly and specifically to the nicotinic acetylcholine (ACh) receptor, producing a nondepolarizing block (*2*). Therapeutic action against cobra bite usually consists of treatment with specific antibody to the venom (*3*, *4*). This action is commonly explained by the fact that one free neurotoxin molecule binds to at most three or four specific immunoglobulin molecules and thus becomes inactive (*5*, *6*).

However, recent data suggest that neurotoxin-specific immunoglobulins may have a role at the level of the neurotoxin–ACh receptor complex. It is known that (i) a neurotoxin molecule associated with its physiological target is not internalized but remains at the surface of the postsynaptic membrane (*7*, *8*) and that (ii) the binding of a neurotoxin to its

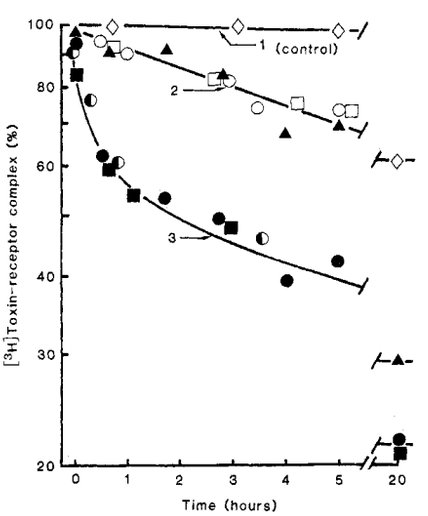

Fig. 1. Effect of excess unlabeled toxin or antibodies to the toxin on dissociation of the [^{3}H]toxin-receptor complex. Acetylcholine receptor–rich membranes from *T. marmorata* (10 n*M* [^{3}H]toxin binding sites) were mixed with [^{3}H]toxin (4 n*M*) in Ringer solution at room temperature. After incubation for 3 hours or overnight the mixture was diluted ten times. Reversal was initiated by adding (▲) unlabeled toxin at a final concentration of 0.7 μM, (■) horse antiserum to the venom (0.3 μM), (●) mouse monoclonal antibody against the toxin, or (◐) its Fab fragments at a final concentration of 0.25 μM; or a preincubated (30 minutes) mixture of toxin (0.7 μM) and either (□) horse antiserum (0.3 μM) or (○) monoclonal antibody (0.25 μM). When the toxin-antibody complex is formed, the amount of free unlabeled toxin remains large (at least 1000 times the concentration of [^{3}H]toxin). The control (◇) was prepared without adding unlabeled toxin or antitoxin immunoglobulins. At different times 0.5 ml of each mixture was taken and filtered through two Millipore filters (0.45 μm), which were washed with 15 ml of Ringer solution at 4°C. The filters were dried and the retained tritium was counted.

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