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10. We attempted to find chloride responses for all four neurotransmitters tested. However, chloride inhibitory responses due to 5-HT and DA are extremely rare in the neurons of *A. californica*.
11. In one unidentified neuron, a response elicited by ACh and presumed to be due to an increase in chloride conductance was attenuated by only 26 percent. The other six cholinergic chloride responses were elicited in identified neurons (medial cells of the pleural ganglia and L2 of the abdominal ganglion) and behaved as illustrated, as did all of the chloride responses to the other neurotransmitters.
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Relaxin: A Disulfide Homolog of Insulin

Abstract. *Relaxin, a peptide hormone responsible for the widening of the birth canal in mammals, has been purified from the ovaries of pregnant hogs. The amino acid sequences of its constituent A and B chains were determined, and the positions of the disulfide cross-links were established. Relaxin was shown to be identical to insulin with respect to its disulfide bond distribution, but significant homology was lacking in other positions. These findings suggest that relaxin and insulin were derived from a common ancestral gene. Since the intrauterine mode of propagation is synonymous with the development of mammals, the genetic distance between insulin and relaxin should therefore permit an estimate of the earliest possible time of commitment of one evolutionary branch to the development of mammals. This event was estimated to have occurred about* 5×10^8 *years ago.*

Relaxin is a peptide hormone produced in the corpora lutea of pregnant mammals and acts in conjunction with estrogen on the structures of the birth canal to provide for the passage of the offspring (*1*). The major target organs include the uterus, uterine cervix, and vagina, as well as the pubic and sacroiliac joints.

The amino acid sequences of the two constituent chains of porcine relaxin (Fig. 2) were determined as reported (*2*, *3*). We have now secured information concerning the disulfide cross-linking pattern in relaxin and have thus demonstrated the existence of the first disulfide homolog of insulin.

The relaxin used for the disulfide cross link study was purified from pregnant hog ovaries (*4*). Tryptic fragments were prepared (under nonreducing conditions) by incubating 5 mg of the purified hormone with 50 μg of trypsin in 500 μl of 0.2*M* *N*-ethylmorpholine buffer (*p*H 8.5) and digested for 4 hours at 37°C. The resultant digest was lyophilized and then dissolved in 0.05*M* ammonium bicarbonate; this solution was injected onto the top of a high-pressure liquid chromatography (HPLC) column (μBondapak C_{18}, Waters Associates) and eluted with a stepwise gradient of acetonitrile in ammonium bicarbonate (0.05*M*). The result is shown in Fig. 1, and the tryptic fragments (outlined and designated Nos. 2 and 3 in Fig. 2) were identified by their amino acid composition as given in Table 1.

Table 1. Amino acid analyses of tryptic peptides derived from unreduced relaxin.

Amino acid	Peptide	
	HPLC No. 2	HPLC No. 3
Aspartic acid		
Threonine		
Serine		1
Glutamic acid	1	1
Glycine	2	1
Alanine	1	
Cysteine	4	2
Valine	1	2
Isoleucine	1	1
Leucine		2
Lysine		
Arginine	2	
Tryptophan*		2

*Determined spectrophotometrically.

In order to determine which cysteine residue (A8 or A9) was linked to residue B10, peptide No. 2 (Fig. 2) was subjected to one cycle of Edman degradation in the absence of reducing agents. Only phenylthiohydantoin alanine was obtained from the degradation cycle. The remaining peptide was recovered from the sequencer cup, dried, and redissolved in 200 μl of 0.2*M* *N*-ethylmorpholine buffer (*p*H 8.5). By means of thin-layer chromatography, it could be demonstrated that the remaining peptide still yielded only one spot when sprayed with ninhydrin or phenanthrenequinone (test for arginine). After treatment with mercaptoethanol to reduce its disulfide bonds the peptide yielded two spots in the same thin-layer chromatography system as described (*3*) for the separation of tryptic fragments derived from the B chain of relaxin. This result indicated that the relaxin A and B chains were linked through cysteine A9 and cysteine B10. Had the cross-link involved cysteines A8 and B10, the Edman step would have released the intrachain disulfide loop (A9 through A13) which would have been seen as a second peptide prior to reduction.

Evidence for a cross-link between the cysteines at A22 and B22 could be deduced from another tryptic peptide obtained from the total digest of relaxin. The tryptic peptide designated HPLC No. 3 in Fig. 1 contained the residues A21 leucine, A22 cysteine, and B17 leucine through B26 serine. The amino acid analysis of this peptide is also shown in

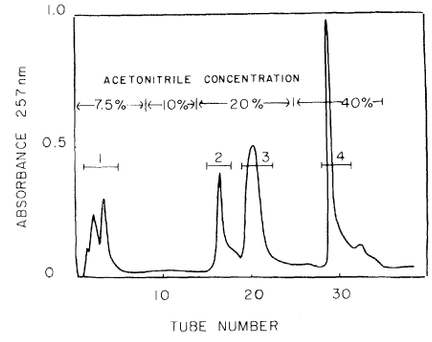

Fig. 1. Elution record of the separation by high-pressure liquid chromatography of tryptic peptides derived from unreduced relaxin. The acetonitrile gradient increased stepwise and peaks were collected as indicated in the figure. Peptides Nos. 2 and 3 were found to contain the disulfide linkages.

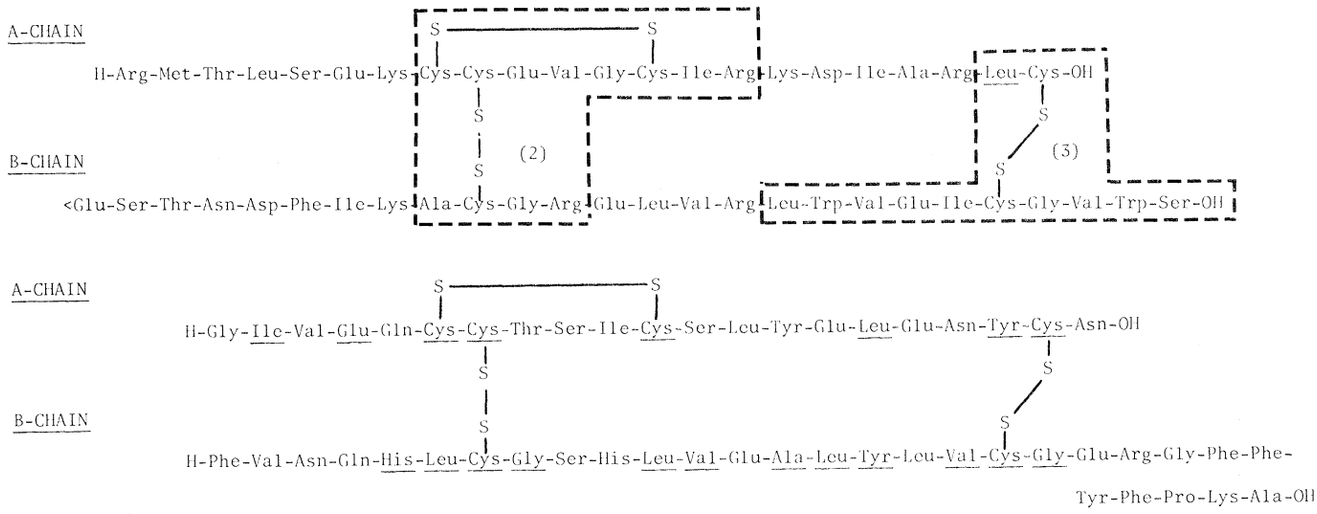

Fig. 2. The primary structures of porcine relaxin (top) and insulin (bottom) with their cysteine residues aligned. The dashed lines in the relaxin structure identify the tryptic peptides designated Nos. 2 and 3 (also Table 1). In the insulin structure, the underlined residues signify those that are either homologous or conservatively replaced with reference to the respective chains in relaxin.

Table 1. Further evidence for the cross-link between the cysteines at A22 and B22 was obtained from experiments in which lysosomal carboxypeptidase B was allowed to act on the intact relaxin molecule. As was recently reported (*3*), lysosomal carboxypeptidase B rapidly removed the COOH-terminal residues Ser, Trp, Val, and Gly as well as the cysteines involved in the cross-link. A time course digest of total relaxin with lysosomal carboxypeptidase B yielded—in addition to serine, tyrptophan, valine, and glycine—leucine and arginine before isoleucine or glutamic acid could be observed. This can only be interpreted as evidence that Leu^{21} and Arg^{20} in the A chain were hydrolyzed before the first glutamic acid (Glu^{20}) and isoleucine (Ile^{21}) could be released from the B chain.

In view of these experiments, we concluded that the cross-linking patterns of insulin and relaxin are superimposable. It is surprising, however, that virtually no homology exists between the insulin and relaxin molecules with respect to the remaining residues. The homology is limited to only five residues in the A chain. In addition, three conservative replacements occur in the A chain. The B chain contains six homologous positions in addition to six conservative replacements relative to insulin. Considering only the two initial bases of the appropriate codons, this would correspond to 51 point mutations. If we assume that the rate of mutation acceptance for relaxin is 1 pauling unit (10^{-9} mutation accepted per amino acid per year) the gene duplication may have occurred as early as 5×10^8 years ago. Insulin accepts mutations at the rate of 0.4 pauling or four residues per 10^8 years (*5*), that is, it has changed its amino acid composition possibly in 20 positions during the same period. This estimate of 5×10^8 years for relaxin seems reasonable when one considers that sharks and bony fishes diverged from the branch of vertebrate evolution which eventually gave rise to the mammals.

It has been suggested (*6*) that the A2 isoleucine in insulin (at which position leucine is found in relaxin), the A16 leucine (which is isoleucine in relaxin), as well as the A19 tyrosine (which is leucine in relaxin), would allow a similar formation of a hydrophobic core in relaxin as it occurs in insulin. Another point of identity or similarity between the B chains is given by the fact that cysteine is followed by glycine in both hormones and is preceded by an aliphatic residue (alanine and isoleucine in relaxin as compared to leucine and valine in the insulin B chain). The identical cross-linking patterns of relaxin and insulin suggest that duplication of an ancestral gene led to the evolution of two hormones with profoundly diverse functions.

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A New Satellite of Saturn?

Abstract. *Analysis of all available observations of faint objects near Saturn during the 1966 passage of the earth through the plane of Saturn's rings suggests the existence of at least one previously undiscovered satellite of Saturn. The data support the previously published orbit for Janus. These satellites may be major members of an extended ring.*

In 1966 the earth passed through the plane of Saturn's rings. Since the ring system is only a few kilometers thick (*1*), there was very little scattered light from the rings at that time, which enabled Dollfus (*2*) to discover a tenth satellite of Saturn (S10), commonly referred to as Janus. Dollfus calculated an orbit based on his own observations as well as those by J. Texereau at McDonald Observatory, Fort Davis, Texas. A later analysis by Franklin *et al.* (*3*) included an additional observation by R. Walker at the U.S. Naval Observatory, Flagstaff, Arizona.

We have reexamined photographs tak-