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The Isolation of Two Proline-Containing Relaxin Species from a Porcine Relaxin Concentrate (40808)[1]

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Several early reports (1–3) suggested that relaxin activity is shared by several low molecular weight, basic proteins. Sherwood and O'Byrne (4) have described the isolation of three fractions with relaxin activity by subjecting acid acetone extracts of pregnant sow ovaries to gel filtration on Sephadex G-50 followed by ion-exchange chromatography on CM-cellulose and hydroxyapatite. The three fractions, designated CM-B, CM-a and CM-a′, had similar biological activities (2500–3000 U/mg) in the mouse interpubic ligament assay, similar amino acid compositions, and their rates of migration in polyacrylamide gels at pH 4.3 were identical. None of the three contained histidine, proline, or tyrosine, and each was shown to consist of two peptide chains linked by disulfide bonds. Schwabe and McDonald (5) have determined the structure of one of the relaxins isolated by the Sherwood–O'Byrne method; the two peptide chains contain 22 and 26 amino acids, respectively. Their proposed structure bears a distinct homology to porcine insulin with respect to the location of the disulfide bridges; however, homology with respect to primary sequence is limited to fewer than half its constituent amino acids. Amino acid sequences for the two peptide chains of another relaxin variant isolated by the Sherwood–O'Byrne method have been described by James *et al.* (6); their sequence differs from that described by Schwabe and McDonald by the presence of Gln instead of Glu in the shorter (a) chain and the replacement of the B-chain C-terminal tripeptide Val–Trp–Ser-OH by the hexapeptide Ser–Val–Ser–Trp–Gly–Arg-OH.[2]

In this paper we describe the isolation of three distinct species of porcine relaxin by chromatography on Bio-Gel P-10, followed by electrophoresis at pH 9.0 on a column of Sephadex G-25. The amino acid composition of one of these corresponds quite closely to the material characterized by Schwabe and McDonald (5). The other two differ most significantly in that each contains one proline residue. In addition, one of the three compounds which we have isolated possesses biological and immunological properties which are significantly different from the others.

Materials and methods. Partially purified porcine relaxin, specific activity 440 guinea pig units (GPU) per milligram, was obtained from the National Institutes of Health. Bio-Gel P-10 and Sephadex G-25 (superfine) were purchased from Bio-Rad Laboratories and Pharmacia, Inc., respectively. Relaxin activities were determined in estrogen-primed guinea pigs as previously described (8); each preparation was tested at 4–7 concentrations, using 9–20 animals at each concentration; NIH relaxin served as a reference standard. Samples of CM-B, CM-a, and CM-a′, prepared by the Sherwood–O'Byrne method, were kindly provided by Dr. Bernard Steinetz of Ciba-Geigy Corporation, Ardsley, New York.

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[2] This sequence was originally described as Ser–Val–Ser–Thr–Trp–Gly–Arg–OH but has since been corrected (7).

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For isoelectric pH determinations the 110-ml LKB electrofocusing apparatus was used. Electrofocusing was conducted in 2% Ampholine solutions for 44–96 hr; gradients were stabilized with glycerol or sucrose. Analytical disc gel electrophoresis was performed in 10% polyacrylamide gels at pH 8.2 (0.025 *M* Tris chloride) or at pH 5.0 (0.025 *M* ammonium acetate). Samples for amino acid analysis were hydrolyzed in redistilled constant boiling HCl (5.7 *M*) *in vacuo* at 110°C for 22 and 48 hr; after the addition of internal standards and removal of the acid, the hydrolysates were analyzed in a Beckman Model 121 amino acid analyzer equipped with a system AA integrating computer. Half-cystine was determined as cysteic acid in hydrolysates prepared after performic acid oxidation. The data for serine and threonine were corrected for destruction during hydrolysis; for the other amino acids the 22- and 48-hr values were averaged. Tryptophane was estimated spectrophotometrically on unhydrolyzed samples by the method of Bencze and Schmid (9).

Estimates of the molecular weights of purified relaxin preparations were made by (a) high speed sedimentation equilibrium in a Beckman Model E analytical ultracentrifuge (C = 0.5 mg/ml in 0.1 M pH 7.0 phosphate buffer) at rotor speeds of 44,770 and 37,020 rpm for at least 70 hr; and (b) gel filtration on a 145 × 1.8-cm column of Bio-Gel P-10 in 0.1 *M* ammonium acetate, pH 5.0. Porcine ACTH, horse heart cytochrome *c*, bovine pancreatic ribonuclease, equine myoglobin, and bovine chymotrypsinogen were used to calibrate the column.

The apparatus used for column electrophoresis was modified from that described by Porath (10). The column (1.87 × 63.5 cm) was charged with Sephadex G-25 (superfine), equilibrated with 0.025 *M* NH_4OAc buffer, pH 9.0; coolant at 4°C was circulated through the column jacket. The sample, dissolved in 1.0–1.5 ml buffer, was layered on the top of the column and allowed to penetrate by collecting 2–3 ml buffer from a capillary delivery tube connected to its lower end. With the delivery tube and the port connecting the upper end of the column to the bridge both closed, the space above the column packing was filled with deaerated 0.025 *M* buffer and sealed. The delivery tube was then removed and the lower end of the column submerged in an electrode vessel containing deaerated 0.050 *M* buffer, pH 9.0. The bridge, with its lower end in the second electrode vessel, was then attached and filled with 0.050 *M* buffer. The two electrode vessels were connected through a stopcock to facilitate equilibration. After the levels of buffer in the two vessels had equalized, the stopcock connecting them was closed and the port connecting the electrophoresis column and the bridge opened. Current was supplied through platinum electrodes by an LKB model 33710 constant voltage power supply. At the conclusion of the experiment, the current was interrupted, the column again sealed, the bridge removed, and the column, with the delivery tube restored, was attached to a buffer reservoir and mounted over a fraction collector. The column was eluted with 0.025 *M* ammonium acetate, pH 9.0, in 2.0- to 2.5-ml fractions.

Results. 1. Purification. When NIH relaxin was subjected to gel filtration on a column of Bio-Gel P-10 in 0.10 *M*, pH 5.0 ammonium acetate buffer, it was resolved into two peaks, with V_e/V_t = 0.35 and 0.62, respectively (Fig. 1). Approximately 90% of the activity recovered from the column emerged with the second peak (shaded

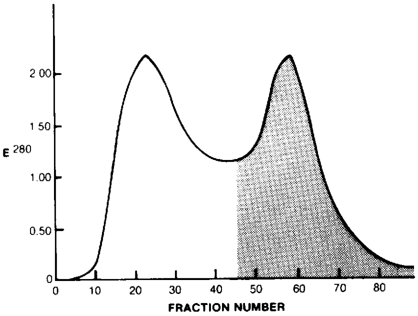

FIG. 1. Exclusion chromatography of NIH relaxin on Bio-Gel P-10. 282 Mg relaxin was applied to a 1.8 × 135-cm column of Bio-Gel P-10 and eluted with 0.10 *M* NH_4OAc, pH 5.0. The eluate was collected in 2.5- to 3.0-ml fractions. The shaded area indicates the relaxin-containing region.

area). When material recovered from the active peak was subjected to polyacrylamide gel electrophoresis at pH 8.2, two discrete bands migrating toward the cathode could be observed (Fig. 2a); at pH 5.0 two fainter, more slowly migrating components could also be discerned (Fig. 2b). When the active Bio-Gel P-10 peak was subjected to isoelectric focusing in a glycerol-stabilized pH 3–11 gradient two major peaks, with isoelectric points of 10.7 and 9.7 were resolved. Both of these had substantial relaxin activity. The electrophoretic behavior of these fractions in polyacrylamide gels at pH 8.2 corresponded to the two cationic species which appear in Fig. 2a.

The observation that the major active constituents of the second P-10 peak had significantly different isoelectric points and were well separated by electrophoresis in polyacrylamide gels suggested that column electrophoresis might effectively be used to separate and purify the several active peptides. Accordingly, experiments were initiated to test the applicability of this procedure. Figure 3 indicates the results which were obtained when 59 mg of Bio-Gel P-10 filtered relaxin was subjected to electrophoresis for 36.5 hr at pH 9.0 on a 62.5-cm column of Sephadex G-25. Analysis of the eluate (absorbance at 280 nm) indicated that the original sample had been separated into three electrophoretically distinct components. Each possessed relaxin activity; they were designated relaxins A, B, and C in order of decreasing mobility on the column (and in polyacrylamide gels at pH 5.0). The amount of sample applied to the column could be increased to 105 mg with no loss of resolution, but the application of 185 mg resulted in apparent overloading, as indicated by overlapping of the peaks corresponding to relaxins A and B.

For further purification, the column effluent was pooled as indicated and the protein was recovered by lyophilization. Corresponding pools from several experiments were combined and refractionated by electrophoresis to provide electrophoretically homogeneous samples of each active species (Fig. 4).

2. *Characterization. Biological and immunological activities.* The activities of electrophoretically homogeneous samples of relaxins A, B, and C, as determined by the guinea pig palpation method, were 2195 ± 310, 1615 ± 215, and 1050 ± 180 GPU/mg

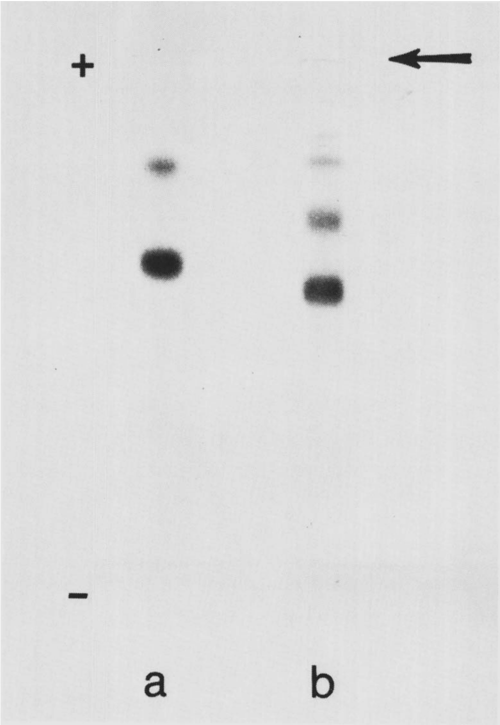

FIG. 2. Polyacrylamide gel electrophoresis of gel-filtered relaxin. Electrophoresis was performed at pH 8.2 (a) and 5.0 (b) with the cathode at the bottom. Gels were stained with Buffalo black. The arrow indicates the point of application of the sample.

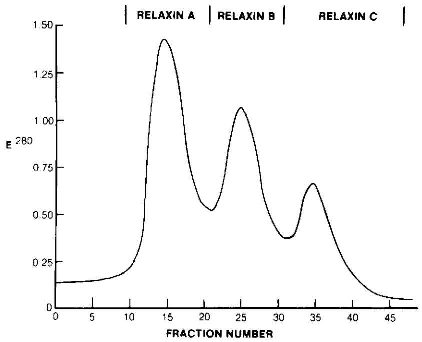

FIG. 3. Column electrophoresis of gel-filtered relaxin. 59 Mg relaxin was applied to a 62.5-cm column of Sephadex G-25 equilibrated in 0.025 *M* NH_4OAc, pH 9.0. *V* = 1200, *I* = 27 mA. Running time was 36.5 hr.

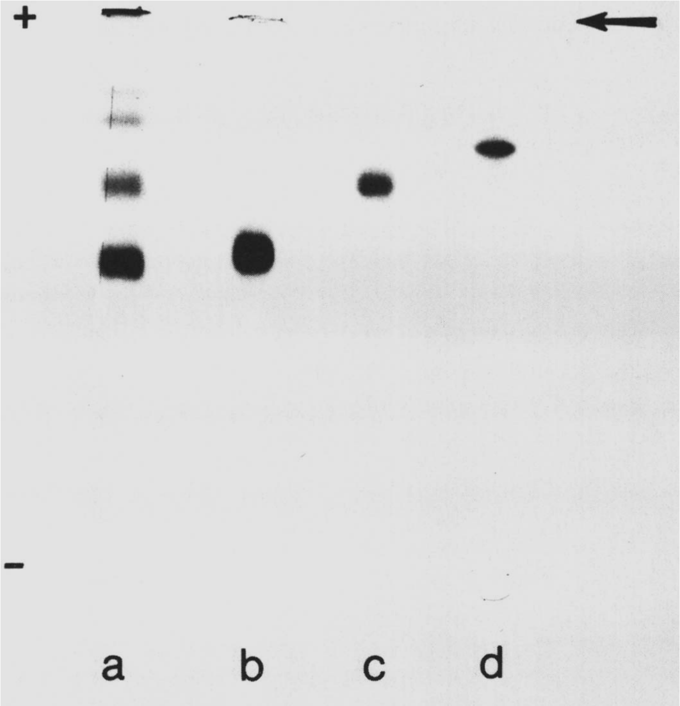

FIG. 4. Polyacrylamide gel electrophoresis of electrophoretically purified relaxins. Electrophoresis was performed at pH 5.0 with the cathode at the bottom. The arrow indicates the point of application of the sample. (a) Bio-Gel filtered relaxin; (b)–(d) relaxins A, B, and C, respectively.

(averages of determinations at 4–7 concentrations for each preparation, ±SE), respectively. All three were capable of inhibiting spontaneous contractions of rat uteri *in vitro* (11, 12). Relaxins C and A appeared to be most active in this respect, the concentrations required for 50% inhibition of contraction amplitude being in the range of 10–30 ng/ml for relaxin C and 40–80 ng/ml for relaxin A. Considerably higher (10- to 40-fold) concentrations were required for a similar degree of inhibition by either relaxin B or NIH relaxin. As judged by their effectiveness in competing with ^{125}I-tyrosylated relaxin for rabbit anti-relaxin antibody (13), relaxins A and B each possessed 1.5 times the immunoreactivity of a mixture of CM-a and CM-B, which was used as a reference standard; the corresponding figure for relaxin C was 0.37.

3. Characterization. Chemical properties. Table I summarizes the results of amino acid analyses of the three relaxin species, compared with similar data for CM-a and CM-B (4) and with the composition of relaxin based upon sequence data reported by Schwabe and McDonald (5) and by James *et al.* (6). Gel filtration experiments on Bio-Gel P-10 indicated that the molecular weights of all three relaxin species were approximately 5600. Estimates of the molecular weights of relaxins A and B by sedimentation equilibrium, using partial specific volumes calculated from their amino acid compositions, were 7640 and 7360, respectively.

Discussion. The combination of gel filtration through Bio-Gel P-10 and column electrophoresis on Sephadex G-25 provides a convenient and reproducible means of isolating electrophoretically homogeneous relaxins from a porcine relaxin concentrate. Typically, of the material recovered from the electrophoresis column, 40% is relaxin A, 33% is relaxin B, and the rest (27%) is relaxin C, based on absorbance at 280 nm.

Estimates of the molecular weight of relaxin from gel filtration data (~5600) were

TABLE I. Amino Acid Analysis of Relaxin Peptides[a]

	Relaxin						
Amino Acid	A	B (mole/mole Phe)	C	CM-a[b]	CM-B[b]	Schwabe and McDonald[c]	James *et al.*[c]
Alanine	2.5	2.6	3.1	2.7	2.3	2	2
Arginine	5.6	5.0	3.9	5.3	4.5	5	6
Aspartic acid	2.9	2.9	3.0	2.8	2.9	3	3
Half-cystine	5.3	6.0	5.4	4.8[d]	4.9[d]	6	6
Glutamic acid	4.8	4.8	4.7	4.7	4.7	5	5
Glycine	3.8	3.6	3.4	3.6	3.2	3	4
Histidine	0	0	0.4	—	—	0	0
Isoleucine	3.6	3.4	2.8	3.3	3.2	4	4
Leucine	4.1	4.1	3.9	3.9	3.8	4	4
Lysine	3.3	3.1	3.3	3.1	3.2	3	3
Methionine	0.85	0.77	0.74	0.83	0.83	1	1
Phenylalanine	1.00	1.00	1.00	1.00	1.00	1	1
Proline	1.15	0	0.96	—	—	0	0
Serine	3.2	3.4	2.8	2.8[e]	2.8[e]	3	4
Threonine	2.1	2.1	1.7	2.3[e]	1.9[e]	2	2
Tryptophan	1.6	1.4	1.0	—	—	2	2
Tyrosine	0	0	0.2	—	—	0	0
Valine	3.7	3.6	3.2	3.7	3.5	4	4

[a] Phenylalanine was assigned one residue.

[b] From Sherwood and O'Byrne (4).

[c] From sequence data.

[d] These samples were not oxidized.

[e] Uncorrected for destruction during hydrolysis.

in good agreement with the calculated value for the 48-residue peptide described by Schwabe and McDonald (5516) and not far from that of the 51-residue peptide described by James *et al.* (5816). Sedimentation equilibrium weights of both relaxins A and B were significantly larger, suggesting that relaxin, like insulin, undergoes association in aqueous solution near pH 7.[3]

The discovery that relaxins A and C each contain one proline residue was unexpected, since this amino acid has not previously been found as a constituent of any porcine relaxin species. However, Sherwood (14) found one residue of proline (as well as histidine and tyrosine) in each of two relaxins isolated from the ovaries of pregnant rats, and shark ovary relaxin has been reported to contain two residues of proline per molecule (15).

Table I indicates that the amino acid compositions of relaxins A and B are similar, except for the presence of proline in the former; further, there appear to be no striking differences in composition between relaxin B and either CM-a or CM-B. The differences in isoelectric points and electrophoretic mobilities of relaxins A and B are most reasonably attributed to differences in the extent of amidination of aspartic acid or glutamic acid residues. We have compared the electrophoretic properties of the several relaxins (CM-a, CM-a′, CM-B, A, B, and C) in ammonium acetate buffer at pH 5.0. As indicated in Fig. 5, CM-a migrates in this system as a single band with mobility indistinguishable from that of relaxin A. CM-a′ consists predominantly of material of mobility very slightly greater than that of relaxin A, plus a small amount of material which migrates like relaxin B, while CM-B consists almost entirely of relaxin B-like material.[4] It should be noted

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[3] The differences between our figures for sedimentation equilibrium molecular weights and those reported by Sherwood and O'Byrne (4) may be due to differences in the conditions (pH and ionic strength) at which the experiments were carried out.

[4] We have confirmed the observation by Sherwood and O'Byrne (4) that the gel electrophoresis system described by Reisfield *et al.* (16) does not separate CM-a, CM-a′, and CM-B. Likewise, relaxins A and B do not separate in this system, while relaxin C migrates somewhat less rapidly toward the cathode.

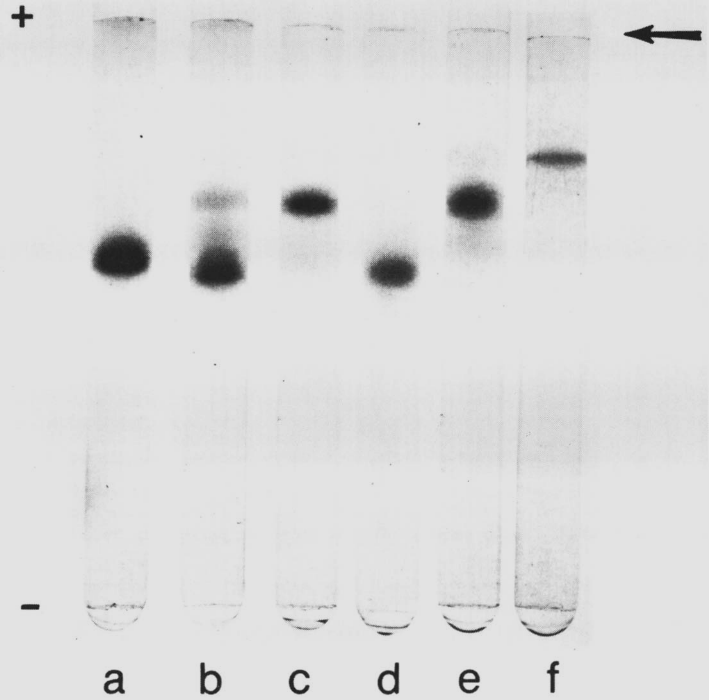

FIG. 5. Comparison of mobilities of relaxin preparations. (a)–(c) Preparations CM-a, CM-a′, and CM-B, respectively; (d)–(f) relaxins A, B, and C, respectively. Gel pH was 5.0, with the cathode at the bottom. Arrow indicates the point of application of the sample.

that although the isoelectric points of relaxin A and CM-a are nearly the same (pH 10.7 and 10.72) that of relaxin B is significantly lower than that reported for CM-B (9.7 vs 10.55).

Like relaxin A, relaxin C contains one proline residue; compared to relaxins A and B its arginine, hydroxy amino acid (serine and threonine) and branched chain amino acid (isoleucine and valine) contents are reduced. Finally, relaxin C appears to contain only a single tryptophan residue. These facts suggest that relaxin C represents a form of relaxin truncated at the C-terminus of the B chain. Schwabe and Braddon (17) have shown that the oxidation of one of the two tryptophans in CM cellulose-purified relaxin (probably that at the C-terminal end of the B-chain) does not reduce its activity in the mouse pubic ligament assay. Whether the traces of histidine and tyrosine found in relaxin C are due to an electrophoretically undetectable impurity or are indicators of possible microheterogeneity must await further characterization.

Relaxins A and B had activities in the guinea pig (2195, 1615 GPU/mg) which appear to be somewhat lower than the value reported by Sherwood and O'Byrne (4) for CM-a, although the significance of the difference is difficult to assess. Relaxin C was clearly less active in guinea pigs than its congeners. In the rat uterine inhibition (RUI) assay, however, relaxin C was at least as active as relaxin A, and considerably more active than either relaxin B or the original relaxin concentrate.

Oliver *et al.* (18) have reported the separation of three fractions with relaxin activity by polyacrylamide gel electrophoresis of acid acetone extracts of ovaries removed from sows at various stages of gestation. Two of these (C-3 and C-2) appear to be similar to relaxins A and B (and CM-a and CM-B) as judged by their electrophoretic mobilities at pH 8.65, although neither contained proline. The third relaxin species identified by Oliver *et al.* differed in several respects from relaxin C: (a) proline was absent; (b) its RUI activity was lowest of the three, and (c) it migrated toward the cathode on polyacrylamide gels at pH 8.65. In contrast, relaxin C either remains at the top of the gel or migrates toward the anode at pH 8.2.

In 1967 Griss *et al.* (3) reported the isolation of a uterine relaxing factor from ovaries of pregnant sows. The product was characterized as a low molecular weight (5,000–10,000) basic protein which, in addition to its myometrial-inhibiting activity, was also very active (~1000 GPU/mg) in the guinea pig pubic symphysis assay. Chamley (15) found that relaxins CM-a, CM-a′, and CM-B all possessed substantial motility-inhibiting activity when tested on the rat uterus *in vitro*. The fact that relaxin isolated by three different procedures is capable of inhibiting rat uterine motility clearly implies that this phenomenon is an intrinsic property of the porcine hormone. The data available also indicate that a number of different (chemically related) peptides share these activities. In this context, we suggest that in view of its relatively low symphyseal relaxation activity and significant motility-suppressing activity, the structure of relaxin C may be especially important in establishing structure-function relationships among this group of ovarian peptides.

Summary. A combination of exclusion chromatography on Bio-Gel P-10 and electrophoresis on a column of Sephadex G-25 at pH 9 has been used to isolate three proteins with relaxin activity from a porcine relaxin concentrate (NIH relaxin). The three proteins had similar molecular weights (~5600) but different mobilities on polyacrylamide gels at pH 5.0 and 8.2. Two of them contained one residue of proline per mole. Bioassays indicated that one of the proline-containing relaxins was significantly less active in the guinea pig palpation assay than the other two, while retaining high activity in the rat uterine motility inhibition assay.

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