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Evidence for a "Pro-relaxin" in Porcine Relaxin Concentrates (39681)

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Porcine relaxin has been characterized as a small (5500–6000 dalton) basic protein devoid of histidine, proline and tyrosine (1–3). Three different compounds with relaxin activity have been isolated from relaxin concentrates, using either ion-exchange chromatography (1) or column electrophoresis (4); these have similar biological activities (2000–2500 GPU [guinea pig units (5)] per mg) but differ in electrophoretic mobility and amino acid composition. Relaxin, like insulin, consists of two peptide chains linked by disulfide bonds. An amino acid sequence for the shorter (22 residue) chain has recently been proposed by Schwabe *et al.* (3).

In this paper, we present evidence for the existence of a larger molecule which may be converted to relaxin by exposure to trypsin. This substance, referred to here as 'prorelaxin', appears to possess intrinsic relaxin activity.

Materials and Methods. Relaxin, in the form of an acid-acetone extract of ovaries of pregnant sows, was obtained from the National Institutes of Health. This material (NIH relaxin) contained 440 Warner-Lambert units/mg[1]. Bio Gel P-10 was obtained from Bio-Rad Laboratories; it was equilibrated with NH_4OAc buffer, 0.10 *M*, pH 5.0, and poured to form a column 1.84 × 140 cm. Relaxin samples were dissolved in water (25–100 mg/ml) and applied to the column; 3–3.5 ml fractions were collected at the rate of 17–18 ml/hr. Cellex D (DEAE Cellulose), also obtained from Bio-Rad, was equilibrated with 0.005 *M* NH_4OAc, pH 7.0 and used in the form of a 1.0 × 57 cm column. Samples were applied in the same buffer; after emergence of the first peak, a linear gradient (0.005 → 0.50 *M* NH_4OAc, pH 7.0) was applied.

The sample to be digested was dissolved in 0.1 *M* NH_4HCO_3 to a concentration of 25 mg/ml. Diphenyl carbamyl trypsin (DCC-trypsin, Sigma), also dissolved in 0.1 *M* NH_4HCO_3 was added in the proportion of 1 μg trypsin/mg substrate. A blank with the same amount of substrate, but containing no enzyme, was also prepared. After incubation at 37° for 30–60 min, the solutions were cooled to 0° and adjusted to pH 5.0 to terminate the action of the enzyme. They were stored frozen.

Disc gel electrophoresis was performed in 10% polyacrylamide gels using the technique described by Davis (6). The buffer [Tris chloride, pH 8.3] concentration in the electrode vessels was 0.05 *M* that in the gel was 0.025 *M*. After electrophoresis for 3 hr (5 ma/gel) the gels were fixed by immersion in 10% trichloroacetic acid for one-half hour, then stained overnight in 0.2% buffalo black. The gels were destained by immersion in MeOH:AcOH:H_2O (50:10:40).

Relaxin was assayed in guinea pigs primed with 5 μg estradiol benzoate (5). Each sample was tested at three or more twofold dilutions, using 9–10 animals for each concentration.

For molecular weight estimations, a P-10 column was calibrated using relaxin, cytochrome *c*, ribonuclease, ovalbumin, and bovine serum albumin as reference proteins.

Results. When NIH relaxin was chromatographed on Bio-Gel P-10, the protein emerged in two peaks, with V_e/V_t = 0.35 and 0.62, respectively (Fig. 1, upper panel). Of approximately 120,000 GPU of relaxin applied to the column, 6000 GPU (5%) appeared with the first peak (pool I, tubes 11–36), and 85,000 GPU (71%) was found in pool III (tubes 43–78). When material recovered from pool I was rechromatographed on the same column (Fig. 1, lower panel) all of the protein emerged in a single peak, with V_e/V_t = 0.35; this contained 80–90% of the activity (10,000 GPU) applied in this experiment, while only a small amount (~10%) was recovered from tubes

[1] The Warner-Lambert unit is approximately equal to the "guinea pig unit" (5).

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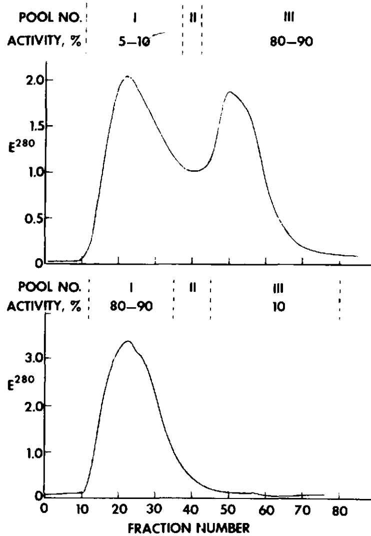

FIG. 1. Upper panel: Chromatogram of NIH relaxin (282 mg) on a 1.84 × 140 cm column of Bio-gel P-10. The buffer was 0.10 M NH_4OAc, pH 5.0. After collection of the first 62 ml of effluent (fraction 1) 3.5 ml fractions were collected. Lower panel: Rechromatography of 200 mg of pool I material, recovered from several experiments, on the same column. Fractions were pooled as indicated and assayed for relaxin activity.

45–80. These experiments suggested that the original preparation contained a substance with intrinsic relaxin activity which has a molecular weight significantly greater than relaxin itself.

Relaxin which has been purified by gel filtration displays a characteristic pattern when subjected to electrophoresis in polyacrylamide gels: two closely-spaced bands which migrate to the cathode at pH 9.0 or lower (Fig. 2, c). These have been identified (2) as proteins with nearly identical biological activities. At a comparable concentration (200 μg), the higher molecular weight material from pool I displayed no evidence for cationic components (Fig. 2, b); however, there appeared to be some diffusely staining material which migrated to the anode at pH 8.3. When this material was exposed to DCC-trypsin for 30–60 min two new components, corresponding in position and mobility to those found in gel-purified relaxin, appeared (Fig. 3, b and c). Furthermore, the relaxin activity of pool I, initially 65 ± 8 GPU/mg, was increased after digestion to 115 ± 20 GPU/mg[2].

Additional evidence for the ability of trypsin to convert a higher molecular weight compound to relaxin was obtained from a comparison of the chromatographic behavior of the presumptive precursor with that of its trypsin-treated product. When 50 mg of untreated material recovered from the first peak of a Bio-gel chromatogram of NIH relaxin was rechromatographed on Bio-gel, only a low shoulder could be observed in the region (tubes 45–80) in which relaxin normally appears (Fig. 4, left). This fraction contained about 8% of the total protein (as estimated spectrophotometrically) and its relaxin content was less than 1000 GPU. Its electrophoretic pattern showed only faint bands corresponding to those appearing in relaxin concentrates. When a second 50 mg sample was treated with tryspin for 30 min, chromatography on Bio-gel revealed a distinct relaxin peak (Fig. 4, right). The protein content in this region was increased to 17% and its relaxin content was also increased, to 8000 GPU. The cationic components which characterize electropherograms of purified relaxin were also much more prominent.

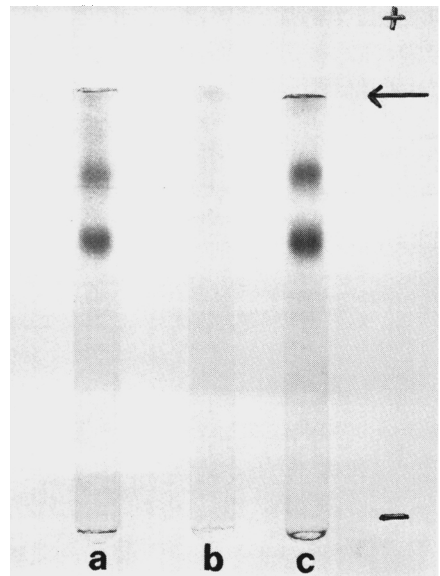

FIG. 2. Polyacrylamide gel electrophoresis of NIH relaxin and fractions recovered from chromatogram on Bio-gel P-10. a: NIH relaxin, b: pool I, c: pool III, Tris chloride buffer, 0.05 M, pH 8.2, cathode at the bottom. The arrow marks the point of sample application. Two hundred microgram samples were applied.

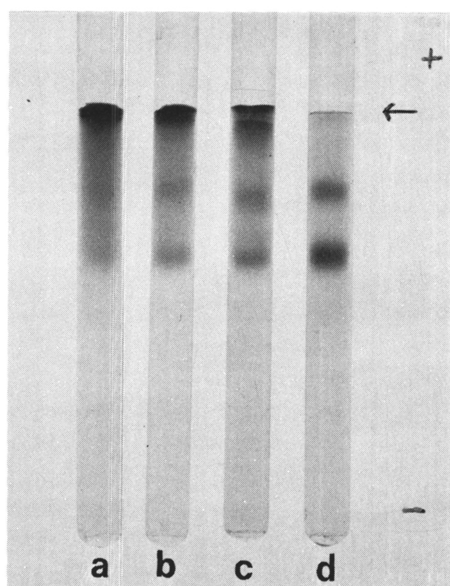

FIG. 3. Polyacrylamide gel electrophoresis of pool I material before and after digestion with DCC-trypsin (1 μg/g). (a) 1 mg pool I, untreated; (b) 1 mg pool I after 30 min trypsin, 37°; (c) 1 mg pool I after 60 min trypsin; (d) 200 μg NIH relaxin.

Another method of separating relaxin from its presumptive precursor is by ion-exchange chromatography on Cellex D (Fig. 5). The first peak (I), which emerged with the solvent front, could be resolved into three cationic species, with electrophoretic mobilities corresponding to three active relaxin species (Fig. 5, a). When a gradient of increasing ionic strength was applied, two additional peaks appeared. Neither of these contained significant amounts of material migrating toward the cathode at pH 8.3 (Fig. 5, b and d). After treatment of material recovered from peak II with DCC-trypsin for 30 min however, the electrophoretic pattern was characteristically relaxin-like (Fig. 5, c). On the other hand, no such transformation occurred when peak III was exposed to trypsin (Fig. 5, e).

To rule out the possibility that noncovalent binding of relaxin to a larger molecule accounts for the experimental results described above, a sample of the material obtained from the first peak of a Bio-Gel P-10 fractionation of NIH relaxin was dissolved in 8 *M* urea (in 0.1 *M* NH_4OAc buffer, pH 5.0) and allowed to remain at room temperature for 24 hr. This procedure resulted in no change in its electrophoretic behavior. Likewise, when pool I material was chromatographed on Bio-gel P-10 which had been equilibrated with 8 *M* urea in 0.1 *M* NH_4OAc, the profile of the chromatogram was indistinguishable from that observed in the absence of urea.

Discussion. The epxeriments summarized above indicate that porcine relaxin concentrates contain a constituent that can be converted to relaxin by digestion with DCC-trypsin. On the basis of its chromatographic behavior on Bio-gel P-10, the apparent molecular weight of the precursor is about 42,000.

Approximately 5–10% of the total activity of NIH relaxin is contained in the "prorelaxin" fraction recovered from Bio-gel; whether this represents an intrinsic property of the precursor or is due to its conversion to relaxin *in vivo* we do not know. Although some separation of "prorelaxin" from other, inactive components can apparently be achieved by ion-exchange chromatography on cellex D, we have so far been unable to resolve the high molecular weight fraction (Bio-Gel pool I) into discrete components by electrophoresis or other means. The relatively low yield of relaxin obtained after 30-min exposure to trypsin (equivalent to 3–4 mg from 50 mg pool I material) may be due to either insufficient exposure time, or the fact that NIH relaxin has been depleted in its pro-hormone content by the preliminary purification procedure.

Since the discovery of proinsulin by Steiner and Oyer in 1967 (7), high molecular weight biologically active or immunoreactive forms of several peptide or protein hormones have been identified in the plasma or endocrine tissues of several species of animals. These include adrenocorticotropin (ACTH) (8), gastrin (9), glucagon (10), growth hormone (11) and parathyroid hormone (PTH) (12). "Large" growth hormone appears to consist of growth hormone noncovalently bound to a larger molecule, since it can be converted to growth hormone by exposure to guanidine or by storage at −20°, while trypsin has no effect. The macromolecular forms of adrenocorticotropin ("big" ACTH), gastrin, and glucagon are

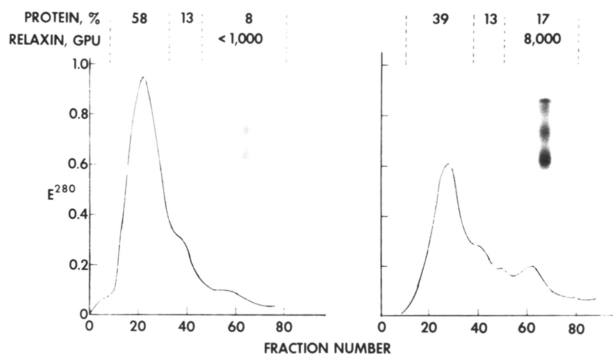

FIG. 4. Left panel: Chromatogram of 50 mg of untreated peak 1 material on Bio-gel P-10, 1.83 × 140 cm. Right panel: Chromatogram of 50 mg of peak 1 material, after treatment with 50 μg. DCC-trypsin for 30 min at 37°, on the same column. In both experiments 3.6 ml fractions were collected after the first 85 ml of effluent had passed through the column. The effluent was pooled as indicated. The superimposed electropherograms are those of 1.1 mg samples (estimated from E^{280} data) of corresponding pools from the two chromatograms.

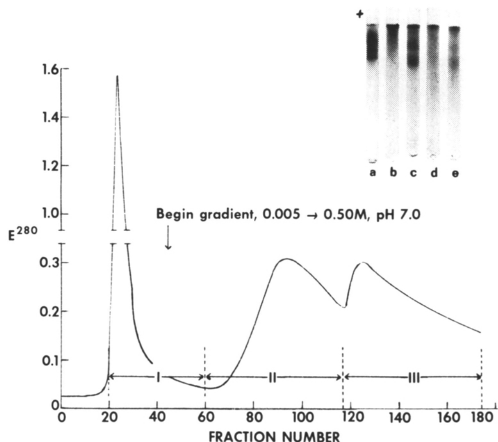

FIG. 5. Chromatogram of 50 mg NIH relaxin on Cellex D (1.0 × 57 cm). The sample was applied in 0.005 *M* NH_4OAc, pH 7.0, followed by a gradient to 0.5 *M*. In the upper right-hand corner are shown electropherograms of material recovered from the chromatogram. (a, b, d): pools I, II, III (untreated), respectively; (c, e) pools II and III, respectively, after 30′ digestion with DCC-trypsin.

converted to the native hormones by digestion with trypsin, while the precursor form of parathyroid hormone is converted to PTH either *in situ* or *in vitro*, by trypsin. At present, the relation of relaxin to its presumed precursor seems to be similar to the case of 'big' ACTH, where the ACTH precursor has been reported to have a molecular weight between that of growth hormone and bovine serum albumin.

Summary. From 5 to 10% of the biological activity of a porcine relaxin concentrate is contained in a fraction with a molecular weight which is considerably higher (~42,000 daltons) than that of relaxin itself. Exposure of this material to a low concentration of trypsin results in the appearance of native relaxin, as judged by an increase in biological activity and changes in its chromatographic and electrophoretic properties.

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