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Chromatographic Separation of Multiple Renin Substrates in Women: Effect of Pregnancy and Oral Contraceptives (39957)

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We recently reported that electrophoretic separation shows the presence of multiple renin substrates in human plasma and that both pregnancy and oral contraceptives alter the quantity of the minor components (1). However, a recent report by Skinner *et al.* (2), referring to separation of substrate in plasma of nonpregnant and pregnant women by means of chromatography on columns of DEAE-Sephadex states that "substrate in plasma from both pregnant and non-pregnant subjects behaved identically." In view of this discrepancy, we decided to check the renin substrate pattern in pregnant and nonpregnant women as well as in women taking oral contraceptives, using Sephadex G-200 as the separatory medium. We also hoped to obtain an estimate of the molecular weights of the renin substrates by this procedure.

Materials and methods. A column of Sephadex G-200, 2.6 × 37.0 cm, was used for gel chromatography. A 1-ml portion of plasma was put on the column and then eluted with 0.067 *M* phosphate buffer, pH 7.9, at a flow rate of approximately 20 ml/hr. Fractions of 2.5 ml were collected. The relative concentration of protein in each fraction was estimated by measuring uv absorbance at 280 nm. Renin substrate was determined by conversion to angiotensin I by prolonged incubation with human renin and measurement of the angiotensin by radioimmunoassay. A 100-μl aliquot of each fraction was added to 600 μl of a suitable incubation mixture. One hundred milliliters of this mixture was made up by combining 0.4 ml of human renin (1 Goldblatt unit/ml), 59.6 ml of sterile saline (0.9 g of NaCl/100 ml), 20 ml of EDTA (disodium salt, neutralized 150 mg/ml), and 20 ml of BAL (a suspension of 0.12 ml of BAL in 20 ml of H_2O). This mixture plus the added aliquot was incubated for 24 hr at 37°, after which a 600-μl aliquot was transferred to a tube containing 1 ml of a solution containing the necessary components for radioimmunoassay of angiotensin I. Slightly more than 100 ml of this radioimmunoassay mixture was made up by combining 100 ml of Tris acetate buffer, 0.1 *M*, pH 7.4, containing 1 mg of lysozyme/ml, 2.5 ml of antiangiotensin I (to make a final serum concentration of 1:40,000), and sufficient ^{125}I-labeled angiotensin I to give 7000 cpm/ml of mixture. The immunoassay mixture plus the added aliquot was kept at 2-5° in a refrigerator for 18 to 24 hr. The radioimmunoassay was completed by adding 1 ml of a suspension of dextran-coated charcoal to each tube, waiting 5 min, centrifuging, and then decanting the supernatant fluid from the precipitated charcoal. Both charcoal and supernatant portions were counted in a gamma scintillation counter, the ratio of bound to free counts was calculated, and the quantity of angiotensin I was estimated from a standard curve of known amounts of unlabeled angiotensin I vs B/F run simultaneously with the unknown samples.

In order to estimate molecular weights, proteins of known molecular weight were run on the same Sephadex column. These were (a) apoferritin (mol wt 460,000), (b) phosphorylase A (mol wt 370,000, (c) aldolase (mol wt 158,000), (d) ovalbumin (mol wt 45,000), and (e) chymotrypsinogen A (mol wt 25,000). Blue dextran 2000 (mol wt 2,000,000) was used to determine the void volume and the partition coefficient, *Kav* (3), for each protein standard was then plotted against the corresponding molecular weight on semilogarithmic graph paper. Points on this curve which corresponded to the *Kav* values for the various substrate peaks were taken to indicate the molecular weight of the renin substrate.

The renin used was prepared from human kidneys, following the procedure of Haas *et al.* (4; steps 1-5 of procedure A). The

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antiangiotensin I used was prepared by immunizing rabbits with angiotensin I coupled to thyroglobulin, following Latta's modification of the method of Skowsky and Fisher (5, 6). Its titer was such that a dilution of the original serum of 1:32,000 would give a B/F ratio of 1.0. The ^{125}I-labeled angiotensin I was purchased from New England Nuclear Co. and the unlabeled angiotensin I from Beckman Instruments, Inc., Bioproducts Department. Blue dextran 2000 and proteins of known molecular weight were purchased from Pharmacia and from Calbiochem.

Blood from nonpregnant women, from pregnant women at term, and also from women taking estrogen containing oral contraceptive medication was kindly provided by Dr. Edward Clark. In addition, some blood samples from nonpregnant women not taking oral contraceptives were obtained from women employed at the Veterans Administration Hospital. All blood was anticoagulated with EDTA and was centrifuged two times in a refrigerated centrifuge to yield cell-free plasma. The plasma was stored frozen in glass tubes at −20° until use.

Results. A total of 20 plasmas from different individuals was run: 6 pregnant females, 6 nonpregnant females, 6 females taking oral contraceptives, and 2 male subjects. In nonpregnant females the renin substrate appeared in the eluate as two peaks of a very dissimilar size: a major peak with an apparent molecular weight of 65,000 daltons and a minor peak which eluted just after the void volume of our column. We estimate its molecular weight as between 450,000 and 500,000 daltons. The amount of renin substrate in the smaller of the two peaks is 3 to 5% of the amount in the larger peak, as estimated from the sum of the amounts of angiotensin in each point. All 6 normal females showed a similar pattern. An example is shown in Fig. 1. To test whether the peaks observed were, in fact, renin substrate, a few control experiments were run in which no renin was added to the incubation mixture. The result, as anticipated, was that no peaks of renin substrate were seen.

In pregnant females there were either

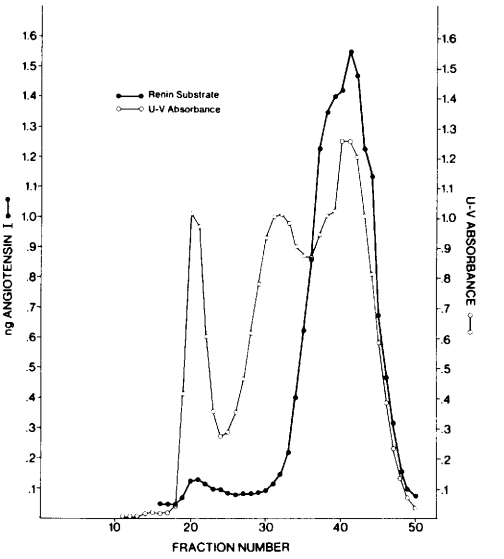

FIG. 1. Renin substrate in plasma of a normal nonpregnant female. A very small peak of high molecular weight substrate is present in addition to the major peak.

two very large peaks of renin substrate or two large ones and a medium or small one of higher molecular weight. Examples are shown in Figs. 2 and 3. The largest peak was the usual one, about 65,000 daltons, but present in much greater quantity than in nonpregnant plasma. The second very large peak has an approximate molecular weight of 350,000 daltons, and its quantity is sometimes greater than the total amount of renin substrate in normal nonpregnant plasma. The other smaller peak corresponds to the high molecular weight peak in normal plasma (mol wt 450,000–500,000). In some pregnant plasma the middle peak is so large that it overlaps any small peak of higher molecular weight and it is only possible to verify the presence of two renin substrates.

In women taking oral contraceptives, as in normal nonpregnant, non-estrogen-treated women, there are two peaks of renin substrate, the major one (mol wt 65,000) and the minor one (mol wt 450,000–500,000). However, the quantities of both peaks are increased and this effect is most obvious for the minor peak, which, in women not taking oral contraceptives, is quite small. There may be, in addition, a very small peak which apparently corre-

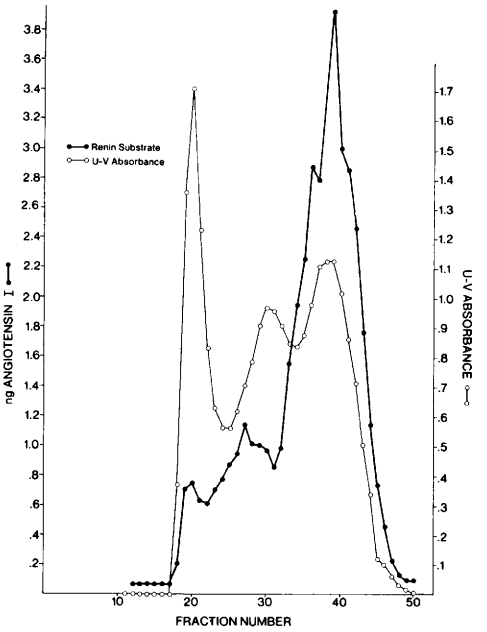

FIG. 2. Renin substrate in plasma of a pregnant woman at term. An intermediate peak of renin substrate (mol wt 350,000) is present in addition to the small and large peaks. Note change in ordinate scale for angiotensin.

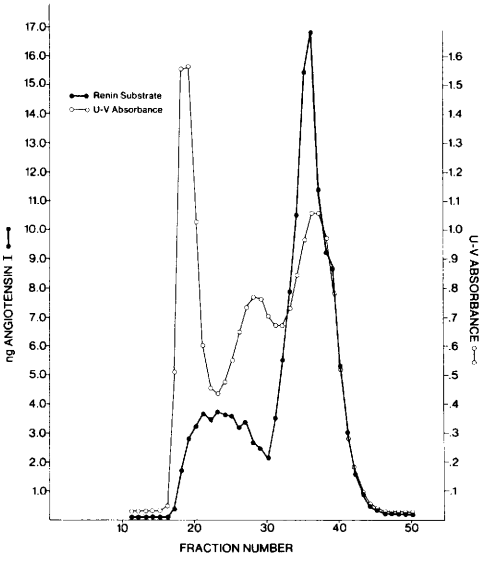

FIG. 3. Renin substrate in plasma of a pregnant woman at term. Intermediate peak is large and may overlap the high molecular weight renin substrate. Note condensed ordinate scale (10× that in Fig. 1).

sponds to the middle peak (mol wt 350,000) in pregnant women. An example is shown in Fig. 4. In the two male plasmas we ran, the

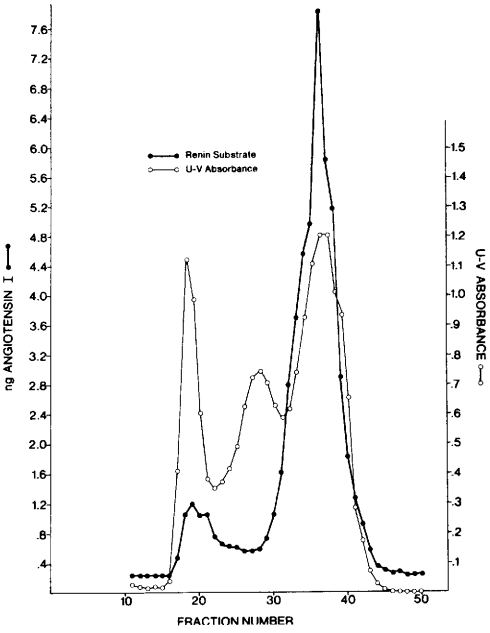

FIG. 4. Renin substrate in plasma of a woman taking oral contraceptive medication. Both peaks of renin substrate are increased.

pattern of renin substrate was apparently not different from that of the normal nonpregnant female.

Discussion. There are multiple renin substrates in human plasma and these are revealed by Sephadex gel chromatography as well as by gel electrophoresis, the technique we previously reported (1). In normal plasma with gel chromatography, only two substrates are seen and the discrepancy in quantity between the major and minor substrate peak is so large that the small one may have been overlooked by previous investigators. However, in pregnancy and in women taking oral contraceptives, the minor substrates, as well as the major one, are strikingly increased. In pregnancy at term, it is the intermediate molecular weight substrate which shows the most obvious change, whereas with oral contraceptives this peak is negligible or absent but the high molecular weight substrate is increased above its normal quantity.

A comparison of the separation of renin substrates by gel chromatography with the separation achieved by polyacrylamide gel electrophoresis indicates that the former gives somewhat less resolution of compo-

nents. The peak designated as E on electrophoresis, i.e., the major substrate component, corresponds with the 65,000 molecular weight peak obtained by gel chromatography. Probably, peaks designated A and B on electrophoresis correspond to the high molecular weight peak found with chromatography, and C and D with the intermediate molecular weight peak.

Eggena *et al.* (7), at a recent meeting, reported that renin substrate in the plasma of pregnant women showed different reaction kinetics with renin than renin substrate in the plasma of nonpregnant women, strongly suggesting the presence of a different substrate.

Margolis and Kenrick (8) reported the appearance of a "pregnancy protein" in sera of pregnant women and of women taking oral contraceptive medication. By gradient gel electrophoresis, they estimated its molecular weight to be about 450,000. It is possible that this pregnancy protein and the high molecular weight renin substrate we find in women taking oral contraceptives are the same substance.

Summary. Proteins in blood plasma of women were separated by chromatography on a column of Sephadex G-200, and the amount of renin substrate in each fraction was determined. The molecular weight of the peaks of renin substrate found was estimated by comparison with the elution position of proteins of known molecular weight. Plasma of normal nonpregnant women shows the presence of two renin substrates: a major one with a molecular weight of 65,000, and a minor one, amounting to 3–5% of the quantity of the major peak, with a molecular weight of 450,000 to 500,000. In women taking oral contraceptives, the same two peaks of renin substrate are seen, but the quantity of each is increased significantly. In pregnant women at term, in addition to the other two renin substrates a third renin substrate of intermediate size is present. Its molecular weight is approximately 350,000 daltons. It sometimes overlaps and obscures the peak of high molecular weight renin substrate. The quantities of the intermediate and low molecular weight renin substrates are greatly increased in pregnancy. The resolution of renin substrates in plasma by gel chromatography is somewhat less than that achieved by polyacrylamide gel electrophoresis which, as previously reported (1), shows the presence of five renin substrates. However, both techniques confirm the presence of multiple renin substrates in human plasma.

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