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Renin Substrate in Plasma of Various Mammalian Species: Electrophoresis on Polyacrylamide Gel (39097)

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In 1971, Nasjletti *et al.* (1) reported their studies of the distribution of renin substrate in sera of four different species of mammals following electrophoresis on starch gel. Subsequently, Sen *et al.* (2) and Menard *et al.* (3) reported on the electrophoretic separation of rat renin substrate. Only Menard *et al.* used polyacrylamide gel as a supporting medium. We (4) independently developed a technique using polyacrylamide gel electrophoresis and radioimmunoassay in place of bioassay. This technique provides greater resolution and greater sensitivity. We have applied this technique to the plasma of eight mammalian species including the four originally reported by Nasjletti *et al.* (1). There are striking differences in the mobility of renin substrate of different mammals. Of the species tested, mouse renin substrate shows the greatest mobility, and sheep renin substrate shows the least.

Methods. *General description.* The method we have developed consists of electrophoresis of plasma proteins on polyacrylamide gel, slicing the gel, incubation of each slice separately with renin and protective agents in a small volume of saline, and transfer of an aliquot of the saline to a test tube containing the necessary components for radioimmunoassay of angiotensin I. The radioimmunoassay technique and the determination of bound (B) and free (F) angiotensin I is described below. The B/F ratio is calculated for each gel slice. A significant lowering of this ratio indicates the presence of angiotensin I, which, in turn, indicates the presence of renin substrate. A standard dose–response curve relating B/F values to the quantity of angiotensin I can be used, but we felt that the B/F values themselves provide equivalent information.

Electrophoresis. Electrophoresis was carried out by a modification of the technique outlined by Nerenberg (5). Plasma was mixed with an equal volume of 0.5 *M* sucrose containing 0.5% bromphenol blue as a marker dye. The bromphenol blue travels in two bands during electrophoresis; the fast moving band indicates the electrophoretic "front" and the slower band indicates the position of albumin. Five microliters of the plasma mixture was applied to 5% polyacrylamide gel cylinders of about 110 mm length and 5 mm diameter. A continuous buffer system was used with glycine-tris buffer adjusted to pH 7.9 with HCl as the chamber buffer.

This buffer was made with 2.9 g glycine, 0.6 g Tris (base) and approximately 0.5 ml 1 *N* HCl per 100 ml and was diluted to 1000 ml before use. The gel buffer, pH 8.1, consisted of 2.9 g glycine and 0.6 g Tris (base) per 100 ml. We diluted 2.5 ml of this buffer to 20 ml by combination with the other ingredients making up the polyacrylamide gel. A constant voltage and time (300 V for 30 min) was used. In early experiments, room temperature was used (22–27°), but in later experiments a constant temperature of 25° was maintained.

After electrophoresis, the gels were removed from the glass tubes and sliced manually into 2.5 mm sections with a scalpel, using lines on graph paper as a guide during cutting. Gels were sliced promptly after completing electrophoresis to minimize diffusion and spreading of bands. Matching gels, not sliced, were stained for 1 hr in a mixture of amido black 0.02%, methanol 20%, and acetic acid 10%, and then, they were destained overnight or longer in 7% acetic acid. In a few experiments, in which more precise localization of renin substrate relative to other proteins was desired, an additional gel was marked by inserting metal points at 2.5 mm intervals, and this gel was also stained. This was done to ensure proper matching of the stained gel to the other (sliced) gel, since in the process of staining and destaining of

the gel there occurs some shrinkage and some swelling.

Incubation with renin. Each gel slice was put into a 12 × 75 mm glass or plastic tube and 0.5 cc of a mixture containing renin, either homologous or heterologous, was added to it. For all species other than the dog and sheep, a homologous renin was used. For dog plasma, hog renin was used, and for sheep plasma, human renin was used. The mixture was made up of 10 ml of an aqueous suspension of BAL (0.15 ml BAL diluted to 25 ml with distilled water), 10 ml EDTA, Disodium salt (150 mg/ml), an amount of renin found, empirically, to give a good conversion of renin substrate to angiotensin, and sufficient saline (0.9% NaCl) to make the total volume 50 ml. The BAL and EDTA block angiotensinase activity. The tubes containing the gel slice and the renin mixture were closed with plastic caps and incubated at 37° for 2 hr or longer with occasional shaking. During this time the renin is able to diffuse into the gel and react with any renin substrate in it. The angiotensin I which is formed diffuses readily out of the gel slice into the surrounding fluid. At the end of incubation, the tubes were placed in crushed ice to lower the temperature to 0° and stop the reaction. The inclusion of the gel slice in the incubation mixture, rather than a separate elution step, provides a considerable simplification of procedure.

Radioimmunoassay. Radioimmunoassay was carried out by a modification of the technique described by Haber *et al.* (6). After completion of the incubation with renin, 0.4 ml of the fluid surrounding each gel slice was transferred to a glass tube containing 1.0 ml of the immunoassay mixture. This consists of 0.1 *M* tris-acetate buffer, pH 7.4 containing 1 mg/ml of lysozyme plus antibody to angiotensin I (final concentration of antiserum 1:50,000) and ^{125}I labelled angiotensin I in sufficient quantity to give 6000–8000 cpm per ml of the mixture. The tubes were capped and refrigerated at 0–2° for approximately 16 hr. Then, keeping all components at the same low temperature, 1.0 ml of a stirred suspension of dextran-coated charcoal was pipetted into each tube, and, after waiting 5 min, the tubes were centrifuged at 1,500*g* for 20 min. Subsequently, the supernatant fluid in each tube was carefully decanted into another correspondingly numbered glass tube. The supernatant fluid contains the labelled angiotensin that is bound to antibody, i.e., the bound fraction (B), while the charcoal remaining in the original tube retains the labelled angiotensin that is not bound to antibody, that is, the free fraction (F). The tubes containing the free and bound fractions were counted in a gamma scintillation counter and the quotient of radioactivity of the bound fraction divided by that of the free fraction, the B/F ratio, was calculated for each pair of tubes.

Materials. Radioactive ^{125}I-labeled angiotensin I was purchased from New England Nuclear Co., Billerica, Massachusetts. Unlabeled angiotensin I (asp-1 ileu-5 form) was purchased from Schwarz–Mann Co. The antibody to angiotensin I was kindly provided by Dr. F. Katz of the University of Colorado School of Medicine. Its final concentration in the radioimmunoassay mixture was 1:50,000.

The various animal renins were prepared according to the procedure of Haas *et al.* (7), using steps 1–3 of procedure A, from kidneys collected and frozen in our laboratory.

The human renin used was the standard human renin preparation provided by the National Institute for Medical Research in London, England. The potency of this preparation is given as 0.1 Goldblatt unit per vial by the supplier. In addition, a preparation of human renin of higher potency was obtained from the laboratory of Dr. Harry Goldblatt.

The materials used for preparation of polyacrylamide gels were obtained from Bio-Rad Co., Richmond, California. BAL (2,3 dimercaptopropanol) was purchased as the pure compound (not a suspension in oil) from Sigma Chemical Co. It was made up on the day of its use as a suspension of 0.15 ml of BAL in water (total volume 25 ml). This suspension was kept at room temperature as refrigeration results in a reduced solubility and precipitation of BAL droplets from an aqueous suspension. The reagents used for radioimmunoassay (other than antibody to angiotensin I and radioactive ^{125}I labeled angiotensin I) were obtained from Schwarz–Mann Co.

Results. Renin substrate in rat plasma and

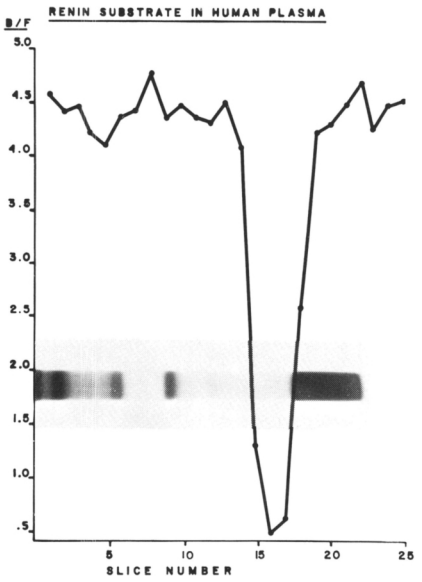

FIG. 1. Polyacrylamide gel electrophoretic pattern of renin substrate (indicated by reduced B/F) in human plasma, superimposed on the pattern of stained plasma proteins. Accurate superimposition of the B/F curve and the protein pattern was ensured by running three gels simultaneously: one stained with amido black, destained and photographed; one sliced into equal 2.5 mm slices and subjected to radioimmunoassay; and one marked by inserting metal pointers at 2.5 mm intervals, and then stained and destained without slicing. The photographic negative of the stained gel was then enlarged and the print aligned so as to match the positions of the origin and of the front of the albumin band with the corresponding slice numbers. Renin substrate is located just behind the albumin band.

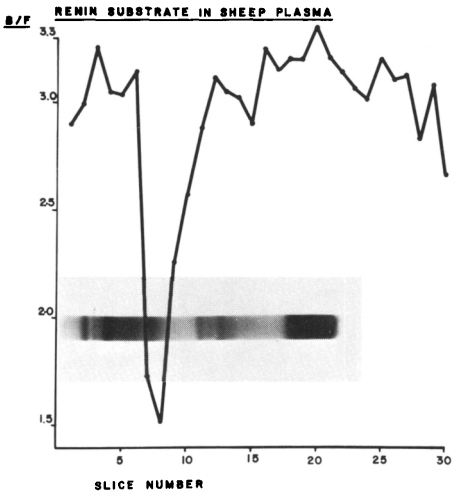

FIG. 2. Renin substrate in sheep plasma appearing far behind albumin and located in the vicinity of the beta-globulins. Details of procedure as in Fig. 1.

in human plasma show a similar mobility, the maximum quantity or "peak" of substrate locating mainly in slice No. 13 (32.5 mm) to No. 16 (40 mm) in different experiments. Rat renin usually peaks at No. 13, human renin at No. 15 or No. 16. Their relationship to the position of albumin is different, but this is because human albumin migrates somewhat faster than rat albumin. Dog renin substrate appears at slices No. 13 through No. 15. It is noteworthy that dog plasma regularly shows two renin substrates: a major component at slice No. 13 and a minor one at slice No. 6. This confirms Nasjletti *et al.*'s finding of dog substrate in two fractions, although they used serum from nephrectomized dogs while our measurements were made on plasma of normal dogs. Human plasma also shows a minor peak at slice No. 4 to No. 7, but this is not consistently observed. In three out of seven experiments with human plasma, we found small peaks of activity that suggested the presence of a second renin substrate in this region. If one grades the eight species tested according to electrophoretic mobility, they separate into two groups; a slow moving group includes sheep, cow, pig, and rabbit, and a faster moving group includes human, dog, rat, and mouse. Renin substrate in the slow moving group is found at slice No. 9 (cow), No. 10 (pig and rabbit), and usually at slice No. 7 in sheep (occasionally, as in Fig. 2, at slice No. 8). The renin substrate of the mouse is unique in exhibiting the highest mobility. The peak of maximum substrate activity shows up at slice No. 18 to No. 20 in different experiments, and mouse albumin occurs in the same region. The relationship of the location of renin substrate to well-recognized proteins stained after electrophoresis is shown in Figs. 1–3. The renin substrates of sheep, cow, pig, and rabbit occur somewhat ahead of the slow-moving globulins; human and dog renin substrate occur somewhat behind their respective albumins, while rat and mouse substrate coincide with their albumins.

Discussion. Whether the different electrophoretic mobilities of renin substrates which we observed have any functional signifi-

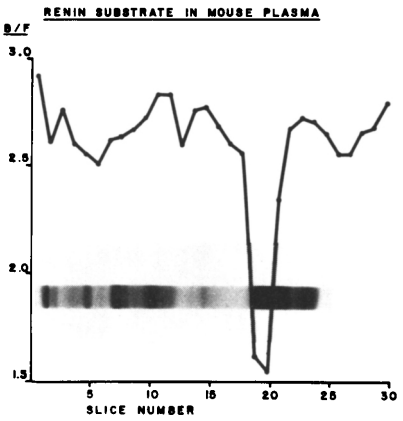

FIG. 3. Renin substrate in mouse plasma occurring in the same region as mouse albumin and farther advanced from the origin (50 mm) than any of the other species tested. A prealbumin band is clearly visible. Details as in Fig. 1.

cance, other than indicating a difference in size of the molecules or net negative charge at an alkaline pH, is unknown. However, we note a possible inverse relationship between electrophoretic mobility and reactivity of the substrate with human renin. Thus sheep substrate, the slowest, reacts very avidly with human renin (8) while mouse substrate, the fastest, does not react at all with human renin. The unique high electrophoretic mobility of mouse renin substrate corresponds to its unique biological specificity. Oliver and Gross (9) have reported that, unlike other mammalian species, the mouse has a renin substrate which reacts with mouse renin but does not react with renins of other mammals. In view of its high mobility, mouse renin substrate may be either a small protein or one with a high net negative charge.

The apparent division of renin substrates of the eight species tested into two groups with different electrophoretic mobilities may reflect a generic or type difference. The slower moving renin substrates of the cow, pig, and rabbit are the only ones fitting the earlier designation of an alpha-2-globulin (10). It is, perhaps, only coincidental that all the herbivores tested fall into the slow-moving group. The faster-moving renin substrates of man and dog should probably be classified as alpha-1-globulins. The renin substrates of the rat and mouse, which occur together with their albumins, cannot be designated as either pre- or postalbumins; perhaps a new term such as coalbumin could be used to describe the position they reach during polyacrylamide gel electrophoresis.

Since our results with polyacrylamide gel electrophoresis showed that human renin substrate migrates closer to albumin than do most alpha-2-globulins, we questioned the designation of human renin substrate as an alpha-2-globulin. Although it is usually stated that renin substrate is an alpha-2-globulin (11), we have not found any reference to support this statement with regard to human renin substrate. Studies with hog renin substrate have shown it to migrate as an alpha-2-globulin (10, 12). Nasjletti *et al.* (1) reported that with starch gel electrophoresis, human renin substrate is located in a fraction close to albumin. To recheck this point, we determined the location of human renin substrate after electrophoretic separation on paper, using veronal buffer pH 8.6, and found that it appears in a position midway between the alpha-1- and alpha-2-globulins.

Summary. The plasma of eight different species was subjected to electrophoresis on polyacrylamide gel, and the position of renin substrate was determined. There are considerable differences in the electrophoretic mobility of the renin substrates tested. Sheep substrate shows the slowest migration and mouse substrate the most rapid. The species tested appear to fall into two groups: slow-moving substrates occurring in the plasmas of sheep, cow, pig, and rabbit and fast moving substrates in man, dog, rat, and mouse. In most species only a single peak of renin substrate appeared, but in man and dog a minor peak was often observed in addition to the prominent major one. The classification of human renin substrate as an alpha-2-globulin is questioned.

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