PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 153, 314-318 (1976)

# Heterogeneity of Renin Substrate in Human Plasma: Effect of Pregnancy and Oral Contraceptives (39536)

D. B. GORDON, I. N. SACHIN, AND V. N. DODD

*Research Laboratory, Veterans Administration Hospital, Livermore, California 94550*

There have been few studies of the electrophoretic mobility of renin substrate in plasma and only two[1] reports of the behavior of renin substrate in human plasma (1, 2). Both of these reports indicate that there may be, in human plasma, a small quantity of a second renin substrate with a lesser mobility than the obvious major component, which migrates to a position just behind albumin. Since our last publication, we have modified our technique, making it more effective in showing the presence of small amounts of renin substrate in plasma. With this modified technique, we have found at least five electrophoretically dissimilar renin substrates to be present in human plasma. In addition, we have found significant variations of the pattern of renin substrates in the blood plasma of pregnant women and of women taking oral contraceptive medication.

*Methods.* Details of our technique are described in our previous publication (2). In brief, the procedure consists of electrophoresis of plasma proteins on a polyacrylamide-gel cylinder, slicing the gel, incubating each slice separately with human renin, EDTA, and BAL in a small volume of saline, and transferring an aliquot of this mixture to a test tube containing the necessary components for radioimmunoassay of angiotensin I. Three modifications of technique have proven to be effective in increasing the "visibility" of small amounts of renin substrate present in blood plasma: (i) increasing the sample size, (ii) prolonging the time of incubation with renin, and (iii) improving the purity of the renin used. We now apply 40 $\mu$l of a 50:50 mixture of plasma and bromphenol blue dye plus sucrose solution (equivalent to 20 $\mu$l of plasma) to the top of each gel prior to electrophoresis, which is eight times as much plasma as we previously used. In order to do this without overloading the gel and without greatly increasing the depth of the layer applied initially to the top of the gel, we use a gel tube twice the diameter, 10-mm i.d. instead of 5-mm i.d., giving four times the cross-sectional area to which the sample is applied. In a few experiments we have used as much as 100 $\mu$l of sample applied to the gel.

In connection with the second and third modifications of technique listed, if one uses a prolonged incubation with renin (for example 24 hr instead of 2 hr), it is necessary to use a renin preparation which is reasonably free of angiotensinase. Otherwise, during the prolonged incubation, destruction of angiotensin keeps pace with its rate of formation. We have used three different human renin preparations in our experiments, two prepared in our own laboratory and one kindly supplied by Drs. E. Haas and H. Goldblatt. All three were made up as a stock solution in saline equivalent to 1 Goldblatt until ml, which was kept frozen. The final concentration was varied but usually was approximately 0.02 GU/ml of incubation mixture. All three renins were prepared by the technique described by Haas *et al.* in 1966 (3) in their procedure A. Our less pure renin was carried through steps 1-3 of this procedure, which still leaves considerable contamination with angiotensinase. Our purer renin and the one from Dr. Goldblatt's laboratory were carried through steps 1-5 of the Haas procedure, which gets rid of most, but not all, of the angiotensinase present in the kidney extract.

*Results.* In normal plasma from male or female subjects, there is a single large peak of renin substrate with a mobility slightly less than that of albumin. There is, in addition, a series of very small peaks of renin substrate with lesser mobilities than the major peak. Usually four small peaks appear

[1] Four, if one counts preliminary abstracts by the same authors.

"before" the major peak and we have labeled these A, B, C, and D and called the major peak E. Their electrophoretic mobilities relative to the front of the albumin band ($R_f$ alb) are A, 0.05–0.10; B, 0.25–0.30; C, 0.40–0.45; D, 0.50–0.55; and E, 0.75–0.80. Figure 1 shows an example of this pattern in a normal male and also shows how the small more slowly moving peaks (especially B) are made to stand out by increasing the sample size and prolonging the time of incubation with renin. Figure 2 shows the same for the pattern of renin substrates in the plasma of a normal female. In this particular example, peaks C and D are absent, but other female plasmas do show one or both of these peaks. Of the four minor peaks, the second (B) is usually present in the largest quantity and is the one previously reported to be present. It is not yet certain from our experiments whether there are consistently demonstrable minor peaks of renin substrate with electrophoretic mobilities greater than that of the major renin substrate peak. In some experiments two or three such rapidly migrating substrates do appear and, if we can confirm their existence, they will be labeled F, G, and H, making a total of eight electrophoretically different renin substrates present in normal human plasma. We have examined the pattern of renin substrates in the plasma of six normal males and eight normal females and found no obvious differences between the individual plasmas.

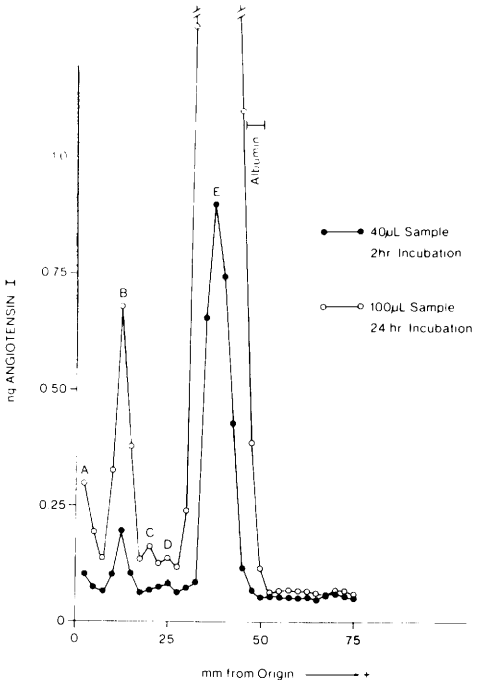


FIG. 1. Pattern of renin substrate after electrophoresis on polyacrylamide gel. Normal male human plasma. The small, slower-moving peaks of renin substrate, especially B, are more prominent when a larger sample and a longer incubation time are used. The position of albumin is indicated by the horizontal bar.

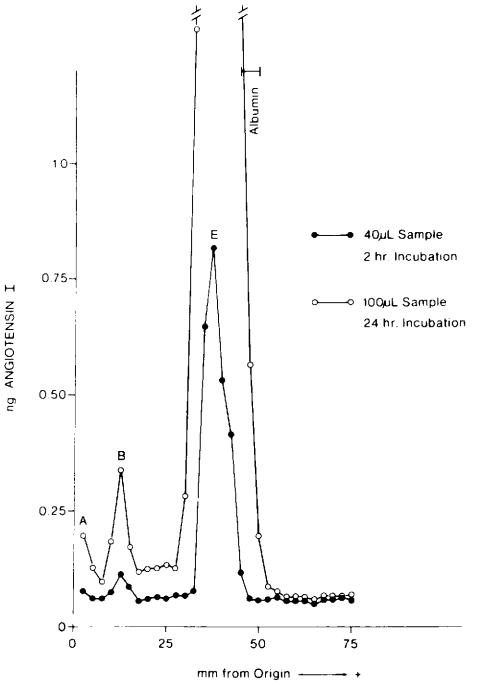


FIG. 2. Pattern of renin substrate after electrophoresis of normal female plasma. Minor peaks show a similar but not identical pattern compared to that of normal male.

*Effect of oral contraceptives.* It has been shown by several investigators (4–10) that the concentration of renin substrate in the blood plasma of women taking oral contraceptive medication (estrogens plus gestagens) is greatly increased. Increases of three to five times normal control values have been reported. We subjected plasma from six women using such medication to the electrophoretic analysis of renin substrate described above and found a distinctly different pattern of renin substrates from that shown by women not taking such medica-

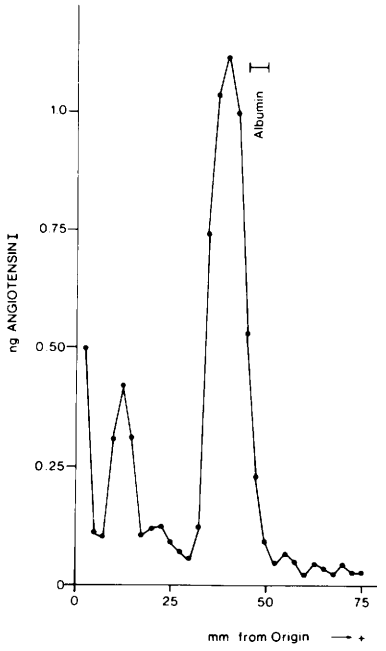


FIG. 3. Pattern of renin substrates in plasma of a woman taking oral contraceptive medication. Note the striking increase of the two slowest-migrating peaks.

tion. An example is shown in Fig. 3. Peaks A and B are especially increased. The major peak (E) may also be increased, but this is not always evident. The pattern of substrates in women taking oral contraceptives is quite characteristic and, although minor variations occur, it can be readily recognized.

*Effect of pregnancy.* The fact that the total amount of renin substrate increases significantly in pregnant women has been well established (5, 11-16). We subjected the plasma of seven pregnant women to electrophoresis and measurement of renin substrate and found a pattern of renin substrates strikingly different from that of nonpregnant women and also, to a lesser degree, different from the pattern of women taking oral contraceptives. Not only are peaks B, C, and D significantly increased but the bases of all the slow-moving peaks run together so that there is not the usual return to baseline between peaks. An example of the pattern of renin substrates in the plasma of a pregnant woman is shown in Fig. 4. In addition, in the same figure, the effect of omitting the renin during the incubation step is shown. The absence of the peaks when renin is omitted indicates that they are, indeed, renin substrates. All but one of the plasmas from pregnant women which we tested were from pregnant women at term or nearly at term. The only plasma from an earlier stage of pregnancy was from a woman 7 months pregnant and in her case the pattern of renin substrates is closer to that seen in women using oral contraceptives. In general, there seems to be a greater variation in the pattern of renin substrates in pregnant women than in the other groups.

*Effect of nephrectomy and of cirrhosis.* In addition to the categories described above, we have examined the renin substrate pattern in two other situations in which renin substrate might be expected to be altered: in patients with cirrhosis of the liver and ascites and in patients subjected to bilateral nephrectomy. In two of four cirrhotic patients, the amount of major substrate peak

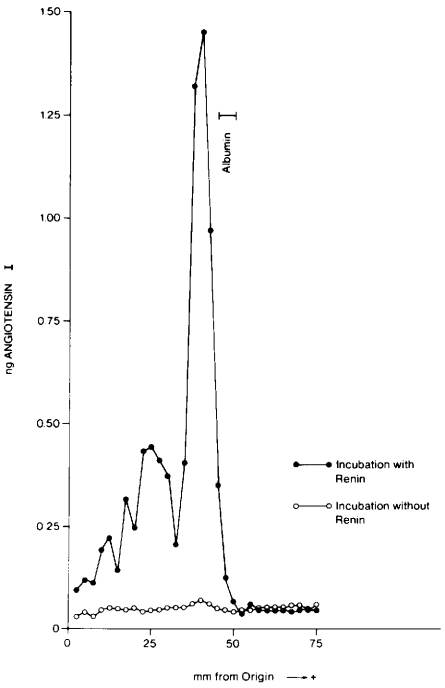


FIG. 4. Pattern of renin substrates in plasma of a pregnant women. All slow-moving peaks are increased, especially the one migrating behind the major renin substrate peak. Open circles show the absence of angiotensin produced from renin substrate when renin is omitted from the incubation.

(E) was greatly diminished and the minor peaks were also reduced. In three bilaterally nephrectomized patients, contrary to our expectation, there was not a significant increase in the major peak and the pattern of minor peaks was not different from normal.

*Control experiments.* In order to confirm the presumption that the peaks of renin substrate activity which we observed were indeed isomeric forms of renin substrate, we felt that it was necessary to show (i) that they did not appear (or were greatly diminished) if the step involving incubation with renin was omitted, (ii) that the product formed by incubation with renin had the characteristic properties of angiotensin, and (iii) that the peaks could be eliminated (i.e., converted to angiotensin) by prolonged incubation with renin prior to electrophoresis. Figure 4 shows that omission of the renin incubation step does prevent the appearance of the renin substrate peaks. The fact that in all experiments we used antibodies against angiotensin I to detect the end product of the renin reaction (by radioimmunoassay) is itself strong evidence that the source of the material we measured is some form of renin substrate. We found that the product formed (a) is absorbed onto charcoal and (b) is not destroyed by 10-min heating to 95–100°; both properties confirm that the active product is not a protein. In addition, we found that we could eliminate the peaks of renin substrate by a 2-hr incubation of the plasma with human renin, thus converting the substrates to angiotensin prior to electrophoresis of the plasma. In sum, the results of all these tests support the view that the peaks of activity we observed are, in fact, variants of renin substrate.

*Discussion.* Our results show that there are five or more electrophoretically distinguishable renin substrates in human plasma. In 1963, Skeggs *et al.* (17) reported the separation of five or more forms of renin substrate from hog plasma by means of DEAE-cellulose chromatography. However, the method used by Skeggs *et al.* involved preliminary chemical treatment including exposure of the plasma to a pH of 2.5, and it has been pointed out that this treatment may have altered the substrate molecule, so that it is uncertain to what extent the results correspond to the situation in unaltered plasma (18). It should be emphasized that in our work the plasma is not exposed to extreme conditions prior to or during electrophoresis on polyacrylamide gels. The buffer we used for electrophoresis is Tris–glycine buffer, pH 7.9.

Whether the heterogeneous nature of renin substrate in human plasma has any biological significance is unknown. It is most interesting that situations such as pregnancy and medication with oral contraceptives result in alterations of the relative amounts of the different renin substrates present. Skinner *et al.* (19) reported that renin substrate in plasma from pregnant and nonpregnant women behaved identically when chromatographed on columns of DEAE-Sephadex. The difference between their results and ours can probably be explained by the different methods used for separation of plasma proteins. If some biochemical differences between the variants of renin substrate can be shown, e.g., differences in rate of reaction with renin, this could be significant, since it might affect the quantity of angiotensin generated by circulating renin *in vivo*. In any case, now that the existence of variants of renin substrate has been shown, future investigations of possible clinical correlation will have to take account of qualitative as well as quantitative variations of this substance.

*Summary.* The electrophoretic mobility of renin substrate in human plasma was determined by electrophoresis of the plasma on a cylinder of polyacrylamide gel, followed by slicing the gel, incubation of each slice with human renin, EDTA, and BAL, and determination of the angiotensin formed by radioimmunoassay. In the plasma of normal males and females, a single large peak of renin substrate was found with an electrophoretic mobility somewhat less than that of albumin. In addition, a series of three or four very small peaks of renin substrate with lesser electrophoretic mobility were also observed, the second peak from the origin being the largest of the minor peaks. The peaks, in order of increasing mobility were labeled A through E, E being the large major peak of renin substrate. Occasionally, but not consistently,

observed were two or three small peaks of renin substrate with electrophoretic mobilities greater than that of the major peak. Thus, there are five and possibly as many as eight electrophoretically distinguishable renin substrates present in normal human plasma.

In women taking oral contraceptives the pattern of renin substrates is different from that of women not taking such medication. Peaks A and B are significantly increased. In pregnant women a different pattern of renin substrates is found; the minor peaks being markedly increased, especially B, C, and D. Plasma of patients with cirrhosis of the liver and of patients subjected to bilateral nephrectomy were also examined. The pattern of renin substrates in these did not differ significantly from normal, except that the quantities of all the variants of renin substrate, including the major peak, were greatly reduced in some of the patients with cirrhosis.

We thank Drs. E. Haas and H. Goldblatt for the gift of renin and Dr. E. Clark for providing us with blood samples from pregnant and nonpregnant women.

1. Nasjletti, A., Lewis, L. A., and Masson, G. M. C., Amer. J. Physiol. **220,** 804 (1971).
2. Gordon, D. B., and Sachin, I. N., Proc. Soc. Exp. Biol. Med. **150,** 645 (1975).
3. Haas, E., Goldblatt, H., Gipson, E. C., and Lewis, L., Circ. Res. **19,** 739 (1966).
4. Laragh, J. H., Sealey, J. E., Ledingham, J. G. G., and Newton, M. A., J. Amer. Med. Ass. **201,** 918 (1967).
5. Helmer, O. M., and Judson, W. E., Amer. J. Obstet. Gynecol. **99,** 9 (1967).
6. Skinner, S. L., Lumbers, E. R., and Symonds, E. M., Clin. Sci. **36,** 67 (1969).
7. Saruta, R., Saade, G. A., and Kaplan, N. M., Arch. Intern. Med. **126,** 621 (1970).
8. Beckerhoff, R., Luetscher, J. A., Wilkinson, R., Gonzales, C., and Nokes, G. W., J. Clin. Endocrinol. Metab. **34,** 1067 (1972).
9. Cain, M. D., Walters, W. A., and Catt, K. J., J. Clin. Endocrinol. Metab. **33,** 671 (1971).
10. Tapia, H. R., Johnson, C. E., and Strong, G. C., Obstet. Gynecol. **41,** 643 (1973).
11. Gould, A. B., Skeggs, L. T., and Kahn, J. R., Lab. Invest. **15,** 1802 (1966).
12. Skinner, S. L., Lumbers, E. R., and Symonds, E. M., Clin. Sci. **42,** 479 (1972).
13. Weir, R. J., Fraser, R., Lever, A. F., Morton, J. J., Brown, J. J., Kraszewski, A., McIlwaine, G. M., Robertson, J. I. S., and Tree, M., Lancet **1,** 291 (1973).
14. Tapia, H. R., Johnson, C. E., and Strong, G. C., Lancet **2,** 847 (1972).
15. Catt, K. J., Baukal, A., and Ashburn, M., *in* "Oral Contraceptives and High Blood Pressure" (M. J. Fregly and M. S. Fregly, eds.), pp. 211–226, Dolphin Press, Gainesville, Fla. (1974).
16. Weinberger, M. H., Petersen, L. P., Herr, M. J., Wade, M. B., and Rosner, D. R. *in* "Oral Contraceptives and High Blood Pressure" (M. J. Fregly and M. S. Fregly, eds. ) pp. 237–246, Dolphin Press, Gainesville, Fla. (1974).
17. Skeggs, L. T., Lentz, K. E., Hochstrasser, H., and Kahn, J. R., J. Exp. Med. **118,** 73 (1963).
18. Lee, M. R. *in* "Renin and Hypertension – A Modern Synthesis," 224 pp. Williams and Wilkins, Baltimore (1969).
19. Skinner, S. L., Dunn, J. R. Mazetti, J., Campbell, D. J., and Fidge, N. H., Aust. J. Exp. Biol. Med. Sci. **53,** 77 (1975).

Received April 19, 1976. P.S.E.B.M. 1976, Vol. 153.