Binding of Angiotensin I and Angiotensin II to Plasma Proteins Precipitated by Ammonium Sulfate (37006)

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Although many hormones are known to bind to plasma proteins (1–3), evidence as to whether angiotensin I or II does or does not bind is still insufficient. Page *et al* (4) found that most of the angiotensin I measurable in human blood plasma by radioimmunoassay was eluted from columns of Sephadex G-25 in the protein containing fraction. They regard this result as due to some sort of artefact. On the other hand, Schweikert, Carey and Liddle (5) reported that a considerable portion of ^{125}I labelled angiotensin I added to plasma and subjected to electrophoresis migrated with an α-2 globulin fraction. They interpret this as clear evidence of binding of angiotensin I to plasma protein and suggest that the "renal pressor substance" of Grollman (6) consists of angiotensin I bound to plasma protein. Houle, Piwonska and Carretero (7) recently reported that angiotensin II shows considerable binding to plasma proteins dialyzed against an external solution of distilled water, but not against Ringer's solution or saline.

We (8) carried out some experiments involving ultrafiltration of plasma which had been acted upon by renin. The results indicated that the pressor product formed was either a protein or was protein bound. We decided to test whether angiotensin I or II added to plasma would be co-precipitated with plasma proteins precipitated by ammonium sulfate. The results clearly show that angiotensin I and II are precipitable along with proteins and that, under the circumstances of our experiments, binding of angiotensin to plasma proteins does occur.

Methods. Assay. In most experiments, angiotensin was measured by bioassay in a ganglion-blocked, saline loaded Long-Evans rat. Unknown solutions were matched against a standard solution of aspartyl-l-angiotensin II after determination of a dose–response curve. In some experiments labeled angiotensin was estimated by measuring the radioactivity of ^{125}I labeled angiotensin I (Schwarz-Mann) or of ^{14}C labeled angiotensin II (Nuclear-Chicago).

Fractional precipitation. Stepwise increments of ammonium sulfate concentration were used to separate plasma proteins into six fractions. Rat plasma was cooled to 0° in crushed ice and angiotensin I or II was added to it. The final concentration of angiotensin I was made 400 ng/ml and of angiotensin II was 200 ng/ml. Additional reagents, such as ethylenediaminetetracetate (EDTA) and 2,3-dimercaptopropanol (BAL) were added when appropriate to the experiment. The ammonium sulfate solution contained 500 g ammonium sulfate/liter and an additional quantity of ammonium bicarbonate sufficient to bring the pH to 7.5. The material precipitated by ammonium sulfate was centrifuged at 15,000*g* for 15 min, at 0° and separated from the supernatant fluid by decanting. To the supernatant fluid was added enough concentrated ammonium sulfate solution to bring it to the next higher concentration. The steps were (a) 150, (b) 200, (c) 250, (d) 300, (e) 350 and (f) 400 g/liter in earlier experiments and (a) 150, (b) 200, (c) 250, (d) 300, (e) 340 and (f) 380 g/liter in later experiments. In order to "wash" each precipitate, it was redissolved in water (made alkaline by the addition of ammonium bicarbonate) and reprecipitated by ammonium sulfate and centrifuged and decanted as before. The final precipitate was drained of excess fluid and then kept frozen at —20° un-

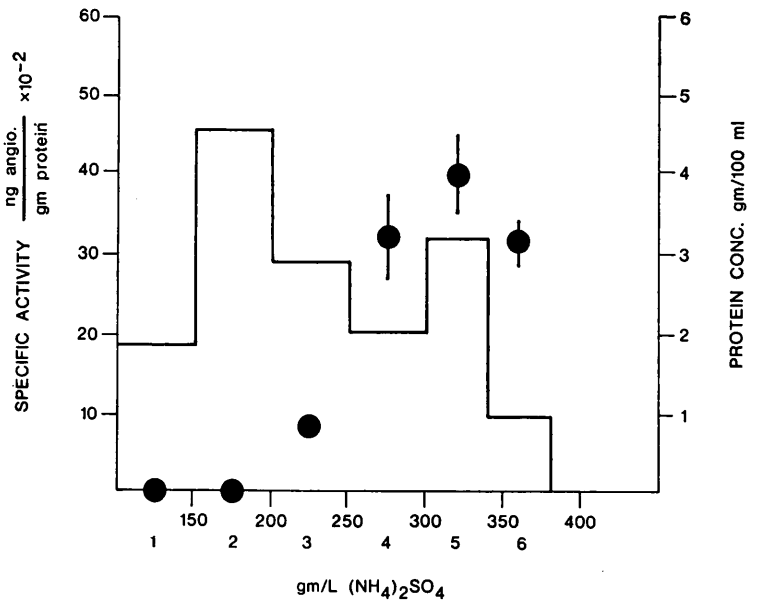

FIG. 1. Binding of angiotensin I to certain proteins in rat plasma precipitated by ammonium sulfate. (●) Specific activity of angiotensin ± SE; (—) protein concentration. Mean of 6 experiments. Fractions numbered 1 to 6 correspond to increasing concentrations of ammonium sulfate.

til testing. Finally it was dissolved in 1 ml of (alkaline) water and an aliquot of 0.01 to 0.1 ml was tested for pressor activity by intravenous injection in a bioassay rat.

Protein concentration. The amount of protein in each redissolved precipitate was measured by diluting it 1:100 in distilled water and measuring its uv absorbance at 280 nm. In 9 experiments the amount of protein was also measured by the biuret method and a conversion factor was calculated for each protein fraction. Subsequently, these factors were used to convert uv absorbance readings to protein concentration. The specific activity of angiotensin in each protein fraction was then calculated as ng angiotensin/ml ÷ g protein/ml = ng angiotensin/g protein.

Electrophoresis. The plasma protein fractions were subjected to electrophoresis on polyacrylamide gel columns using a modification of the original method of Davis (9). No stacking gel was used. After electrophoresis, the gel was stained with amido black, destained in acetic acid and examined visually.

Miscellaneous procedures. A comparison was made between the binding properties of rat plasma alone and rat plasma to which EDTA (1.5 mg/ml) or EDTA plus BAL had been added. In a few experiments human blood plasma was used instead of rat plasma. In other experiments solutions of purified human albumin (electrophoretically pure 100%) and of purified rat albumin (Cohn fraction V > 95% pure) were used. In each case the results obtained with angiotensin I were compared with those obtained with angiotensin II.

Results. Both angiotensin I and angiotensin II were found to coprecipitate with proteins of rat plasma precipitated by ammonium sulfate. They do not precipitate with the earlier fractions, but only with the fractions precipitated by concentrations of ammonium sulfate greater than 200 g/liter and in significant amounts only with concentrations of 300 g/liter and higher. (The earliest fractions of protein precipitate out between 100 and 150 g/liter). The fractional precipitation pattern of angiotensin I (av of 6 experiments) is shown in Fig. 1. The same for angiotensin II is shown in Fig. 2. It can be seen that the patterns differ, the amount of angiotensin II relative to the total amount of protein, *i.e.*,

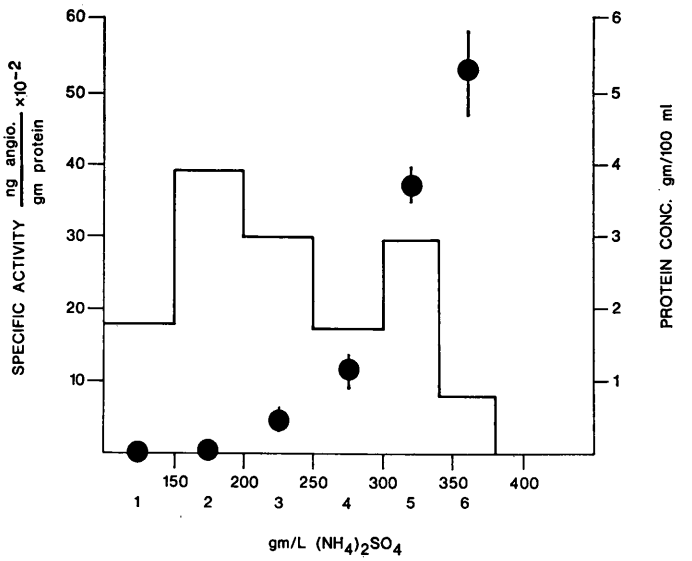

FIG. 2. Binding of angiotensin II to certain proteins in rat plasma precipitated by ammonium sulfate. (●) specific activity of angiotensin ± SE; (—) protein concentration. Mean of 6 experiments.

its specific activity, is greatest in fraction 6, whereas the specific activity of angiotensin I is approximately equal in fractions 4, 5, and 6. Electrophoresis of each of the fractions on polyacrylamide gel shows each of them to consist of various mixtures of the plasma proteins but fraction 5 is primarily albumin and fraction 6 is practically pure albumin. The specific activity of the angiotensin II is proportional to the amount of albumin in the latter fractions. These facts suggested to us that angiotensin II binds to albumin. To test this, in 2 experiments we added angiotensin II to purified rat albumin and in 3 experiments we added angiotensin II to purified human albumin. In all experiments the albumin, when precipitated by ammonium sulfate and then washed once, reprecipitated and redissolved, contained a large proportion of the added angiotensin. Angiotensin I also coprecipitates with purified albumin in similar experiments. Thus it is clear that both angiotensin I and angiotensin II precipitate with albumin when it is precipitated by ammonium sulfate. It also seems likely that when plasma proteins are similarly precipitated, angiotensin II coprecipitates only with albumin, whereas angiotensin I coprecipitates with albumin and also some other plasma protein.

In six experiments EDTA was added to rat plasma, in addition to angiotensin I, and fractional precipitation carried out as before. This resulted in the maximum specific activity shifting to fraction 4, as shown in Fig. 3. This suggests that the EDTA may interfere to some extent with the binding of angiotensin

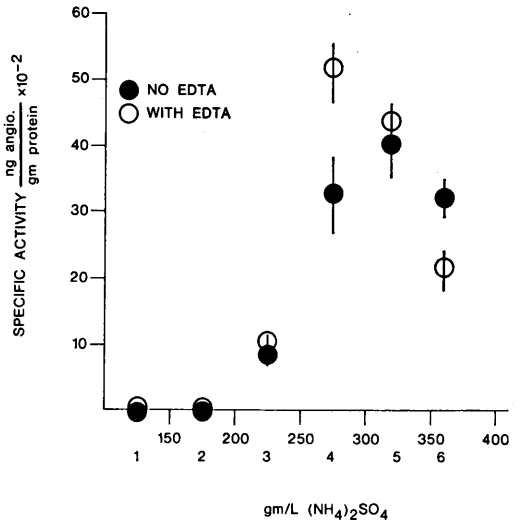

FIG. 3. Effect of EDTA on binding of angiotensin I to proteins in rat plasma. (○) with EDTA; (●) without EDTA. Mean of 6 experiments.

TABLE I. Angiotensin/Protein[a] in Fractions Obtained by Ammonium Sulfate Precipitation of Plasma Proteins.

Mixture	*n*	Original sample	Fr.: 1	2	3	4	5	6
Angio I	6	25.17	0	0	7.98	32.20	40.03	31.41
+ rat plasma		±2.25		0	±1.00	±5.40	±5.20	±2.80
Angio II	6	18.71	0	0.48	4.73	11.86	37.22	53.20
+ rat plasma		±3.76		±0.48	±1.07	±2.31	±2.67	±5.96
Angio I	6	21.43	0.55	0	10.11	50.75	43.06	21.07
+ rat plasma		±2.44	±0.26		±0.92	±4.08	±2.43	±2.79
+ EDTA								
Angio II	5	17.70	0	0	4.83	12.21	42.00	41.01
+ rat plasma		±3.69			±1.05	±1.98	±2.99	±4.61
+ EDTA								

[a] Values are mean ± standard error (ng angiotensin/g protein) × 10^{-3}.

I to albumin but not with its binding to some other protein. A summary of the data on precipitation of angiotensin I and II with and without EDTA is given in Table I.

Contrary to our original expectations, EDTA added to rat plasma does not prevent the binding of angiotensin I or angiotensin II to proteins in rat plasma, that is when the proteins are precipitated by ammonium sulfate. As indicated above, EDTA does alter the pattern of binding of angiotensin I. It has little influence on the fractional precipitation pattern of angiotensin II.

The presence of BAL in addition to EDTA, a combination which effectively blocks angiotensinase action, also permits binding of angiotensin I and angiotensin II to plasma proteins precipitated by ammonium sulfate. It yields a fractional precipitation pattern similar to that produced when only EDTA is added.

In several experiments, ^{125}I labeled angiotensin I and ^{14}C labeled angiotensin II were added to plasma (in addition to the corresponding "cold" angiotensin). After fractional precipitation by ammonium sulfate, fractions 1 through 6 were tested by bioassay and also by measurement of radioactivity in a gamma counter (for ^{125}I) or a liquid scintillation counter (for ^{14}C). The measurements of radioactivity gave a good parallelism with the bioassay measurements. An example is shown in Fig. 4. One advantage of the radioassay over the bioassay is that the angiotensin concentration of the supernatant fluid from each ammonium sulfate precipitation could be measured. (In the bioassay technique, the high concentration of ammonium sulfate in the supernatant fluid interferes with the blood pressure raising effect of the angiotensin). By measuring the radioactivity of the supernatant fluid after precipitating fraction 6, it could be shown that most of the angiotensin had been removed and that the angiotensin present in the precipitate could not be accounted for by trapping of supernatant fluid along with protein in the precipitate.

In 5 experiments human plasma was used instead of rat plasma. Some difficulty was experienced in separating the precipitated proteins in fractions 3 and 4 from the supernatant fluid because of the tendency of the precipitate in these fractions to float up during centrifugation (presumably due to its high lipoprotein content). Nevertheless, separations were achieved and the results were similar to those with rat plasma. Angiotensin I and angiotensin II do bind to plasma proteins in human plasma and are precipitated with the later fractions 5 and 6 but not with the earlier ones.

An attempt was made to identify the protein which binds angiotensin I by adding ^{125}I labeled angiotensin I to plasma and subjecting the mixture to electrophoresis. However, no clear evidence of association of radioactivity

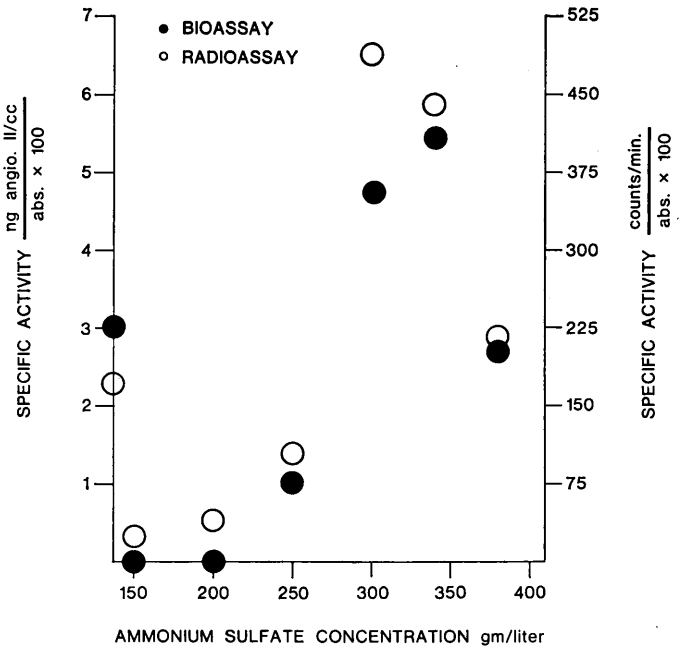

FIG. 4. Bioassay vs radioassay. Binding of angiotensin I to proteins in rat plasma with EDTA. (○) radioactivity (cpm) divided by the protein concentration in terms of uv absorbance at 280 nm. (●) angiotensin concentration measured by bioassay divided by the protein concentration. The two values on the left hand ordinate are those for the original (unfractionated) plasma. One experiment.

with any specific band of protein was obtained. Apparently under the conditions we used for polyacrylamide gel electrophoresis no binding of angiotensin I occurs. The results with angiotensin II were also negative. Unfortunately, the available evidence is still inadequate to answer this question.

Discussion. Our results together with those of Schweikert, Carey and Liddle (5) and of Houle, Piwonska and Carretero, (7), show that under some circumstances angiotensin does bind to certain plasma proteins. However, whether it also binds under the circumstances which prevail *in vivo* is still uncertain. It is worth noting that Houle, Piwonska and Carretero (7) found binding to occur only under conditions involving very low ionic strength while our experiments show binding under the opposite condition of high ionic strength due to the high concentration of ammonium sulfate. Perhaps additional experiments using ultrafiltration can answer the question of whether binding of angiotensin occurs when ionic strength and protein concentrations are in the range normally found in blood plasma.

It is interesting that there are differences in the binding properties of angiotensin I and angiotensin II. As described under results, with ammonium sulfate precipitation, especially in the presence of EDTA, the maximum specific activity of angiotensin II occurs in a different fraction than the maximum specific activity of angiotensin I. This difference may indicate that angiotensin I binds to some plasma protein to which angiotensin II does not bind, whereas both bind nonspecifically to albumin. Houle, Piwonska and Carretero (7) reported that Cohn fraction V of human plasma, which is mainly albumin, bound angiotensin II in their dialysis experiment, but fraction IV-4 did also. Whether the differences in the binding properties of the two angiotensins play any role in their biological activity is unknown, but one might speculate that the binding of angiotensin I might reduce its pressor potency and might slow down its conversion to angiotensin II. On

the other hand, there is no *a priori* reason to exclude the possibility that angiotensin bound to a plasma protein might be *more* potent as a vasoconstrictor than free angiotensin. The fact that angiotensin II is more effective in causing constriction of isolated aortic strips (10) or of the perfused isolated rabbit ear artery (11) when blood plasma is present would support such a possibility.

Summary. Angiotensin I and angiotensin II, when added to rat or human plasma, coprecipitate with the later protein fractions, when the plasma is subjected to fractional precipitation by ammonium sulfate. The maximal specific activity of angiotensin II is with the last step (which is mainly albumin) while that of angiotensin I is with an intermediate step. Both angiotensins also coprecipitate with purified albumin when it is precipitated by ammonium sulfate. These results lead us to the conclusion that both angiotensin I and angiotensin II bind to albumin and that angiotensin I also binds to some other protein. We were unable to identify this other protein. Chelating agents, such as EDTA and BAL, do not prevent the binding of either angiotensin to proteins precipitated by ammonium sulfate. Whether a similar binding occurs *in vivo* still remains unknown.

I thank Miss Carrie Lee for her assistance during the early phases of this investigation.

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Received Sept. 26, 1972. P.S.E.B.M., 1973, Vol. 142.