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Further Evidence for the Existence of Angiotensinogen Stimulating Activity (ASA) after Nephrectomy[1] (39476)

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After preincubation with plasma from nephrectomized animals liver slices from normal rats released larger amounts of angiotensinogen than slices which had been preincubated with normal plasma (1). This observation together with results of liver perfusion (2) led to the suggestion that the increase in circulating angiotensinogen seen after nephrectomy is due mostly to an increase in synthesis elicited by an angiotensinogen stimulating factor. Some possibility remained, however, that the larger amounts of angiotensinogen present in the incubation medium might represent release of material taken up during the preincubation period from the angiotensinogen rich plasma. Preliminary experiments in which liver slices were preincubated with semipurified angiotensinogen were inconclusive (1). The present paper demonstrates that stimulation of angiotensinogen synthesis can be obtained with a portion of plasma virtually free of angiotensinogen. It also includes further data on the nature of the stimulus.

Physical methods. DEAE Cellulose chromatography. The method of Cook and Lee (3) for purification of angiotensinogen was used with the hope that it might permit separation of the angiotensinogen from angiotensinogen stimulating activity (ASA). Serum from 48 hr nephrectomized rats was partially purified by ammonium sulfate precipitation at 40% saturation and then subjected to chromatography on DEAE cellulose. Elution was carried out with a linearly increasing concentration of sodium chloride in 0.01 *M* Tris-HCl buffer pH 7.5 at a rate of 60 ml per hour, and was monitored by UV absorption at 280 nm. The eluate was collected in fractions of 12 ml in 136 tubes. Protein composition of the fractions was determined by polyacrylamide gel electrophoresis (4). Angiotensinogen and ASA were determined by bioassay. Angiotensinogen was determined directly on 1 ml of fluid. For ASA determination, the remaining content from each tube was concentrated by evaporation to half its volume and dialyzed against Robinson solution (5). Then aliquots of 3 ml were preincubated with normal liver slices for measurement of ASA.

Starch powder electrophoresis. The electrophoretic method of Kunkel and Slater (6) was carried out on 5 ml portions of serum from nephrectomized rats and on equivalent amounts of the active fraction obtained by precipitation with ammonium sulfate. In the latter instance, the precipitate was dissolved in water and dialyzed against 0.9% NaCl solution. Serum required no prior treatment. At the end of the electrophoresis, the protein pattern was identified from a paper strip which had been applied on the starch and stained for proteins. From the pattern obtained, the starch block was cut into segments which were suspended in saline. The suspension was centrifuged and the supernatant was concentrated by evaporation to the original serum volume. After dialysis against Robinson solution, the eluted material was analyzed for content of angiotensinogen and ASA, nitrogen content by Kjeldahl digestion and nesslerization, and protein composition by cellulose acetate electrophoresis.

Chromatography of Sephadex. The ASA-active portions of the eluate obtained by chromatography on DEAE cellulose were combined and concentrated by evaporation from 10 ml to 1.5 ml. This amount was applied on a 30 ml column of Sephadex G-

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200 that had been equilibrated with Robinson solution. Elution was carried out with Robinson solution at a rate of 3 ml/hr, and monitored by uv absorbance at 280 nm. Fractions (1 ml) of the effluent solution were collected for a total of 30 tubes. All proteins were contained in tubes 10 to 30. These 1 ml fractions were combined to give seven subfractions with volumes ranging from 2 to 4 ml in approximate proportion to the breadth of protein boundaries evident from electrophoresis. The subfractions were made up to a volume of 4 ml and were preincubated with normal liver slices for determination of ASA.

Biological methods. Female Sprague-Dawley rats weighing approx 200 g were fed a commercial chow and given tap water to drink. Normal and 48 hr nephrectomized animals were used to provide plasma, serum, or liver slices. Plasma was obtained by withdrawing blood during amobarbital anesthesia (9 mg/100 g) in a syringe moistened with 0.3 *M* disodium EDTA. Plasma and serum were used fresh or after freezing.

Determination of ASA. After laparotomy during amobarbital anesthesia (9 mg/100 g) a PE 60 plastic cannula was inserted into the portal vein. Aorta and vena cava were sectioned above the diaphragm and the liver was flushed with 30 ml of saline followed by 30 ml of cold Robinson solution. Slices 0.5 mm thick were cut, washed three times in 15 ml of Robinson solution, and drained on filter paper. Portions weighing 200 mg were placed in Warburg flasks containing the material to be tested, dissolved in 3 ml of Robinson solution. In control experiments the medium consisted only of 3 ml of Robinson solution or of normal plasma or serum. The flasks were then placed in a Gibson differential respirometer at 37°C, shaken for 1 hr at a frequency of 120 times per minute and ventilated with a mixture of 95% O_2 and 5% CO_2. Following this preincubation the medium was discarded, the slices were washed twice with 50 ml of Robinson solution, suspended in 3 ml of fresh Robinson solution and incubated for a period of 4 hr under conditions identical to those described above. At the end of the incubation, the content of each flask was centrifuged and the supernatant fluid used for determination of angiotensinogen. Rates of angiotensinogen released and presumably formed during incubation are expressed in nanograms of angiotensin per gram of liver per hour of incubation (ng angiotensin/g/hr). Rates exceeding the control value indicate presence of ASA in the preincubating medium.

Determination of angiotensinogen. Angiotensinogen concentration was estimated from the amount of angiotensin generated following incubation of samples with an excess of renin (7). Angiotensin was bioassayed in pentolinium treated rats (5 mg of the tartrate/100 g) using angiotensin II (Hypertensin, Ciba) as standard. Angiotensinogen concentration is expressed in nanograms of angiotensin per milliliter (ng angiotensin/ml). Values are means ± SD.

Effect of Renin on ASA. The purpose of the experiment was to explore the possibility of a cause-effect relationship between the low renin and the high ASA associated with nephrectomy. Plasma (3 ml) from 15 hr nephrectomized rats was incubated for 15 min in the presence of 25 Goldblatt units of semipurified renin contained in 0.1 ml of saline (2). At the end of the incubation it was cooled in an ice bath and dialyzed against Robinson solution for 24 hr to remove angiotensin and other products which may have resulted from the proteolytic action of renin and other peptidases. It was then assayed for angiotensinogen concentration ASA. Control tubes contained plasma incubated with renin which had been inactivated by boiling.

Results. Validation of the procedure for determination of ASA is supported by the following results. Without preincubation, normal liver slices incubated for 4 hr in Robinson solution released angiotensinogen at the rate of 9.3 ± 0.92 ng angiotensin/g/hr. Similar values were obtained with preincubation for 1 hr in Robinson solution or normal plasma, averaging respectively 8.7 ± 1.04 and 9.7 ± 1.47 ng angiotensin/g/hr. However, rates were markedly increased by preincubation in plasma from nephrectomized animals to average 21.0 ± 2.72 with 5 hr nephrectomized plasma and 41.94 ± 4.66 with 15 hr nephrectomized plasma.

DEAE cellulose chromatography. Chro-

matographs of two batches of serum from nephrectomized animals gave similar results. With the first batch angiotensinogen was found in tubes 30 to 40 with a peak amounting to 1825 ng of angiotensin per ml (138 ng of angiotensin per mg protein) in tube 36 (Fig. 1). On the other hand, ASA was present in tubes 30 to 33 with a peak of 29.7 ng angiotensin/g/hr in tube 32. The tubes containing the largest amounts of angiotensinogen did not show significant ASA; values varied between 10.2 and 11.7 ng angiotensin/g/hr as compared with control values between 9.7 and 11. With the second batch, both peaks were slightly displaced to the left. Angiotensinogen was present in tubes 24 to 34 with a maximum of 2000 ng/ml in tube 30, while ASA was present in tubes 23 to 27 with a peak of 33.7 ng angiotensin/g/hr in tube 26. Again, the tubes (28 to 31) which contained the largest amount of angiotensinogen did not show significant ASA. The peak of angiotensinogen was eluted with a chloride concentration of 0.094 *M* and that of ASA with a concentration of 0.078 *M*.

Electrophoresis of the various fractions obtained by chromatography (Fig. 2) showed that the fractions rich in ASA and angiotensinogen contained numerous components, some of which varied in apparent proportion to ASA and angiotensinogen concentrations. However, we could not associate any single component with each of the two biological activities.

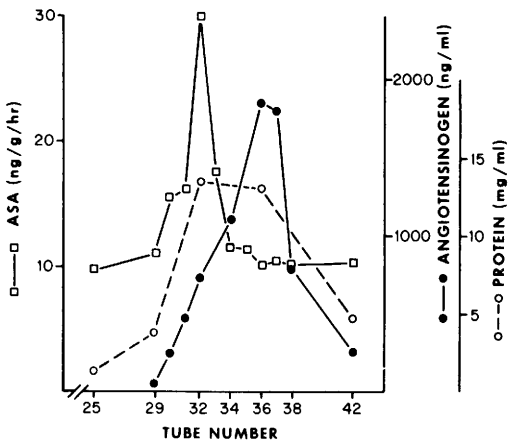

FIG. 1. Separation of angiotensinogen from ASA by gradient elution from DEAE-cellulose.

Starch powder electrophoresis. Following electrophoresis of serum from nephrectomized animals, significant amounts of ASA were found only in the $\gamma 2$-globulin fraction (Table I). Rates of angiotensinogen formation in the other fractions did not significantly differ from the control value. The concentration of ASA in the $\gamma 2$-globulin fraction is high both in absolute value and in terms of protein concentration. In contrast, angiotensinogen activity was found in the fraction with albumin-$\alpha 1$-globulin mobility. Results obtained by electrophoresis of the material precipitated at 40% ammonium sulfate saturation were not informative because the activity was more or less evenly distributed between all three of the major protein zones: albumin, $\alpha + \beta$ globulins, and $\gamma 1 + \gamma 2$ globulins.

Chromatography on G 200 Sephadex. The active fractions rich in ASA derived from DEAE cellulose chromatography were separated according to molecular size of its constituents by chromatography on Sephadex G 200 (Fig. 3). Assay of effluent for ASA showed that the bulk of activity was eluted in the fractions containing proteins of molecular weights around 60,000.

Effects of renin on ASA. Results from two experimental series using the same pool of plasma were similar, and differences between values were less than 10%. As summarized in Table II, they show that incubation with active renin (tubes 4 and 5) caused the destruction of both angiotensinogen and ASA, while addition of boiled renin had no effect on either angiotensinogen or ASA. Pretreating plasma by dialysis (tubes 1 and 2) lowered ASA but not angiotensinogen.

Discussion. The present experiments confirm our previous observation (1) that after nephrectomy the plasma acquires the property of stimulating angiotensinogen formation, hence that this property is likely related to the spectacular rise in circulating angiotensinogen. They also demonstrate that ASA is amenable to purification by electrophoresis and chromatography.

It has been shown that the liver does not store any appreciable amounts of angiotensinogen and that whatever is present in a liver perfusate (2) or in the incubating medium of liver slices (7) represents newly synthesized material. Nevertheless, since most

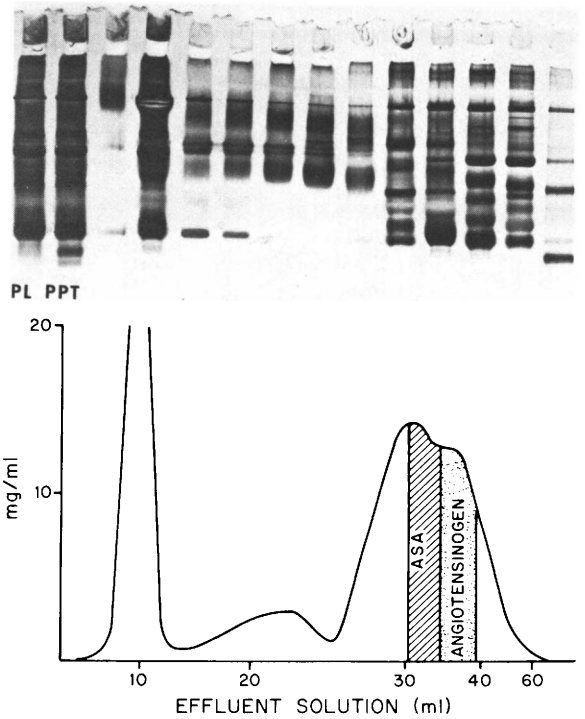

FIG. 2. DEAE chromatography of ammonium sulfate precipitate containing ASA and angiotensinogen. The elution profiles of the various components in the starting material are portrayed by the superimposed polyacrylamide-gel electrophoresis patterns. The abscissa of the chromatogram was drawn in nonlinear scale to achieve alignment with the electrophoretic patterns. As judged by resemblance to patterns obtained with both plasma (PL) and the precipitate (PPT), tubes 9–11 contained protein applied in excess of binding capacity of the DEAE. Absence of ASA and angiotensinogen in this fraction indicated they were fully retained. The crosshatched and stipled areas depict location of peak activities of ASA and angiotensinogen in the eluate.

TABLE I. EFFECTS OF FRACTIONS FROM STARCH-POWDER ELECTROPHORESIS ON ANGIOTENSINOGEN FORMATION.

Fractions	Protein[a] concentration (mg/ml)	Angiotensinogen[a] concentration (ng angiotensin/ml)	Rates of angiotensinogen formation (ng angiotensin/g/hr)
Albumin + α-globulin	10.50	200	9.5
α2-globulin	1.32	34	9.0
β-globulin	2.45	30	9.2
γ1-globulin	0.38	32	9.7
γ2-globulin	0.10	32	15.7
Original serum	64	2850	43.2
Control	–	–	9.7

[a] Measurements made on preincubation medium.

of the active materials which were previously tested for ASA contained large amounts of angiotensinogen and since the amounts of angiotensinogen released by slices are relatively small, one could not discard the possibility that some angiotensinogen might be absorbed during the preincubation to be later released during the final incubation. Another possibility, however remote, is that angiotensinogen itself or an altered form of it may act as a stimulus. The first possibility may be discarded on the following evidence. First, plasma contained more ASA than serum although both were obtained from the same animals and had the same angiotensinogen concentration (1). Secondly, on electrophoresis of serum the ASA was present in the γ2-globulin fraction whereas angiotensinogen moved with the albumin-α1-globulin. Thus, ASA and angiotensinogen have opposite positions in the electrophoretic pattern (8). Lastly, chromatography on DEAE cellulose revealed that the ASA and angiotensinogen were eluted separately, thus eliminating the possibility

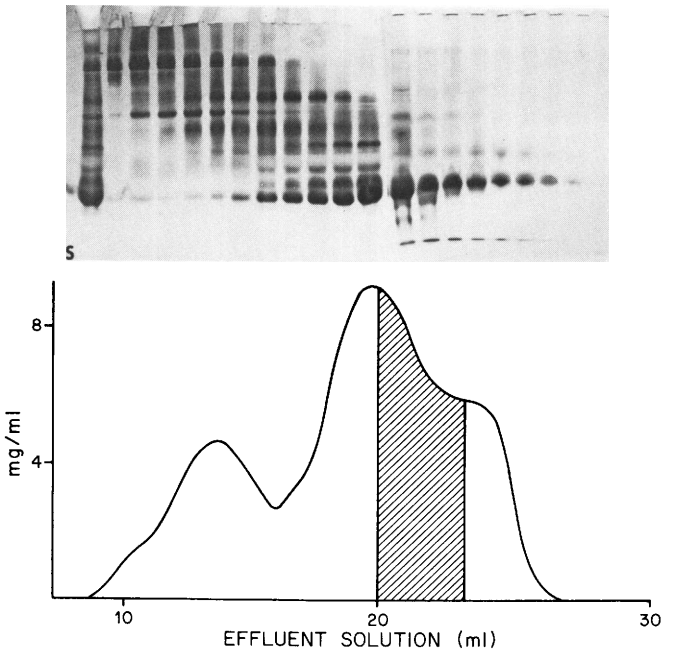

FIG. 3. Gel filtration chromatography on G 200 Sephadex of DEAE fractions containing ASA. The cross-hatched area demarcates portion of the effluent solution with peak ASA. The accompanying polyacrylamide-gel electrophoresis patterns showed that this portion contained small sized proteins such as albumin.

TABLE II. EFFECTS OF RENIN ON ASA AND ANGIOTENSINOGEN IN PLASMA FROM NEPHRECTOMIZED ANIMALS.

Tubes[a]	1	2	3	4	5
Active renin	0	0	0	+	+
Inactive renin	+	+	+	0	0
Incubation	0	0	+	+	+
Dialysis	0	+	+	0	+
Angiotensinogen (ng angiotensin/ml)	3600	3500	3300	N.M.[b]	N.M.[b]
Rates of angiotensinogen formation (ng angiotensin/g/hr)	39.2	26.1	25.5	6.9	7.6

[a] All tubes contained 3 ml of plasma from 15 hr-nephrectomized rats.
[b] Not measurable.

that angiotensinogen was responsible for ASA.

Although the observed differences in physical properties dispel the previous suggestion that ASA might somehow be due to angiotensinogen itself, the possibility of an altered form of angiotensinogen possessing ASA warrants consideration. If alteration is conjectured, the change would have to be (i) related to nephrectomy by virtue of appearance of ASA in nephrectomized plasma, (ii) unrelated to the assay system or concentrations of angiotensinogen incubated with liver slices because of lack of ASA in purified angiotensinogen, and (iii) also unrelated to changes occurring in plasma because ASA did not increase on storage. It had also been noted before that incubation with a semipurified renin preparation destroyed ASA in plasma. Since these considerations largely eliminate possibility of change occurring in plasma and liver, the principal sources of angiotensinogen, we presently view the conjectured alteration as only a remote possibility.

Except that it is very likely a protein, we have little information on the nature of the substance responsible for ASA. Electrophoresis and ultracentrifugation of serum suggest a high molecular weight protein behaving like a γ-globulin. On the other hand, gel filtration of material that had been purified

by ammonium sulfate precipitation and DEAE chromatography indicates a smaller molecule behaving like albumin. This contradiction may, however, be only apparent if we postulate that the substance has either high density, possibly corresponding to a glycoprotein, or exists as a complex with other protein, seemingly γ-globulin in serum. The latter postulate is based on the following observation. Electrophoresis of concentrates of ASA prepared by ammonium sulfate precipitation and dialysis gave results that differed from electrophoresis of serum on each of two trials in which serum and concentrates were compared. The observed association of ASA with all of the major protein fractions separated by electrophoresis subsequent to ammonium sulfate precipitation suggests that binding affinities were altered by the precipitation. The apparent alteration of binding affinities by the precipitation procedure do not invalidate distinctions made in properties of ASA and angiotensinogen because they were separable from each other both before and after the precipitation, the electrophoretic separation being performed before, and the DEAE chromatography after.

The specific activity of ASA purified by the electrophoresis of serum far exceeded that achieved by all the other attempted purification methods combined. The high efficiency of this separation method is attributable to the migration of ASA with the γ2-globulins which comprise only a small percentage of the serum proteins. The active subfractions from DEAE chromatography and gel filtration subsequent to ammonium sulfate treatment contained numerous electrophoretic components, albumin being a major one. Since large losses in activity did not occur, the low specific activity achieved by the latter methods was undoubtedly due to the high content of albumin in the subfractions. From observations made thus far, it would appear that a very high degree of purification might be achieved by electrophoresis followed by ammonium sulfate precipitation and gel filtration, but this possibility remains to be tested pending availability of material.

The substance responsible for ASA is not unique. Estrogens (2), cortisol (7), and angiotensin (9), are also known to directly stimulate angiotensinogen formation. The report (7) that cortisol injected into normal rats mimicked the rate of angiotensinogen formation elicited by nephrectomy is not in accord with results from experiments in which cortisol was added to the medium perfusing a normal liver: Rates were increased but not as much as after nephrectomy (10). It is very likely that the greater rates found after cortisol injections in nonnephrectomized animals were due to the stimulating activity of angiotensin superimposed on that of the steroid. Indeed any procedure which would result in a stimulation of the pituitary adrenal system should be considered of questionable value for the detection of ASA, especially when a rise in plasma angiotensinogen is used as criterion (11).

ASA is undoubtedly an extrarenal factor, because of its appearance in blood after nephrectomy. It is not clear whether formation of ASA might be specifically associated with the uremic state or might possibly represent the accumulation of a physiologically formed substance which under normal conditions is excreted or destroyed by the kidney. The present observations on the inactivating effect of renin could provide an explanation for its destruction when renin levels are high or normal and its accumulation when renin secretion ceases. However, this point will require study of kinetics of the inactivation.

The evidence that many factors including the one appearing after nephrectomy have the property of stimulating angiotensinogen synthesis and that some of these factors are able to affect directly both angiotensinogen formation and renin secretion emphasize the complexity of angiotensinogen regulation as well as the complexity of the interrelationships existing between the various components of the renal–pressor system (10). The renal–pressor system is known to be well integrated and to possess multiple compensatory pathways for maintenance of vascular turgor. The extent to which ASA may participate in autoregulation cannot be predicted at present because of the unex-

plored possibility that its appearance in blood might specifically depend on nephrectomy.

Summary. After nephrectomy, plasma or serum acquires the property of stimulating angiotensinogen (A) synthesis in liver slices from normal rats. The possibility that this angiotensinogen stimulating activity (ASA) may be an artifact arising from exposure of the tissue to the high levels of A in the test material has been examined. It has now been found that ASA and A can be separated by electrophoresis on starch powder and gradient elution from DEAE cellulose. The identification of ASA as a distinct component of the renal-pressor system raises new prospects for investigation of control mechanisms within the system.

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