CHARACTERIZATION OF THE RENIN-ANTIRENIN SYSTEM*

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The enzyme renin, found in the kidney (1) and extracts of other organs and blood vessels (2), has been shown to induce an elevation in blood pressure (3), by acting on a plasma protein substrate to release a decapeptide, angiotensin I, which is subsequently converted to the potent pressor substance, angiotensin II (4). In addition, this enzyme system has been shown to play a role in the control of aldosterone secretion (5). Developing knowledge of the physiologic role of renin has been summarized in a recent symposium (6). Impeding progress in this area is the lack of a sensitive, practical assay for renin. The several assays which have emerged measure, in the main, angiotensin and are complicated and the results obtained contradictory (6). The experiments reported herein record our study of the renin-antirenin system and involve (*a*) characterization of antienzyme antibodies elicited by hog renin in the dog and rabbit, (*b*) purification of a precipitating rabbit anti-hog renin antibody, (*c*) consequent development of a sensitive and specific hemagglutination assay for both renin and antirenin, and (*d*) the localization of renin in hog and dog tissues as determined by immunofluorescent techniques with the purified specific antirenin antibody.

Materials and Methods

Renin.—Renin was obtained from hog and dog kidneys using in part the method of Haas, Lamfrom, and Goldblatt (7). Dog kidneys were taken through the second step and hog kidneys through the fifth step of this renin purification procedure. Following fractionation, the preparations were dialyzed for 2 days at 6°C against distilled water, lyophilized, and stored at −20°C until use. Prior to injection they were dissolved in phosphate-buffered saline (PBS)

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pH 7.5 (0.02 N $Na_2HPO_4 \cdot H_2O$ and 0.15 N NaCl). The specific activity of the renin preparations, i.e., vasopressor units of renin/mg of lyophilized preparations, varied in the individual studies undertaken and will be stated in results.

Antirenin.—Antirenin was induced in Dutch-belted rabbits by intraperitoneal injection of 10 units of hog renin on each of the first 4 successive days of the week over at least a 4 wk period. Dogs, which included several previously immunized with hog renin by Dr. G. E. Wakerlin, were given intramuscular injections of 100 units of hog renin/day in like manner. With both species, animals were bled 7 days after the completion of their inoculation series, the sera brought to 56°C for ½ hr, and stored in the presence of 1:10,000 merthiolate at −20°C until use. The immunization schedule was repeated at monthly intervals. Renin at specific activities of 1 and 5 units/mg was utilized for injection.

Inactivated Renin Preparations.—Renin preparations were inactivated essentially by the method of Lamfrom, Haas, and Goldblatt (8), by bringing renin in PBS, pH 7.5, to pH 11.5 with 0.3 N NaOH for 10 min at room temperature. At the end of the incubation period, the preparation was readjusted to pH 7.5 with an approximately equal volume of 0.3 N HCl. The loss of vasopressor activity was documented by assay in the dog.

Assays for Renin and Antirenin.—The in vivo assay was performed in dogs; the arterial pressure was determined by means of a manometer connected to the femoral artery by a cannula and recorded on a kymograph. Test preparations were injected into the femoral vein. Dogs were anesthetized with 1 ml Nembutal/kg. Pentolinium bitartrate (Wyeth Laboratories, Philadelphia), a ganglionic blocking agent, 10 mg/ml and 0.1 ml/kg was administered to stabilize the blood pressure. 5 ml heparin, (120 international units/mg, 10 mg/ml) was injected to prevent the formation of clots. Administration of these preparations was repeated as needed to assure meaningful results. Since it was known that Nembutal (9) as well as pentolinium bitartrate (10) could affect the animals' response to renin, a standard renin preparation, obtained from Dr. O. Helmer, Eli Lilly and Co., Indianapolis, was utilized as a control in all in vivo assays. The dog unit (DU) of renin was defined as that quantity of renin required to raise the mean femoral systolic blood pressure of a dog by 30 mm Hg (11). The assay of any given unknown preparation was preceded and followed by the administration of standard renin to determine the validity of the assay.

Antirenin titers were measured by mixing 0.5 to 1 ml of either undiluted or diluted serum with 1 unit of renin and injecting the mixture into dogs after 15 min of incubation at room temperature. One unit of antirenin (ADU), equaled that amount of antiserum required to just block the pressor effect of 1 DU of renin. It was found that errors up to 30% were obtained on assay of test preparations in different dogs. Duplicate assay procedures performed in the same dog were subject to errors of up to 15%.

In Vitro Precipitation Reactions.—Unless stated otherwise, all precipitation reaction mixtures were incubated for 7 days at 4°C, in the presence of merthiolate at a dilution of 1:10,000. Before analysis, any precipitate formed was washed twice with chilled 0.15 N NaCl.

Antirenin Absorption.—Rabbit antirenin was absorbed at 4°C with 2 mg samples of inactivated renin in PBS, pH 7.5, at 2-day intervals until precipitation no longer occurred. After each 2-day interval, the sera were centrifuged before the addition of further inactivated renin. The system was incubated for 7 days at 4°C after the final addition of inactivated renin.

Agar Gel Diffusion Analysis.—Double diffusion was carried out for 7 days by the method of Ouchterlony (12), in 1% Ionagar (Oxoid Ionagar, No. 2. Consolidated Lab., Chicago), in PBS, pH 7.5 in the presence of merthiolate, 1:10,000.

Immunoelectrophoretic Analysis.—A modification of the method of Grabar and Williams (13) was employed, using 76 × 26 mm glass slides covered with buffered (0.1 N barbital-HCl buffer, pH 8.2) 1% Ionagar, to a height of 3 mm. Electrophoresis was carried out in the same buffer for 45 min. at approximately 40 ma, the voltage being adjusted so that approximately 10 v/cm across each slide was attained. After the addition of antiserum to the troughs,

the slides were incubated for 24 hr at 4°C, washed overnight in PBS, pH 7.5, and stained with amido black.

Hemagglutination and Hemagglutination Inhibition Studies.—To 10 ml of a 2.5% suspension of washed sheep red blood cells in pH 7.2 PBS was added 10 ml of tannic acid, at a dilution of 1:10,000 in 0.15 N NaCl. After incubation for 10 min at 37°C and washing with cold PBS, pH 7.2, the cells were resuspended to a final volume of 10 ml in PBS, pH 7.2. Renin at a specific activity of 5 and at a concentration of 0.5 mg/ml was added to the cells in 40 ml of PBS, pH 6.4. The mixture was incubated for 15 min at room temperature, washed once with 1:250 normal rabbit serum in saline, and resuspended in 10 ml of 1:250 normal rabbit serum so as to yield a 2.5% suspension of cells. Assays were carried out utilizing 2-fold dilutions of antisera in saline containing 1:100 normal rabbit serum. To each dilution of antiserum was added 0.05 ml tanned, renin-treated cells. Negative controls consisted of tanned, sensitized cells in PBS, pH 7.2, and antisera added to tanned, unsensitized cells. The positive controls utilized are described in the experimental results.

Hemagglutination inhibition studies were performed using the test described above excepting that dilutions were made of the renin preparations, with a constant amount of antirenin being added to each tube. 0.2 ml of antirenin, which was equal to 20 times the hemagglutination titer of the serum utilized, was added in each case. The renin dilutions were preincubated with the antirenin at 37°C for ½ hr before the addition of sensitized cells. Tests were then read after 1 hr at 37°C and 2 hr at room temperature. Button or doughnut shaped patterns were considered negative for hemagglutination and positive for hemagglutination inhibition. Evenly distributed "dust" patterns, or doughnutlike patterns with ragged edges, were considered positive for hemagglutination and negative for hemagglutination inhibition.

It was found essential that all sera utilized be tested for the presence of nonspecific substances capable of inducing hemagglutination or hemagglutination inhibition. Filtration of whole sera through a Seitz filter eliminated those nonspecific effects.

Fluorescent Antibody Studies.—Sheep anti-rabbit gamma globulin was precipitated from serum by a 40% concentration of $(NH_4)_2$ SO_4, brought to pH 7.2 with 0.01 M PBS, and 20 ml containing 28 mg protein/ml was conjugated with fluorescein isothiocyanate at 4°C for 18 hr after addition of 5.6 ml 0.5 M $NaHCO_3$ buffer, pH 9, 30.4 ml 0.15 N NaCl, and 6.72 mg fluorescein isothiocyanate. The resultant solution was equilibrated to pH 6.3 in PBS and chromatographed according to the method of Riggs et al. (14). Column eluates containing a fluorescein:protein ratio of between 1.5 and 5 were pooled as suggested by Goldstein et al. (15), and equilibrated with 0.001 M PBS, pH 7.2, by means of gel filtration. A final fluorescein to protein ratio of 3:1 was obtained; the final concentration of protein in the preparation was 1.44 mg/ml.

Dog and hog frozen tissue sections were cut by microtome to between 4 and 6 mμ in a cryostat set at −15°C. Tissues were fixed in acetone at room temperature for 15 min and then incubated to dryness for ½ hr at 37°C. Tissues were first treated with the unlabeled purified antirenin preparation, washed for 10 min in three changes of PBS, pH 7.2, and the reacted tissues exposed to the fluorescein isothiocyanate conjugated sheep anti-rabbit gamma globulin for ½ hr. After a final wash, the slides were mounted in buffered glycerol (1 part glycerol, 9 parts 0.8% NaCl at pH 7.0 with 0.01 M phosphate buffer) and observed by ultraviolet microscopy.

Absorption of sheep anti-rabbit gamma globulin with lyophilized hog serum removed a factor which reacted nonspecifically with hog glomeruli.

EXPERIMENTAL RESULTS

Characterization of Antibodies Reacting with Renin.—Previous publications (8, 16) had reported that antirenin would not precipitate, but would neutralize renin. In our system precipitation did occur between the renin preparations

utilized and immune sera obtained from Dutch-belted rabbits.[1] However, these antisera possessed antibodies to many of the contaminating substances present in the impure renin preparations; therefore, proof of a precipitating renin-antirenin complex necessitated the demonstration of either renin or antirenin in the precipitate. Accordingly, attempts were made to elute antirenin from washed

TABLE I

Precipitation of Rabbit Antirenin by Varying Amounts of Renin

Renin* added	Rabbit antirenin serum No.	Total‡ antirenin added	Antirenin dissociated§ from formed precipitate
DU		*ADU*	*ADU*
1	1	8.5	0
5		8.5	1
10		8.5	1.4
20		8.5	0.3
30		8.5	0
1	9	8.5	0
5		8.5	1
10		8.5	0
20		8.5	0
30		8.5	0

* The renin utilized had a specific activity of 5. Volumes, as well as the pH of all reactants were kept constant.

‡ The antirenin was in a concentration of 25 ADU/ml.

§ Antibody was dissociated by treatment of the reaction mixture with alkali and assayed for capacity to neutralize the pressor activity of renin in the dog. Direct injection of untreated renin-antirenin precipitates into dogs elicited no pressor effect.

precipitates utilizing a modification of the technique used by Lamfrom, Haas and Goldblatt (8) to dissociate nonprecipitating antirenin.

To constant amounts of several rabbit antirenin sera were added increasing amounts of renin. The resultant precipitates were washed twice with PBS, pH 7.5 and brought to pH 11.5 with 0.3 N NaOH. The alkali-treated preparations were then incubated for 1 hr at 4°C before being readjusted to pH 7.5 with 0.3 N HCl. Any precipitate which reformed at this point was removed, and the resultant supernatant fluids were assayed in a dog for the presence of antipressor activity.

As can be seen in Table I, under the proper condition, antipressor activity was dissociated from the precipitate, indicating the existence of antirenin complexed with the enzyme renin in the precipitate. Although visible precipitation occurred in the presence of excess renin, under this condition antirenin could not be

[1] Immunization of New Zealand white rabbits for a period of over 1 yr with renin injected by several routes in a variety of doses, as well as the use of adjuvants, did not give rise to any antirenin activity.

dissociated from the precipitate. A significant difference between the two antirenin sera investigated was noted, in that precipitation of the renin-antirenin complexes occurred over a much narrower range of renin addition in the serum from rabbit 9 than in the serum from rabbit 1. Both sera contained 25 ADU/ml, and their volumes as well as pH were similar. In a like experiment utilizing antirenin serum from rabbit 9 after 8 additional months of immunization, similar results were noted.

To determine whether the data obtained by means of the antibody dissociation procedure were reproducible, renin was added to antirenin sera in three replicate tubes. The amount of antibody dissociated by alkali was determined for each of the precipitates that formed by assaying for antipressor activity in a dog. From each of the 3 tubes 2 ADU were eluted, indicating that the quantity

TABLE II

Precipitation of Renin-Antirenin Complexes in Antirenin Excess

Renin* added	Antirenin‡ added	Antirenin dissociated from precipitate§
DU	*ADU*	*ADU*
10	17	8
10	100	16

* Renin at a specific activity of 5 was used.

‡ Antirenin at 45 ADU/ml, obtained from rabbit 9 was used.

§ Antibody was dissociated by treatment with alkali and tested for capacity to neutralize pressor activity of renin in the dog.

of antirenin dissociated from renin-antirenin precipitates, under similar conditions, was reasonably constant.

Experiments testing two variables that could conceivably affect the formation of precipitating complexes, antirenin excess, and increased reaction volume, were investigated. It had been reported by Schlamowitz, in studies with dog intestinal phosphatase (17), and by Zamecnik and Lipmann using lecithinase (18), that excess antibody when added to these enzymes, inhibited the formation of precipitating complexes. However, a 6-fold excess in antipressor units of antirenin to renin did not inhibit the precipitation of antirenin by renin (Table II). Likewise, evidence had been presented that the precipitation of antigen-antibody complexes could be inhibited to some degree, by excess reaction volume (19). Since experiments performed in the present study at times involved up to a 3-fold dilution of renin-antirenin reaction mixtures, the effect of such dilution on the precipitation of renin-antirenin complexes was investigated and found not to affect the precipitation of the renin-rabbit antirenin complexes.

Studies similar to those above, designed to permit the precipitation of renin-

dog antirenin complexes, were unsuccessful. Varying conditions of renin or antirenin excess in no instance resulted in specific, precipitating antirenin. Thus, these results confirm the report that antirenin prepared in dogs against hog renin (8), reacted with renin to form only nonprecipitating complexes.

TABLE III

The Coexistence of Precipitating and Nonprecipitating Antirenin Antibodies in Immune Rabbit Sera

Renin* added	Rabbit serum No.	Initial serum titer	Supernatant‡ fluid analysis		Antirenin dissociated§ from	
			Renin	Antirenin	Reacted supernatant fluid	Formed precipitate
DU		*ADU/ml*	*DU*	*ADU*	*ADU*	*ADU*
15	1	30	10	—	7	7
15			10	—	5	7
0			—	30		
0			—	30		
15	11	40	2	—	5	30
15			1	—	5	30
0			—	40		
0			—	40		
15		0	15			
15			15			

* Renin at a specific activity of 5 was added in increments of 5 DU until precipitation no longer occurred.

‡ After centrifugation, the supernatant fluids of the reactants were assayed in vivo for the presence of renin or antirenin in excess.

§ Antibody was dissociated by treatment of the reaction mixture with alkali, and tested for capacity to neutralize the pressor action of renin in the dog.

With successful demonstration of precipitating antirenin in the rabbit, the existence of nonprecipitating complexes of renin with rabbit antirenin also was sought under conditions avoiding the formation of soluble complexes caused by antigen excess.

Renin was added in small increments to 2 rabbit antirenin sera, and any precipitate which formed was removed by centrifugation before the addition of further increments of renin. Addition of renin was continued in this manner until precipitation no longer occurred. The precipitates which had formed were pooled, washed, dissociated by treatment with alkali, and assayed for antirenin. The supernatant fluids that remained were divided into equal aliquots, with half being tested directly by dog assay for free antirenin or renin and half being analyzed in this regard for antirenin in soluble complex form, detectable only after dissociation with alkali.

The results, recorded in Table III, indicated the coexistence of nonprecipitating with precipitating antibody, in that antipressor activity was dissociated

from the supernatant fluids as well as the precipitates of these preparations. Thus, although free renin was present in the former as revealed by direct inoculation into dogs, treatment with alkali of an equal aliquot of the supernatant fluid, released reactive antirenin from nonprecipitating complexes. Similar experiments performed with antirenin sera from 6 other rabbits also showed the coexistence of precipitating and nonprecipitating antirenin in their sera.

A third type of antibody reacting with the actual enzyme was revealed following absorption experiments originally designed to obtain a specific serum containing only precipitating antirenin. Toward this end, the pressor activity of renin preparations was destroyed by subjecting them to pH 11.5 for 10 min at room temperature. This procedure had previously been shown to inactivate renin pressor activity (8), and this was confirmed by bioassay of these preparations in our laboratory. These preparations containing renin devoid of enzymatic activity were utilized to absorb 4 rabbit antisera, with the objective of precipitating antibodies against impurities in the renin preparations, but leaving in the supernatant fluid antibodies neutralizing the enzymatic activity of renin.

TABLE IV

The Reactivity of Several Rabbit Antirenin Sera with Inactivated Renin

Rabbit serum No.	Initial titer	Titer after absorption*
	ADU/ml	*ADU/ml*
1	20	10
2	6	6
9	45	45
11	7	4

* Inactivated renin, originally at a specific activity of 5, was added in 1 mg increments to each preparation until precipitation no longer occurred.

The results (Table IV) demonstrated that rabbit antirenin sera differed in their reactivity to inactivated renin preparations. Although all 4 sera were tested at the same time under identical conditions, the antirenin titer of 2 of the sera (1 and 11) were reduced to approximately one-half after absorption with inactivated renin preparations, while 2 others (2 and 9) were not altered by this procedure. Additional tests with sera from 4 different rabbits showed 2 which were not affected by absorption with inactivated renin, 1 which dropped 40% in titer, and 1 which dropped 25% in titer as a result of absorption with increments of inactivated renin. Thus, in addition to precipitating and nonprecipitating antirenin, a third antibody reacting with this enzyme was revealed in some sera, having the characteristic of being able to be precipitated by the *inactivated* enzyme preparation. This antibody was presumed reacting with a determinant group on the actual enzyme but away from the enzymatic site, inas-

much as absorption of these sera with renin preparations devoid of pressor activity resulted in a reduced capacity to neutralize the pressor activity of the enzyme in the dog.

As no loss in antirenin titer resulted from the absorption with inactivated renin of 4 out of 8 sera, it was unlikely that the drop in neutralizing titer observed in the other 4 sera was due to the presence of residual enzyme activity in the inactivated renin preparation. To acquire further assurance for this contention the inactivated preparations were assayed in the rat, an animal more responsive to renin than the dog. By rat assay, only 0.0024 active units/original DU were detected. Thus, 500 DU equivalents of inactivated renin would have had to have been used in order to have reduced the neutralizing titer of any serum by one unit. At no time in the above experiments were more than 30 DU equivalents of inactivated renin added to any of these sera.

Purification of Precipitating Neutralizing Antirenin.—In an attempt to obtain by absorption procedures an antiserum containing precipitating antibody directed solely against the pressor activity of the enzyme, serum from rabbit 9 was chosen as it contained a high initial titer of antirenin that was not reduced by the addition of inactivated renin. When inactivated renin was used in increments to absorb this serum to the point where no further precipitation occurred, the final absorbed anti-hog renin serum reacted with hog renin, at a specific activity of 5, to form but one line of precipitation when assayed by both simple and double agar diffusion. The absorbed serum likewise formed one line of precipitation with dog renin. A reaction of partial identity with a single spur was obtained between the hog and dog renin preparations.

To determine whether the single precipitate obtained in the gel diffusion studies represented the specific reaction of renin with antirenin, an attempt was made to dissociate antirenin from this precipitate. Two different lots of rabbit 9 sera, absorbed with inactivated renin, were precipitated with renin. The precipitates that formed were washed and treated with alkali to dissociate antirenin, and the resultant solutions were assayed in a dog. The successful dissociation of approximately 40% of the antirenin capable of neutralizing the pressor activity of renin indicated that the precipitates that formed did contain antirenin (Table V). Double diffusion analysis, utilizing as reactants this absorbed and dissociated antirenin preparation together with hog renin at a specific activity of 5, gave one line of precipitation between the renin and antirenin preparation; no precipitate formed between the antiserum and inactivated renin or hog serum (Text-fig. 1). Immunoelectrophoretic analysis of the interaction between hog renin and absorbed rabbit antirenin likewise showed the presence of but one line between the impure renin preparation utilized and the purified antirenin preparation (Text-fig. 2). It appeared, therefore, that a precipitating and neutralizing antirenin had been obtained in purified form.

Dissociation of Renin from Renin-Antirenin Complexes.—It had been reported

that when antirenin reacted with renin, the renin was irreversibly inactivated (8). This result differed with evidence obtained in other systems in which active enzyme could be recovered from enzyme-antienzyme complexes (20, 21). Therefore, an attempt was made to dissociate active renin from neutralized renin-

TABLE V

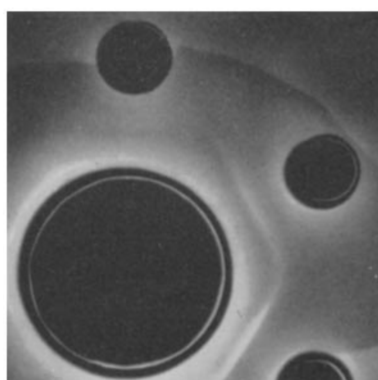
*Reactivity of Rabbit 9 Antirenin Serum with Inactivated Renin and Active Renin**

Rabbit serum No.	Initial serum titer	Titer‡ after adsorption	Total antirenin§ dissociated from		Antirenin dissociated from precipitate
			Supernatant	Precipitate	
	ADU/ml	*ADU/ml*	*ADU*	*ADU*	%
9	33	33	13	12	36
	30	28	13	12	42

* Dissociation of the absorbed sera by alkali was performed after reaction with renin.

‡ The antirenin titer of rabbit serum 9 after absorption with inactivated renin.

§ That quantity of antirenin dissociated from the absorbed immune serum after reaction with renin.



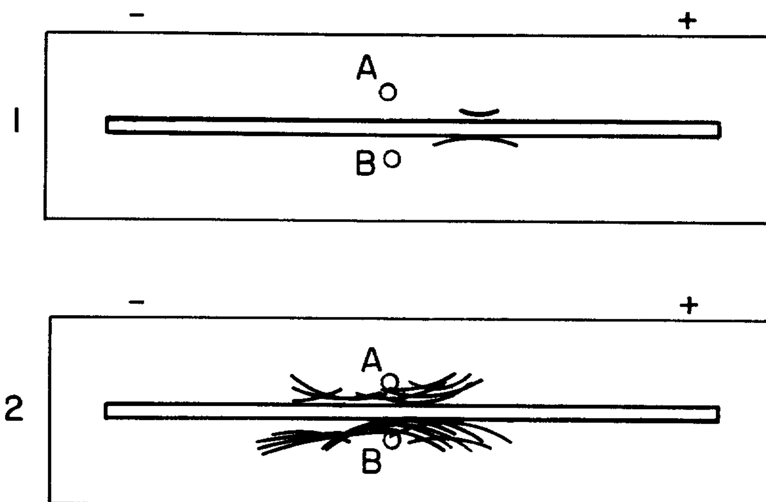
TEXT-FIG. 1. Reactivity in double diffusion in agar of a purified rabbit anti-hog renin preparation with varying quantities of hog kidney antigens. Large center well contained purified rabbit antirenin, 16 ADU/ml; surrounding wells reading from top clockwise, hog renin 6 DU/ml, 12 DU/ml, and 25 DU/ml respectively.

antirenin complexes. Dog antirenin was used as it was felt that the active enzyme would be easier to dissociate from a nonprecipitating system.

To 800 ADU of dog antirenin at 150 ADU/ml, was added 400 DU of renin at a specific activity of 5. A second tube containing 200 ADU was reacted with 100 DU of renin. After 2 days of incubation at 4°C, the reaction mixture of the first tube, was adjusted to pH 2.15 with 4 N H_2SO_4 at 1°C. This was followed by the addition of ethanol to a final concentration of 10% (v/v). In a repeat study, reaction mixtures were incubated for 1 hr at pH 2.15 and

at 1°C before the addition of ethanol. The resultant supernatant fluids, after overnight dialysis against changes of PBS, pH 7.5, were assayed in a dog. The untreated reaction mixture of the second tube was examined for the presence of renin or antirenin excess.

The untreated reaction mixture was found to contain over 10 ADU/ml of antirenin, the treated reaction mixtures were found to contain active renin (Table VI). The dissociated substance was verified as being renin by neutralization of its' pressor activity upon the addition of purified antirenin. When the reacted sera were incubated for 1 hr before the addition of ethanol, a 22% yield



TEXT-FIG. 2. The reactivity of purified, 1, and unpurified, 2, rabbit antirenin with renin as determined by microimmunoelectrophoresis. At renin concentrations of 60 and 300 DU/ml (A and B respectively) one line was obtained on reaction with purified rabbit antirenin. As many as eleven lines were discernible on reaction of the same renin preparations with unabsorbed rabbit antirenin sera.

of renin was obtained. A 2% yield of renin was obtained when ethanol was added to the reaction mixture without prior incubation.

Passive Hemagglutination Test for Renin and Antirenin.—With the availability of antirenin purified by absorption with inactivated renin, and subsequently precipitated with active renin and dissociated with alkali, it became feasible to attempt to establish in vitro assays for both renin and antirenin using hemagglutination and hemagglutination inhibition tests. In a preliminary experiment, it was found that 0.5 mg/ml of renin at a specific activity of 5 was needed to sensitize tannic acid–treated sheep red blood cells in order to induce hemagglutination in the presence of the rabbit antirenin. Subsequent testing showed (Table VII) that the reciprocal hemagglutination titers obtained for antirenin sera of similar neutralizing titer were essentially the same. In addition,

TABLE VI

*The Dissociation of Active Renin from Renin-Dog Antirenin Complexes**

DU Renin added	ADU antirenin added	Total dissociated renin	
		A‡	B§
		DU	*DU*
400	800‖	7	89

* Renin dissociation was achieved by subjecting renin-antirenin complexes at 1°C, to a pH of 2.15 with 4 N H_2SO_4, followed by the addition of ethanol to a final concentration of 10% (v/v). Dissociated renin was identified by its pressor effect in dogs and its ability to be neutralized by purified rabbit antirenin.

‡ In this case, the reaction mixture was not incubated at pH 2.15 before the addition of ethanol.

§ In experiment B, the reaction mixture was incubated for 1 hr at pH 2.15 and at 0°C before the additional of ethanol.

‖ The reaction of 800 ADU with 400 DU was proven to result in an antirenin excess.

TABLE VII

Hemagglutination (HA) Assay for Antirenin

Serum used	Antirenin titer	Tanned cells sensitized with	Reciprocal HA titer	Minimum antirenin units detected
	ADU/ml			
Antirenin	5	Renin A*	160	0.016
"	5	" "	320	0.008
"	5	" "	160	0.016
"	5	" "	160	0.016
"	5	" "	160	0.016
"	5	Renin B‡	160	0.016
"	5	None	0	
"	5	Inactivated renin	10	
NRS	0	Renin A	0	

* Tanned cells were treated with renin at a specific activity of 5, containing 0.5 mg/ml, (2.5 DU/ml), except in one instance (renin B).

‡ In this instance, renin at a specific activity of 0.5 containing 5 mg/ml, (2.5 DU/ml), was used to sensitize the tanned sheep red blood cells. In all instances, the first tube contained 0.25 ADU in a final volume of 0.5 ml.

it was found that 0.008 to 0.016 ADU was the minimum amount of antirenin producing definite hemagglutination.

When renin was assayed by the hemagglutination inhibition test, the results obtained were reproducible with different lots of purified antirenin and with renin preparations of 2 different specific activities (Table VIII). It was found

that between 0.003 to 0.006 DU of renin could be detected by this technique. Hemagglutination inhibition did not occur when inhibition of the reaction was attempted with inactivated renin.

In a further effort to establish the validity of the assays, the following experiments were performed. Available lots of absorbed and dissociated antirenin were pooled, lyophilized, and dissolved in PBS, pH 7.5. The purity of the pooled preparation was ascertained by double diffusion analysis in which only one line was observed between the purified antirenin and renin. Assay of the pooled purified antirenin of unknown titer revealed a reciprocal hemagglutination titer

TABLE VIII

Assay for Renin by the Hemagglutination Inhibition (HI) Technique

Antigen titrated	Antiserum added*	Tanned cells sensitized with	Reciprocal HI titer‡	Minimum units renin detected
	Incubation for ½ hr at 37°C			
Renin A§	Antirenin	Renin	160	0.006
" "	"	"	160	0.006
" "	"	"	320	0.003
" "	"	"	320	0.003
" "	"	"	160	0.006
Renin B	"	"	160	0.006
Inactivated Renin	"	"	0	

* In each instance 0.2 ml, containing 0.32 ADU (equivalent to twenty 100% HA units) of purified antirenin was added to each tube.

‡ HI titers describe the highest dilution of renin capable of inhibiting agglutination between a constant amount of antirenin and tanned renin sensitized sheep red blood cells.

§ The first tube contained 0.1 DU in a final volume of 0.5 ml after the addition of antirenin. The renin used at an initial titer of 2 DU/ml had a specific activity of 5 except in one instance (renin B), where renin with a specific activity of 0.5 was used.

of 640 in a total volume of 0.5 ml, a titer which, on the basis of the minimum amount of antirenin detectable being 0.016 ADU, corresponded to an initial titer of 20 ADU/ml (Table IX). Addition of an unknown amount of renin inhibited the above hemagglutination reaction to the extent that a concentration of 16 DU renin/ml (Table IX) was calculated. This was verified by dog assay.

Finally, verification of the hemagglutination inhibition test as a valid assay for renin was furthered with the results of a blind test on 5 different samples of renin prepared and assayed for dog unitage in a different laboratory. In Table X the amounts of renin in these unknowns calculated from testing by hemagglutination inhibition are compared with those determined by assay for pressor activity in the dog. A good correlation between the 2 tests is apparent.

It appeared therefore, that both the hemagglutination and hemagglutination inhibition techniques were of value as a means of assaying both antirenin and renin in vitro when a purified antirenin was available. Assays performed utilizing absorbed sera that had not been purified further by precipitation with active renin and dissociation at pH 11.5 did not give as reproducible results.

TABLE IX

*Comparative in Vitro and in Vivo Assays for Unknown Quantities of Renin and Antirenin**

Assay for	Reciprocal HA titer	Reciprocal HI titer	Renin and antirenin detected	
			in vitro	in vivo
			units/ml	*units/ml*
Renin‡		1280	16 DU	16 DU
Antirenin§	640		20 ADU	16 ADU

* Unknown renin and antirenin preparations were prepared and first assayed in vitro. In vitro assay results were then verified by in vivo dog assay.

‡ Renin at a specific activity of 5, was used.

§ A pooled purified rabbit antirenin preparation was used.

TABLE X

Comparison of Renin Content in Unknown Samples Determined by Hemagglutination Inhibition and with Dog Assay

Unknown renin sample	Theoretical value (DU renin)	Actual value HI test (DU renin)
A	0.1	0.16
B	0.8	0.64
C	Negative	Negative
D	0.07	0.08
E	0.02	0.04

In Vitro Assay of Circulating Renin.—With these in vitro assay methods specific for the enzyme renin and antirenin, a model for the detection of renin in circulating fluids was tested by the intravenous injection of 20 DU of hog renin into rabbits. It was found by the hemagglutination inhibition method that serum obtained immediately after the injection of renin contained 0.1 DU of renin/ml of serum. This was validated by dog assay. On the other hand, serum harvested from a rabbit inoculated with renin and bled after 4 hr failed to show the presence of renin by the hemagglutination inhibition technique or dog assay, indicating a rapid inactivation of this enzyme. Neither normal rabbit serum nor inactivated renin preparations induced inhibition of hemagglutination between renin-sensitized cells and a constant amount of antirenin. In addition, hemagglutination did not occur between antirenin and cells sensitized with inactivated renin.

Normal hog serum, in contrast to normal rabbit serum, did show reactivity in the hemagglutination inhibition test for hog renin, as is illustrated in Table XI. A reciprocal hemagglutination inhibition titer of 64 was obtained, apparently indicating the presence of 0.2 to 0.4 DU of renin/ml of normal hog serum. As a test of the specificity of the above reaction, hemagglutination was attempted with cells sensitized with inactivated renin. No hemagglutination occurred in this system or in a system which utilized bovine gamma globulin

TABLE XI

Renin in Normal Sera as Indicated by the Inhibition of Renin-Rabbit Antirenin Hemagglutination by Normal Hog Serum

Serum titrated	Lot No.*	Antiserum added‡	Tanned cells sensitized with	HA	Reciprocal HI titer§
		Incubation for ½ hr at 37°C			
Normal hog serum	1				64
	1	Antirenin	Renin		32
	2				64
	1	None	Renin	Negative	
	1	Anti-BGG	BGG‖	Positive	
	1	None	BGG‖	Negative	
	1	Antirenin	Inactivated renin	Negative	

* Two lots of pooled normal hog sera were assayed; each lot represented the pooled serum of three hogs.

‡ In each instance, 0.32 ADU (equivalent to twenty 100% units) of purified antirenin was added to each tube. Likewise, twenty 100% (HA) units of anti-BGG were added to each tube in the test utilizing anti-BGG.

§ HI titers describe the highest dilution of hog serum capable of inhibiting hemagglutination between a constant amount of antirenin and renin tanned sensitized sheep red blood cells. No HI occurred on the reaction of renin sensitized cells and antirenin in normal rabbit serum.

‖ BGG refers to bovine gamma globulin used as a control system.

sensitized cells. In addition, normal hog sera neutralized the antipressor activity of purified rabbit anti-hog renin. Thus, when 1 ADU of antirenin was added to 1 ml of hog serum, the antipressor activity of the antirenin was diminished in 3 of the 4 hog sera studied (Table XII). In control experiments, it was shown that pooled normal *rabbit* sera did not neutralize the antipressor activity of *rabbit* anti-hog renin preparations with which they were incubated. It was shown also that normal hog and rabbit sera, when incubated with renin, did not diminish the vasopressor effect of this enzyme. On the basis of this evidence it was concluded that a substance antigenically similar to renin was present in normal hog sera in a serologically active form.

Renin Localization in the Kidney by Immunofluorescence.—In 1939, Goormaghtigh (22) presented evidence which indicated that the juxtaglomerular cells were the site of renin production in the kidneys. His original proposal has since been strengthened by the findings of other investigators (23, 24). However, it now appeared feasible to confirm the site of localization of renin in the kidney by means of the purified rabbit anti-hog renin. In this series of experiments, the kidneys of two rats, two normal dogs, two sodium-deficient dogs, and four normal hogs were studied for the presence of renin by means of the immunofluorescent technique. Approximately 100 sections from the kidneys of each of these animals were examined in compiling the data described below.

TABLE XII

Renin in Normal Sera as Indicated by the Inhibition of Neutralizing Rabbit Antirenin by Normal Hog Serum (NHS)

Serum used*	Antirenin added	Renin added	Renin detected
	15 min incubation	15 min incubation	
	ADU	*DU*	*DU/ml NHS*
A	1	1	0.8
B	1	1	0.3
C	1	1	0
D	1	1	0.5

* Serum A refers to a pool of three hog sera. Sera B to D represent three individual different hog sera. When antirenin was preincubated with normal rabbit serum, before the addition of renin, no resultant reduction in antirenin titer was noted.

Renin, in both the dog and the hog, was found localized in the cytoplasm of the juxtaglomerular cells of the afferent arteriole and in the region of the macula densa of the distal convoluted tubule (Figs. 1 and 2). Pretreatment of hog, or dog kidney sections with dog anti-hog renin, blocked the ability of the rabbit antirenin to localize in the region of the juxtaglomerular apparatus. In addition, hog and dog liver, heart, spleen, and kidney medulla were negative for specific immunofluorescence. Normal rabbit serum, used in place of rabbit antirenin likewise did not give rise to immunofluorescence in kidney sections when followed by treatment with the labeled sheep anti-rabbit gamma globulin. Finally, rat kidney sections, which contain renin that does not cross-react with rabbit anti-hog renin, did not fluoresce when treated with the purified rabbit antirenin and the fluorescein isothiocyanate-labeled sheep antirabbit globulin. It appeared, therefore, that the fluorescence observed in the juxtaglomerular cells and in the region of the macula densa of hog and dog kidney sections was specific for renin present in these regions of the kidney.

Kidney sections obtained from normal dogs, while fluorescing specifically in

the region of the vascular pole, did not fluoresce as brightly or over as great an area as did sections obtained from sodium-deficient dogs. With hogs, however, it proved unnecessary to institute a sodium-deficient diet to obtain bright specific fluorescence in kidney sections. This is probably attributable to a more ready reaction of purified rabbit anti-hog renin with homologous hog renin than with heterologous dog renin.

The relative number of times specific fluorescence was observed in hog and dog kidney cortex sections is recorded in Table XIII. In over 300 instances, areas with sufficient histologic integrity were obtained to permit identification

TABLE XIII

*The Sites of Localization of Renin in Hog and Dog Kidneys by the Fluorescent-Antibody Technique**

Histologic site	No. of fields positive for specific immunofluorescence	Total fields positive for site‡
		%
Macula densa (MD)	142	8
Juxtaglomerular cells (JGC)	93	5
MD and JGC	96	5
Vascular pole	583	33
Other regions	0	0

* Sections were first treated with purified rabbit antirenin and then with fluorescein isothiocyanate-labeled sheep anti-rabbit globulin. Dog and hog liver, heart, lung, spleen, and kidney medulla, as well as rat kidney sections, were negative for immune fluorescence.

‡ A total of 1743 microscopic fields showing glomeruli were observed that possessed sufficient histological integrity to enable identification of the site of immunofluorescent localization. Immunofluorescence was not observed in the periglomerular regions in 829 (47%) of these fields.

of the regions in which the purified antirenin had localized. In over 200 fields of individual glomeruli, localization of renin was found to occur in the region of the macula densa of both dogs and hogs. In 189 individual fields, localization of renin was found to occur in the cytoplasm of the juxtaglomerular cells. At no time did specific fluorescence occur outside of these regions. Often, however, because of the variable tangential angles at which the glomeruli were sectioned, it was not possible to state definitely the region in which immunofluorescence occurred. In the latter cases, immunofluorescence was localized to the vascular pole of the glomeruli studied. An additional point of interest was the lack of specific immunofluorescence in approximately 50% of the glomeruli studied. This, in part, occurred because glomeruli may be cut to show neither the juxtaglomerular cells nor the macula densa. Nevertheless, even in some fields which showed these structures, specific fluorescence was not seen. In fact, the impression was gained that the specific renin content in the region of each glomerulus varied.

DISCUSSION

Inoculation of animals with heterologous kidney extracts containing renin had been shown by Johnson and Wakerlin (25), as well as others (8, 16, 26), to result in antirenin antibodies. Although the antigenic preparations were impure, it was demonstrated that the antisera could reduce the blood pressure of chronically hypertensive animals in certain instances of experimental hypertension (26–28). In addition, Deodhar has shown that the pressor activity of angiotensin II can be neutralized by antibody produced with benzylangiotensin II-azo-bovine gamma globulin (29). Our earlier attempts at serologic characterization of renin-antirenin interaction employed dog antisera and were not successful (30). This was due most likely to the fact that the antisera utilized neutralized renin, but did not precipitate, fix complement, or participate in passive hemagglutination. However, as reported herein, the elaboration of a precipitating, neutralizing antirenin by some rabbits permitted the development of what appears to be an effective serologic assay for renin. Although previous studies employing rabbits immunized with renin had reported that only nonprecipitating antibody was formed, the absorption procedures used might have removed precipitating, neutralizing antirenin. This would be true particularly when absorption of the immune sera was performed with serum fractions homologous to the animals from which the renin preparations were obtained. That normal sera contain renin has now been established (6) and verified by our data. The finding of 3 different types of antirenin antibodies in rabbits, each capable of neutralizing the enzymatic activity of renin, is not surprising in view of the fact that specific antibodies directed against several distinct sites on a single antigen had been established (31–33). The use of a monovalent antiserum, however, was found essential in our tests for adequate assay of renin content of unknown samples.

Experiments designed to determine the site of localization of renin in the kidney by means of the immunofluorescent technique have been carried out by Nairn, et al. (34), and Edelman and Hartroft (24). Although absorption of the immune sera was performed in these studies, the completeness of the absorption procedures could not be determined because of the nonprecipitating nature of the antirenin utilized. In addition, the results obtained using impure antirenin preparations were not absolute and in fact were contradictory (24, 34). Our finding of renin in the cells of the macula densa is at variance with the very extensive work of Hartroft and her group on this subject (35). It is possible that our use of 4 to 6 mμ sections in contrast to their very thin sections might have permitted some cell overlay. However, evidence of a direct nature also indicating the presence of renin in cells outside of the juxtaglomerular cells, has been reported (36, 37). In addition, Latta and Maunsbach (38), and Oberling and Hatt (39), in studies with the electron microscope have demonstrated the intimate relationship of the macula densa to the granular cells of the juxtaglomerular apparatus.

The knowledge derived necessarily from study of the hog renin-antirenin system is now being applied to the human system. As has been pointed out by Conn, et al., a practical assay for human renin would facilitate markedly detection of the approximately 20% of the hypertensive human beings who can be cured by surgical procedures (40). Early experiments have indicated the ability of Dutch-belted rabbits to respond with serologically active human antirenin. The assay of renin in human body fluids with this system will be the subject of a further report.

SUMMARY

Rabbit antibody capable of neutralizing the pressor activity of the enzyme renin, derived from hog kidneys, was characterized with respect to type and capacity to react serologically. Three antirenin antibodies were detected, both a precipitating and nonprecipitating, neutralizing antibody as well as a neutralizing antibody capable of being absorbed by inactivated renin. The precipitating, neutralizing antibody was purified and used successfully in an assay for hog renin by means of a hemagglutination inhibition test. By immunofluorescent methods utilizing the purified antiserum, renin was found to be localized in the macula densa of the distal convoluted tubules and the juxtaglomerular cells of the afferent arteriole in both hog and dog kidneys.

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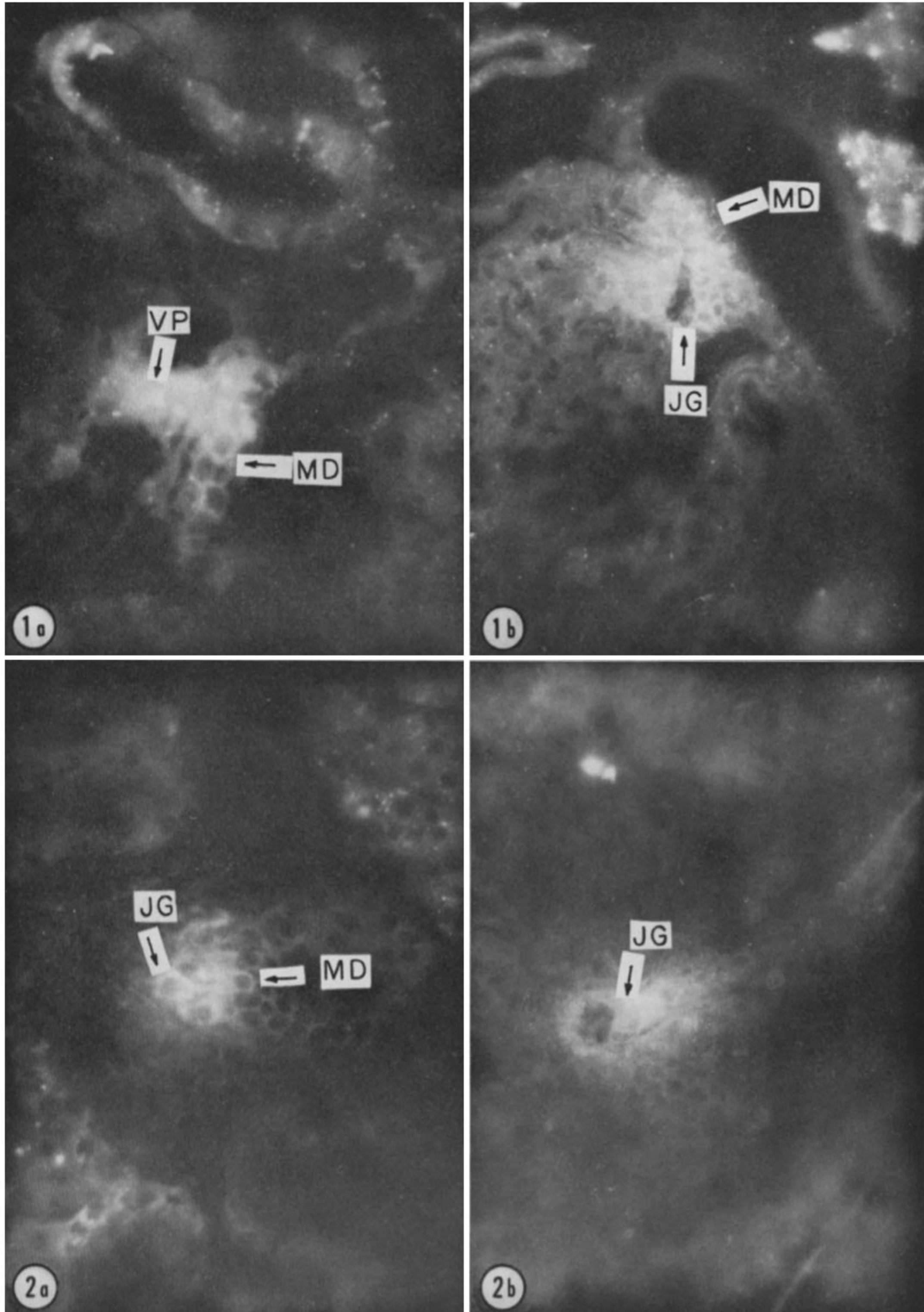
EXPLANATION OF PLATE 108

In all cases, staining was carried out by the indirect method, using purified rabbit anti-hog renin and fluorescein isothiocyanate-labeled sheep anti-rabbit globulin. × 325.

FIGS. 1 *a* and 1 *b*. Photomicrographs of a kidney section from a dog kept on a sodium deficient diet for 3 wk. Note the specific immunofluorescence in the region of the macula densa (*MD*), the region of the vascular pole (*VP*), at the hilus of the glomeruli and in the granular cells of the juxtaglomerular apparatus (*JG*) at the insertion of the afferent arteriole into the glomerulus.

FIGS. 2 *a* and 2 *b*. In sections prepared from normal hog kidneys, immunofluorescence was found in both the region of the macula densa (*MD*), and in the cytoplasm of granular cells of the juxtaglomerular apparatus (*JG*).



(Warren et al.: Renin-antirenin system)