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BRADYKININOGEN, ANGIOTENSINOGEN AND KALLIDINOGEN

BY

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The plasma globulins which produce the vasoactive polypeptides, bradykinin, kallidin and angiotensin, have been compared. After incubation of plasma with kallikrein and exhaustion of its kallidinogen, subsequent incubation with trypsin did not result in formation of bradykinin, showing that bradykininogen had also been exhausted and suggesting that kallikrein and trypsin use the same substrate. Kallidin and bradykinin formation was not prevented by acid-treatment of plasma, though heat-denatured substrate produced kallidin less readily. Kallikrein could exhaust plasma bradykininogen without affecting levels of angiotensinogen. Following nephrectomy of dogs, plasma angiotensinogen levels rose whereas bradykininogen levels did not. These results confirm the belief that bradykininogen differs from angiotensinogen but not from kallidinogen.

When plasma is incubated with trypsin the nonapeptide bradykinin is formed (Rocha e Silva, Beraldo & Rosenfeld, 1949; Boissonnas, Guttmann & Jaquenoud, 1960; Elliott, Lewis & Horton, 1960), whereas when plasma is incubated with kallikrein the decapeptide kallidin is formed (Werle & Berek, 1950; Pierce & Webster, 1961). The substrates upon which trypsin and kallikrein act have been called bradykininogen and kallidinogen respectively. Renin forms the decapeptide angio-.ensin I when incubated with plasma (Braun-Menéndez, Fasciolo, Leloir & Muñoz, 1939; Page & Helmer, 1940; Skeggs, Marsh, Kahn & Shumway, 1954). The renin substrate has been called angiotensinogen.

Bradykininogen, kallidinogen and angiotensinogen all occur in the globulin fraction of plasma but it is unknown whether they are one and the same protein. However, Espada, Fasciolo, Binia & Cabut (1961) showed that a partial separation of angiotensinogen and bradykininogen could be achieved by ammonium sulphate-fractionation of ox and dog plasma. Moreover, they observed that renin can exhaust plasma angiotensinogen without modifying the bradykininogen content. These results suggest that bradykininogen and angiotensinogen are different proteins. Bradykininogen and kallidinogen have not convincingly been shown to be different, though some differences have been reported (Werle, 1963).

In the present investigation these plasma substrates have been compared by studying the action of kallikrein upon bradykininogen and angiotensinogen and the effect of nephrectomy upon plasma bradykininogen and angiotensinogen levels. The results confirm the belief that angiotensinogen and bradykininogen are probably different globulins, whereas kallidinogen and bradykininogen could not be distinguished.

METHODS

Estimation of plasma bradykininogen. The method of Fasciolo, Espada & Carretero (1963) was used. Dog plasma (0.1 ml.) was diluted to 3.5 ml. with 0.9% saline buffered to *p*H 7.4. The tubes were placed in a boiling-water bath for 3 min. After cooling, 0.5 mg of crystalline trypsin (Worthington Biochemical Corp.), dissolved in 0.5 ml. of 0.9% saline, was added and the mixture was incubated for 30 min at 37° C. The amount of bradykinin formed was estimated by its depressor action on the dog perfused leg preparation (Fasciolo, Halvorsen, Itoiz & Paladini, 1958) by bracketing assay. A unit of bradykininogen is the amount which yields 1 U of bradykinin on incubation with trypsin. One unit of bradykinin is the amount which produces a 50 mm Hg fall in the pressure of the perfused leg preparation. It corresponds to 0.1 μg of synthetic bradykinin (Sandoz).

Estimation of plasma angiotensinogen. Dog plasma (2 ml.) was diluted to 4 ml. with 0.9% saline (*p*H 7.4) and incubated with 20 U of renin for 15 min at 37° C. The contents of the tubes were then acidified to *p*H 6.5 and placed in a boiling-water bath for 3 min. The precipitate thus formed was separated by centrifugation and discarded. The angiotensin concentration of the supernatant fluid was assayed on the anaesthetized rat blood pressure. In the nephrectomy experiments dog plasma was stored at −20° C until the determination of angiotensinogen and bradykininogen was carried out.

Preparation of dog plasma. Blood was obtained by puncture of the jugular vein of unanaesthetized dogs. It was collected in cooled plastic centrifuge tubes containing heparin, and centrifuged for 10 min at 0° C.

Nephrectomy. Mongrel dogs of either sex were anaesthetized with ether and nephrectomized through a mid-line abdominal incision.

Materials. The angiotensin standard was synthetic "Hypertensin, Ciba," 5-valyl angiotensin amide II, of which 6 ng=1 rat unit. A standard preparation of bradykinin was made by incubating heated dog-plasma with crystalline trypsin and purifying on alumina by the method of Hamberg & Deutsch (1958). " Padutin " (Bayer) was used as the source of kallikrein; the commercial preparation contains peptidases (chymotrypsin and aminopeptidase) which rapidly destroy any kallidin formed by kallikrein. To inactivate these peptidases the kallikrein solution was heated for 30 min at 62° C before the release of kallidin was to be measured. Under these conditions most of the kallikrein is preserved. An extract of renin containing 500 Buenos Aires units/ml. was prepared from hog kidneys by the method of Braun-Menéndez, Fasciolo, Leloir, Muñoz & Taquini (1943). The extract was almost free of angiotensinase. A Buenos Aires unit was defined by Leloir, Muñoz, Braun-Menéndez & Fasciolo (1940) as the amount of renin which, on incubation for 2 hr at 37° C and at *p*H 7.4 with an excess of angiotensinogen in 20 ml. total volume, releases 0.5 U of angiotensin. One unit of angiotensin is the amount which raises the arterial pressure of a dog, anaesthetized with chloralose, by 30 mm Hg.

RESULTS

Effect of kallikrein on bradykininogen. If kallidin and bradykinin are formed from the same substrate, treatment of plasma with kallikrein should reduce the amount of bradykinin which can subsequently be formed by incubation with trypsin. This possibility was tested as follows. Four tubes were prepared each containing 1 ml. of dog plasma diluted to 7 ml. with 0.9% saline. The contents of tube 1 were boiled for 4 min and then 0.4 U of unheated kallikrein (in 1 ml. of 0.9% saline) were added. The same amount of kallikrein was added to tubes 2 and 3 which were incubated for 15 and 30 min respectively and then boiled for 4 min. Tube 4 was incubated for 30 min after addition of 1 ml. of 0.9% saline without kallikrein; it was then boiled for 4 min. The contents of each tube were divided into two 4 ml. fractions. One fraction was used for bradykininogen estimation and the other was used as a control to show that any kallidin formed had been inactivated. The

TABLE 1

DESTRUCTION OF BRADYKININOGEN BY KALLIKREIN

Four units of kallikrein were incubated with 1 ml. of plasma in 8 ml. of total volume. After incubation, tubes were boiled for 4 min, their content divided, and 0.5 mg of trypsin was added to one fraction (to estimate the bradykininogen) and 0.5 ml. of saline to the other (to verify if all the kallidin formed has been destroyed)

Time of incubation (min)	Depressor effect of		Bradykininogen	
	Trypsin (mm Hg)	Saline (mm Hg)	Content (U)	Destroyed (U)
0 (Kallikrein)	−45	−6	45	—
15 (Kallikrein)	−27	−6	16	29
30 (Kallikrein)	−23	−5	11	34
30 (Saline)	−41	−4	47	—

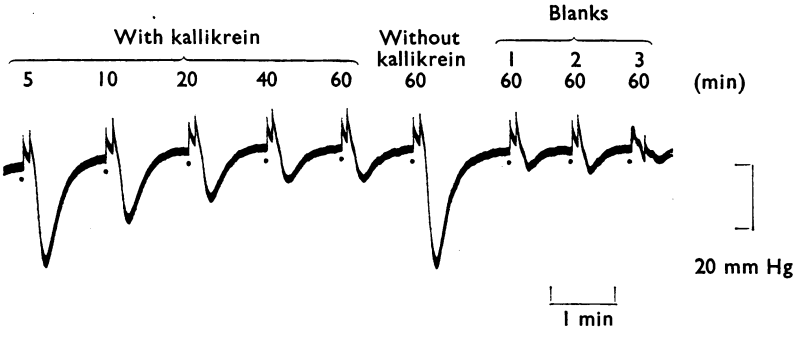

Fig. 1. Utilization of bradykininogen by kallikrein. Tubes with 0.1 ml. of dog plasma in 3.5 ml. of saline were incubated with 0.2 U of kallikrein at 37° C for different periods of time. After 4 min of boiling, the bradykininogen contents were estimated from the falls in blood pressure of the dog perfused leg due to bradykinin released by incubation with 0.5 mg of trypsin. Blanks of plasma (1), trypsin (2) and kallikrein (3) were also run.

results are shown in Table 1. Incubation with kallikrein for periods of 15 min and 30 min reduced the plasma bradykininogen level by 64% and 76% respectively. A similar experiment is illustrated in Fig. 1; bradykininogen gradually diminished in content and almost completely disappeared after 40 min of incubation.

Effect of temperature on kallidinogen. Tubes containing 0.25 ml.-aliquots of dog plasma diluted to 3.5 ml. with 0.9% saline were heated at 62° C and 70° C for 30 min and at 97° C for 5 min. After cooling, 0.5 mg of trypsin and 0.005 and 0.2 U of heated kallikrein were added and the mixtures were incubated for 30 min. Kallikrein formed more kallidin from plasma heated at 62° C than from plasma heated at 70° C. After heating at 97° C only the higher dose of kallikrein formed kallidin (Table 2). Trypsin, on the other hand, formed similar amounts of bradykinin from each of the three plasmas, and the vasodilator responses were greater than those obtained with kallikrein (Fig. 2, left-hand record).

TABLE 2

EFFECT OF HEATING UPON KALLIDINOGEN

Dog plasma was heated at different temperatures, incubated with kallikrein (previously heated to 62° C during 30 min) and with trypsin during 30 min. Duplicate experiments were performed. The values given are the fall of the perfusion-pressure of the isolated dog leg in mm Hg

	Dog plasma (0·25 ml. in 3·5 ml. of saline)						Blanks	
Incubated with	62° C Expt. 1	30 min Expt. 2	70° C Expt. 1	30 min Expt. 2	97° C Expt. 1	5 min Expt. 2	Trypsin	Kallikrein
Kallikrein (0·005 U)	−29	−30	−8	−19	−5	−3	−2	−3
Kallikrein (0·2 U)	−37	−38	−31	−28	−13	−14	−2	−6
Trypsin (0·5 mg)	−54	−44	−53	−46	−50	−49	−3	−4
Saline	−3	−6	−5	−3	−4	−4		

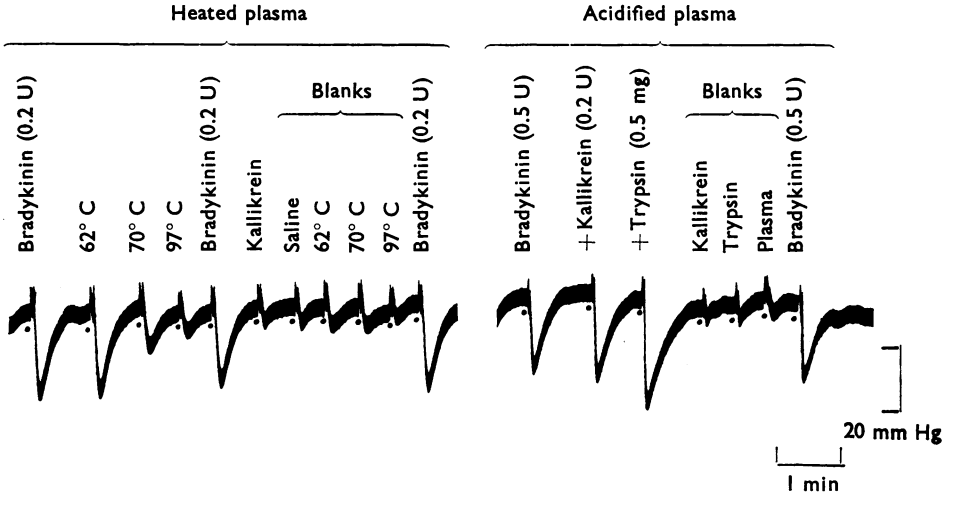

Fig. 2. Effects of heating and of acidification on kallidinogen content of plasma, assayed on the blood pressure of the dog perfused leg. Left-hand record: fractions of 0.5 ml. of dog plasma in 3.5 ml. of saline were heated at 62° C and at 70° C for 30 min, and at 97° C for 5 min, and were incubated with 0.002 U of kallikrein (heated at 62° C for 30 min). Right-hand record: dog plasma was incubated at *p*H 2.5 and 37° C for 30 min. After dialysis, 0.2 ml. fractions were incubated with 0.2 U of kallikrein and 0.5 mg of trypsin. Tests with standard solution of bradykinin at the beginning and at the end of the records show that there was no change in the sensitivity of the preparation.

Effect of acid on kallidinogen. Dog plasma was diluted with an equal volume of 0.9% saline, adjusted to *p*H 2.5 with phosphoric acid, and incubated at 37° C for 30 min. After neutralization with sodium hydroxide the plasma was incubated again for 60 min and then dialysed for 72 hr to remove any kallidin which had been formed. One aliquot was incubated with 0.2 U of kallikrein and another with 0.5 mg of trypsin. Kallikrein and trypsin were each able to form vasodilator substances from this acid-treated substrate, though trypsin yielded larger amounts (Fig. 2, right-hand record).

Angiotensinogen was found to be more labile than kallidinogen, being rapidly destroyed both by acidification to *p*H 2.5 and by heating at 60° C.

Effect of kallikrein on angiotensinogen. Two 2-ml. aliquots of dog plasma were each diluted to 7 ml. with buffered 0.9% saline. To one, 5 U of unheated kallikrein were added and the other was used as a control. Both mixtures were incubated at 37° C for 90 min. Then angiotensinogen and bradykininogen contents of each were estimated. Previous incubation with kallikrein completely exhausted the bradykininogen content whereas the angiotensinogen level was unaffected (Fig. 3).

Effect of nephrectomy on angiotensinogen and bradykininogen levels. The plasma angiotensinogen and bradykininogen levels of eight dogs were estimated before and at intervals after bilateral nephrectomy. The angiotensinogen level 24 hr after nephrectomy was more than double the control level, and after 48 hr it was about three times higher. Bradykininogen levels, on the other hand, were little if at all changed (Figs. 4 and 5).

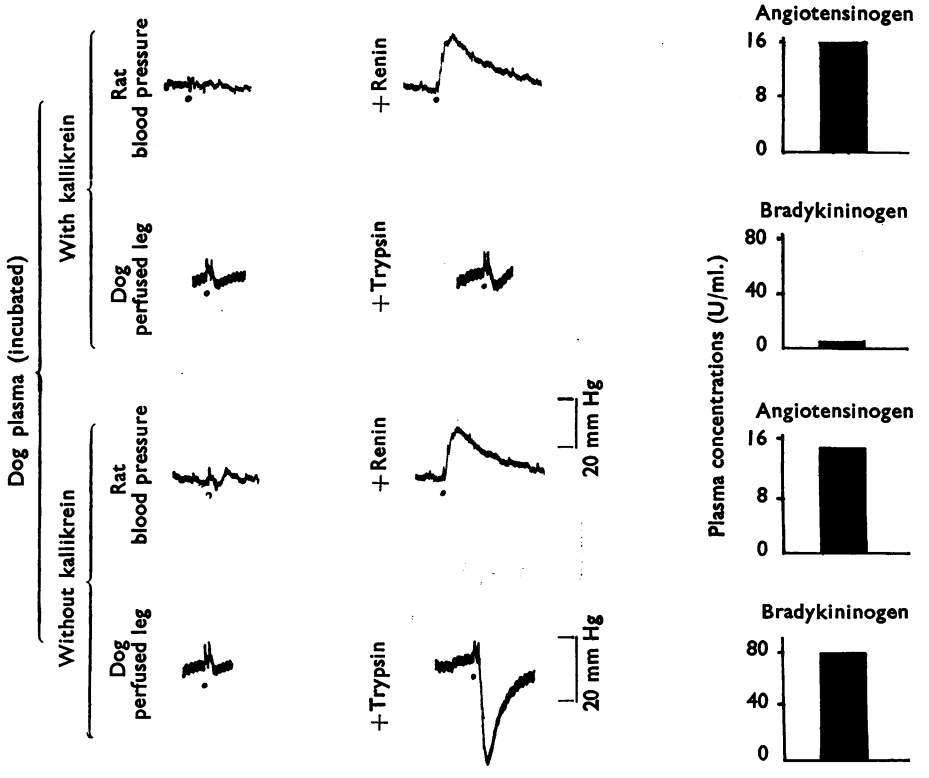

Fig. 3. Effect of kallikrein on angiotensinogen content of plasma, assayed on blood pressure of the rat and of the dog perfused leg. After incubating 2 ml. of dog plasma with 5 U of kallikrein for 90 min at 37° C, its angiotensinogen content was estimated with renin (assayed on the rat blood pressure) and its bradykininogen content with trypsin (assayed on the dog perfused leg). The bradykininogen content was exhausted, while the angiotensinogen content remained unchanged.

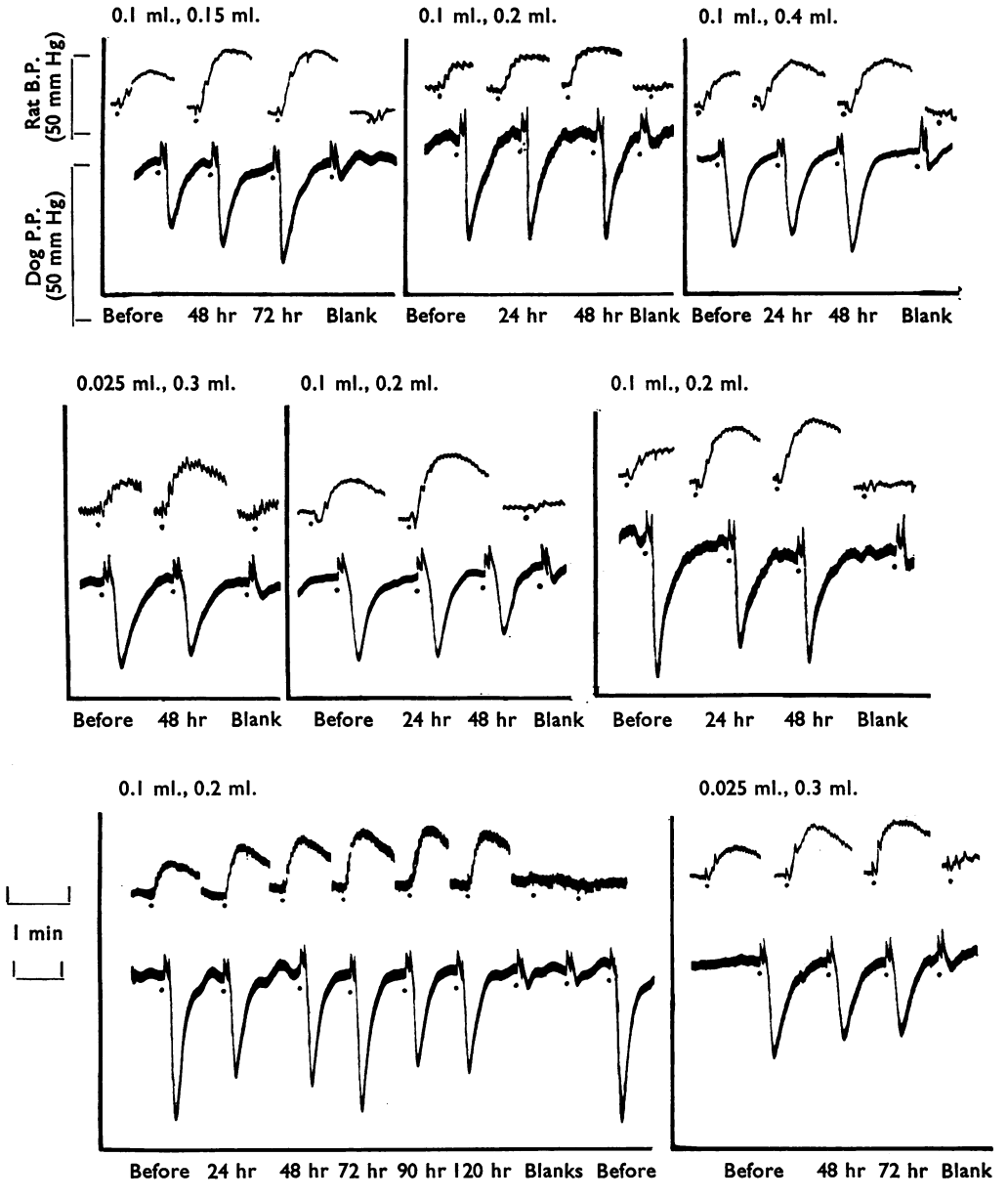

Fig. 4. The bradykininogen and angiotensinogen contents of the plasma of eight nephrectomized dogs. In each experiment the upper record shows the angiotensinogen assay on the rat blood pressure, and the lower record shows the bradykininogen assay on the perfusion pressure of the dog leg. Estimations were of contents before, and at indicated times after, nephrectomy. Blanks were with plasma without renin or trypsin. Volumes indicated above the records refer to the amounts of plasma used for the angiotensinogen and bradykininogen assays respectively. Time scales refer to the upper and lower records.

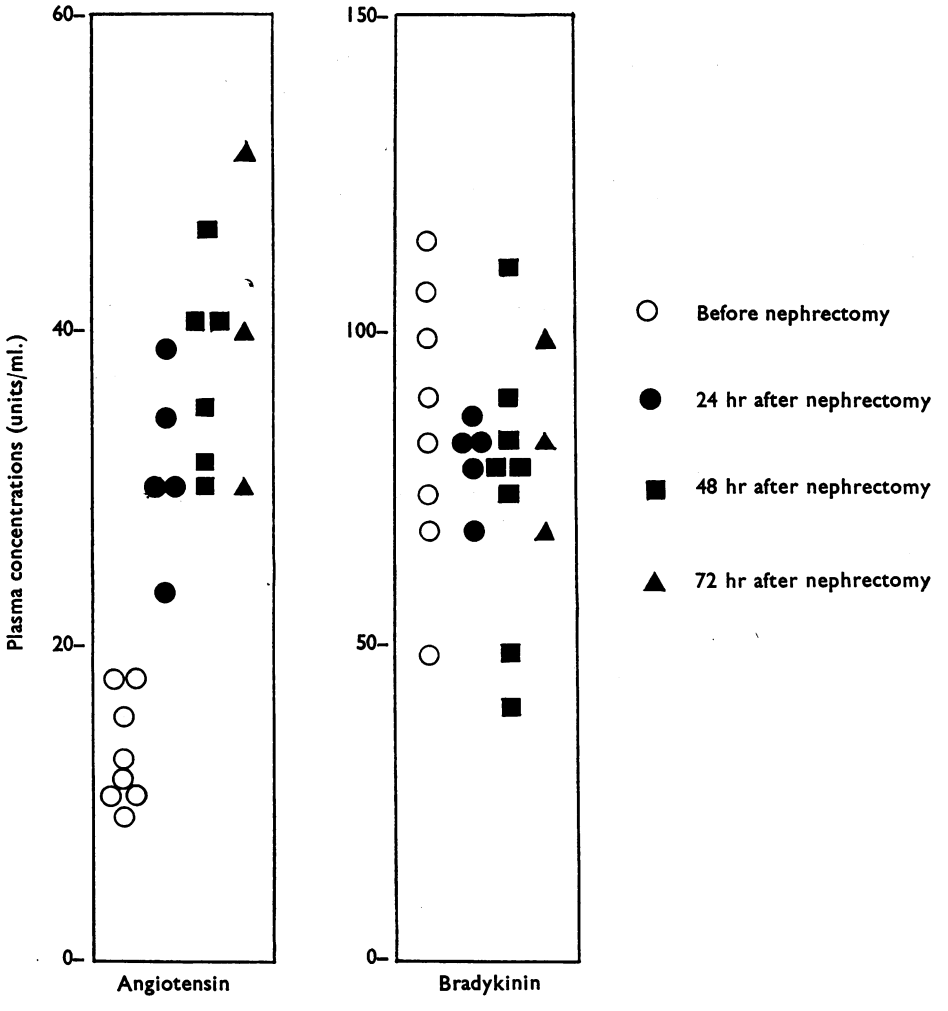

Fig. 5. Angiotensinogen and bradykininogen contents of the plasma after bilateral nephrectomy. The angiotensinogen and bradykininogen contents are expressed in units/ml.

DISCUSSION

Kallikrein can reduce or even exhaust the bradykininogen content of plasma. This result suggests that kallidin and bradykinin are derived from the same plasma globulin. Van Arman (1955) has reported that kallikrein will produce kallidin from Cohn's fraction IV-6 of plasma which has been boiled for 15 min. He also showed that the trypsin substrate is similarly unaffected by boiling at acid *p*H. In the present experiments heat-denatured substrate formed kallidin less readily, though bradykinin formation was unimpaired. Trypsin can digest denatured proteins, and these results suggest that its ability to form bradykinin is similarly unaffected by

heat-denaturation of substrate. Kallikrein, on the other hand, acts less readily on the denatured substrate. This difference in activity does not provide evidence that the two substrates, bradykininogen and kallidinogen, are different.

Acid-denaturation of dog plasma has been used by Horton (1959) for the preparation of kallikrein substrate, free from kinin-inactivating enzymes. The present experiments confirm that acid-denaturation of plasma prevents neither the formation of kallidin by kallikrein, nor the formation of bradykinin by trypsin. None of these experiments provides any evidence that kallidinogen and bradykininogen are different proteins.

In contrast there is good evidence that renin and kallikrein act upon different substrates. The renin substrate is readily denatured by boiling and by acid (Braun-Menéndez *et al.*, 1943). According to Van Arman (1955), serum which has been incubated with renin and so depleted of its angiotensinogen cannot thereafter form bradykinin on incubation with trypsin. This has not been confirmed in the present investigation. After depletion of kallidinogen (and bradykininogen) by kallikrein, plasma can still form angiotensin on incubation with renin.

After nephrectomy, plasma angiotensinogen levels rose whereas bradykininogen levels remained constant. This result provides additional evidence for the lack of identity of the two substrates. The mechanism by which bilateral nephrectomy raises the angiotensinogen levels of plasma is still unknown. It has been postulated that this effect may be due to lack of substrate consumption because of the absence of renin.

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