Biochemical Pharmacology, Vol. 21, pp. 2779–2792. Pergamon Press, 1972. Printed in Great Britain.

PARTIAL PURIFICATION AND CHARACTERIZATION OF A RENIN-LIKE ENZYME FROM RAT SUBMANDIBULAR GLAND

E. HACKENTHAL, CHR. KOCH, TH. BERGEMANN and F. GROSS

Pharmakologisches Institut der Universität Heidelberg, Heidelberg, Deutschland

(*Received* 9 *February* 1972; *accepted* 8 *May* 1972)

Abstract—A renin-like enzyme from rat submandibular gland was purified 40-fold by repeated chromatography on DEAE cellulose columns. The reaction product of this enzyme with a purified renin substrate from rat plasma was identified as angiotensin II by paper chromatography, high voltage electrophoresis and radioimmunoassay. The rate of angiotensin release from substrate is proportional to enzyme concentration, is maximal between pH 5·8 and 6·1 and obeys first order kinetics with regard to substrate concentrations up to 3200 ng/ml. The rate of angiotensin generation declines during the incubation period, reaching almost zero at 30 min. A variety of possible causes for this behaviour such as angiotensinases, substrate consumption, substrate specificity, formation of an inhibitor and changes in the incubation conditions could be excluded. An unusual stimulation of the enzyme substrate reaction by bifunctional SH reagents was observed. Direct intravenous injection of the enzyme to rats produced a transient rise in blood pressor, which differs qualitatively and quantitatively from that produced by renin. It is concluded that the rat submandibular enzyme is clearly distinct from renin as well as from isorenin of mouse submandibular gland. Comparison with pseudorenin is not possible due to lack of kinetic data for the latter enzyme.

IN A previous report, the presence of a renin-like enzyme* in rat submandibular gland has been demonstrated.[1] When compared on the basis of angiotensin-like pressor substance generation, the concentration of this enzyme in rats is much less than that of isorenin found in submandibular glands of male mice.[2–4] In addition, no sexual dimorphism in enzyme activity was observed in rats. Indirect evidence suggested that the pressor substance formed during the incubation of rat submandibular gland extracts with rat plasma is identical with angiotensin.

The present paper describes the partial purification of the renin-like enzyme, some of its kinetic characteristics and the identification of the reaction product as angiotensin.

MATERIALS AND METHODS

Preparation of crude extracts from submandibular glands. Male Wistar rats weighing 250–300 g were used. The animals were fed a standard commercial diet and given tap water *ad lib.*

Submandibular glands were removed under ether anaesthesia, freed from adherent fat and connective tissue and stored at −20°. The thawed tissue was homogenized in 9-parts of 2 mM dimercaptopropanol (BAL) in water by means of a knife-blade homogenizer (Ultra-Turrax, Jahnke and Kunkel, Germany) 4 times for 45 sec with intermittent 1 min periods of cooling. The homogenate was centrifuged at 5000 *g*

* The term "renin-like enzyme" is used for the sake of convenience and is not intended to suggest a close relationship to either renin or isorenin.

for 15 min, the clear yellow supernatant decanted and the pellet re-extracted with approximately 3 vol. of 2 mM BAL in water.

Purification procedure. Dry DEAE-cellulose powder (Cellex D, Biorad Laboratories, 1·2 g per gram wet tissue) was added to the combined supernatants. The slurry was stirred for 60 min and subsequently centrifuged at 3000 *g* for 10 min. The supernatant was discarded, and the cellulose pellet washed twice with 5 vol. of 5 mM Tris–HCl buffer (pH 7·4). The enzyme was eluted with a solution of 0·8 M NaCl in 10 mM Tris–HCl (pH 7·4, 10 ml per gram original tissue). The elution was repeated and the combined eluates were dialysed for 24 hr against several changes of 10 mM Tris–HCl buffer (pH 7·4) containing 2 mM BAL. During the last 6 hr of dialysis, BAL was omitted to exclude possible interference of BAL in the subsequent column fractionation.

The dialysed material was fractionated on a DEAE-cellulose column (50 × 2·5 cm) previously equilibrated with 10 mM Tris–HCl (pH 7·4) by elution with a linear gradient of NaCl from 0 to 1·5 M in the same buffer. Elution was monitored by continuous recording of protein absorption at 280 nm. Fractions of 2·5 ml were collected at a flow rate of 10 ml per hr.

The fractions were analysed for renin-like activity, esterolytic activity, chloride, protein and, in some cases, angiotensinase activity (see below).

During the first steps of enzyme purification, extraction of angiotensin by the ion exchange column procedure of Boucher *et al.*[6] was performed to eliminate substances interfering with the bioassay of angiotensin. During the later stages of enzyme purification and in all subsequent kinetic experiments the standard enzyme assay was used, as described below.

Fractions with renin-like activity were pooled, dialysed for 24 hr against 10 mM Tris–HCl (pH 7·4), reduced in volume by partial lyophilization and re-dialysed against the same buffer. The resulting preparation was chromatographed a second time on the same column under the same conditions. The bulk of renin-like activity was usually eluted in one or two fractions, which were stored at −20° and used, without further purification, for all subsequent kinetic experiments. All procedures described above were performed at temperatures between 0°–5°.

Esterolytic activity. This was estimated by the procedure described by Trautschold and Werle[5] utilizing benzoyl-arginine-ethylester (BAEE) as a substrate. Results are expressed as μmoles BAEE hydrolysed per min.

Substrate preparation. Crude renin substrate (angiotensinogen) was prepared as described by Boucher *et al.*[6] from the plasma of rats which had been nephrectomized 24 hr previously. This procedure yields a preparation containing 50–80 ng substrate per milligram protein, expressed as ng angiotensin liberated by an excess of hog renin. Though virtually free of angiotensinase activity, this preparation is not satisfactory for kinetic experiments, since systems requiring high substrate concentrations would become overloaded with high concentrations of inert protein (up to 50 mg protein per millilitre). Therefore, the substrate was further purified by successive fractionation on DEAE–cellulose and phosphorylated cellulose (Cellex P, Biorad) columns, to yield preparations containing from 500 to 1000 ng substrate per milligram protein. Unless otherwise stated, for all kinetic experiments described in this study, a substrate preparation with a specific concentration of 560 ng per milligram protein was used. This preparation was free of measurable angiotensinase and renin activity (see below).

Standard enzyme assay. The reaction mixture contained 30 mM BAL, 500 ng renin substrate (560 ng/mg protein) in a final volume of 0·25 ml of 60 mM citric acid–phosphate buffer (pH 5·8). After a pre-incubation of 4 minutes at 37°, the reaction was started by the addition of 50 μl of the enzyme preparation. The tubes were incubated for 15 min at 37° and were then immersed in boiling water for 5 min after 50 μl of 0·02 N HCl had been added. Subsequently, the tubes were placed in an ice bath and the volume was adjusted to 1·0 ml by the addition of 1% NaCl. Insoluble matter was removed by centrifugation (10 min, 10,000 *g*) and pressor activity in the supernatant was determined in the nephrectomized, pentolinium-treated rat by comparison with val-5-angiotensin II amide.[7,8]

Angiotensinases. The activity of angiotensin inactivating peptidases was measured by incubating the enzyme preparation with 50 ng of val-5-angiotensin II amide (Hypertensin, Ciba) in a total volume of 0·3 ml 60 mM citric acid–phosphate buffer (pH 5·8) for variable periods at 37°. The amount of angiotensin remaining after the incubation was measured by rat bioassay. A given preparation was considered free of angiotensinases when neither val-5-angiotensin II amide, val-5-angiotensin II nor ileu-5-angiotensin I was inactivated during 30 min of incubation at 37° at either pH 5·8 or 7·4.

Protein. Was determined by the method of Lowry *et al.*[9] and chloride by the mercury–chloranilate method described by Renschler.[10] All substances used were reagent grade. The Wellcome Research Laboratories kindly provided 5-ileu-angiotensin I; val-5-angiotensin II amide (Hypertensin) and val-5-angiotensin II were a gift from Ciba, Basle. Hog renin was obtained from Nutritional Biochemicals Corp., Cleveland, Ohio.

RESULTS

Enzyme purification. Crude extracts of rat submandibular glands contain considerable amounts of peptidases which rapidly hydrolyse angiotensin and are not completely inhibited even by high concentrations of BAL and/or EDTA. Furthermore, during incubation of extracts with renin substrate, large quantities of vasodepressor material are formed, probably by the action of kallikrein and related enzymes which are present in rat submandibular glands in high concentrations.[11]

The latter complication, which does not permit the direct pressor assay for angiotensin in supernatants after incubation, can be circumvented by isolation of angiotensin on resin columns.[6] However, this procedure is tedious and not sufficiently accurate for kinetic studies. Therefore, an attempt was made to purify the renin-like enzyme. The main concern was to obtain a preparation devoid angiotensinases and kallikreins and with a minimum loss of enzyme activity, rather than to obtain the enzyme in a highly purified state. Results of a typical purification experiment are listed in Table 1. Starting with 15·6 g of frozen submandibular glands, the overall purification from the crude extracts was about 40-fold with a recovery of 70 per cent. Although kallikreins contribute only about 50 per cent to the total esterolytic activity of submandibular gland extracts,[11] this measurement was used for the estimation of kallikrein. By the purification procedure described, more than 99·5 per cent of the esterolytic activity present in crude extracts were eliminated. The final enzyme preparation was free of angiotensinases, as judged by the criteria already described.

Identification of the pressor product as angiotensin. Renin substrate was incubated

TABLE 1.

Purification step	Volume (ml)	Total protein (mg)	Renin-like enzyme		Total esterolytic activity units† (mMol BAEE/10 min)
			Total units* (μg angiotensin/15 min)	Specific activity (units/mg protein)	
Homogenate	156	2380	2472	1·04	1060
Crude extract	226	2305	3155	1·37	1157
Cellex-D batch eluate	254	736	2774	3·76	584
Active pool from cellex-D-column I	41	182	2384	13·1	69
Active fractions from cellex-D-column II	7	42·6	2304	54·0	3

* 1 unit of renin-like activity is defined as the amount of enzyme preparation, which liberates 1000 ng of angiotensin in 15 min at 37° under standard assay conditions.

† 1 unit of esterolytic activity is defined as the amount of enzyme preparation, which hydrolyses 1 ml of BAEE in 10 min at 22° under standard assay conditions. For details see Materials and Methods.

with an excess of enzyme preparation under standard conditions and the pressor principle formed was purified on a small column (7 × 1 cm) of Sephadex G-10 previously equilibrated with 5 mM pyridinium-acetate (pH 4·5). The eluted fractions were assayed for pressor activity; the pooled active fractions were then lyophilized, dissolved in a small volume of pyridinium-acetate buffer and applied to chromatography paper (Whatman No. 1). As a reference, 10 μg each of angiotensin I and II were used. After descending chromatography (butan-2-ol: 3% NH_3; 25 : 11, v/v) or high voltage electrophoresis (0·15 M pyridinium-acetate, pH 4·5, 70 min, 40 V/cm) the outer margins of the chromatograms, containing the reference standards, were cut off and stained with Folin's reagent followed by 50% saturated sodium carbonate. The angiotensins appear as dark blue spots on a light background. This method has a lower limit of sensitivity of approximately 2 μg angiotensin. Since the total amount of unknown pressor activity applied to the paper was too low to allow location by staining, the centre parts of the chromatogram were cut into vertical strips (1 cm wide), which were eluted at room temperature by shaking for 30 min with 1 ml of 1% NaCl in 50 mM phosphate buffer (pH 7·4). Pressor activity in the extracts was determined by bioassay.

When enzyme and substrate had been incubated in the presence of 2 mM EDTA the unknown pressor product migrates like angiotensin II, in the chromatographic as well as in the electrophoretic system. When the EDTA concentration was raised to 10 mM, an additional active peak occurs, which migrates like angiotensin I. This can be taken as additional evidence for the identity of the pressor product with angiotensin, since it is well documented that the enzymatic conversion of angiotensin I–II is inhibited by high concentrations of EDTA.[12] Further evidence was obtained by radioimmunoassay of the pressor principle. In a radioimmunoassay system for angiotensin II,[13] all of the pressor material generated by the enzyme during incuba-

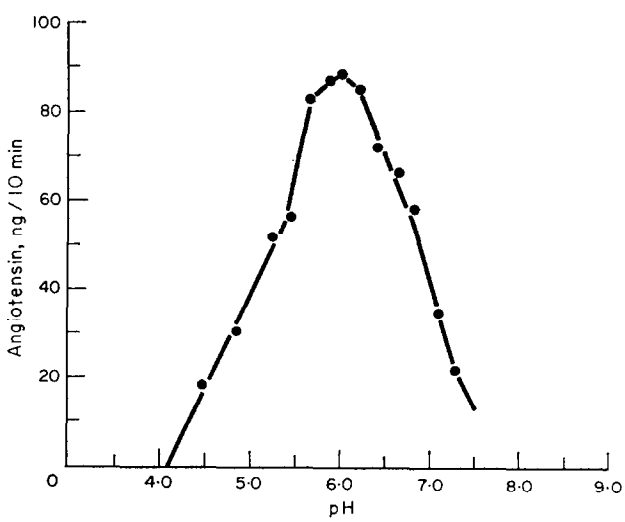

FIG. 1. Effect of pH on the rate of angiotensin formation. The reaction mixtures contained 100 mM citric-acid–phosphate buffer at different pH, 795 ng substrate, 3·3 μg enzyme preparation and 10 mM BAL, in a final volume of 0·25 ml. The mixtures were incubated at 37° for 10 min. Each point represents the mean of three experiments.

tion in the absence of EDTA could be accounted for as angiotensin II. No angiotensin I was detectable in a system specific for angiotensin I. Finally, the characteristics of blood pressure response to the purified incubation product are indistinguishable from that of angiotensin.

Effect of pH and enzyme concentration. The enzyme exhibits maximum activity between pH 5·7 and 6·2 (Fig. 1). Half maximal activities were observed at pH 5·1 and 6·9.

Angiotensin generation is proportional to the enzyme concentration, as can be seen from Fig. 2.

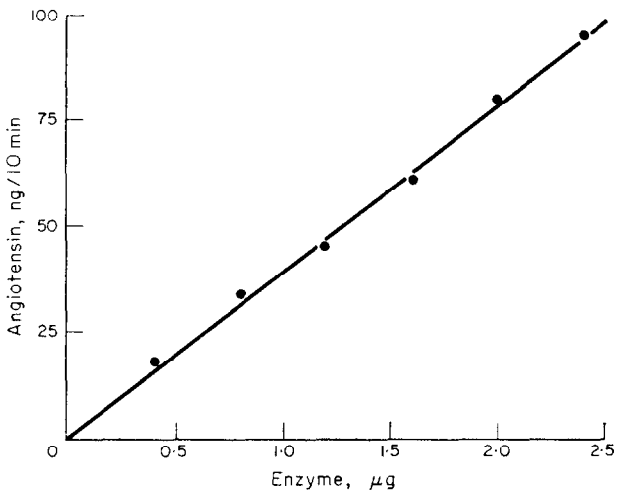

FIG. 2. Effect of enzyme concentration on the rate of angiotensin formation. Standard incubation conditions as described in Materials and Methods.

Effect of substrate concentration. Figure 3 demonstrates that angiotensin generation is proportional to the initial substrate concentration up to 3200 ng substrate per ml, i.e. the reaction follows first-order kinetics with regard to substrate concentration in this range. Consequently, no K_m could be established.

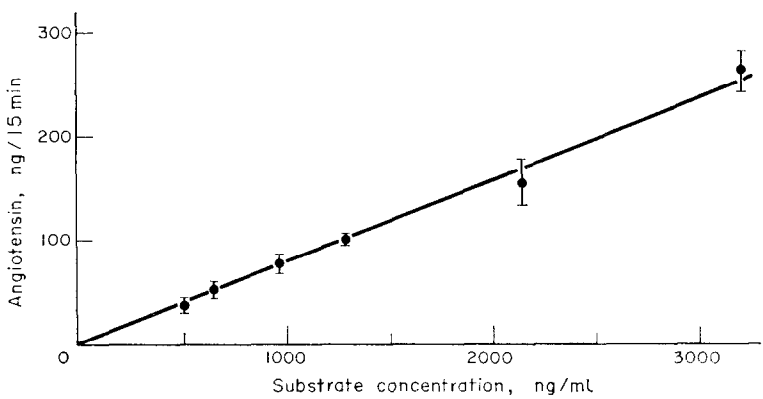

FIG. 3. Effect of substrate concentration on the rate of angiotensin formation. Standard incubation conditions except for variation of substrate concentration. 2·6 μg enzyme preparation per incubation mixture. Points represent means ±S.D. of four experiments.

B.P. 21/20—H

TABLE 2. ANGIOTENSIN GENERATION FROM DIFFERENT SUBSTRATES

Substrate preparation	Substrate concentration (ng/250 μl incubation mixture)	Specific substrate concentration (ng/mg protein)	Enzyme added (μg)	Angiotensin formed (ng/10 min)	Angiotensin formed (ng/10 min/μg enzyme)
1	500	74	3·2	32	10·0
2	430	185	1·6	37	23·1
3	470	560	0·7	57	81·3

Substrate preparations 1–3 were those obtained at different stages of the purification and were free of angiotensinase activity. The amount of enzyme preparation added to the different incubation mixtures was adjusted to produce sufficient angiotensin for accurate bioassay, without leading to excessive substrate consumption Since angiotensin generation is proportional to enzyme concentration (cf. Fig. 2) the results can be calculated as angiotensin generation per μg enzyme. The incubation mixtures containing the substrate preparations and the amount of enzyme indicated were incubated in a final volume of 250 μl 60 mM citric-acid–phosphate buffer pH 5·8 for 10 min at 37°.

It was observed that the rate of angiotensin release per μg of enzyme preparation varied with different substrates. Since the initial substrate concentrations were the same in these incubations or varied only within narrow limits, the variations observed do not reflect differences due to first-order kinetics. Table 2 demonstrates that the rate of angiotensin release per μg of enzyme preparation is roughly proportional to the specific substrate concentration (expressed as ng angiotensin per mg protein). All substrate preparations used were free of angiotensinase activity.

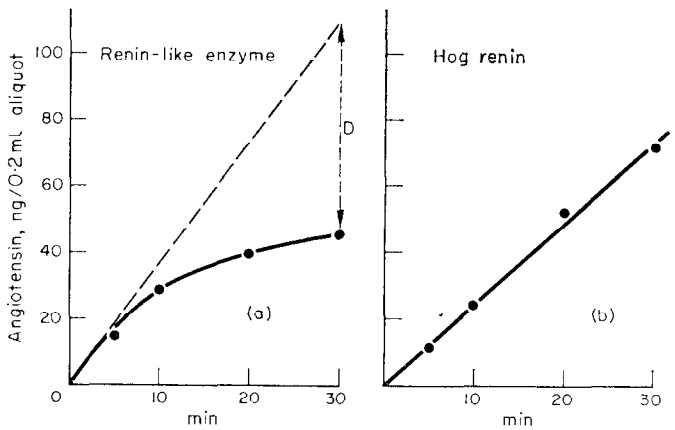

FIG. 4a and b. Time course of angiotensin formation by renin-like enzyme (4a) and hog renin (4b). The incubation mixture contained 5000 ng substrate, 20 mM BAL, 60 mM citric-acid–phosphate buffer (pH 5·8) and 16 μg renin-like enzyme or 12 mU hog renin in a total volume of 2·0 ml. During incubation at 37°, 0·2 ml aliquots were taken at the time intervals indicated and the angiotensin formed was estimated by bioassay. Ordinate: Angiotensin found in 0·2 ml aliquot. Dashed line: extrapolation of initial velocity. For explanation see text.

Effect of incubation time. When angiotensin generation was measured under standard conditions at different time intervals, it was consistently found to decline during the incubation. After 30 min of incubation, little additional angiotensin was formed (Fig. 4a). The pH of the incubation mixture had not changed during this period. Hog renin, when incubated under the same conditions shows linear time-dependence of angiotensin formation (Fig. 4b). Thus, it seems reasonable to conclude that factors other than the incubation conditions must be responsible for this phenomenon. Some other possible causes could be ruled out by the following experiments or considerations.

(1) Although there was no detectable angiotensinase activity either in the enzyme or in the substrate preparation, this was further ascertained as follows. If one assumes that the apparent decline in angiotensin generation is due to enzymatic degradation of angiotensin, whereas the true generation proceeds at a constant rate, it is possible to calculate the amount of angiotensin degraded during the 30 min incubation by extrapolating the initial velocity to 30 min. This hypothetical situation is depicted in Fig. 4a by the dashed line. In this case, the difference (D) between the total amount generated and the amount actually found after 30 min is about 50 ng per 0·2 ml aliquot, i.e. 10 per cent of the initial substrate. Thus, if this assumption were valid, the sum of angiotensin found after 30 min plus the remaining substrate should be 90 per cent of the initial substrate. However, by converting the remaining substrate

quantitatively to angiotensin by addition of an excess of hog renin, 98–103 per cent of the initial substrate were recovered as angiotensin in 3 experiments. One can therefore conclude that enzymatic degradation of angiotensin does not account for the decline in velocity.

(2) Since the reaction–velocity at the substrate concentration used (2000 ng/ml) is first-order with respect to substrate, a decrease in substrate concentration might be considered responsible for the decline in product formation. This possibility is excluded by the fact that substrate consumption is less than 10 per cent during the 30 min incubation period.

(3) From the work of Skeggs *et al.*,[14] it is known that the renin substrate in plasma is not represented by a single homogeneous protein, but comprises several distinct protein fractions. The renin-like enzyme from rat submandibular gland may have a relative or absolute specificity for only one or two of these fractions. In this case, a time-dependence with the same characteristics as shown in Fig. 4a would result from exhaustion of this particular substrate. In order to clarify this point, a standard incubation experiment was performed, in which small aliquots were withdrawn for the estimation of angiotensin at 5, 10, 20 and 30 min. At 31 min, an additional amount of enzyme was added in a small volume so that the enzyme concentration in the incubation mixture was exactly twice that after 30 min. Again, small aliquots were withdrawn during the following 30 min for angiotensin assay. If the assumption that exhaustion of a substrate fraction with a higher affinity to the enzyme is responsible for the decline in angiotensin generation were valid, then a 2-fold increase of enzyme concentration at 31 min would produce only a 2-fold increase in the slope of angiotensin generation at this point (depicted by the dashed lines in Fig. 5). Actually, more

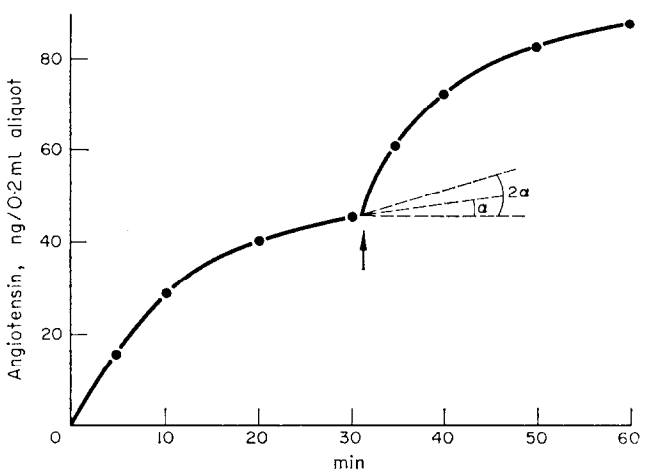

FIG. 5. Time course of the formation of angiotensin with repeated addition of enzyme. The incubation mixture contained 5000 ng substrate, 10 mM BAL, 60 mM citric-acid–phosphate buffer (pH 5·8) and 16 μg enzyme preparation. At 5, 10, 20 and 30 min 0·2 ml aliquots were taken for angiotensin assay. At 31 min, 9·6 μg enzyme preparation in 20 μl buffer were added (arrow) which exactly doubles the enzyme concentration at this point. Incubation with 0·2 ml sampling was continued as indicated. Figures on the ordinate represent the amounts of angiotensin found in the 0·2 ml aliquots. The dashed lines indicate the slope of the curve at 31 min and a 2-fold increase of this slope respectively. For further explanation see text.

than an 8-fold increase was observed (Fig. 5). Moreover, the second part of the curve is virtually identical to the first. Thus, exhaustion of substrate can be ruled out. Furthermore, the results of this experiment permit the conclusion that all possible causes for the decline in angiotensin generation which are related to substrate and the incubation conditions, such as evaporation of BAL which stimulates the enzyme (see below) and formation of a competitive inhibitor during the incubation, can be ruled out.

Sulfhydryl reagents and chelators. When the time course of the enzyme–substrate reaction in the presence or absence of BAL was compared, a striking stimulatory effect of BAL became evident (Fig. 6). With EDTA and chlorhexidingluconate, both frequently used as inhibitors of angiotensinases and converting enzyme,[15,16] no comparable effect was demonstrable.

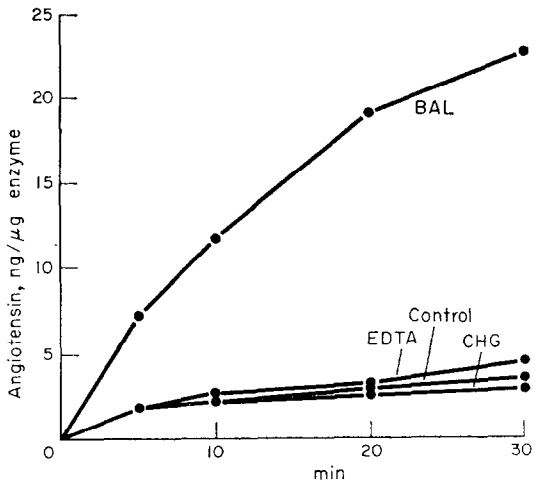

FIG. 6. Effect of BAL, EDTA and chlorhexidingluconate on the time course of angiotensin formation. The incubation mixture contained 1600 ng substrate, 19 μg enzyme preparation, 60 mM citric-acid–phosphate buffer (pH 5·8) and either 10 mM EDTA, 110 μg/ml chlorhexidingluconate (CHG), 10 mM BAL or no addition, in a total volume of 1·2 ml. At the time intervals indicated, 0·2 ml aliquots were taken and the angiotensin formed was estimated by bioassay. The incubation mixture, to which BAL was added contained only 7 μg of enzyme preparation to prevent substrate exhaustion. For comparison, angiotensin formation is plotted per μg enzyme preparation. The amounts of angiotensin actually measured after 30 min of incubation were 159, 87, 68·5 and 54 ng in incubations containing BAL, EDTA, no addition and chlorhexidingluconate, respectively.

The influence of varying concentrations of BAL and other bifunctional SH-reagents on angiotensin formation is shown in Figs. 7 and 8. An almost linear increase in angiotensin formation with increasing concentrations of BAL (up to 40 mM) or dithioerythrol or dithiothreitol (up to 10 mM) was observed. Cysteine and glutathione (5 mM) had no stimulatory effects.

Direct blood pressure effect. The intravenous injection of a small dose (5 mU) of hog renin elicits a typical, sustained increase in blood pressure, which has been used for the identification and quantification of renin preparations. Isorenin from submandibular glands of mice produces a blood pressure response which is indistinguishable from that of renin.[2–4] When the renin-like enzyme from rat submandibular gland is given to rats intravenously, the observed increase in blood pressure is of short duration

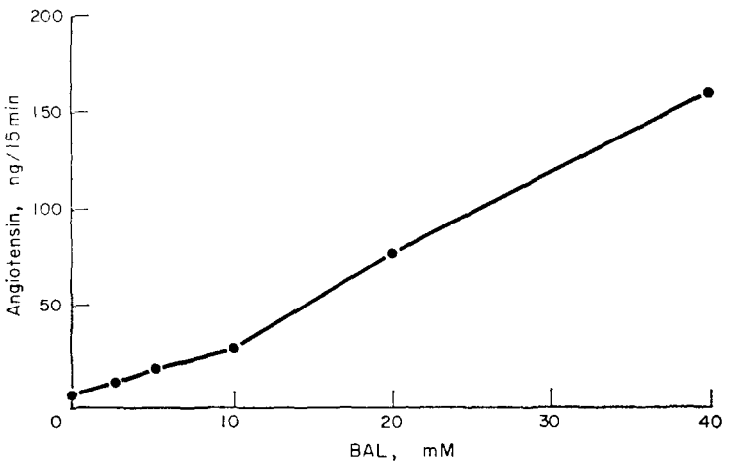

FIG. 7. Influence of dimercaprol (BAL) on the generation of angiotensin. Standard incubation mixtures containing 2·2 μg enzyme preparation, 640 ng substrate and different concentration of BAL. Incubation for 10 min at 37°.

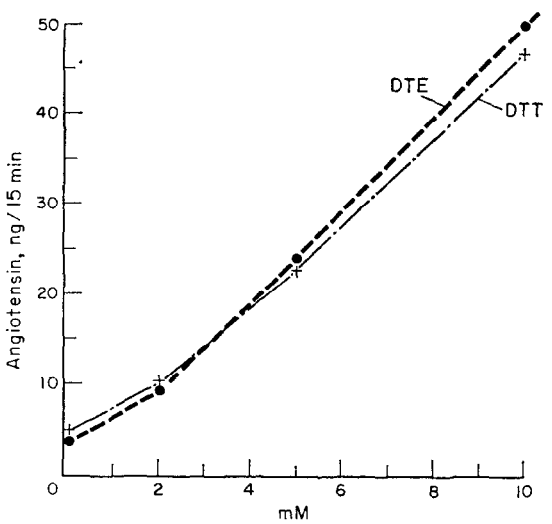

FIG. 8. Influence of dithiothreitol (DTT) and dithioerythrol (DTE) on the generation of angiotensin. Standard incubation mixtures containing 560 ng substrate, 1·5 μg enzyme preparation and DTE or DTT in different concentrations. Incubation for 15 min at 37°.

(Fig. 9). Furthermore, to obtain a blood pressure response comparable in height to that seen after the injection of 5 mU of hog renin, a dose of renin-like enzyme is required which corresponds to 100 mU of hog renin as compared on the basis of angiotensin formation under standard incubation conditions in the absence of BAL. The same amount of renin-like enzyme, when incubated with substrate in the presence of 20 mM BAL (which strongly stimulates this enzyme but not renin), generates angiotensin at a rate which corresponds to more than 1 U of hog renin.

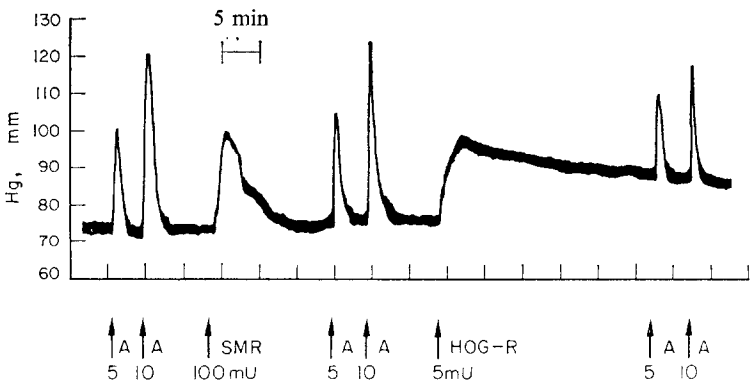

FIG. 9. Direct blood pressor effect of renin-like enzyme. A = val-5-angiotensin II amide (Hypertensin Ciba) figures in ng. SMR = renin-like enzyme 100 mU, Hog-R = hog-renin 5mU. For explanation see text.

DISCUSSION

Renin-like enzymes of extra-renal origin and distribution have been demonstrated in various tissues including rabbit and hog placenta and uterus,[17–19] hog arterial wall[20,21] and mouse submandibular gland.[2–4,22] The renin-like enzyme from mouse submandibular gland is of particular interest, since it is present in the gland in very high concentrations, reaching 20,000 Goldblatt Units per gram of tissue in adult male mice. Its concentration is much higher in mature males than in females, and is negligible in immature mice.[3,23] Based on indirect immunological evidence, Werle *et al.*[4] recently classified this enzyme as an isoenzyme of renin and proposed the term "isorenin". Bing and Farup[24] suggested that a relationship exists between the granules of the striated ducts and renin-like activity in the salivary glands, analogous to the granular location of renin in the juxtaglomerular apparatus of the kidney.

In view of these observations, it was disturbing that no renin-like activity was detected in rat submandibular glands[15] which contain granulated cells similar to those seen in mice.[11] Erdös *et al.*[11] isolated these granules and measured a high concentration of kallikrein, but did not analyse them for their possible content of renin-like activity. In a recent report from this laboratory,[1] the presence of a renin-like principle in rat submandibular glands has been demonstrated. This enzyme activity has escaped recognition by other investigators, probably because its concentration in the gland is much less than that of isorenin in mice glands. The pronounced sexual dimorphism observed in the isorenin content in mice[3,23] was not demonstrable in rats.

In view of the results presented here, it seems reasonable to conclude that the renin-like enzyme from rat submandibular gland is clearly distinguishable from renin as well as from mouse isorenin. This conclusion is based on the qualitative and quantitative differences in the direct blood pressure effects (Fig. 9), the stimulation by bifunctional SH reagents of the renin-like activity from rat gland but not of renin activity, and differences in other kinetic characteristics, such as the influence of substrate concentration and of incubation time. So far, the only property shared by the rat enzyme and renin (or isorenin) seems to be the capability to generate angiotensin from plasma substrate.

Recently Skeggs *et al.*[25] have described the presence of an angiotensin-forming enzyme in various tissues of the rat and have proposed the term "pseudo-renin". This enzyme, which was found in the highest concentrations in the submandibular gland, preferentially utilizes the synthetic tetradecapeptide as a substrate, to a much lesser degree (less than 1 : 2000) purified substrate A and does not generate angiotensin from crude plasma substrate. This type of substrate specificity obviously resembles the increase in angiotensin formation with increasing specific concentration of the substrate observed in this study. Whether this phenomenon in fact represents substrate specificity or removal of an inhibitor during substrate purification cannot be determined at the present time. The observation by Skeggs *et al.* that the reaction of pseudo-renin with tetradecapeptide is strongly inhibited in the presence of serum is in favour of the latter explanation. However, the lack of sufficient kinetic data for pseudo-renin makes it impossible either to ascertain or exclude the identity of pseudo-renin with the renin-like enzyme described here. At least one point of difference seems to be the dependence of the reaction velocity on substrate concentration. With purified substrate A, pseudo-renin exhibits a K_m of 700–2500 ng per ml,[11] whereas the renin-like enzyme generates angiotensin proportional to substrate concentration up to 3200 ng/ml. Among the properties of the renin-like enzyme described here, the non-linearity of angiotensin generation with time and the striking stimulatory effect of bifunctional SH-reagents are particularly noteworthy. Since various possible causes for the decline in angiotensin formation with increasing incubation time, such as change of pH, substrate exhaustion, angiotensinase activity, and an endogenous inhibitor could be excluded, the most likely explanation remains that the enzyme itself loses activity during the incubation, possibly by aggregation. The stimulatory effect of SH-reagents may then be via prevention of such aggregation. It seems unlikely that simple protection of SH-groups in the enzyme is involved, since this can be accomplished with most enzymes at much lower concentrations of Cleland's reagent.[26] Clearly, more experimental work is required to establish identity or non-identity with pseudo-renin and also to explain the unusual kinetic properties of the renin-like enzyme in rat submandibular gland.

REFERENCES

1. Ch. Koch and J. Unger, *Naunyn-Schmiedebergs Arch. exp. Path. Pharmak.* **264,** 257 (1969).
2. E. Werle, R. Vogel and L. F. Goldel, *Naunyn-Schmiedebergs Arch. exp. Path. Pharmak.* **230,** 236 (1957).
3. I. Trautschold, E. Werle, A. Schmal and N. Graf Hendrikoff, *Hoppe-Seyler's Z. Physiol. Chem.* **334,** 232 (1966).
4. E. Werle, I. Trautschold, K. Krammer and A. Schmal, *Hoppe-Seyler's Z. Physiol. Chem.* **349,** 1441 (1968).
5. I. Trautschold and E. Werle, in *Methoden der enzymatischen Analyse* (Ed. U. Bergmeyer), p. 880, Chemie Weinheim (1962).
6. R. Boucher, J. Menard and J. Genest, *Canad. J. physiol. Pharmac.* **45,** 5 (1967).
7. W. S. Peart, *Biochem. J.* **59,** 300 (1955).
8. F. Gross and F. Sulser, *Naunyn-Schmiedebergs Arch. exp. Path. Pharmak.* **229,** 374 (1956).
9. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193,** 265 (1951).
10. H. E. Renschler, *Klin. Wschr.* **40,** 484 (1962).
11. E. G. Erdös, L. L. Tague and L. Miwa, *Biochem. Pharmac.* **17,** 667 (1968).
12. P. A. Khairallah, F. M. Bumpus, I. H. Page and R. R. Smeby, *Science* **140,** 672 (1963).
13. E. Hackenthal, in preparation.
14. L. T. Skeggs, K. E. Lentz, H. Hochstrasser and J. R. Kahn, *J. exp. Med.* **118,** 73 (1963).
15. I. H. Page and J. W. McCubbin, in *Renal Hypertension*, p. 29, Year Book Medical Publishers, Chicago (1969).

16. J. W. RYAN, J. K. MCKENZIE, M. R. LEE, *Biochem. J.* **108,** 679 (1968).
17. F. GROSS, G. SCHAECHTELIN, M. ZIEGLER and M. BEYER, *Lancet* **1,** 914 (1964).
18. M. ZIEGLER, B. RINIKER and F. GROSS, *Biochem. J.* **102,** 28 (1967).
19. P. C. ESKILDSEN, *Acta Path. Microbiol. Scand.* **78,** 473 (1970).
20. H. DENGLER, *Naunyn-Schmiedebergs Arch. exp. Path. Pharmak.* **227,** 481 (1956).
21. A. B. GOULD, L. T. SKEGGS and J. R. KAHN, *J. exp. Med.* **119,** 389 (1964).
22. H. TURRIAN, *Helv. Physiol. Acta* **18,** 259 (1960).
23. W. J. OLIVER and F. GROSS, *Am. J. Physiol.* **213,** 341 (1967).
24. J. BING and P. FARUP, *Acta Path. Microbiol. Scand.* **64,** 203 (1965).
25. L. T. SKEGGS, K. E. LENTZ, J. R. KAHN, F. E. DORER and M. LEVINE, *Circulation Res.* **25,** 451 (1969).
26. N. HAUGARD, *Physiol. Rev.* **48,** 311 (1968).