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# KINETICS OF THE REACTION OF HUMAN RENIN WITH NATURAL SUBSTRATES AND TETRADECAPEPTIDE SUBSTRATE

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## SUMMARY

1. The reaction of purified human renin (EC 3.4.4.15) was studied with four substrates, two homologous (unpurified and partially purified plasma human substrate), one heterologous (hog substrate), and one of synthetic origin (tetradecapeptide substrate) representing the N-terminal sequence of the other three substrates.

2. The activity of renin was determined by specifically measuring with a radioimmunoassay the formation of angiotensin I, the direct product of the enzymatic reaction. The effects of pH, ionic strength, temperature and substrate concentration, were examined for each substrate, and the respective optimum pH, activation energies, maximal velocities and Michaelis constants were determined.

3. Human renin presented with the tetradecapeptide substrate a 100-times faster reactivity than with the other substrates, but accompanied by a 10-times lower affinity. With human substrate at physiological plasma concentration, a first-order reaction was obtained at optimum pH (5.5) but which tended to a zero-order at physiological pH. This indicates that whereas the substrate is a rate-limiting factor in the *in vitro* conditions of most methods of measurement of renin activity, it appears to be in a sufficient amount for the *in vivo* reaction.

4. Two factors known to inhibit *in vivo* renin secretion, angiotensin II and potassium ion, were demonstrated as well as angiotensin I not to influence *in vitro* the activity of human renin.

## INTRODUCTION

For the measurement of renin activity, it is of importance to assess the factors affecting the enzymatic reaction, such as pH, temperature, ionic strength, incubation time, concentrations of substrate and enzyme. The purpose of the present study was to determine the role of each parameter of the reaction of purified human renin with different substrates, two being homologous (human substrate in plasma and partially

Abbreviation: GU, Goldblatt units.

purified), one heterologous (hog substrate) and one of synthetic origin (1,14-(Ile$^5$)-tetradecapeptide) which represents the common N-terminal sequence of the other substrates. Many reports concerning renin kinetics have been published, using bioassays in which conversion activity was not always inhibited and whose methodology differed[1–11]. The great difference of the data thus obtained led to contradictory interpretations. In the present study the rate of formation of angiotensin I, the direct product of the enzymatic reaction, was measured to assess the renin activity with different substrates, the conversion of angiotensin I to angiotensin II being totally inhibited during incubation and a specific radioimmunoassay for angiotensin I being used[12,13].

## MATERIALS AND METHODS

### *Enzyme*

Human renin, kindly supplied by Dr E. Haas, Cleveland, Ohio, was a purified extract from kidneys, free of angiotensinases, which was verified not to contain converting enzyme activity, and which possessed a specific activity of 0.15 Goldblatt units (GU) per mg.

### *Substrates*

Plasma human substrate consisted of unpurified plasmas of pregnant woman and anephric subjects which contained large amounts of substrate (about 4000 pmoles/ml in the former and 2000 pmoles/ml in the latter). The substrate content of plasma was determined by a 2-h incubation at 37 °C and pH 5.5, in the presence of an excess of human renin ($2 \cdot 10^{-2}$ GU for 50 $\mu$l plasma), and was expressed by the concentration of the angiotensin I formed (pmoles/ml). The converting enzyme and the angiotensinases present in plasma were inhibited by EDTA and DFP, which were demonstrated not to modify the renin activity. The endogenous renin concentration was evaluated from the slope of the generation of angiotensin I in the presence of increasing small amounts of exogenous renin[6,13].

Partially purified human substrate was prepared from human plasma by precipitation with $(NH_4)_2SO_4$ according to Skeggs *et al.*[10]. This purification led to the elimination of renin, converting enzyme and angiotensinases.

Partially purified hog substrate purchased from Miles Laboratories, Kankakee, Ill., had a specific activity of 12.5 nmoles/ml. It contained no detectable renin, converting enzyme nor angiotensinases.

(Asp$^1$–Ile$^5$)-tetradecapaptide was obtained from Schwarz-Bioresearch, Orangeburg, N.Y.

### *Kinetic studies*

Incubations of the different substrates with renin were carried out in 1 ml 1/7 M barbital–acetate buffer (ionic strength of 0.16) at a temperature of 37 °C. Renin was added at zero time and sampling was effected at intervals of 3–5 min, the volume of the aliquots varying from 10 – 500 $\mu$l depending on the expected content of generated angiotensin I. The enzymatic reaction was stopped by immediately cooling the aliquots in the buffer at pH 7.5 and 4 °C used for the immunoassay. The absence of further formation of product was verified with the appropriate controls in each

experiment. Each experiment was performed twice and each measurement made in duplicate. The coefficient of variation was 10–20%. The details of the study of the pH effect have been described previously[14]. The effect of temperature was assessed at 27, 32, 37, 42 and 47 °C, with the concentrations of enzyme and substrates indicated in Table I. The influence of ionic strength ($I$) was studied, with barbital buffer ($I$ = 0.01–0.16) and acetate buffer ($I$ = 0.0125–0.2) ,upon the reaction of human renin and tetradecapeptide substrate at 37 °C and pH 4.5. For the determination of the Michaelis constant ($K_m$) and maximal velocity of the reaction ($V$), human renin at about 5 times the physiological concentration ($5 \cdot 10^{-4}$ GU/ml) was incubated for 15 min at 37 °C with the four substrates over a wide range of concentrations in barbital buffer, at physiological ionic strength ($I$ = 0.16), and at optimum pH for each substrate. $K_m$ and $V$ were estimated by the statistical method of Wilkinson[15].

The possibility of a product inhibition by angiotensin I or II upon renin activity was investigated by adding either the decapeptide or the octapeptide in concentrations of the same order or larger than that of the tetradecapeptide substrate.

The influence of $K^+$ upon the same enzymatic reaction was examined with 0.4, 4 and 40 mM KCl added to the barbital buffer at an ionic strength of 0.16.

*Radioimmunoassay of angiotensin I*

The volume of the aliquots pipetted out of the incubation medium was brought up to 1 ml with 0.1 M Tris buffer (pH 7.5). The angiotensin I content was determined by radioimmunoassay as previously described[13], but without prior extraction since the small content of protein of the substrates at the concentrations used did not interfere. No angiotensin II, as measured by specific radioimmunoassay[13], was found as a product of the enzymatic reaction. Whereas the natural renin substrates did not cross-react with the angiotensin I of the antiserum, the tetradecapeptide substrate showed a weak cross-reaction of 4% and the results were corrected accordingly.

## RESULTS

*Effect of time of incubation*

With each substrate the formation of angiotensin I was a linear function of time during the 15-min incubation, as shown previously with human plasma substrate[13]. The initial velocity of the reaction is obtained from the slope of this line.

*Effect of pH*

The optima of the human renin activity were at pH 4.5 with tetradecapeptide substrate, pH 5.5 with plasma human substrate, pH 6 with partially purified human substrate and pH 7.5 with hog substrate.

*Effect of temperature (Table I, Fig. 1)*

The elevation of the temperature from 27 – 47 °C accelerated the human renin activity upon the four substrates. The temperature coefficient ($Q_{10}$) was the highest with tetradecapeptide substrate (6.6 between 27 and 37 °C, and 2.8 between 37 and 47 °C). With the three other substrates, the reaction was accelerated about 5 times ($Q_{10}$ = 4.5–5.4) from 27–37 °C, and 2.3 times from 37–47 °C ($Q_{10}$ = 2.2–2.5).

TABLE I

EFFECT OF TEMPERATURE ON HUMAN RENIN ACTIVITY WITH FOUR DIFFERENT SUBSTRATES

The values indicate the velocity of angiotensin I formation (pmoles/ml per min), at the concentrations of enzyme and of substrate mentioned, during an incubation at optimum pH for each substrate.

| *Substrate* | *Substrate concn (pmoles/ml)* | *Renin concn (GU/ml)* | *27 °C* | *32 °C* | *37 °C* | *42 °C* | *47 °C* |
|---|---|---|---|---|---|---|---|
| Tetradecapeptide substrate | 220 | $5 \cdot 10^{-4}$ | 0.81 | 2.55 | 5.33 | 11.2 | 15.0 |
| Human substrate in plasma | 206 | $5 \cdot 10^{-3}$ | 0.45 | 1.45 | 2.42 | 3.76 | 5.52 |
| Partially purified human substrate | 188 | $5 \cdot 10^{-3}$ | 0.45 | 0.94 | 2.04 | 2.91 | 4.48 |
| Hog substrate | 250 | $5 \cdot 10^{-3}$ | 0.43 | 0.98 | 2.21 | 2.85 | 5.48 |

Tetradecapeptide substrate required the highest activation energy (38 kcal/mole), hog substrate an intermediate energy (29 kcal/mole) and human substrate the lowest energy (26 kcal/mole for plasma substrate and 26.6 kcal/mole for partially purified substrate).

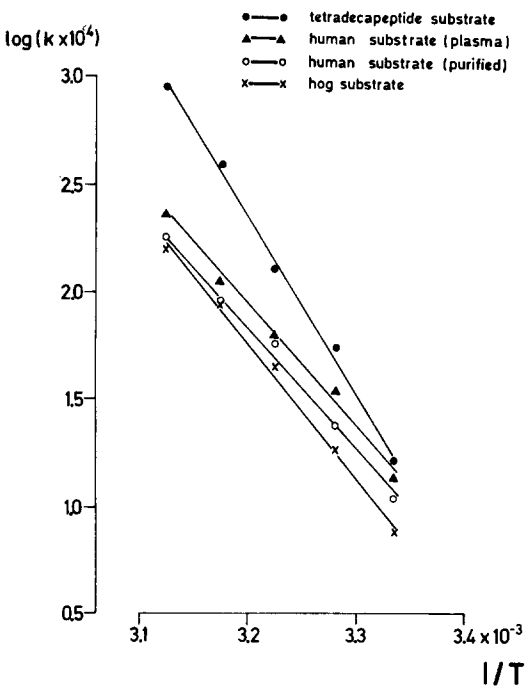


Fig. 1. Effect of temperature on human renin activity with four different substrates at the temperatures of 27, 32, 37, 42 and 47 °C. For the construction of the Arrhenius diagram, the logarithms of the rate constants (log $k$) of substrate hydrolysis calculated from data of Table I are plotted *vs* the reciprocals of the absolute temperatures ($1/T$). Activation energies ($E_a$) are obtained from the slopes, according to the Arrhenius equation: $E_a = -2.3\, R\, \mathrm{d} \log k / \mathrm{d}\, 1/T$.

*Effect of ionic strength (Fig. 2)*

An increase in the ionic strength from 0.01 – 0.2 resulted in a 2-fold increase of the rate of angiotensin I formation. The same effect was observed whether barbital or acetate buffer was used.

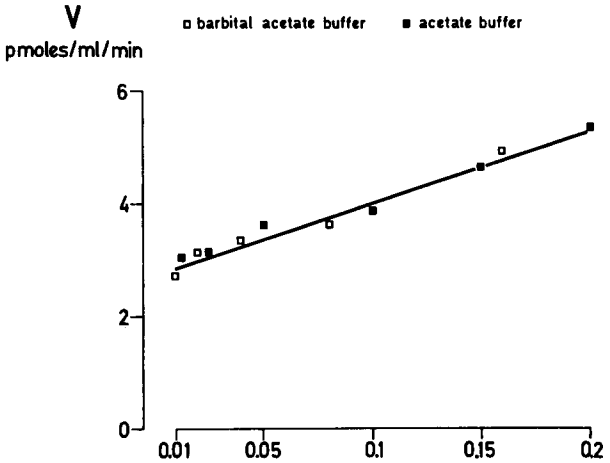


Fig. 2. Effect of ionic strength ($I$) on human renin ($5 \cdot 10^{-4}$ GU/ml) activity upon tetradecapeptide substrate (220 pmoles/ml), with acetate buffer (closed squares) and barbital buffer (open squares) at pH 4.5. The renin activity is indicated by the velocity of angiotensin I formation (pmoles/ml per min).

*Effect of substrate concentration (Table II, Fig. 3)*

Under similar conditions, except that the optimum pH for each substrate was selected, human renin presented a different reactivity with its four substrates. The highest values of $K_m$ and $V$ were found with tetradecapeptide substrate and the lowest with hog substrate. $K_m$ and $V$ were slightly superior with human substrate as compared to hog substrate. Thus human renin acted upon the tetradecapeptide

TABLE II

MICHAELIS CONSTANTS ($K_m$) AND MAXIMAL VELOCITIES ($V$) FOR REACTION OF HUMAN RENIN WITH FOUR DIFFERENT SUBSTRATES

The formation of angiotensin I was determined in similar conditions, at the optimum pH indicated for each substrate, and also at the physiological pH for human substrate. Values of $V$ are given for the renin concentration used ($5 \cdot 10^{-4}$ GU/ml) and also calculated for 1 GU of enzyme (last column).

| *Substrate* | *pH* | $K_m$ *(pmoles/ml)* | $V$ *(pmoles/ml/min)* | *V/GU* |
|---|---|---|---|---|
| 1,14-(Ileu$^5$)-tetradecapeptide | 4.5 | 6204 | 108 | 216 000 |
| Human (plasma) | 5.5 | 800 | 1.04 | 2 080 |
| Human (partially purified) | 6 | 544 | 1.03 | 2 060 |
| Hog | 7.5 | 380 | 0.80 | 1 600 |
| Human (plasma) | 7.5 | 400 | 0.40 | 800 |
| Human (partially purified) | 7.5 | 251 | 0.40 | 800 |

substrate with a 100-times greater velocity but exhibited an affinity which was one order of magnitude lower than with the other substrates.

With the two preparations of human substrate, a similar $V$ was obtained but the partially purified substrate presented a 1.5-times lower $K_m$. At physiological pH

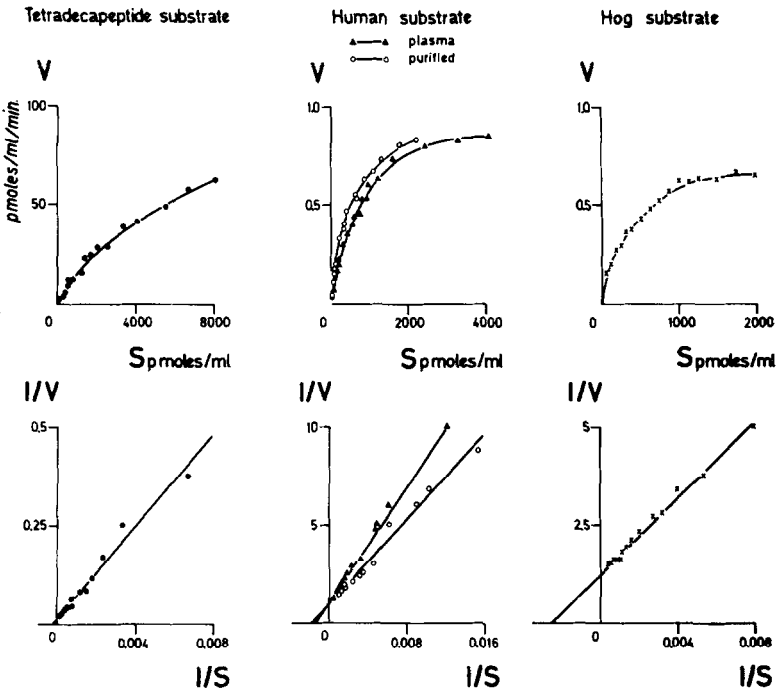


Fig. 3. Effect of substrate concentration on the velocity of human renin reaction with four different substrates. Renin concentration $5 \cdot 10^{-4}$ GU/ml. Upper figure: The velocity of angiotensin I formation, plotted *vs* the substrate concentration, was determined during a 15 min incubation at 37 °C in a barbital buffer at optimum pH for each substrate and at constant ionic strength $I = 0.16$. Lower figure: Lineweaver–Burk plots with the data of upper figure (in ordinate: reciprocal velocity of angiotensin I formation; in abscissa: reciprocal substrate concentration).

(7.5), a 2.5-times decrease of $V$ and a 2-fold increase in affinity as compared to the optimum pH were observed.

Substrates concentrations in human plasma lower than 2000 pmoles/ml at optimum pH and lower than 1000 pmoles/ml at physiological pH, were found insufficient to produce a maximal rate of angiotensin I formation.

*Effect of angiotensin I and angiotensin II (Fig. 4)*

Neither the decapeptide nor the octapeptide at the concentrations used, modified the activity of human renin upon tetradecapeptide substrate.

*Effect of $K^+$ (Table III)*

$K^+$ at physiological (4 mM) or at 10-times lower (0.4 mM) and higher (40 mM) concentrations did not influence the renin activity upon tetradecapeptide substrate.

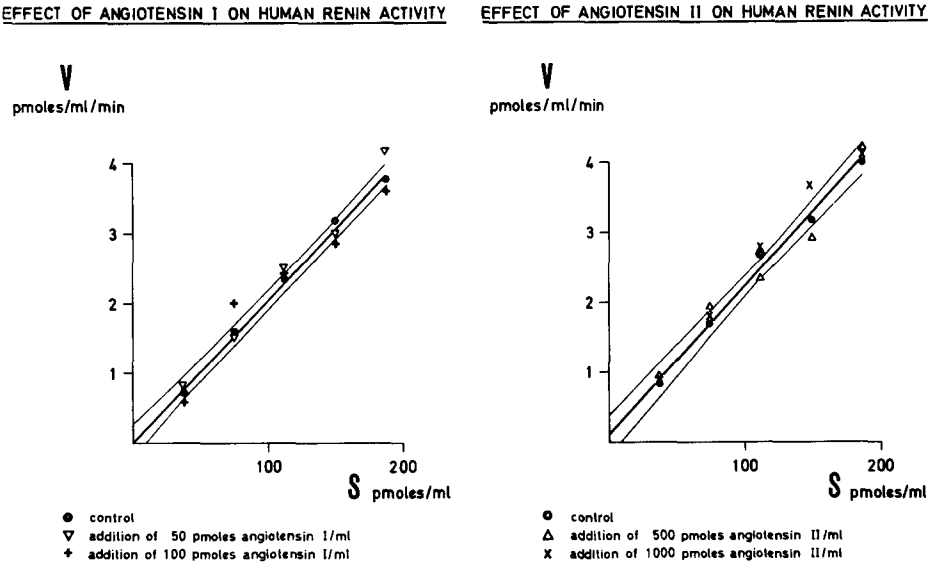


Fig. 4. Effect of angiotensin I (left) and II (right) on human renin activity with tetradecapeptide substrate. The velocity of angiotensin I formation, plotted *vs* the substrate concentration, was determined during an incubation at pH 4.5 and 37 °C, with a constant renin concentration ($5 \cdot 10^{-4}$ GU/ml). The regression line of the linear part of the Michaelis–Menten curve is indicated with its 95% confidence limits. The statistical calculations were performed on the CDC 3800 computer of the University of Geneva (Centre Cantonal d'Informatique) using the REGFIT programme conceived by Dr R. Scherrer.

TABLE III

EFFECT OF $K^+$ ON HUMAN RENIN ACTIVITY WITH TETRADECAPEPTIDE SUBSTRATE

The values indicate the velocity of angiotensin I formation (pmoles/ml/min) obtained with the enzyme concentration of $5 \cdot 10^{-4}$ GU/ml and the different substrate concentrations mentioned, during an incubation at pH 4.5 and 37 °C.

| | *Substrate concn (pmoles/ml)* | *Renin activity (pmoles/ml/min)* | | | | |
|---|---|---|---|---|---|---|
| | | *30* | *60* | *100* | *120* | *180* |
| control without KCl | | 0.42 | 0.92 | 1.67 | 1.92 | 3.0 |
| 0.4 mM KCl | | 0.50 | 0.83 | 1.67 | 1.83 | 2.83 |
| 4 mM KCl | | 0.50 | 1.08 | 1.92 | 2.0 | 2.92 |
| 40 mM KCl | | 0.50 | 0.92 | 1.75 | 1.83 | 2.75 |

DISCUSSION

Human renin is thus found to act upon the four substrates studied with different kinetic characteristics. The difference between the optimum pH obtained with each substrate has been the subject of another publication[14]. The reaction of renin with its substrates has been found to be more accelerated by elevation of the temperature than other enzymatic reactions reported in the literature, with the coefficients $Q_{10}$ rising up to 6.6. Also the elevated activation energies obtained (from 26 kcal/mole with human substrate to 38 kcal/mole for the tetradecapeptide substrate)

were comparable to the ones reported in non-enzymatic catalysis. A different energy of activation was required for each substrate. The dependence of the renin activity upon the ionic strength of the incubation medium is in agreement with the data obtained by Rosenthal *et al.*[9] with barbital buffer.

The velocity of the enzymatic reaction depended on the substrate concentration according to the hyperbolic relation of Michaelis–Menten. $K_m$ and $V$ were determined for each substrate in similar conditions of incubation in the presence of human renin at about 5 times the physiological concentration. Kinetics of the reaction with tetradecapeptide substrate clearly distinguished it from the other substrates, the velocity being 100-times faster and the $K_m$ about 10-times higher. This indicates a low affinity of renin for this synthetic substrate together with a large rate of hydrolysis. Hog substrate presented the lowest $V$ and $K_m$, while human substrate had intermediate values. Human renin showed a higher affinity for the partially purified human substrate than for the unpurified human substrate in plasma, but hydrolysed the two substrates with a similar velocity. These results indicate the possibility of the presence of a competitive inhibitor in whole plasma. The kinetics of human renin with its homologous substrate was also examined at physiological pH, where a 2-fold decrease of both $V$ and $K_m$ was observed. The values of $K_m$ published[4,5,7–10] for the reaction of human renin with a natural or purified homologous substrate are between 68 (ref. 8) and 1320 (ref. 10) pmoles/ml*, and of $V$, between 202 (ref. 8) and 1494 (ref. 9) pmoles/ml per min/per GU*. The present results with the plasma substrate at optimum pH are close to the $K_m$ found by Gould and Green[4] (931 pmoles/ml), and to the $V$ of Rosenthal *et al.*[9] (1494 pmoles/ml per min/per GU*) obtained with a purified substrate. The differences between the published data are to be attributed to the great diversity of incubation conditions (pH, ionic environment) and methods of angiotensin measurement since most authors used bioassays which could not distinguish between angiotensin I and II. For hog substrate the present value of $K_m$ is close to the values published[1,2,16]. Concerning the tetradecapeptide substrate, Gould *et al.*[3] reported a $K_m$ of about 5000 pmoles/ml, 5-times higher than with human substrate, but this was measured at pH 7.5 at which we found an extremely low activity[14]. Skeggs *et al.*[10] studying the kinetics of the reaction of pseudo-renin with tetradecapeptide substrate observed a $V$ 2280-times faster than with hog substrate, although the affinity was of the same order of magnitude ($K_m$ = 1850 pmoles/ml). Waldhäusl and Lewandowski[16] found that it did not follow Michaelis–Menten kinetics.

Since the value of $K_m$ varies with pH, in this enzymatic system, one can distinguish the reaction at optimum pH *in vitro* and the reaction which occurs *in vivo* at physiological pH. The present results indicate that a first-order reaction occurs at optimum pH (5.5) for the reaction of human renin with plasma human substrate at its physiological concentration. The value of $K_m$ (800 pmoles/ml) was close to the plasma content of substrate obtained in ten normal subjects (1040 pmoles/ml), the reaction velocity at this concentration representing 56% of $V$. A decrease of $K_m$ was measured at physiological pH as previously reported[7], and the velocity, although slower than at optimum pH, reached 76% of $V$, the reaction tending thus to a zero order. Therefore, it can be said in agreement with the previous work[5,7,9], that

* Published values were all converted to the same molar unit for the sake of comparison.

plasma contains a sufficient amount of renin substrate to sustain *in vivo* a near maximum reaction rate. However, in the conditions of the methods used to determine plasma renin activity without the addition of an excess of exogenous substrate, the reaction velocity is clearly limited by the plasma content of the substrate as previously demonstrated[4,11,17], and the renin activity does not only reflect the renin concentration of plasma[18]. As the $K_m$ with partially purified substrate appears to be lower than with natural substrate, experimental values with purified substrate should not be used for an estimation of the kinetics of the enzymatic reaction as occurring *in vivo*.

The fact that multiple forms of renin have recently been described[19] in addition to pseudo-renin should be taken into consideration. Renin purified from kidney contains probably many of these forms and the kinetic data obtained with it are the resultant of these enzymatic actions. These data, therefore, reflect more closely the physiological *in vivo* situation.

A product inhibition of converting enzyme by angiotensin II has recently been reported[20]. Since it is well known that angiotensin II inhibits *in vivo* the secretion of renin[21], the *in vitro* effect of angiotensins I and II upon renin activity was studied for the first time and no product inhibition could be demonstrated.

$K^+$, because of its known influence *in vivo*[21], was also studied as a possible inhibitor of renin activity, but did not induce any modification of the reaction velocity.

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