SIMULTANEOUS DETERMINATION OF PLASMA CONVERTING ENZYME AND ANGIOTENSINASE ACTIVITY BY RADIOIMMUNOASSAY

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SUMMARY

1. Homologous angiotensin-I was added to untreated plasma. Angiotensin-II which is formed by plasma converting enzyme and subsequently degraded by angiotensinases was determined as a function of time by using radioimmunoassay. Having determined the kinetics, the activities of converting enzyme and angiotensinases were calculated by a least-square fit of the theoretical curve to the experimentally measured values.

2. The method gives a simple measurement of converting-enzyme activity in untreated plasma; in plasma from salt-depleted and renal hypertensive rats this was found to be slightly increased but was normal in plasma from nephrectomized rats.

3. The half-lives for angiotensin-I in normal rat and human plasma were found to vary between 0-8 and 2-1 min, and the possibility that plasma converting enzyme participates in the regulation of the formation of angiotensin-II in vivo cannot be excluded.

4. The angiotensinase activity in rat plasma gave half-lives for angiotensin-II between 1-5 and 3-3 min; the half-life in normal human plasma was 10 min.

Various methods for the determination of converting enzyme have been described. Bioassay was used by Helmer (1957), Andersen (1967), Ng & Vane (1968), Barrett & Sambhi (1969) and Boucher, Kurihara, Grisé & Genest (1970). Chemical methods have been described by Huggins, Corcoran, Gordon, Henry & John (1970), Piquilloud, Reinharz & Roth (1970) and Dorer, Skeggs, Kahn, Lentz & Levine (1970). Radioactive tracer techniques have been used by Osborn, Hodges, Pickens, Willicombe & Mahler (1970) and Oparil, Sanders & Haber (1970).

The usefulness of most of the assays of converting enzyme in plasma is limited by the abundance of angiotensinases and other peptidases in plasma.

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which utilizes the specificity of the radioimmunoassay for angiotensin-II, which allows the angiotensinases to be fully active, and also their activities to be measured.

**METHODS**

Homologous angiotensin-I was prepared as described by Poulsen & Bing (1970). More than 95% of the angiotensin is angiotensin-I, the concentration being measured in bioassay and radioimmunoassay.

Blood is drawn in non-siliconized tubes, placed in ice–water and immediately separated at 0–4°C, as serum, citrate plasma (sodium citrate, final concentration 0.3%) and heparin-plasma (final concentration 10 units/ml) and is stored frozen at −20°C.

For the enzyme reaction 10 µl of angiotensin-I solution is incubated with 60 µl of plasma dilution (0.2 M-tris–HCl buffer, pH 7.5, containing 1% human albumin) for various periods of time (0–120 min). The final angiotensin-I concentration was varied between 25 and 150 ng/ml, the final plasma dilution was between 1 : 1.2 and 1 : 12. The reaction was stopped by cooling to 0°C and by adding 200 µl of labelled angiotensin-II and 150 µl of anti-angiotensin-II dilution. After 18 h at 4°C the free and antibody-bound tracer was separated and the concentration of angiotensin-II was determined as in the radioimmunoassay described by Poulsen (1969a).

The calculation of the activities of converting enzyme and angiotensinases was performed by simultaneously determining the values of \((k\cdot CE)\) and \((k'\cdot A)\) which gave the least deviation \(s_{y-x}^2 = \sum_n(Angio-II - Angio-II_{theor})^2/N\), in which Angio-II is the experimentally measured concentration of angiotensin-II, Angio-II_{theor} is the theoretical concentration at the same time calculated from eqn. (2), and the summation is over all N measured values.

**RESULTS**

**Kinetics**

Angiotensin-I is added to untreated plasma and is converted into angiotensin-II by converting enzyme. The concentration of angiotensin-II as a function of time depends on the rate at which it is formed and the rate at which it is degraded by the angiotensinases. For low concentrations of angiotensin the rate of formation (Poulsen, 1969b) as well as degradation (Poulsen, 1971) of angiotensin-II are known to be of first order with respect to substrate. This gives,

\[
\frac{d \text{Angio-II}}{dt} = (k \cdot CE) \text{Angio-I} - (k' \cdot A) \text{Angio-II}
\]

in which \((k \cdot CE)\) and \((k' \cdot A)\) are the first-order rate constants, CE and A being the concentrations of converting enzyme and angiotensinases respectively. Integration of eqn. (1) gives the concentration of angiotensin-II as a function of time:

\[
\text{Angio-II} = \text{Angio-I}_0 \frac{(k \cdot CE)}{(k' \cdot A) - (k \cdot CE)} \left[ e^{-(k \cdot CE)t} - e^{-(k' \cdot A)t} \right]
\]

in which Angio-I_0 is the known initial concentration of angiotensin-I at time \(t = 0\). For each plasma sample the concentration of angiotensin-II is measured with time as shown in Fig. 1. By a systematic variation of \((k \cdot CE)\) and \((k' \cdot A)\) an electronic computer constructs the theoretical curve (eqn. 2) which best fits the experimental angiotensin-II values. This gives
the converting enzyme activity \((k \cdot CE)\) and the angiotensinase activity \((k' \cdot A)\). The early part of the ascending curve is predominantly determined by \((k \cdot CE)\) and the late part of the descending curve by \((k' \cdot A)\). However, converting enzyme activity and angiotensinase activity take place during the whole incubation period, and therefore all measured concentrations are taken into account in the determination of \((k \cdot CE)\) as \((k' \cdot A)\) as well. Fig. 1 shows that the progress of the reactions obeys eqn. (2), in which the converting enzyme and angiotensinase reactions are expected to be of first order with respect to substrate concentrations and to be consecutive. Plasma incubated without angiotensin-I caused no detectable angiotensin-II formation over the time studied.

**Effects of variation of enzyme concentrations**

By using different dilutions of plasma and a fixed initial angiotensin-I concentration the converting enzyme activity \((k \cdot CE)\) and the angiotensinase activity \((k' \cdot A)\) were determined. The increase in the activities with enzyme concentration is shown in Fig. 2(a).

**Effects of variation of substrate concentration**

By increasing the initial angiotensin-I concentration with a fixed enzyme concentration (fixed plasma dilution) \((k' \cdot A)\) remained unchanged whereas \((k \cdot CE)\) decreased, indicating a substrate concentration approaching saturation of converting enzyme (Fig. 2b). The lowest concentration in Fig. 2(b), which was shown to obey eqn. (2), was used in the following experiments.
Activities of converting enzyme and angiotensinase in plasma

The activities of converting enzyme and angiotensinase in plasma were determined in plasma from normal, salt-depleted, renal hypertensive, and 24 h nephrectomized rats, as in Fig. 1. The results are shown in Table 1. Since no difference was found the results from citrate- and heparin-plasma and serum were mixed.

Fig. 2. (a) Converting enzyme activity ($k \cdot$ CE) (●) and angiotensinase activity ($k'$ $\cdot$ A) (○) plotted against the plasma dilution for fixed initial angiotensin-I concentration (25 ng/ml). (b) ($k \cdot$ CE) (●) and ($k'$ $\cdot$ A) (○) plotted against various initial angiotensin-I concentrations at constant enzyme concentrations (plasma dilution 1 : 3-5).

The converting enzyme activities were identical in plasma from normal and nephrectomized rats but a significant slightly increased activity was found in plasma from salt-depleted rats ($t = 3\cdot6, \ P = 0\cdot001$) and renal hypertensive (having one renal artery clipped) ($t = 8\cdot2, \ P < 0\cdot001$) rats.

The angiotensinase activities were slightly but significantly ($t = 3\cdot2, \ P = 0\cdot005$) decreased in plasma from renal hypertensive rats.

In human plasma the converting-enzyme activity was of the same magnitude as in rat plasma, whereas the angiotensinase activity was lower.

The reproducibility was repeatedly determined by using the same plasma for several months. The SD ($n = 8$) was found to be 12% for ($k \cdot$ CE) and 23% for ($k'$ $\cdot$ A). Since more attention was paid to the experimental determination of the increasing part of the progress curve, ($k \cdot$ CE) was determined more accurately than ($k'$ $\cdot$ A).
Table 1. Converting enzyme and angiotensinase activity was determined as in Fig. 1. The half-life for the angiotensin-I concentration used (25 ng/ml) was calculated for undiluted plasma by using $t_1 = \frac{0.286}{(k \cdot CE)}$, analogous for angiotensin-II

<table>
<thead>
<tr>
<th></th>
<th>Converting enzyme activity (plasma dilution 1:3·5) ($k \cdot CE \pm SD$ (min$^{-1}$))</th>
<th>Angiotensin-I half-life (undiluted plasma) $t_1 \pm SD$ (min)</th>
<th>Angiotensinase activity (plasma dilution 1:3·5) ($k' \cdot A \pm SD$ (min$^{-1}$))</th>
<th>Angiotensin-II half-life (undiluted plasma) $t_1 \pm SD$ (min)</th>
<th>$n$</th>
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<tr>
<td>RATS</td>
<td></td>
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<td>Normal</td>
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<td>24 h nephrectomized</td>
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<td>1·6 ± 0·2</td>
<td>0·09 ± 0·02</td>
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<td>2·1 ± 0·4</td>
<td>0·019 ± 0·008</td>
<td>10 ± 4</td>
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</table>
DISCUSSION

The formation of angiotensin-II in plasma \textit{in vivo} is always accompanied by its simultaneous degradation. \textit{In vitro} these conditions are similar as the activities of converting enzyme and angiotensinases are determined in untreated plasma.

The specificity of the method depends on the antibody against angiotensin-II used in the radioimmunoassay. The cross-reactivity of this antibody with angiotensin-I is less than 2\% (Poulsen & Bing, 1970), and accords with the fact that the initial angiotensin-II concentration is undetectable (Fig. 1). The cross-reactivity against metabolites of angiotensin-II is not known for this particular antibody, but generally antibodies against angiotensin-II react to some degree with the metabolites, especially with the hexapeptide. Such cross-reactivity does not influence the generation rate of angiotensin-II, but it could lead to falsely low values for its degradation rate. Thus, converting enzyme will be measured more accurately than angiotensinase, as a change in the rate of hexapeptidase activity would be falsely reflected as a change in the rate of angiotensinase activity. The agreement between the experimental and theoretical concentrations of angiotensin-II is in accordance with a complete conversion of angiotensin-I into angiotensin-II. Hypothetically, however, a small part of angiotensin-I could be degraded through a route which does not include angiotensin-II, for example by angiotensinases. In that case the activities of converting enzyme will be relative ‘effective’ activities, rather than absolute terms.

The absolute half-life of angiotensin-I in plasma (1–2 min) is much shorter than the half-life in heparinized plasma (15 min) or in citrate plasma (30 min) as determined by Oparil \textit{et al.} (1970) by using tracer techniques. Fig. 2(b) shows that the half-life for angiotensin-I is dependent on the initial angiotensin-I concentration; this might perhaps explain this marked difference. By using whole blood Oparil \textit{et al.} (1970) found a half-life of about 3 min. The difference in reaction rate for converting enzyme between heparin plasma and citrate plasma could not be reproduced in this work. This is in accordance with Boucher \textit{et al.} (1970).

Further, the use of a homologous and biological angiotensin-I can be important for determination of absolute activities of converting enzyme in plasma. Poulsen & Bing (1970) have shown that species differences in converting enzyme as well as in angiotensin-I play a role as mouse angiotensin-I is split slowly as well in rat as in human plasma. Piquilloud \textit{et al.} (1970) have shown that the synthetic substrate Z-Phe-His-Leu ($Z = \text{benzhydroxycarbonyl}$) is split more rapidly than angiotensin-I.

On the basis of intravenous injections of large amounts of angiotensin-I Ng & Vane (1968) and Oparil \textit{et al.} (1970) have shown that angiotensin-I is converted into angiotensin-II on a single passage through the pulmonary circulation. From this fact together with long half-lives for angiotensin-I in plasma the authors concluded that the conversion in plasma probably plays no important physiological role. However, endogenous renin has a long half-life (15 min) and therefore recirculates. Thus by its action on renin substrate in the blood, renin generates angiotensin-I throughout the circulation. The half-life of angiotensin-I, of 1–2 min found in this study and which is perhaps even shorter for the lower physiological plasma concentrations of angiotensin, is of about the same magnitude as a single circulation time. Therefore, it is possible that plasma converting enzyme may take part in a local formation of angiotensin-II.

By the present method for the determination of converting enzyme in plasma, surprisingly
small variations were found between the physiological and pathological conditions studied. This fact does not support the hypothesis that plasma angiotensin converting enzyme plays a physiological role.

ACKNOWLEDGMENT

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REFERENCES


