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KININ FORMATION AND KININOGEN DEPLETION IN RATS AFTER INTRAVENOUS INJECTION OF ELLAGIC ACID

BY

K. M. GAUTVIK AND H. E. RUGSTAD

From The Institute of Physiology, University of Oslo, Norway

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Contact with glass will initiate in blood both the mechanism leading to coagulation and that leading to the release of plasma kinins. These two chains of reactions seem to have their first event in common—namely, the activation of factor XII or Hageman factor (Margolis, 1958, 1960). Ellagic acid (4, 4′, 5, 5′, 6, 6′, hexahydroxydiphenic acid 2, 6:2′, 6′ dilactone), a compound which accelerates clotting of plasma *in vitro*, apparently causes activation of Hageman factor in a manner similar to glass surfaces (Ratnoff & Crum, 1964). It should be possible, therefore, to use this compound as a tool in research on plasma kinin formation. Its addition to plasma might represent a convenient way of initiating, not only the coagulation of blood, but also the release of kinins from plasma proteins. Intravenous injections of ellagic acid in dogs produces a transient fall in arterial blood pressure (Botti & Ratnoff, 1964). This might be due to the activation of Hageman factor. When activated this factor induces vasodilatation, possibly by activating plasma kallikrein (Webster & Ratnoff, 1961).

Jacobsen (1966a) described the presence of two different substrates for plasma kinin-forming enzymes in rat plasma. One of the substrates (substrate 1) formed kinins with plasma kallikrein as well as with glandular kallikrein, whereas only glandular kallikrein caused rapid kinin formation from the other substrate (substrate 2). He also observed the development of kinin-like activity when a buffered solution of ellagic acid was incubated with plasma from man, rat or guinea-pig. At the same time the substrate for plasma kallikrein (substrate 1) disappeared from plasma.

Substrate 1 was also apparently absent in plasma which he obtained from rats into which ellagic acid had been injected intravenously 30 min earlier. No detectable amount of kinin was formed by such plasma on contact with glass or on incubation with plasma kallikrein.

The purpose of the present investigation was to examine further the active substance formed when ellagic acid was added to plasma. It was also intended to study the elimination and, if possible, also the reappearance in plasma of substrate 1, following the intravenous injection of ellagic acid.

METHODS

Plasma kinin activity

This was tested on the isolated rat uterus preparation. Virgin rats, weighing 150–200 g, were given 30 μg stilboestrol intraperitoneally 20 hr before being killed. The isolated uterus was stored for 24 hr at 4° C in de Jalon solution before use, in order to make it insensitive to kallikreins and reduce the tendency to spontaneous contractions. A 2 cm piece of one uterine horn was suspended in a 5 ml. organ bath containing aerated de Jalon solution at 29° C. The test samples were left in contact with the tissue for 1 min and the interval between tests was 4 or 5 min. The preparation was sensitive to the addition of as little as 1 ng bradykinin in tests for kinin formation.

In blood, or in mixtures of blood and ellagic acid, kinin activity was tested after inactivation of kininase with absolute ethanol. Three millilitres of whole blood, taken directly from the rat, or a mixture of 2 ml. citrated blood and 1 ml. solution of ellagic acid (2×10^{-4} M) were poured into 40 ml. absolute ethanol. After 10 min this was centrifuged at $1{,}500\times g$ for 30 min. The supernatant was then dried under reduced pressure at 40° C in a rotary evaporator. The residue was dissolved in 2 ml. 0.05 M phosphate buffer (pH 7.35) and tested for kinin activity. This treatment completely eliminated the destruction of kinin by kininase, because when synthetic bradykinin was added to citrated rat blood and the mixture treated in this way, the bradykinin could be recovered without detectable loss.

Kininase activity

This was estimated by incubating synthetic bradykinin (BRS 640, Sandoz, Basle, Switzerland) at 37° C with the fluid to be tested. Aliquots of 0.1 ml. of the mixture were tested on the rat uterus every 5 min.

One unit of kininase is defined as the amount of kininase which destroys 75% or more of 500 ng bradykinin in 11 min (but not in 6 min) under standardized conditions (Rugstad, 1966).

Plasma

Plasma (standard plasma) was obtained from citrated blood (one part of 3.1% sodium citrate dihydrate solution to 9 parts of blood) that had been withdrawn with silicone-treated apparatus from healthy human males or from rats. To obtain the plasma all blood samples were centrifuged at $1{,}500\times g$ for 30 min.

Plasma kallikrein preparation

Pseudoglobulin preparations were used as a source of plasma kallikrein. Citrated human plasma was precipitated with ammonium sulphate between 33 and 46% saturation as described by Lewis (1958). The precipitate was dissolved in distilled water and dialysed against running tap water for 24 hr. The final volume of the preparation was about one-third of the original plasma volume. The pseudoglobulin samples, which could be shown to contain no substrates for plasma kinin-forming enzymes, were stored at −20° C.

Glandular kallikrein

Rat saliva was used as a source of glandular kallikrein. It was collected during anaesthesia after stimulation of salivation by an intraperitoneal injection of pilocarpine (2 mg/kg body weight). The saliva was centrifuged and treated with hydrochloric acid in order to remove kininase activity as described by Amundsen & Nustad (1964).

5-Hydroxytryptamine inhibition

In some experiments methysergide (Sandoz), 10^{-7} g/l., was added to the de Jalon solution to block response to any 5-hydroxytryptamine present.

Kininase inhibition

A standard solution of disodium edetate dihydrate (Fluka, A. G., Buchs, Switzerland) 2.7×10^{-2} M in 0.05 M Tris-HCl buffer and with a pH of 7.35 was used.

Determination of kininogens in plasma

The amounts of substrates for plasma kallikrein (substrate 1) and for glandular kallikrein (substrate 2) (Jacobsen, 1966a) were estimated separately by bracketing. The amount of each substrate was expressed in terms of the amount of kinin that could be developed /ml. plasma (see Fig. 1). For the determination of substrate 1, 0.2 ml. plasma and 0.1 ml. standard solution of disodium edetate were preincubated for 5 min. This mixture was then incubated at 37° C with 0.1 ml. plasma kallikrein preparation and the amount of kinin formed was estimated by testing 0.1 ml. portions of the mixture on the rat uterus preparation. In preliminary experiments it was shown that maximal kinin level was reached within 5 min. In the subsequent experiments tests were made after 1 and 5 min and the highest of the two estimates was used.

Substrate 2 was determined in plasma depleted of substrate 1: 0.4 ml. plasma was incubated with 0.2 ml. plasma kallikrein preparation at 37° C for 15 min. At that time no kinin activity could be detected in the mixture and it contained no more substrate 1. Then 0.1 ml. disodium edetate was added giving a final concentration of 0.68×10^{-2} M and the mixture was kept for another 5 min at 37° C and then 0.1 ml. rat saliva was added. This final incubate was tested for kinin activity after 1 and 5 min.

In vivo *injection of ellagic acid and blood pressure recording*

Rats weighing between 170–210 g were anaesthetized by intraperitoneal injection of 40–50 mg/kg body weight pentobarbitone (Abbott Laboratories, London). Anaesthesia was maintained by further injections of 5×10 mg/kg body weight, as needed. The trachea was cannulated and injections were made through a catheter in the external jugular vein. The blood pressure was recorded by a cannula in the carotid artery with a pressure transducer (P 23 De, Statham) connected to a Sanborn model 320 Dual channel DC amplifier-recorder (Sanborn Company, Massachusetts, U.S.A.) equipped with a Sanborn preamplifier. The paper speed and the calibration of the recorder were so arranged that the heart rate as well as the frequency of respiration could be measured.

Ellagic acid

(4, 4′, 5, 5′, 6, 6′ Hexahydroxydiophenic acid 2.6:2′.6′ dilactone, "practicum" grade (Fluka, A. G., Buchs, Switzerland) was used in a standard solution of 2×10^{-4} M in a 0.15 M Tris-HCl buffer of pH 7.35. This solution was injected in doses of 0.5 ml.

Determination of ellagic acid

This was performed spectrophotometrically and also by its ability to initiate kinin formation. The spectrophotometric readings were made in a Zeiss model PMQ-II spectrophotometer at 20° C and with quartz cuvettes with a 1 cm light path.

Ellagic acid in Tris-HCl buffer at pH 7.35 has a maximal absorbancy at 270 mμ, with a molar extinction of 2.45×10^{4}. Accordingly, we measured the absorption of 270 mμ, but had first to precipitate the proteins in the plasma samples by adding perchloric acid (final concentration 0.5 M), centrifuging at $3{,}000\times g$ for 30 min and then diluting the supernatant 1:2 with distilled water. The optical density was then measured and compared with that of standards prepared from plasma containing known amounts of ellagic acid. The lowest concentration of ellagic acid in plasma which could be detected by such a procedure was 1×10^{-5} M. The absorption appeared to comply with Beer's law.

The presence of ellagic acid in plasma was also evaluated biologically by examining its ability to form kinins from the standard plasma. A mixture of 0.2 ml. standard plasma and 0.1 ml. standard solution of disodium edetate had been preincubated for 5 min at 37° C. To this mixture was added 0.2 ml. of the plasma sample to be tested. Aliquots of 0.1 ml. from this final mixture were taken out after 1 and 5 min incubation and assayed for kinin-like activity on the rat uterus.

Disc electrophoresis

Disc electrophoresis on polyacrylamide gel was carried out with the Canalco Model 12 system (Canalco Corporation, Bethesda, Maryland, U.S.A.) with the procedures described in "Canalco Chemical Formulation and Instructions for disc electrophoresis" (1965).

Dialysis

This was carried out with Visking dialysis tubing 18/32 (Visking Department, Union Carbide International Co., New York, U.S.A.).

Siliconizing

Siliconizing of glassware and needles was carried out with Siliclad (Clay-Adams Inc., New York, U.S.A.). A 1% solution was used for glass and a 5% solution for metal, followed by drying overnight at 100° C.

RESULTS

In vitro *studies of kinin formation by ellagic acid*

The results confirm the observations by Jacobsen (1966a) that ellagic acid causes the release of material with kinin-like activity from rat plasma. A final concentration in plasma of 5×10^{-7} M was needed for significant formation of kinin-like activity to take place.

With less ellagic acid present the amounts of active substance formed were no more than in the control test, in which Tris-HCl buffer and disodium edetate only were added to plasma.

We have studied the time course for formation of the kinin-like substance in more detail. Rat plasma, 0.2 ml., was preincubated with 0.1 ml. 2.7×10^{-2} M solution of disodium edetate at 37° C for 5 min, after which 0.1 ml. ellagic acid 2×10^{-4} M (see Methods) was added, and the appearance of kinin-like activity was followed. Such activity could be detected after $\frac{1}{2}$ min of incubation and reached a maximum after 2–4 min. The average of the maximal values from samples of plasma from 30 animals corresponded to 350 ng synthetic bradykinin/ml. plasma. When ellagic acid and plasma were incubated without disodium edetate, much less kinin-like activity could be detected, and all of it was inactivated within 6 min. Contact with glass or the addition of a plasma kallikrein preparation did not then cause any further development of kinin-like activity in this mixture, which was thus apparently devoid of substrate 1. The amount of substrate for glandular kallikrein (substrate 2) was, however, apparently not reduced (Fig. 1).

The following additional observations indicated that the active substance formed in plasma in the presence of ellagic acid was kinin-like: (1) The contraction of the rat uterus caused by the substance was slow and similar to that produced by synthetic bradykinin. (2) It was not antagonized by the addition to the organ bath of methysergide. (3) The substance was rapidly inactivated by plasma from humans and from rats. This inactivation was very much delayed by the presence of disodium edetate. (4) Incubation of the active substance formed with a kininase from *Pseudomonas aeruginosa* (Rugstad, 1967a) abolished the effect on the rat uterus.

In vitro *and* in vivo *elimination of ellagic acid from blood*

As a preliminary experiment 4 ml. standard solution of ellagic acid (2×10^{-4} M) was incubated at 37° C with 4 ml. citrated blood. After 5 min, and at various subsequent

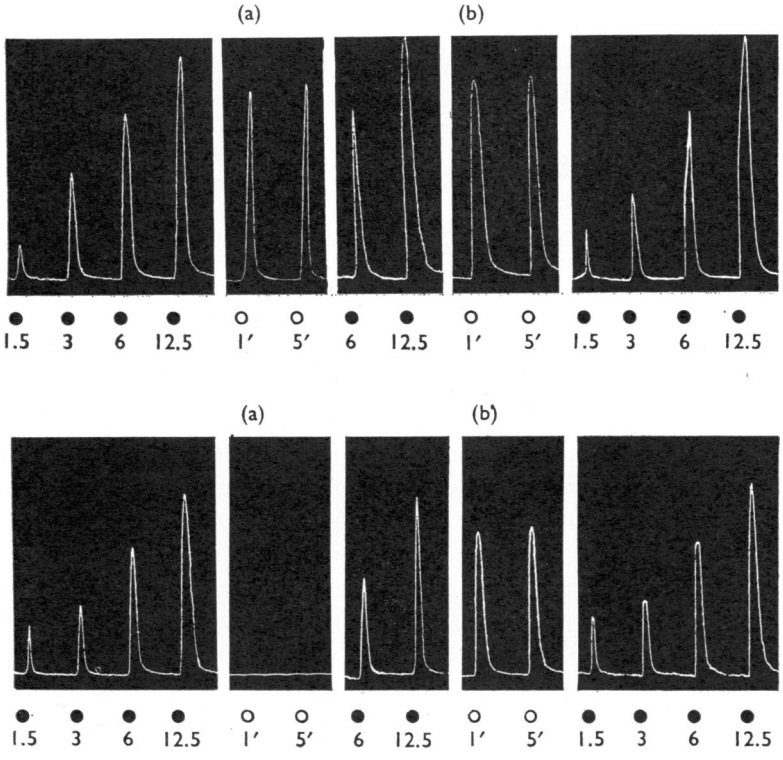

Fig. 1. Evaluation of the content of substrate 1 and substrate 2 in rat plasma before (upper tracings) and after (lower tracings) incubation of this plasma with ellagic acid. The substrates were assayed as the amount of kinin that could be developed/ml. plasma by addition of a plasma kallikrein and a glandular kallikrein preparation respectively. The amount of kinin was again evaluated by addition of samples of the mixture to the rat uterus preparation, the contraction of which is shown. Known doses of synthetic bradykinin were used for reference (filled circles). (a) Determination of substrate 1 (substrate for plasma kallikrein) by incubation of plasma, a solution of disodium edetate and a plasma kallikrein preparation (see Methods). Aliquots of 0.1 ml. of the mixture were taken out after 1 and 5 min incubation and added to the rat uterus preparation (open circles). (b) Determination of amount of substrate 2 (substrate for glandular kallikrein) by incubating plasma depleted of substrate 1, a solution of disodium edetate and rat saliva (see Methods). Aliquots of 0.1 ml. of this mixture were taken out after 1 and 5 min and added to the rat uterus preparation (open circles).

intervals, samples of this mixture were taken out, centrifuged and the supernatant examined for its content of ellagic acid spectrophotometrically and biologically (see Methods). Both methods showed that the concentration of the substance was the same in a sample taken after 3 hr as in the first sample taken. Some " inactivation " of ellagic acid, nevertheless, appeared to occur initially in blood. As stated above, a concentration in plasma of 5×10^{-7} M ellagic acid was sufficient to cause kinin formation, if the ellagic acid had been added directly to the plasma. If ellagic acid (in Tris-HCl buffer) was added first to citrated blood, and the plasma of this mixture was again added to another portion of plasma (standard plasma), a much higher calculated concentration of ellagic acid, 4×10^{-5} M, was needed in this final mixture for kinin formation to take place. The

reason for the apparent disappearance of some ellagic acid in the first blood portion is not known.

In whole animals, however, ellagic acid seems to disappear rapidly from the circulation. Three doses of 0.5 ml. 2×10^{-4} M ellagic acid were injected at 5 min intervals into the jugular vein of an anaesthetized rat. Although each dose in itself should produce a plasma concentration of about 10^{-5} M ellagic acid, if the drug is distributed equally throughout the blood, no ellagic acid could be detected, biologically or spectrophotometrically, in samples taken from the carotid artery 15 min after the last injection.

Kininase activity in rat plasma

The kininase activity in rat plasma is very marked and was found to vary between 30 and 50 u./ml. (see Methods). The addition of disodium edetate (final concentration of 0.68×10^{-2} M) did not eliminate the kininase activity of plasma, but reduced it to a level corresponding to less than 1 u./ml.

In order to see if the three injections of ellagic acid altered the kininase activity of the animals' plasma, blood samples taken before, and 10, 30 and 60 min after such injections were examined, but the kininase activity was found to be the same in all of them.

Effects of intravenous injection of ellagic acid on blood pressure, respiration and plasma level of free kinins

The effects of injecting ellagic acid into the jugular vein were examined in 15 rats, and the results were very uniform. Three injections of 0.5 ml. 2×10^{-4} M solution were given, usually at intervals of 5–7 min. Figure 2A shows a blood pressure record during a typical experiment. From 15 to 20 sec after the first injection the blood pressure fell rapidly. The reduction was of the order of 60–70 mm Hg and the hypotensive period usually lasted 4–6 min, after which the blood pressure gradually returned to the initial value or was stabilized on a level 20–30 mm Hg below the initial value. During the larger part of the hypotensive period, the frequency of respiration was nearly doubled.

The second injection of ellagic acid caused a fall in arterial blood pressure which was less pronounced and of shorter duration than that seen after the first injection. The third injection caused hardly any fall in the blood pressure. The hypotensive effects of intravenously injected synthetic bradykinin or of histamine, were, in contrast, apparently unchanged after the three injections of ellagic acid.

Injections of 0.5 ml. Tris-HCl buffer, the medium in which the ellagic acid had been dissolved, did not lower the blood pressure at any stage of the experiment. Injection into the jugular vein of 50 μg/kg synthetic bradykinin gave approximately the same fall in blood pressure as did a first injection of ellagic acid.

A fourth injection of ellagic acid had a moderate hypotensive effect when given 4 hr after, but not when given 2 or 3 hr after, the third injection.

In 5 rats the kinin-inactivating ability of the blood was increased at the outset of an experiment by injecting intravenously a dose of 3,000 u. of a purified kinin-inactivating enzyme from *Pseudomonas aeruginosa* (Rugstad, 1967a). This amount of that particular enzyme has previously been found to increase the kininase activity in plasma of 200–250 g rats 3–5 times (Rugstad, 1967b). When the standard dose of 0.5 ml. 2×10^{-4} M ellagic

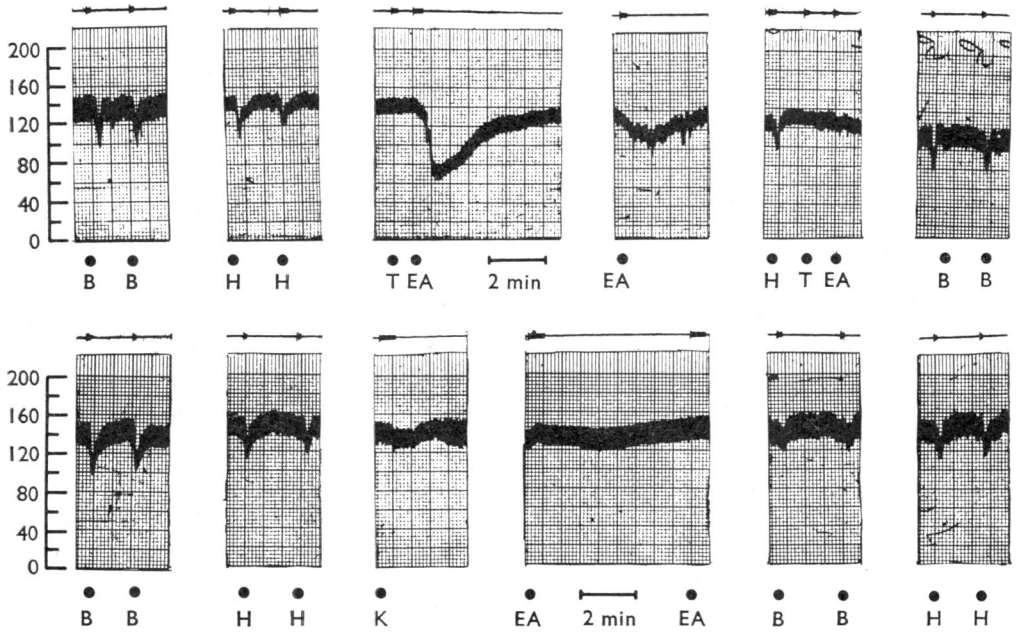

Fig. 2. The effect of intravenous injections of ellagic acid on the blood pressure of an anaesthetized rat before (upper tracing) and after (lower tracing) an intravenous injection of kininase. At filled circles intravenous injections of: synthetic bradykinin 2 μg/kg (B), histamine 2 μg/kg (h), Tris-HCl buffer (T), ellagic acid 2×10^{-4} M in Tris-HCl-buffer (EA) or a kininase (3,000 u.) from *Pseudomonas aeruginosa* (K). The interval between the individual pieces of tracing is 1 min.

acid was injected 3–5 min later, no hypotensive effect could be detected in any of the 5 animals (Fig. 2B).

The kininase activity in the blood of a rat should return to near normal level 2 hr after such an injection of enzyme (Rugstad, 1967b) and a first injection of ellagic acid given to an animal at that time again induced the usual marked fall in arterial blood pressure. This was seen in all animals tested.

In an attempt to recover possible free kinin activity, arterial blood samples were drained directly into absolute ethanol $\frac{1}{2}$, 1 and 2 min after the first injection of ellagic acid (see Methods). In spite of the fact that the samples were taken at a time when the hypotension was most pronounced, kinin activity could not be detected in any of the samples from 10 rats. On incubating equal volumes of citrated blood and a standard solution of ellagic acid at 37° C for 2 min *in vitro*, and then stopping the reaction in absolute ethanol, we were able to demonstrate kinin activity corresponding to 50 ng bradykinin/ml. blood.

Kininogen levels before and after intravenous injection of ellagic acid

To test the effects of ellagic acid on kininogen levels in plasma, 1 ml. samples of blood were taken from the carotid artery of 25 rats before and after the intravenous injection of 3 doses of 0.5 ml. 2×10^{-4} M ellagic acid at 5 min intervals. One sample was taken

1 hr after the last dose of ellagic acid and one or two additional samples were taken subsequently from each rat.

The amounts of substrate 1 and substrate 2 in the various samples were determined as described in Methods. The plasma level of substrate 1 was given the value 100% in each individual rat before injection of ellagic acid, and all later values were related to this level. The actual initial substrate 1 levels were equivalent to 100 to 240 ng bradykinin/ml. plasma, with an average of about 200 ng/ml. plasma. The amount of kinin which developed/ml. plasma did not increase significantly when the amount of plasma kallikrein in the incubate was doubled.

In one rat ellagic acid did not cause complete depletion of substrate 1, some substrate being present 1 hr after the injections. The values from this rat have therefore been excluded in Fig. 3, which gives the mean blood levels of substrate 1 in the remaining 24 rats at various time intervals after the ellagic acid injections. No substrate 1 could be demonstrated in the samples taken within the first 6 hr after the injections, whereas some substrate 1 was again detected in the 8 hr sample. In 8 rats examined 24 hr after the injections the average plasma level of substrate 1 had reached 61% of the initial value. The amount of substrate 2 was apparently not altered by these procedures.

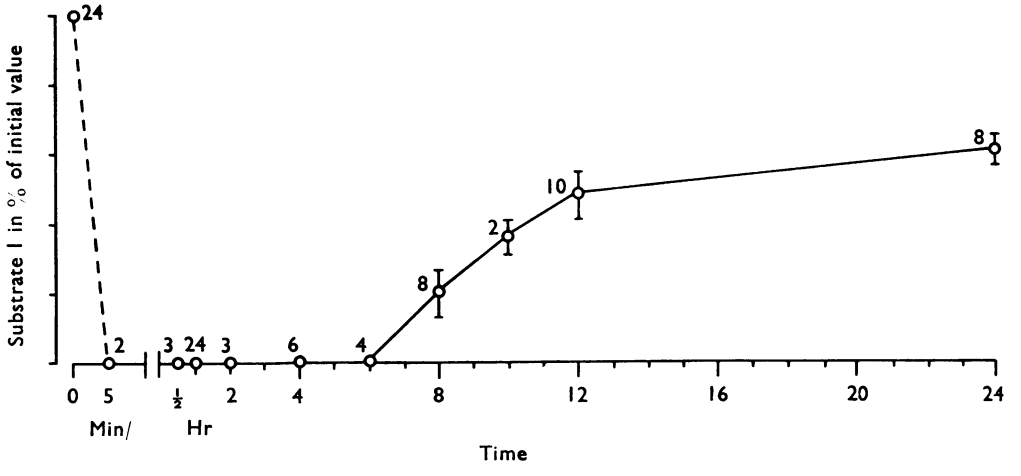

Fig. 3. Disappearance and reappearance of the substrate for plasma kallikrein (substrate 1) in the plasma of rats after intravenous injections of ellagic acid. Results from 24 rats are shown in this Figure. The last of three injections of 0.5 ml. buffered 2×10^{-4} M solution of ellagic acid had been given at zero time. From each rat 2 to 4 arterial blood samples were then obtained at different time intervals after the injections. The mean values of the substrate 1 level in samples taken at the various points (open circles) and the standard deviation of the mean (vertical lines) are given, the substrate level being expressed as per cent of the initial value. The number of rats from which blood samples were taken and analysed at each different point is given in italics.

In order to see if the disappearance of substrate 1 from plasma was followed by any changes in the plasma protein pattern, disc electrophoresis on polyacrylamide gel was carried out as described in Methods. No visible differences in the protein bands could be detected between plasma samples taken before and after the injections of ellagic acid.

DISCUSSION

The most reasonable interpretation of the effects of ellagic acid on the blood pressure of rats is that it causes the release of plasma kinin from substrate 1 and that repeated injections exhaust the supplies of this substrate and hence fail to produce an effect on the blood-pressure.

Free, circulating kinin activity could not, however, be demonstrated in blood samples obtained from the carotid artery after the first intravenous injection of ellagic acid, even when the blood sample was taken at the moment of maximum reduction in blood pressure. This may in part be due to the pronounced kininase activity in rat plasma, which will cause significant kinin elimination even during the time needed for blood sampling into ethanol. Trautschold, Fritz & Werle (1966) similarly obtained a very low recovery in venous blood of intra-arterially injected bradykinin. Their low recovery could not, however, be explained only as a result of inactivation of the kinins by plasma kininase and binding of kinin to some receptor site(s) will probably take place in situations where smooth muscle effects are displayed. Such binding may in part account for the indetectable or low amounts of circulating kinin.

The amount of kinin formed from rat plasma was larger when the plasma was incubated with ellagic acid (average 350 ng/ml. plasma) than when incubated with the plasma kallikrein preparation (average 200 ng/ml. plasma). The reason for this discrepancy is not understood. It is not due to additional formation by ellagic acid of kinin from the substrate for glandular kallikrein (substrate 2), as the level of that substrate was left unchanged by ellagic acid.

Although ellagic acid is stable in blood *in vitro*, it could not be detected *in vivo* 15 min after injection. The nature of this rapid disappearance has not been studied, but it could be due to rapid elimination by the kidney.

Jacobsen (1966b) found that plasma kallikrein in an inactive form was present in dog lymph. Contact with glass caused activation of this kallikrein. Ellagic acid with its small molecule will probably rapidly appear in the interstitial fluid and may activate the kallikrein present there. A depletion of substrate 1 in the interstitial fluid and in lymph would then probably take place, together with the disappearance of the substrate from plasma.

Changes in circulating kininogen have also been studied by Webster & Clark (1959) who examined the effect in dogs of intravenous injections of kallikrein preparations from urine and pancreas. They showed that the concentration of circulating kallidinogen was temporarily lowered after such injections. Werle (1955) has described similar experiments in dogs. The injection of ellagic acid has, however, the advantage in this type of studies that it will specifically remove substrate 1, leaving the amount of substrate 2 unchanged.

The hypotensive effect of intravenously given ellagic acid returned 4 hr after the substrate 1-depleting injections of the reagent. A small amount of substrate 1 had thus apparently reappeared in the circulating blood by this time. In arterial blood samples taken from the animals, however, substrate 1 was not detectable until 8 hr after the ellagic acid injections. One reason for this discrepancy might be that disodium edetate does not inhibit the kininase activity of rat plasma completely.

Substrate 1 moves electrophoretically with the β-globulins (Jacobsen & Kriz, 1967). *In vivo* injections of ellagic acid seem to be a relatively harmless way of depleting an

animal of one of its plasma proteins, and it might be useful in studies on the site of production of a β-globulin and on the regulation of its synthesis.

With ellagic acid injections animals can also be depleted of substrate 1 for a period of time. Such animals could be employed in studies on the possible role of this substrate and of the kinins formed from it in various situations—for example, in an inflammatory reaction.

SUMMARY

1. Ellagic acid has been found to release a kinin-like material when added to rat blood *in vitro* and *in vivo*.

2. Intravenous injections in rats of 0.5 ml. 2×10^{-4} M buffered solution of ellagic acid caused a pronounced fall in arterial blood pressure. A second injection 5 min later had much less hypotensive effect, and a third injection was without such effect.

3. When the blood kininase activity in the rats had been increased by injecting a large dose of a purified kininase from *Pseudomonas aeruginosa*, the hypotensive effect of a first injection of ellagic acid was abolished.

4. Ellagic acid appears to initiate kinin formation from the substrate in blood for plasma kallikrein (substrate 1) only. After three injections of ellagic acid (0.5 ml. 2×10^{-4} M solution) no substrate 1 could be detected in the plasma of the rats, whereas the level of substrate for glandular kallikrein (substrate 2) was unchanged.

5. The hypotensive effect of ellagic acid reappeared 4 hr after the substrate 1 depletion of plasma by previous injections of the acid.

6. On analysis of arterial blood samples, no substrate 1 could be detected 6 hr after an ellagic acid depletion. After 8 hr the average amount of substrate 1 in 24 rats was about 20% and after 24 hr about 60% of the initial value.

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