Br. J. Pharmac. Chemother. (1966), **28**, 64–72.

SUBSTRATES FOR PLASMA KININ-FORMING ENZYMES IN RAT AND GUINEA-PIG PLASMA

BY

STEN JACOBSEN

From the Institute of Physiology, University of Oslo, Norway

(*Received June* 14, 1966)

Two different substrates for plasma kinin-forming enzymes have been identified in plasma from man, dog and rabbit (Jacobsen, 1966a). One of the substrates forms kinins with plasma kallikrein as well as with glandular kallikrein, whereas only glandular kallikrein will cause rapid kinin formation from the other substrate. It would be of interest to know the characteristics and amounts of substrates for kinin-forming enzymes also in the plasma of other animal species.

Rats and guinea-pigs are thus often used in experiments on plasma kinins, and some observations seem to indicate that the plasmas of these animals are peculiar as regards their content of substrates for kinin-forming enzymes. Bhoola & Schachter (cit. Schachter, 1960) found that guinea-pig saliva did not release kinin from guinea-pig plasma, which thus apparently lacked the ability to react with glandular kallikrein. Fasciolo & Halvorsen (1964) were not able to release kinin activity from rat or guinea-pig plasma by incubation with hog pancreas kallikrein, an enzyme preparation which caused kinin formation on incubation with human and dog plasmas. Rat plasma may thus also be lacking a substrate which will react with the glandular type of kallikrein, although species specificity of enzymes and substrates may play a role when there is no reaction of a plasma with kallikrein from another animal.

In the present study plasma from the guinea-pig and the rat have been analysed for their content of different substrates for kinin-forming enzymes. The same separation techniques as have been described for human, dog and rabbit plasma (Jacobsen, 1966a, 1966b) were employed.

METHODS

Plasma. Plasma was obtained from citrated blood (one part of 3.1% sodium citrate dihydrate solution to nine parts of blood) taken with silicone technique. Human blood was obtained from healthy males; rat blood from rats of both sexes weighing from 200 to 300 g; and guinea-pig blood from guinea-pigs of both sexes weighing from 300 to 500 g. Blood from humans was obtained by vein puncture, and blood from rats and guinea-pigs by heart puncture of ether-anaesthetized animals. All blood samples were centrifuged at 1,300 g for 30 min.

Glass activation of plasma. This was carried out as described by Jacobsen (1966a).

Ellagic acid (Fluka A.G., Buchs, Switzerland). A 1×10^{-4} M solution in a 0.025 M sodium barbital buffer of *p*H 7.4 with 0.125 M sodium chloride was used.

Gel filtration of plasma and plasma fractions. This was carried out on Sephadex G 200 (A.B. Pharmacia, Uppsala, Sweden) columns at room temperature (20° C). The columns were equilibrated

with a 0.1 M 2-amino-2(hydroxymethyl)propane-1,3-diol hydrochloride (tris) hydrochloric acid buffer of *p*H 8 containing 0.2 M sodium chloride. The same buffer was used for elution.

For rat plasma portions of 45 ml. were applied to a 120×2.5 cm column and eluted at a rate of 20 ml./hr, fractions of 3.5 ml. being collected with a LKB fraction collector. Fractions of rat plasma obtained from chromatography on DEAE A 50 columns (see below) were also exposed to gel filtration on the same Sephadex column. Twenty ml. of such fractions were then applied to the column and elution carried out as for rat plasma.

Guinea-pig plasma in portions of 17.5 ml. was applied to a 43×2.7 cm column and eluted at a rate of 10 ml./hr. Again collection of 3.5 ml. fractions was carried out.

Elution volumes (V_e) for the various proteins and void volumes (V_o) of the different columns were determined as described by Andrews (1965). The relationship V_e/V_o, which, according to Andrews (1965), is correlated to log molecular weight, has been calculated for the different substrates.

DEAE anion exchange. A DEAE-Sephadex A 50 column (A.B. Pharmacia, Uppsala, Sweden) (10×1.0 cm) equilibrated with 0.05 M 2-amino-2(hydroxymethyl) propane-1.3-diol hydrochloride (tris) hydrochloric acid buffer of *p*H 8, containing 0.1 M sodium chloride was used. One volume of the same buffer, but here without sodium chloride, and two volumes of plasma were mixed. Five 3 ml. portions of this mixture were applied to the column and thereafter 10 ml. of the equilibration buffer. The same buffer was then used for elution, which was performed at room temperature (20° C) by continuously and linearly increasing the sodium chloride content of the buffer (see Results).

Protein content. Fractions from gel filtration were examined for protein content with a Zeiss spectrophotometer (Model PMQ II) at 280 mμ, and in a 1:16 dilution.

Pseudoglobulin preparations. These were made from plasma as described previously (Jacobsen, 1966a).

Plasma kinin activity. This was estimated on the rat uterus preparation kept in de Jalon solution at 29° C in a 5 ml. siliconized organ bath. The uterus was usually stored for 24 hr at 4° C in de Jalon solution before being used for the biological tests. The various test samples were added to the siliconized organ bath with siliconized pipettes.

Kininase activity. This was estimated by incubating at 37° C 0.1 ml. of plasma with a solution of 250 ng of synthetic bradykinin in 0.35 ml. of a 0.1 M 2-amino-2-(hydroxymethyl) propane-1,3-diol hydrochloride (tris) hydrochloric acid buffer of *p*H 7.35. At intervals samples were taken out and tested on the rat uterus preparation, and the time when all bradykinin activity had disappeared was noted.

Bradykinin. Synthetic bradykinin (BRS 640; Sandoz A.G., Basel, Switzerland) was used.

Glandular kallikreins. Dog, rabbit, rat and guinea-pig saliva was collected during anaesthesia. Salivation was stimulated by intramuscular injection of pilocarpine (2 mg/1,000 g body weight). Saliva from these animals and from humans was usually centrifuged at 2,500 *g* for 30 min to remove kininase activity (Amundsen & Nustad, 1964).

Padutin® (Bayer, Leverkusen, Germany), a hog glandular kallikrein preparation, was used in some experiments in a concentration of 0.4 biological units (Frey, Kraut & Werle, 1950)/ml. incubation mixture.

Rat urine was collected by catetherization of the bladder in anaesthetized female rats. The urine obtained was dialysed against tap water for 18 hr. The various kallikrein preparations were kept at −20° C before being used.

Kallikrein inhibitors. Soya bean trypsin inhibitor, SBTI (Nutritional Biochemicals Corporation, Cleveland, Ohio, USA) was used.

Kininase inhibitors. Solutions of disodium edetate dihydrate (A.G. Fluka, Buchs, Switzerland), 2.7×10^{-2}M and of phenanthroline (1,10-Phenanthroline, Sigma Chemical Company, St. Louis, Missouri, USA), 4×10^{-3}M were used.

Dialysis. This was carried out with a Visking dialysis tubing 18/32 (Visking dept., Union Carbide International Company, New York, USA).

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

RESULTS

Contractions of rat uterus provoked by addition of kinin-forming enzymes

Certain kinin-forming enzymes will cause smooth muscle preparations to contract (Margolis, 1958 ; Werle, 1937, 1963). In the present investigation portions of a number of incubation mixtures, containing various kinin-forming enzyme preparations, had to be added to the rat uterus organ bath. Human, dog or rabbit saliva never produced contractions of the rat uterus in the present investigations, not even when 0.5 ml. of undiluted saliva was added to the uterus in the 5 ml. organ bath. As observed by Margolis (1958) glass-activated human and rat plasma will, however, cause the rat uterus to contract. Similarly, a freshly prepared preparation of isolated rat uterus contracts on addition to the organ bath of pseudoglobulin preparations prepared from human or rat plasma. Contractions induced by addition of pseudoglobulin preparations or of glass-activated plasmas were inhibited by addition to the organ bath fluid of SBTI to a final concentration of 10 μg/ml. The rat uterus also contracted on the addition of rat saliva, rat urine or of trypsin. The contractions of a freshly prepared uterus preparation diminished and eventually disappeared after repeated additions to the organ bath of activated plasma, pseudoglobulin, rat saliva, rat urine or trypsin. If the rat uterus preparation had been stored at 4° C for 24 hr in de Jalon solution none of the above-mentioned enzyme-containing preparations caused contractions on addition to the organ bath.

The contractions induced by the preparations mentioned, which all contain plasma kinin-forming enzyme, may be explained by the presence of one or more substrates for these enzymes in the uterine tissue. These substrates are apparently non-reacting with the kallikrein of human, dog or rabbit saliva, and they disappear on storage at 4° C or on repeated enzyme additions.

Kininase activity in rat and guinea-pig plasma

Kininase activity is pronounced in both rat and guinea-pig plasma, and this activity must be inhibited if kinin formation is to be studied. Bradykinin (250 ng) was inactivated (see Methods) by 0.1 ml. of guinea-pig plasma in less than 30 sec and by 0.1 ml. of rat plasma in less than 90 sec. The corresponding times for dog and rabbit plasma were about 8 and 3 min respectively (Jacobsen, 1966c). The kininase activity in rat plasma could be efficiently inhibited by disodium edetate in a final concentration of 0.68×10^{-2}M. In guinea-pig plasma efficient kininase inhibition was obtained by phenanthroline in a concentration of 1×10^{-3}M.

Effect of plasma kallkrein on rat and guinea-pig plasma

Marked kinin activity developed when 0.1 ml. of a pseudoglobulin preparation was incubated at 37° C with 0.2 ml. of rat plasma and 0.1 ml. of 2.7×10^{-2}M disodium edetate.

The kinin activity developed in the course of 2 min equalled 400 ng of bradykinin/ml. Similar kinin activity developed when glass-activated rat or human plasma was incubated with untreated rat plasma. Pseudoglobulin preparation made from rat plasma also induced kinin formation in the plasma.

When a pseudoglobulin preparation was incubated with guinea-pig plasma at 37° C in the presence of phenanthroline (0.2 ml. plasma, 0.1 ml. of phenanthroline solution (4×10^{-3}M) and 0.1 ml. of pseudoglobulin preparation) kinins were produced in amounts corresponding to 300 ng of bradykinin per ml. of plasma. Glass-activated guinea-pig or human plasma also induced kinin formation when added to untreated guinea-pig plasma.

According to Margolis (1958) Hageman-factor-activation is the first step in activation of the plasma kallikrein system. Ratnoff & Crum (1964) found that ellagic acid could activate the Hageman factor. Addition of one volume of buffered ellagic acid solution (1×10^{-4}M) to one volume of rat or guinea-pig plasma was therefore tried, and such addition resulted in formation of kinin activity in both plasmas and also in human plasma. In control incubations of the plasmas with the buffer in which the ellagic acid was dissolved, there was no significant formation of kinin. In plasma portions of guinea-pig, rat and human origin which had been incubated with ellagic acid for 4, 6 and 16 min respectively, and which by then contained no kinin activity, addition of pseudoglobuln as a plasma kallikrein preparation resulted in no further kinin formation. This disappearance in plasma of substrate for plasma kallikrein could be observed also in *in vivo* experiments. Five ml. of a buffered solution containing 1×10^{-4}M of ellagic acid were injected intravenously into anaesthetized rats weighing 250 to 300 g. In plasma obtained from these animals 30 min later, no kinin formation was observed either on glass-activation or on incubation with a pseudoglobulin preparation.

Effect of different glandular kallikreins on rat and guinea-pig plasma

The finding of Fasciolo & Halvorsen (1964) that no kinin activity could be developed from rat plasma by hog pancreas kallikrein was confirmed. Similarly no kinin formation could be revealed on incubation of rat plasma with human, dog or rabbit saliva, not even in the presence of disodium edetate as kininase inhibitor. Similarly the three types of saliva did not cause kinin formation in rat plasma that had been heated to 56° C for 1 hr.

Kinin formation was, however, induced in rat plasma on incubation with rat saliva. When 0.2 ml. of rat plasma was incubated with 0.1 ml. of the 2.7×10^{-2}M disodium edetate solution and 0.1 ml. of undiluted rat saliva, kinin activity (corresponding to 200 ng bradykinin/ml. of plasma) was developed. The formation took place within 2 min. Further kinin formation could not be produced by adding more saliva to the mixture. Similar kinin formation took place when rat plasma was incubated with rat urine.

Human saliva did not induce kinin formation in guinea-pig plasma either. Some kinin formation was observed, however, when guinea-pig saliva was incubated with guinea-pig plasma in the presence of phenanthroline (0.2 ml. of guinea-pig plasma, 0.1 ml. of the 4×10^{-3}M phenanthroline solution and 0.1 ml. of guinea-pig saliva). In this incubation mixture, kinin activity corresponding to some 100 ng of bradykinin/ml. of plasma was formed. The formation took place within 2 min. With saline in the incubation mixture,

instead of saliva, no such formation could be detected. SBTI did not inhibit the kinin formation induced by saliva.

Evidence for existence of two different substrates for kinin-forming enzymes in rat and guinea-pig plasma

Both rat and guinea-pig plasma will thus develop kinin activity with plasma kallikrein as well as with glandular kallikrein, provided that the glandular kallikrein is derived from the animal itself. It would be of interest to know whether the ability of the plasmas to react with the two types of kallikreins was due to their content of two different substrate entities, such as is the case for human, dog and rabbit plasmas (Jacobsen, 1966a).

After incubating 0.2 ml. of rat plasma with 0.2 ml. of the ellagic acid solution at 37° C for 6 min, human pseudoglobulin preparation could not induce any further formation of kinin activity, whereas rat saliva did induce such formation. After incubating 0.3 ml. of rat plasma for 6 min at 37° C with 0.15 ml. of a human pseudoglobulin preparation and 0.1 ml. of the disodium edetate solution, a subsequent addition of 0.15 ml. of rat saliva induced some further kinin formation in the mixture, whereas 0.15 ml. of pseudoglobulin did not.

When guinea-pig plasma was incubated for 6 min at 37° C with guinea-pig saliva in the presence of phenanthroline, additional amounts of guinea pig saliva did not induce more kinin activity, whereas pseudoglobulin preparations caused additional kinin formation corresponding to 300 ng of bradykinin/ml. of plasma. When guinea-pig plasma was

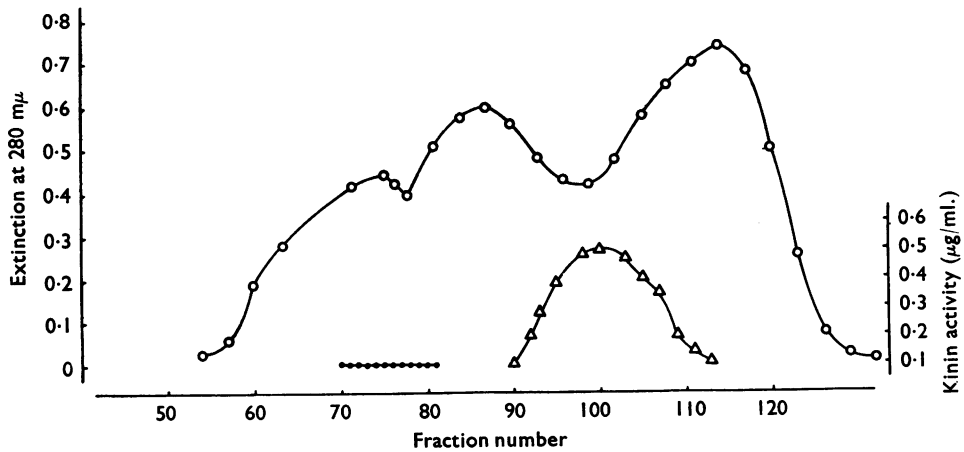

Fig. 1. Content of substrates for kinin-forming enzymes and of protein in consecutive fractions (3.5 ml.) of rat plasma from a Sephadex G 200 column (120×2.5 cm.) O——O=content of protein ; △——△=content of substrate reacting with glandular kallikrein (substrate 2). Substrate is expressed in terms of kinin that could be developed/ml., synthetic bradykinin being used as reference. Protein contents are expressed as extinctions at 280 mμ, using Zeiss Spectrophotometer (Model PMQ 11) and 1:16 dilution of fractions. Fractions containing substrate for plasma kallikrein (substrate 1) were obtained from separation on DEAE-Sephadex A 50 column (see Methods) and ●——●——● gives position at which this substrate was then eluted when applied to Sephadex G 200 column.

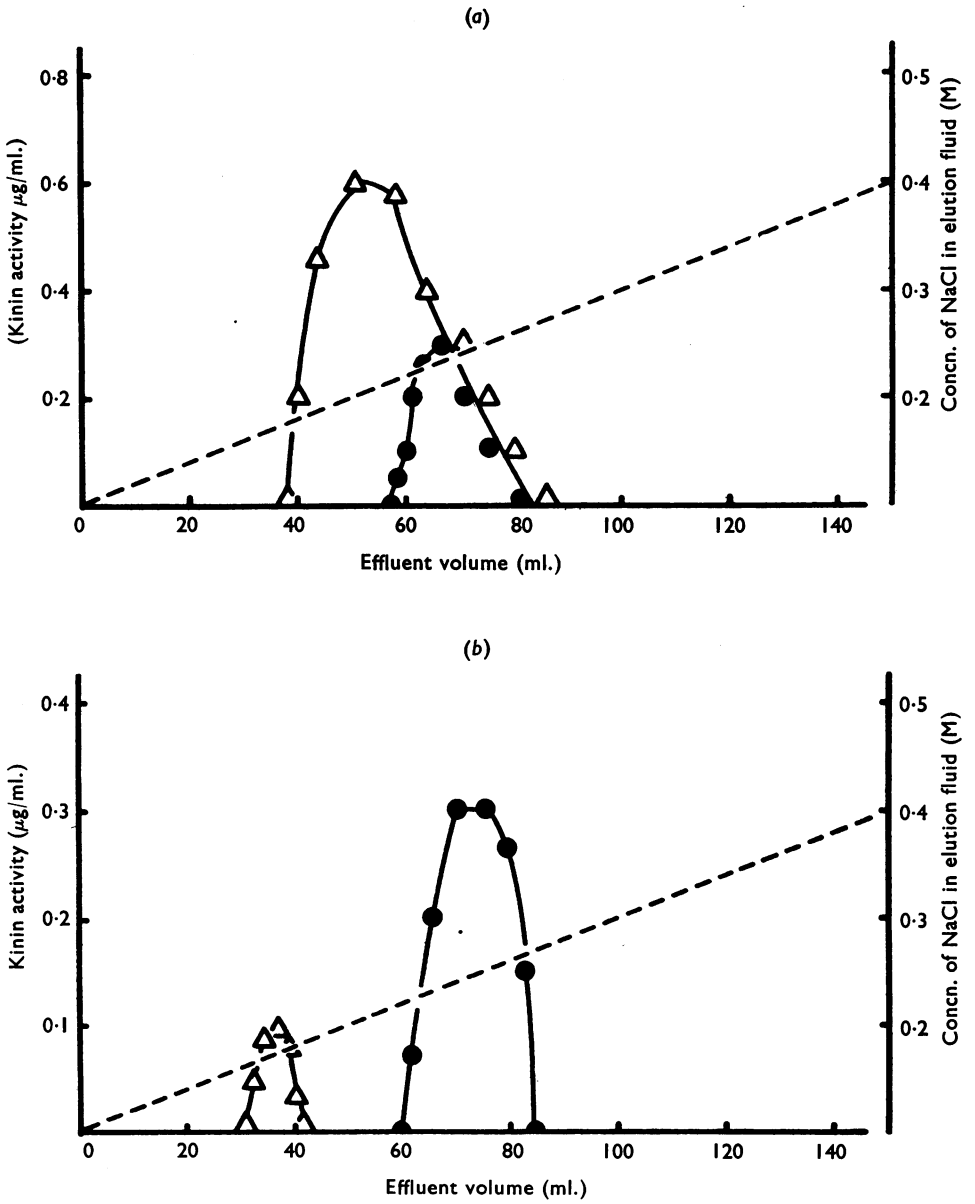

Fig. 2. Content of substrates for kinin-forming enzymes in consecutive fractions of rat (*a*) and guinea-pig (*b*) plasma eluted from a DEAE-Sephadex A 50 column by linearly increasing sodium chloride concentration (see Methods). △——△=content of substrate which reacts with glandular kallikrein (substrate 2); ●——●=content of substrate which reacts with plasma kallikrein (substrate 1). Substrates are expressed in terms of kinins that could be developed/ml., synthetic bradykinin being used as reference. Increasing gradient of sodium chloride shown by broken line.

initially incubated with pseudoglobulin preparations or with ellagic acid, however, no further kinin formation could be seen on addition of saliva.

These findings indicate that both rat and guinea-pig plasma develop kinin activity with plasma kallikrein from a plasma precursor which is apparently different from that which reacts with glandular kallikrein. It was consequently of interest to see if the presence of different substrates could be demonstrated in the two plasmas by various protein separation techniques.

Separation of two different substrates for plasma kinin-forming enzymes

When rat plasma was applied to a Sephadex G 200 column, as described in Methods, eluted fractions did not react with plasma kallikrein, whereas some fractions formed kinin activity on incubation with rat saliva. The pattern of elution as to kinin forming ability and protein content is shown in Fig. 1. The V_e/V_o relationship for the substrate protein present could be calculated to be about 2.1.

Rat plasma was then applied to a DEAE anion exchange column, and elution was carried out by linearly increasing the sodium chloride content of the buffer, as described in Methods. At concentrations between 0.18 M to 0.21 M of sodium chloride, fractions were eluted which reacted with plasma kallikrein only. At concentrations between 0.22 to 0.26 M, fractions were eluted which reacted with plasma kallikrein as well as with glandular kallikrein (Fig. 2*a*). These last fractions, which were now without plasma kallikrein, were applied to a Sephadex G 200 column, and their substrate was eluted as indicated on Fig. 1. These substrate fractions formed kinin with plasma kallikrein preparations as well as with rat saliva, and the V_e/V_o relationship for the substrate protein was calculated to be about 1.6.

In order to see if two different substrates for plasma-kinin-forming enzymes could be separated from guinea-pig plasma, such plasma was applied to a DEAE anion exchange column and was eluted by a buffer with a continuous linear gradient of sodium chloride concentration as described above. One type of substrate was then eluted at concentrations of sodium chloride from 0.16 M to 0.18 M and another at concentrations from 0.22 to 0.27 M (Fig. 2*b*). The fraction first eluted formed kinin with guinea-pig saliva as well as with glass activated (human or guinea-pig) plasma or with pseudoglobulin preparations. The last fraction, however, developed kinin activity only with activated plasmas or with pseudoglobulin preparations, not with guinea-pig saliva.

When guinea-pig plasma was exposed to gel filtration on a Sephadex G 200 column, as described in Methods, elution of fractions with the same two types of substrates was obtained. The V_e/V_o relationship for the substrate protein reacting with glandular as well as with plasma kallikrein was calculated to be about 2. The V_e/V_o relationship for the other substrate protein was about 1.7.

DISCUSSION

One main problem with the kinin-forming ability of guinea-pig and rat plasma has been whether they lack substrate for glandular kallikrein or not. In this investigation the presence of such a substrate (substrate 2) in both rat and guinea-pig plasma has been demonstrated. Both plasmas showed species specificity as regards the glandular

kallikrein and its substrate, in that glandular kallikrein from the animal itself developed kinin from its plasma, whereas kallikreins from some other species were ineffective.

In both plasmas the substrate for glandular kallikrein could be separated from another substrate (substrate 1) which reacted with plasma kallikrein. The separation pattern of the two substrates on gel filtration on a Sephadex G 200 column indicates that substrate 1 has larger molecular dimensions than substrate 2, as has also been found in other plasmas investigated (Jacobsen, 1966a). The rat substrate 1, but not the guinea-pig substrate 1, reacted also with the animal's own saliva. The guinea-pig substrate for glandular kallkrein (substrate 2), but not the corresponding substrate from rat plasma, developed kinin activity also with plasma kallikrein preparations.

There are thus important qualitative differences between rat and guinea-pig plasma as regards their two types of substrates. The substrate types in both these plasmas again differ somewhat from those found in human, dog and rabbit plasma. Table 1 illustrates the interactions of the two substrate types from different plasmas with various types of kinin-forming enzymes.

TABLE 1

KININ DEVELOPMENT ON INCUBATIONS OF DIFFERENT KALLIKREINS WITH SUBSTRATES 1 AND 2 FROM DIFFERENT PLASMAS

Incubations of kallikreins with fractions containing different substrates (see text) were carried out as described in Methods, and kinin activity was tested on isolated rat uterus preparation. + = marked formation of kinin activity; 0 = no kinin activity found; (+) = a small formation of kinin activity took place. As to designations substrate 1 and substrate 2, see Discussion

	Rat plasma		Guinea-pig plasma		Human, dog and rabbit plasmas	
Enzyme preparation	Substrate 1	Substrate 2	Substrate 1	Substrate 2	Substrate 1	Substrate 2
Rat saliva	+	+	0	0	+	+
Guinea-pig saliva	(+)	(+)	0	+	+	+
Human, dog and rabbit saliva	0	0	0	0	+	+
Pseudoglobulin preparation (human)	+	0	+	+	+	0
Hog pancreas kallikrein	0	0	0	(+)	+	+

Rat and guinea-pig plasmas were different from human, dog and rabbit plasmas also in some other respects (Jacobsen, 1966a). The total amounts of substrates for kinin-forming enzymes were considerably smaller in the former two plasmas than in the latter group, and there was especially a very small amount of substrate 2 in the guinea-pig plasma.

The significance of the differences in types and amounts of substrates for kinin-forming enzymes in plasmas from various species is not known. The knowledge of the various substrate characteristics must, however, be of considerable practical interest.

SUMMARY

1. By DEAE-Sephadex anion exchange columns and gel fitration on Sephadex G 200 columns two different substrates for plasma kinin-forming enzymes can be separated from rat and guinea-pig plasma.

2. One of the substrates, substrate 2, reacted with glandular kallikrein. The reaction involved species specificity in that this substrate reacted with the animal's own saliva, but not with saliva from several other species.

3. Substrate 2 from guinea-pig plasma, but not substrate 2 from rat plasma, formed kinin also with plasma kallikrein.

4. The other substrate, substrate 1, has a larger molecular dimension than substrate 2. Substrate 1 from guinea-pig plasma formed kinin activity with plasma kallikrein only. The rat substrate 1, however, reacted with saliva as well as with plasma kallikrein.

5. The total amounts of substrates for kinin-forming enzymes are smaller in rat and guinea-pig plasmas than in several other plasmas examined.

This work was carried out when the author was a Research Fellow of the Norwegian Research Council for Science and the Humanities. Financial support from this Council, from Eli Lilly and Company, Indianapolis, USA, from the Nansen Foundation, from the Norwegian Council on Cardiovascular Diseases and from Anders Jahre's Foundation for the Promotion of Science is gratefully acknowledged. I also wish to thank M. Kriz for skilful technical assistance.

REFERENCES

AMUNDSEN, E. & NUSTAD, K. (1964). Kininase activity of human saliva. *Nature*, (*Lond.*), **201**, 1226–1227.

ANDREWS, P. (1965). The gel-filtration behaviour of proteins related to their molecular weights over a wide range. *Biochem. J.*, **96**, 595-606.

FASCIOLO, J. C. & HALVORSEN, K. (1964). Specificity of mammalian kallidinogen. *Am. J. Physiol.*, **207**, 901–905.

FREY, E. K., KRAUT, H. & WERLE, E. (1950). *Kallikrein* (*Padutin*). Stuttgart: Ferdinand Enke Verlag.

JACOBSEN, S. (1966a). Substrates for plasma kinin-forming enzymes in human, dog and rabbit plasmas. *Br. J. Pharmac. Chemother.*, **26**, 403–411.

JACOBSEN, S. (1966b). Separation of two different substrates for plasma kinin-forming enzymes. *Nature, Lond.*, **210**, 98–99.

JACOBSEN, S. (1966c). Observations on the content of kininogen, kallikrein and kininase in lymph from hind limbs of dogs and rabbits. *Br. J. Pharmac. Chemother.*, **27**, 213–221.

MARGOLIS, J. (1958). Activation of plasma by contact with glass: evidence for a common reaction which releases plasma kinin and inititates coagulation. *J. Physiol., Lond.*, **144**, 1–22.

RATNOFF, O. D. & CRUM, J. D. (1964). Activation of Hageman factor by solutions of ellagic acid. *J. Lab. clin. Med.*, **63**, 359–377.

SCHACHTER, M. (1960). *Some properties of Kallidin, Bradykinin and Wasp Venom Kinin. Polypeptides Which Affect Smooth Muscles and Blood Vessels*, p. 237. London: Pergamon Press.

WERLE, E. (1937). Über die Wirkung des Kallikreins auf den isolierten Darm und über eine neue darmkontrahierende Substanz. *Biochem. Z.*, **289**, 217–233.

WERLE, E. (1963). Über Plasmakinine. *Naunyn-Schmiedeberg's Arch. exp. Path. Pharmak.*, **245**, 254–262.