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SUBSTRATES FOR PLASMA KININ-FORMING ENZYMES IN HUMAN, DOG AND RABBIT PLASMAS

BY

S. JACOBSEN

From the Institute of Physiology, University of Oslo, Norway

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Margolis & Bishop (1963) showed that the kininogen of plasma reacted differently with different types of enzymes. Kinin formation with plasma kallikrein involved only about one third of the total plasma kininogen, whereas trypsin or glandular kallikrein released kinin from the whole of the kininogen. Recently two different substrates for plasma kinin-forming enzymes were separated from human plasma (Jacobsen, 1966). One of the substrates, substrate 1, yielded kinins with plasma kallikrein as well as with glandular kallikreins, whereas from the other, substrate 2, only glandular kallikrein released significant amounts of kinins. Preliminary investigations on lymph (Jacobsen & Waaler, 1965) indicated that both substrates were present in dog lymph, whereas only the substrate for glandular kallikrein seemed to be present in rabbit lymph. The apparent qualitative difference in substrate content of dog and rabbit lymph prompted the present analyses of plasma from these animals. By a separation procedure using gel-filtration on a Sephadex G 200 column (Jacobsen, 1966) it has been possible to demonstrate that both types of substrates are present in dog as well as in rabbit plasma. The amounts of substrates found have been compared to the values for human plasma.

Pseudoglobulin preparations of plasma are commonly used as sources of substrate in work on kinin-forming enzymes. This reagent has been shown to contain a precursor of a plasma kinin-forming enzyme (Lewis, 1960). Active kinin-forming enzyme may also be present (Jacobsen & Waaler, 1965). In the present investigation pseudoglobulin preparations have been treated by a variety of procedures and more closely examined for content of plasma kinin-forming enzymes and their substrates.

METHODS

Plasma. Plasma was obtained from citrated blood (one part of 3.1% sodium citrate dihydrate to nine parts of blood) taken with silicone technique. Human blood was obtained from healthy males (aged 20 to 24 years) and dog blood from mongrels (males), weighing from 15 to 20 kg, by vein puncture. Albino rabbits (males) weighing between 2.7 and 3.5 kg were anaesthetized by intravenous injections of 90 to 180 mg of pentobarbitone sodium (Nembutal Natrium V.M., Abbott Laboratories, London) and 150 mg of chloralose (α-Chloralose puriss., E. Merck, Darmstadt, Germany) and blood taken by heart puncture using siliconed equipment. All blood samples were centrifuged at 1,300 *g* and 4° C for 30 min.

Glass activation of plasma. Equal volumes of plasma and glass ballotini (0.1 mm in diameter) were agitated for 3 min, and the plasma was used immediately.

Massive contact exposure of plasma. Plasma was shaken with 100 mg/ml. of silicate powder for 10 min at 37° C. After centrifugation at 2,200 *g* for 5 min the supernatant fluid was again shaken with 50 mg/ml. of silicate powder for 60 min at 37° C. Further centrifugation at 2,200 *g* for 10 min was followed by filtration of the supernatant fluid through a Whatman No. 1 paper.

Silicate powder. "Speed plus" (Great Lakes Carbon Corp., Los Angeles, U.S.A.) containing particles with a mean diameter of 9 μ was used.

Gel-filtration of plasma. This was carried out out with 50 ml. plasma samples on a Sephadex G 200 (A. B. Pharmacia, Uppsala, Sweden) column (120 × 2.5 cm) equilibrated with a 0.1 M-2-amino-2-(hydroxymethyl)propane-1,3-diol hydrochloride (tris) hydrochloric acid buffer of *p*H 8.0 containing 0.2 M-sodium chloride. Elution was carried out with the same buffer and a flow rate of 20 ml./hr. Fractions of 5 ml. were collected with the use of an LKB fraction collector.

Protein content of fractions from gel-filtration. This is expressed as extinction at 280 mμ, using a Zeiss spectrophotometer (Model PMQ II) and 1:16 dilution of the fractions.

Pseudoglobulin preparations. These were made from dog, rabbit or human citrated or heparinized plasmas by precipitation 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. The final volume of the dialysed solution was about one-third of the original plasma volume. The pseudoglobulin preparation samples were stored at —20° C.

Plasma kinin activity. This was estimated on the rat uterus preparation and sometimes also on the guinea-pig ileum preparation. Virgin rats weighing between 150 and 200 g were used for the stilboestrol 18 hr before being killed. The uterus was usually stored for 24 hr at 4° C in de Jalon solution at 29° C. The rats were injected intraperitoneally with 0.5 ml. of a 0.01% solution of stilboestrol 18 hr before being killed. The uterus was usually stored for 24 hr at 4° C in de Jalon solution before being used for biological tests. When guinea-pig ileum was used, it was suspended in a 5 ml. organ-bath containing Tyrode solution at 35° C.

Test samples were applied to the organ-bath with a siliconized pipette and at intervals of 4 min. The contact time of each sample was 60 sec. In this bath the uterus was usually sensitive to as little as 2 to 3 ng of bradykinin.

Rat duodenum was sometimes used as an additional test organ for plasma kinin. The proximal 2 to 3 cm of the duodenum from rats weighing 150 to 200 g were suspended in a 5 ml. organ-bath containing de Jalon solution at 30° C. Test samples were applied every 3 min and the contact time was 30 sec.

Mepyramine maleate (10^{-4} g/l.; Anthisan, May & Baker) and methysergide (10^{-7} g/l.; Deseril, Sandoz A. G., Basel, Switzerland) were used in the organ-bath fluid for inhibition of histamine and 5-hydroxytryptamine respectively.

Substrates for kinin-forming enzymes. Amounts of substrates in plasma or in fractions from Sephadex G 200 gel-filtration were estimated in the following ways: (1) Substrate for plasma kallikrein: 0.2 ml. of plasma or of a plasma fraction was incubated at 37° C with 0.1 ml. of 2.7 × 10^{-2} M-disodium edetate and 0.1 ml. of a human pseudoglobulin preparation which had been dialysed for more than 48 hr. A sample of the mixture was taken out and tested on the rat uterus preparation after 1 min, at which time the maximal amount of kinin had been formed. (2) Substrate for glandular kallikrein: 0.1 ml. of plasma or of a plasma fraction, 0.7 ml. of 0.9% saline, 0.1 ml. of 6.8×10^{-2}M-disodium edetate and 0.1 ml. of undiluted cell-free saliva were incubated at 37° C. A sample of the mixture was taken out and tested on the rat uterus preparation after 4 min, at which time the maximal amount of kinin had been formed.

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

Glandular kallikrein. Human saliva without kininase activity (Amundsen & Nustad, 1964) was used as source for glandular kallikrein. Occasionally also Padutin (Bayer, Leverkusen, Germany) was used.

Kallikrein inhibitors. Soya bean trypsin inhibitor (Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.) and Trasylol (Bayer, Leverkusen, Germany) were used. Amounts of Trasylol are given in Kallikrein-Inaktivator-Einheit (KIE) (Trautschold & Werle, 1961).

Kininase inhibitor. Disodium edetate dihydrate (A. G. Fluka, Buchs, Switzerland) in concentrations of 2.7×10^{-2} and 6.8×10^{-2}M and *p*H of 7.35 was used.

Dialysis. This was carried out with a Visking dialysis tubing 18/32 (Visking Dept., Union Carbide International Co., New York, U.S.A.) against tap water that had been passed through a glass wool filter.

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

RESULTS

Substrate content and kinin-forming activity of pseudoglobulin preparation

Pseudoglobulin, the fraction of plasma proteins precipitated between 33 and 46% saturation with ammonium sulphate, developed considerable amounts of plasma kinin on incubation with human or rabbit saliva, with urine, with Padutin or with trypsin. When 0.2 ml. of the preparation was incubated with a small amount (0.1 ml.) of freshly glass-activated plasma, however, hardly any kinin formation could be detected. These observations indicate that, whereas there is substrate for salivary kallikrein, Padutin or trypsin in the pseudoglobulin preparation, no substrate for plasma kallikrein seems to be present.

Marked kinin formation occurred when a small amount of a pseudoglobulin preparation was added to a plasma which had been taken with the silicone technique, the incubation being carried out in a siliconed glass tube (Fig. 1, *a*). The enzyme responsible for the kinin formation is apparently present in the pseudoglobulin fraction and its action was inhibited by soya bean trypsin inhibitor and by Trasylol (Fig. 1), agents which are known to inhibit plasma kallikrein (Habermann & Klett, 1965). The enzyme in pseudoglobulin is thus apparently active plasma kallikrein.

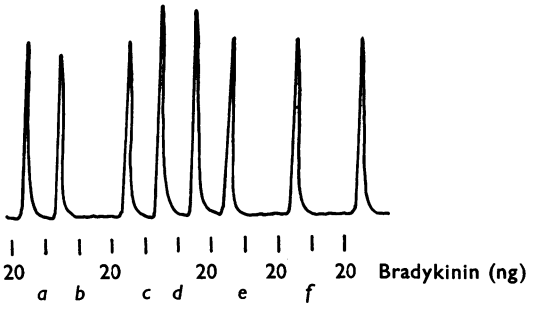

Fig. 1. The effect of soya bean trypsin inhibitor and Trasylol on the kinin-forming activities of pseudoglobulin and saliva. 0.1 ml. of pseudoglobulin from human plasma (dialysed for more than 48 hr) or of human saliva was first incubated for 5 min at 37° C with 0.1 ml. of saline or of a solution containing the inhibitor. The final concentrations of soya bean trypsin inhibitor and Trasylol were 100 μg/ml. and 12.5 KIE/ml. of initial incubation mixture respectively. 0.2 ml. of fresh human plasma was then added and the incubation continued for 1 min, when pseudoglobulin was present, and for 4 min with saliva. 0.1 ml. of the mixture was then tested on the rat isolated uterus preparation. Standard doses of synthetic bradykinin were added between as indicated. Initial incubation mixtures: (*a*) pseudoglobulin and saline; (*b*) pseudoglobulin and soya bean trypsin inhibitor; (*c*) saliva and soya bean trypsin inhibitor; (*d*) saliva and saline; (*e*) saliva and Trasylol; and (*f*) pseudoglobulin and Trasylol.

The reaction between the substrate for glandular kallikrein and the active enzyme in pseudoglobulin is very slow (Jacobsen, 1966) and a good substrate concentration may be retained for some time in the preparation. However, when a pseudoglobulin preparation is kept at 20° C its substrate will gradually disappear in the course of about 2 days. If disodium edetate is added to the preparation the presence of kinins that are slowly being formed can be detected. Also dialysis of the preparation for more than 48 hr (at 8 to 10° C) instead of as usual for 24 to 36 hr, caused the substrate for glandular kallikrein to disappear. The enzyme activity of the preparation was, however, retained after such long-lasting dialysis. The highest plasma kallikrein activity was in pseudoglobulin batches prepared from outdated citrated human bank plasma. The kinin-forming activity was less pronounced in pseudoglobulin made from dog than from human plasma.

The kinin-forming activity of pseudoglobulin could be removed by heating the preparation to 60° C for 1 hr or by exposing the plasma from which it was prepared to massive contact with silicate powder as described in Methods. After such treatment the preparation did not cause kinin formation with plasma taken with the silicone technique. It would, however, still release kinin on addition of glandular kallikrein, some of its substrate being preserved.

Kininase activity was present in all pseudoglobulin preparations and more in preparations made from human plasma than in those made from dog plasma.

Mixtures containing pseudoglobulin from human or dog plasma tended to cause contractions of the rat uterus also when kinins were not thought to be present. These " unspecific " contractions were inhibited by the presence of soya bean trypsin inhibitor or Trasylol in the organ-bath, or by storing the rat uterus for 24 hr at 4° C in the de Jalon solution before using it as test organ.

Substrates for kinin-forming enzymes in human, dog and rabbit plasma

Two different substrates for kinin-forming enzymes can be separated by gel-filtration of plasma on a Sephadex G 200 column (Jacobsen, 1966). Fig. 2 gives the amounts of the two substrates in the various fractions from such a gel-filtration of standard portions (50 ml.) of human, dog and rabbit plasmas. It will be seen that the plasma from all the three species contain both substrates, the greatest amount being obtained from human plasma and the least from rabbit plasma. All three plasmas yielded considerably more of substrate 2 than of substrate 1. The two types of substrates occurred in about the same filtration fractions from all three plasma types, and the relationship to the protein curve was about the same (Fig. 2).

Action of various kinin-forming enzymes on the substrates

Incubation of substrate 1 with either pseudoglobulin or saliva caused the development of equal amounts of kinin activity, as tested on the rat uterus (Fig. 3). After this first incubation, addition to the mixture of the other enzyme source caused no further kinin formation. Pseudoglobulin from human plasma would release the maximal amount of kinin from this substrate in the course of 1 min, and saliva in the course of about 4 min.

When pseudoglobulin was added to substrate 2 no detectable kinin was formed within 15 min of incubation. On addition to this substrate of saliva, however, large amounts

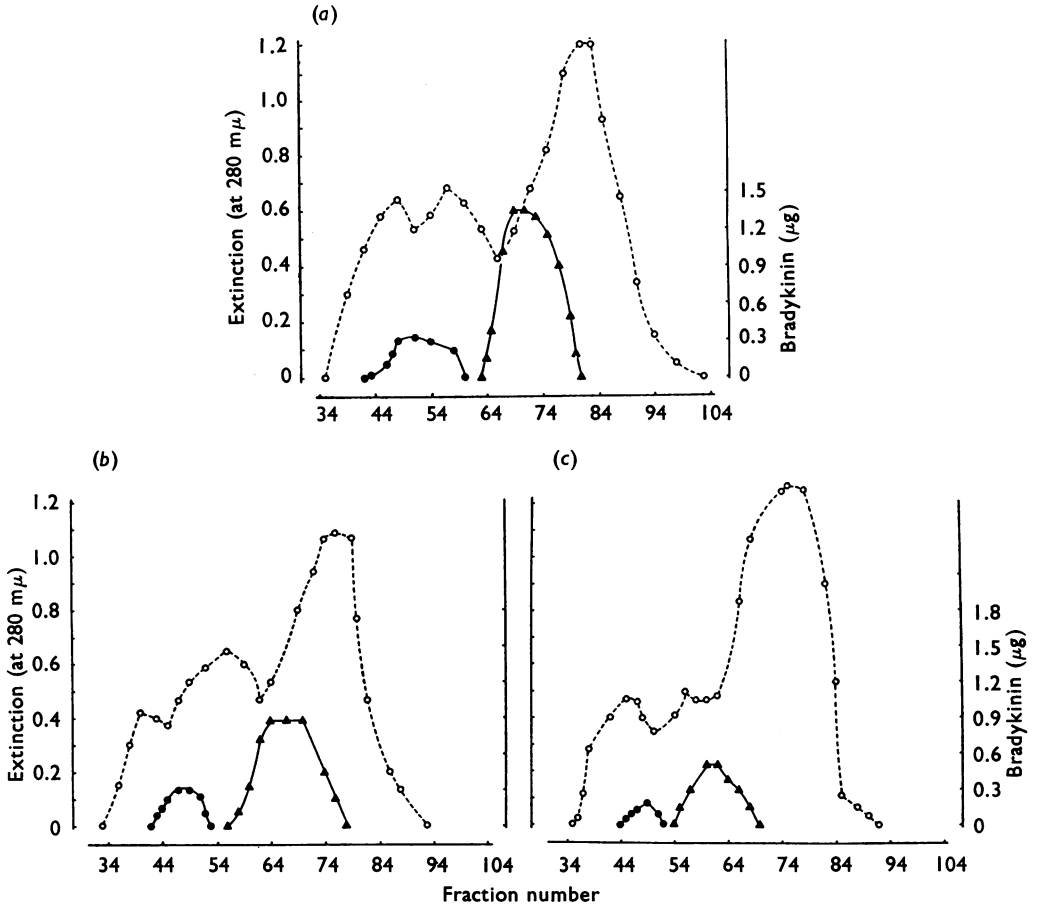

Fig. 2. Content of substrates for kinin-forming enzymes and of protein in consecutive fractions (5 ml.) of human, dog and rabbit plasma from Sephadex G 200 column (see text). (*a*) Human plasma ; (*b*) dog plasma ; (*c*) rabbit plasma. ○- - - -○, content of protein ; ●——●, content of substrate 1 ; ▲——▲, content of substrate 2. Substrates are expressed in terms of kinin that could be developed per ml., synthetic bradykinin being used as reference. Protein contents are expressed as extinctions at 280 mμ, using a Zeiss Spectrophotometer (Model PMQ 11) and 1:16 dilution of the fractions.

of kinin were formed, the maximal amount again being reached in the course of 4 min (Fig. 3). The activities formed on incubation of substrate 1 with pseudoglobulin or with saliva and that from substrate 2 on incubation with saliva all caused contraction of the rat uterus as well as of the guinea-pig ileum, relaxation of the rat duodenum and a fall in rabbit blood pressure on intravenous injections. The activities were furthermore not inhibited by methysergide or mepyramine maleate, and they can thus confidently be characterized as due to plasma kinins. No attempt was made to see if different kinins, or mixtures of kinins, were developed from the two substrates.

In the experiments mentioned above human saliva was used as the source of glandular kallikrein in incubations with fractions from human plasma as well as with fractions from

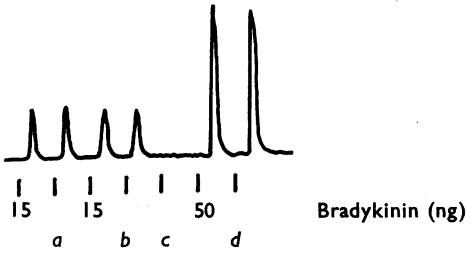

Fig. 3. Maximal kinin activity developed on incubation of substrate 1 or 2 with either pseudoglobulin or saliva. The pseudoglobulin preparation had been dialysed for more than 48 hr. Incubation mixtures: (*a*) 0.2 ml. of a substrate 1-containing fraction and 0.1 ml. of the pseudoglobulin preparation (plasma kallikrein); (*b*) 0.2 ml. of a substrate 1-containing fraction and 0.1 ml. of saliva; (*c*) 0.2 ml. of a substrate 2-containing fraction and 0.1 ml. of pseudoglobulin; (*d*) 0.2 ml. of a substrate 2-containing fraction and 0.1 ml. of saliva. 0.1 ml. of the various mixtures was added to the rat isolated uterus preparation after incubation at 37° C for 1 min in (*a*) and after incubation at 37° C for 4 min in (*b*), (*c*) and (*d*). Standard doses of synthetic bradykinin were added between as indicated. Both substrate fractions (0.2 ml.) were incubated for several hr with 0.1 ml. of saline as controls. No kinin development occurred on such incubations. Similarly no kinin development was observed when 0.1 ml. of the pseudoglobulin preparation was incubated for several hr with 0.2 ml. of saline, not even when sodium edetate (0.1 ml. of 0.027M) was present.

rabbit or dog plasma. With the possibility of species specificity of glandular kallikreins in mind (Bhoola, Morley, Schachter & Smaje, 1965), saliva from dog and rabbit were also examined. No difference could be observed in the kinin formation from dog and rabbit plasma, or from the substrate fractions prepared from these plasmas, when incubated with dog or rabbit saliva instead of with human saliva.

Margolis (1958) has pointed out that plasma kallikrein is very rapidly inactivated in plasma. This was confirmed in the present investigations. Equal volumes of a pseudoglobulin preparation and of citrated plasma from human, dog or rabbit were incubated at 37° C, and 0.2 ml. of this mixture was taken out at intervals and mixed with 0.2 ml. of a substrate 1 preparation and 0.1 ml. of a solution of 0.027M-disodium edetate. Kinin development on the second incubation was taken as an indication of active kallikrein still being present in the first mixture. With rabbit plasma in the first mixture no active kallikrein could be detected after 2 to 3 min. With human plasma no kallikrein could be demonstrated after 3 to 4 min, whereas kallikrein activity persisted for 7 to 8 min of incubation when dog plasma was used. The phenomenon of kallikrein inhibition or inactivation was thus less marked in dog than in human and rabbit plasma. Little kallikrein inhibition was seen in the substrate fractions from Sephadex gel-filtration. Because of the marked kallikrein inhibition, as much as 0.1 ml. of pseudoglobulin from human plasma had to be added to 0.2 ml. of human or rabbit plasma in order to get the maximal amount of kinin formed. With less pseudoglobulin added less kinin was formed, and additional kinin formation was seen when more pseudoglobulin was subsequently added. After heating the plasma to 56° C for 1 hr, much less pseudoglobulin was needed for maximal kinin formation, confirming the finding of Werle (1934) that heating destroys an inactivator of kallikrein.

In previous experiments incubation of rabbit lymph with pseudoglobulin preparations did not result in any kinin formation (Jacobsen & Waaler, 1965). This might be explained by kallikrein inhibition, which is very marked in rabbit plasma. In fact, after heating rabbit lymph to 56° C for 1 hr its incubation with pseudoglobulin resulted in kinin formation. Some substrate for plasma kallikrein is therefore present in rabbit lymph.

Content of substrates in plasma and the effect of storage

The amount of the two substrates can also be estimated in samples of whole plasma by incubation with excess of saliva or pseudoglobulin as described in Methods. The results of such analyses of fresh human, dog and rabbit plasmas are shown in Table 1.

TABLE 1

RANGES FOR THE CONTENT OF SUBSTRATES FOR KININ-FORMING ENZYMES IN FRESH PLASMAS FROM DIFFERENT SPECIES

In parentheses are given the number of individual plasmas on which each range is based. All plasmas were tested immediately after preparation. Incubations and estimations of kinin activity were carried out as described in Methods. The amounts of kinin developed are given as the doses of synthetic bradykinin that had the same effect on the isolated rat uterus preparation

Origin of plasma	Amount of kinin (μg/ml. of plasma) that could be developed	
	With saliva	With plasma kallikrein
Human (10)	2·8–4·5	0·5–1·0
Dog (5)	2·5–4·0	0·5–0·8
Rabbit (3)	1·5–2·5	0·25–0·4

On storage of plasma in ordinary glass tubes, substrate 1 diminishes and eventually disappears completely, as judged from the amount of kinin that could be developed with pseudoglobulin. It disappears more rapidly at 4° C than at higher temperatures (Table 2). In siliconed glass tubes the substrate content is much better preserved.

The content of substrate 1 in plasma which was kept in untreated glass tubes was gradually reduced on repeated freezings and thawings. After five such procedures no kinin could be developed on addition of pseudoglobulin. In plasma that was kept in siliconed glass tubes the ability to release kinin on addition of pseudoglobulin was almost unchanged after three freezings and thawings. On subsequent freezings and thawings moderate reduction in the content of substrate 1 occurred.

TABLE 2

INFLUENCE OF STORAGE ON PLASMA CONTENT OF SUBSTRATE FOR PLASMA KALLIKREIN (SUBSTRATE 1)

Incubation with pseudoglobulin and evaluation of kinin activity were carried out as described in Methods. The amounts of kinin developed in each situation are given as the dose of synthetic bradykinin that had the same effect on the rat isolated uterus preparation

Storage temperature (° C)	Type of glass tube	Kinin (μg/ml. of plasma) developed after storage				
		0 hr	4 hr	6 hr	26 hr	56 hr
4	Siliconed	0·8	0·75	0·7	0·6	0·3
	Untreated	0·8	0·3	0·15	0	
20	Siliconed	0·8	0·8	0·75	0·75	0·6
	Untreated	0·8	0·3	0·2	0	
37	Siliconed	0·8	0·8	0·8	0·8	0·6
	Untreated	0·8	0·8	0·5	0·3	0·1

TABLE 3

INFLUENCE OF STORAGE ON PLASMA CONTENT OF SUBSTRATE FOR GLANDULAR KALLIKREIN (SUBSTRATE 2)

Incubation with saliva and evaluation of kinin activity was carried out as described in Methods. The amounts of kinin developed are given as the doses of synthetic bradykinin that had the same effect on the rat isolated uterus preparation

Storage temperature (° C)	Type of glass tube	Kinin (μg/ml. of plasma) developed after storage		
		0 hr	26 hr	54 hr
4	Siliconed	3·5	2·8	2·8
	Untreated	3·5	1·3	1·0
20	Siliconed	3·5	2·8	2·8
	Untreated	3·5	2·5	2·0
37	Siliconed	3·5	3·0	2·8
	Untreated	3·5	3·0	2·0

The amount of kinin that could be developed in incubation with saliva was moderately reduced on storage of plasma, indicating that substrate 2 is fairly stable (Table 3). The reduction in the amount of kinin developed can mainly be ascribed to the disappearance of substrate 1. Substrate 2 also seemed to be fairly stable on repeated freezings and thawings.

DISCUSSION

Two different substrates for kinin-forming enzymes are present in human as well as in dog and rabbit plasma. There is an apparent difference in molecular size, substrate 1 being larger than substrate 2, as judged from their elution pattern from the Sephadex G 200 column. Also the different behaviours of the two substrates on an anion-exchange column (Jacobsen, 1966) indicate that one is dealing with two different protein entities. The qualitative difference of the two substrates is demonstrated also by their different susceptibility towards enzymes from various sources. Substrate 1 reacts with plasma kallikrein as well as with glandular kallikrein, but apparently more quickly with the former. Substrate 2 will hardly react with plasma kallikrein. Many hours are needed for depletion of this substrate by plasma kallikrein. A rapid kinin formation occurs, however, with saliva.

It had previously been found that substrate for plasma kallikrein was apparently not present in rabbit lymph (Jacobsen & Waaler, 1965). In the present investigation rabbit lymph, as well as rabbit plasma, was found to contain small amounts of this substrate. The previous failure to demonstrate kinin formation in rabbit lymph on incubation with plasma kallikrein has probably been due to the marked inhibition of plasma kallikrein exhibited by rabbit lymph and plasma. Also the high kininase activity of rabbit lymph and plasma may have interfered.

Substrate 1 in plasma is unstable on storage, especially in nonsiliconed glass tubes. This instability is presumably related to activation of plasma kallikrein. The very low affinity of plasma kallikrein for substrate 2 explains the relative stability of this substrate on storage.

The disappearance of substrate 1 but not substrate 2 from a pseudoglobulin preparation can be explained in the same way, as this reagent too contains active plasma kallikrein.

The present experiments show that when treated in different ways the pseudoglobulin fraction may be useful either as a source of plasma-kinin forming enzyme or as substrate for glandular kallikrein. By long-lasting dialysis of pseudoglobulin a preparation is obtained which contains no substrate for kinin-forming enzymes, but with considerable plasma kallikrein activity. On the other hand heating of the preparation to 60° C for 1 hr or exposure of the original plasma to massive contact with silicate powder will yield a preparation with no kinin-forming activity, but with a reasonable content of substrate 2.

The significance of the apparent difference in amounts of the two substrates in human, dog and rabbit plasma is not known.

SUMMARY

1. By gel-filtration on a Sephadex G 200 column two different substrates for plasma kinin-forming enzymes can be detected in dog and rabbit as well as in human plasma.

2. One of the substrates, substrate 1, forms kinins with plasma kallikrein as well as with glandular kallikrein. Kinin formation from this substrate is apparently more rapid with plasma kallikrein than with glandular kallikrein.

3. The other substrate, substrate 2, gives rapid kinin formation only with glandular kallikrein. Plasma kallikrein caused a very slow kinin formation from this substrate.

4. Substrate 1 is relatively unstable on storage and on freezing and thawing.

5. Pseudoglobulin preparations from human and dog plasma contain active plasma kallikrein and some substrate 2, but no substrate 1.

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