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A STABLE SUBSTRATE FOR THE ASSAY OF PLASMA KININ-FORMING ENZYMES

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A substrate plasma for estimation of plasma kinin-forming enzyme activity in biological fluids has been prepared from human blood. Fresh citrated plasma was treated by a brief contact with a large amount of silicate powder, followed by heating to 56° C for 1 hr, and finally by lowering its *p*H to 2 for 10 min. As an alternative to the final treatment with acid, disodium edetate was added to the plasma. The resulting final plasma did not show "spontaneous" plasma kinin activity on contact with glass, contained no kininase activity and did not inhibit plasma kinin enzyme activity of added specimens. The content of plasma kinin precursor in this final plasma was such that it could yield an amount of plasma kinin which corresponded to between 1 and 4 μg of synthetic bradykinin/ml.

The plasma kinin-forming enzyme activity of a biological fluid is usually estimated by incubation of the fluid with a preparation of plasma and the assay of the kinins so formed. Three factors might interfere with this estimation (Lewis, 1960). First, kinin-forming enzymes which are normally present in plasma as inactive precursors might become active on contact with glass or on dilution, for instance, and give rise to "spontaneous" kinin formation. Secondly, kinin-formation might be reduced by inhibitors of enzymes in plasma. Finally the kinins formed during the incubation might be inactivated before estimation by the kininase of plasma.

Several attempts have been made to control these factors in order to produce a substrate suitable for the assay of kinin-forming enzymes in biological fluids or extracts. Werle (1934) observed that heating plasma from various mammals to 58° C for 45 min destroyed what he then called "kallikrein inactivator." Horton (1959) used acid-treatment of dog plasma to eliminate plasma kininase activity and the kallikrein inactivator of plasma, whereas Armstrong, Jepson, Keele & Stewart (1955) showed that plasma kininase activity could be reduced by addition of disodium versenate. However, none of these procedures alone or in combination has yielded a satisfactory substrate, because either the kinin-forming or the kinin-destroying activities of the plasma itself have not been fully controlled or too much of the substrate has been lost during the preparation.

In the present investigation a method has been developed to remove factors from human plasma rendering it incapable of generating kinin activity on contact with glass or on dilution. Subsequent steps in the preparation of the substrate plasma could then be carried out with little loss of plasmakinin precursor.

METHODS

Human plasma. 225 ml. of blood were drawn by venepuncture into each of two siliconized chilled centrifuge bottles, each containing 25 ml. of 3.1% sodium citrate dihydrate. The citrated blood was centrifuged in an "International" refrigerated centrifuge at 1300 *g* for 30 min at 5° C. The plasma was transferred to a siliconized and chilled beaker with a siliconized and chilled pipette.

Silicate powder. "Speed plus" (Great Lakes Carbon Corp., Los Angeles), a powder which contains 90% of silicate particles with a mean diameter of 9 μ and a density of 18.5 to 19 pounds/cu ft, was used for the contact exposure.

Disodium edetate dihydrate. This compound (A/G Fluka, Switzerland), molecular weight 372.5, was dissolved in distilled water and titrated with 0.1 N-sodium hydroxide solution to *p*H 7.4. The concentration of disodium edetate in the stock solution used in the kininase inhibition studies was 5%.

Plasma kinin-forming enzymes. Several sources of plasma kinin-forming enzymes were used. Human saliva was diluted 1:10 with 0.9% saline and centrifuged for 20 min at about 1400 *g*. 0.2 ml. of the supernatant fluid was used as the standard type and amount of enzyme added to each ml. of plasma specimen to be investigated. In some experiments, undiluted human saliva, a preparation of pancreatic kallikrein (Padutin; Bayer, Leverkusen) or human urine was used as a source of plasma kinin-forming enzymes.

Estimation of plasma-kinin activity. Mixtures of plasma preparation and fluids containing enzyme were incubated at 37° C and tested on the rat isolated uterus suspended in de Jalon solution to which methysergide (Deseril; Sandoz A.G.), 0.2 μg/1000 ml., and mepyramine maleate (Anthisan; May & Baker), 0.1 mg/1000 ml., had been added. The contact time was 45 sec, and the interval between each application was 5 min. Contractions were compared with those caused by synthetic bradykinin (Sandoz A.G.). The rats had been given intraperitoneal injections of 0.3 ml. of an 0.01% solution of stilboestrol 16 to 24 hr before being killed.

RESULTS

"Spontaneous" plasma kinin activity of human plasma. Only five out of twelve human plasmas examined in this investigation exhibited plasma kinin activity when brought into contact with the glass wall of a test-tube, and this activity differed in intensity and duration between plasmas. The measurable "spontaneous" kinin activity subsided within 1 hr when stored at room temperature in the same test-tube. Plasma kinin activity could be induced anew, however, by transferring the plasma into another glass tube. This procedure could be repeated several times.

In those plasmas which did not show development of measurable "spontaneous" plasma kinin activity the kinin was nevertheless continuously formed as in the spontaneously active plasmas but was more rapidly inactivated. Addition of 0.1 ml. of the 5% edetate solution/ml. of plasma, to inhibit kinin-inactivation, resulted in the development of pronounced and lasting plasma kinin activity in these plasmas. Addition of the same amount of edetate to plasmas which had exhibited "spontaneous" plasma kinin activity resulted in increased and persistent kinin activity. Both types of plasma could be exhausted of their plasma kinin precursor by storage in the same glass tube for 72 hr or less at room temperature. After this storage, addition of saliva to the plasma did not cause any development of plasma kinin (Fig. 1).

Contact-exposure of human citrated plasma. The effect of a brief contact between plasma and a huge silicate surface was tested in the following way: a siliconized

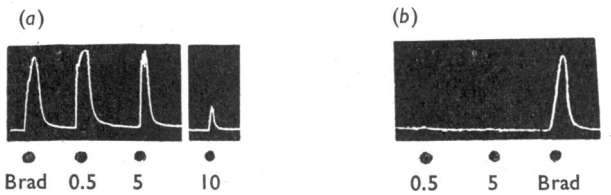

Fig. 1. Plasma kinin precursor in fresh and stored plasma. Incubation at 37° C of 1 ml. of plasma with 0.2 ml. of a 1 : 10 dilution of saliva in 0.9% saline. Contractions of rat isolated uterus suspended in 10 ml. of de Jalon solution on addition of 0.1 ml. of incubated mixture at various intervals. Time of incubation given below in min. (*a*) Freshly drawn citrated plasma; (*b*) same plasma after storage at room temperature for 48 hr. At Brad, addition to 10 ml. organ-bath of a standard dose of 10 ng of synthetic bradykinin.

glass column was packed with 3 g of " Speed plus " on top of a thin layer of glass wool. 100 ml. of fresh citrated plasma were then sucked through this column. After passage through the column neither contact with glass, nor dilution with saline nor addition of edetate induced any plasma kinin activity in the plasma.

The contact-exposure did not noticeably affect either the kininase activity or the content of kinin precursor of the plasma. Incubation of contact-exposed plasma

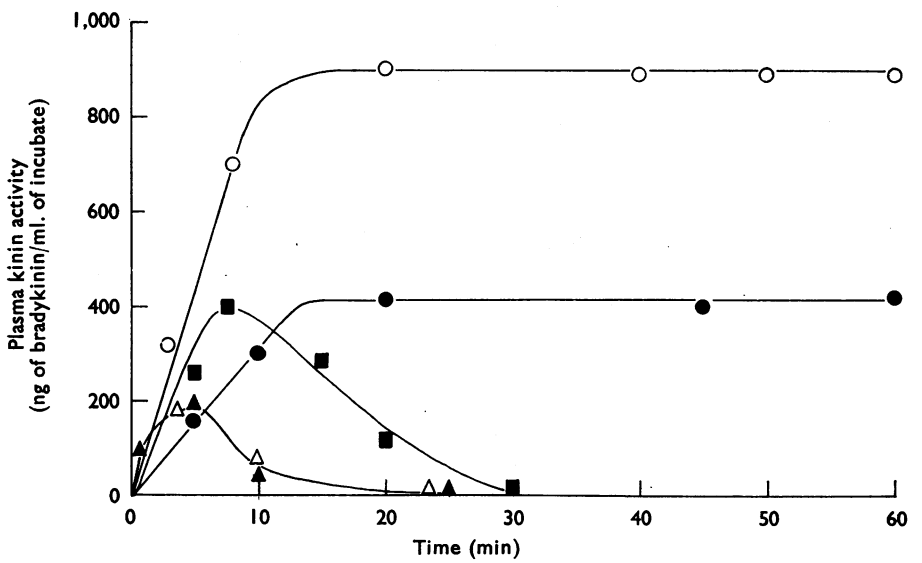

Fig. 2. Development of plasma kinin activity in mixtures of saliva and human plasmas treated in various ways. Incubation at 37° C of 1.0 ml. of plasma with 0.2 ml. of a 1 : 10 dilution of saliva in 0.9% saline; 0.05 ml. volumes of incubates were tested on rat isolated uterus at intervals. △ — △ Fresh, untreated citrated plasma; ▲ — ▲ citrated plasma that had been exposed to silicate powder; ■ — ■ citrated plasma that had been exposed to silicate powder and heated to 56° C for 1 hr; ● — ● citrated plasma that had been exposed to silicate powder and had had 0.1 ml. of a 5% solution of disodium edetate added for each ml. of plasma; and ○ — ○ final substrate plasma (exposed to silicate powder, heated to 56° C for 1 hr and with edetate added as given above). Plasma kinin activity developed is expressed in terms of the activity caused by known amounts of synthetic bradykinin.

with the standard amount of diluted saliva resulted in a development and disappearance of plasma kinin activity which closely resembled that seen with untreated citrated plasma (Fig. 2).

Heating of contact-exposed plasma. The contact-exposed plasma was heated to 56° C for 1 hr to destroy inhibitors of plasma kinin-forming enzymes. When the standard amount of diluted saliva was then added to the plasma, the resulting maximal level of plasma kinin activity was considerably higher than that reached in untreated or in contact-exposed plasma which had not been heated. The plasma kinin activity which developed disappeared from the incubated mixture within 30 min (Fig. 2).

Heating of plasma had some effect on its kininase activity, as shown in Fig. 3. At 4° C, the kininase activity was greater in unheated than in heated plasma. At

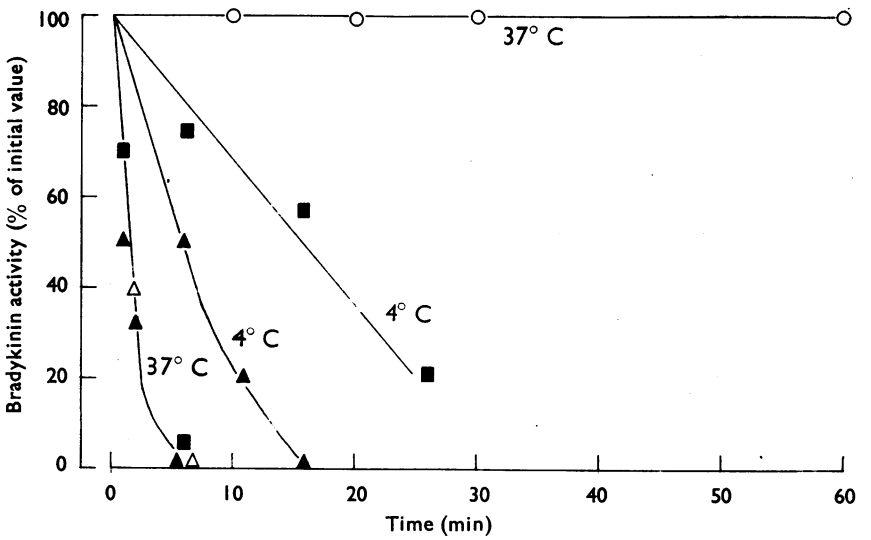

Fig. 3. Kininase activity in heated and nonheated human plasma. Incubation at 4° C or 37° C of 0.9 ml. of plasma with 0.1 ml. of saline containing 5 μg/ml. of synthetic bradykinin; 0.1 ml. volumes of incubates were tested on rat isolated uterus at intervals. △ — △ Fresh, untreated citrated plasma; ▲ — ▲ citrated plasma that had been exposed to silicate powder; ■ — ■ citrated plasma that had been exposed to silicate powder and heated to 56° C for 1 hr; and ○ — ○ final substrate plasma (exposed to silicate powder, heated to 56° C for 1 hr and *p*H lowered to 2 for 10 min).

37° C, however, the kinin activity of added bradykinin disappeared no more slowly than in unheated plasma.

Treatment of contact-exposed and heated plasma with acid. The *p*H in contact-exposed and heated plasma was lowered to 2 by addition of 2N-hydrochloric acid for 10 min and then returned to 7.4 by titration with 2N-sodium hydroxide solution. When the standard amount of diluted saliva was added to plasma treated in this way much more plasma kinin activity developed than with untreated plasma or with plasma that had been contact-exposed and heated only (Figs. 2 and 4). The plasma

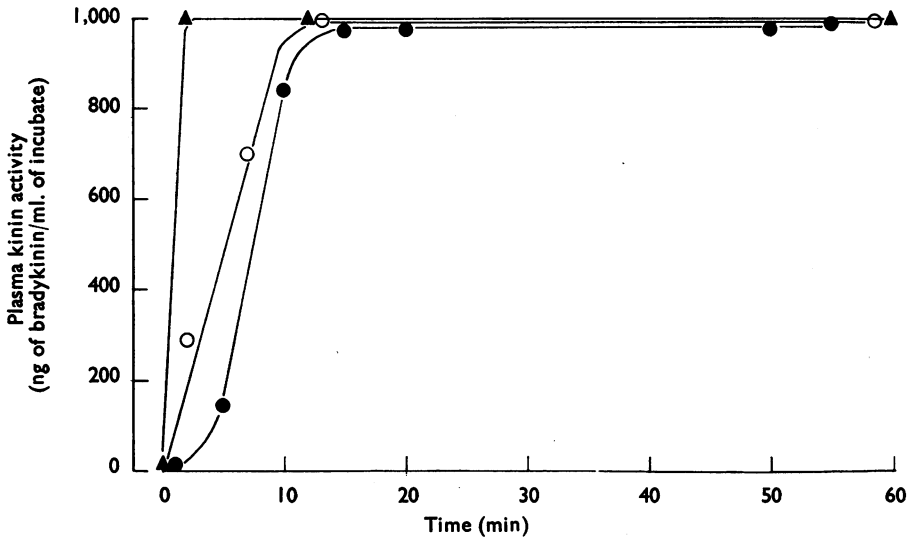

Fig. 4. Plasma kinin activity developed in final substrate on incubation with undiluted human saliva, diluted saliva or human urine. Substrate plasma had been exposed to silicate powder, heated to 56° C for 1 hr and treated with acid (*p*H lowered to 2 for 10 min). Saliva had been similarly heated and treated with acid. Urine was fresh and unheated. 0.05 ml. volumes of incubates were tested on rat isolated uterus at intervals. Incubates: ● — ● 0.9 ml. of plasma substrate with 0.1 ml. of urine; ○ — ○ 0.9 ml. of plasma substrate with 0.1 ml. of saliva diluted 1 : 10 with 0.9% saline; and ▲ — ▲ 0.9 ml. of plasma substrate with 0.1 ml. of undiluted saliva. Plasma kinin activity developed is expressed as in Fig. 2.

kinin activity was still maximal several hours after incubation at 37° C. If undiluted instead of diluted saliva was used as the source of plasma kinin-forming enzyme the same final level of plasma kinin activity was reached within a much shorter time (Fig. 4).

On incubation with the standard amount of diluted saliva, plasma that had been exposed to contact and then treated with acid, but not heated, developed much less plasma kinin activity than did a portion of the same plasma that had been heated as well (Fig. 5). The level of activity reached was stable in both instances. There was no difference however in maximal plasma kinin activity reached in these two types of plasma when undiluted saliva was used as the source of plasma kinin-forming enzymes (Fig. 5).

Addition of disodium edetate. Addition of 0.1 ml. of 5% solution of edetate to each ml. of contact-exposed and heated plasma had the same effect as had treatment with acid. In both instances the final plasma substrate, on incubation with saliva or urine, yielded a plasma kinin activity corresponding to about 1 to 4 μg of synthetic bradykinin/ml. (Figs. 2 and 4). The plasma kinin activity developed was just as stable on storage in plasma which had had edetate added to it as it was in plasma treated with acid. There was one important difference between these two types of plasma substrates, however. When plasma that had had edetate added to it was

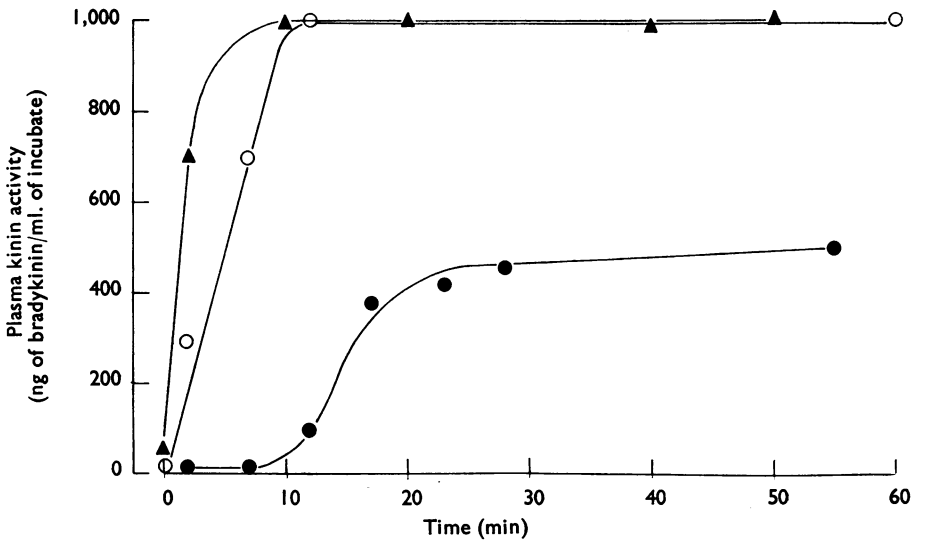

Fig. 5. Effect of heating plasma substrate on development of plasma kinin activity on subsequent incubation with saliva. Incubation of 0.9 ml. of plasma with 0.1 ml. of saliva or diluted saliva at 37° C. 0.05 ml. volumes of incubates were tested on rat isolated uterus at intervals. Saliva had been heated to 56° C for 1 hr and had its *p*H lowered to 2 for 10 min to eliminate its kininase activity. Incubates: ● — ● plasma exposed to silicate powder and treated with acid (*p*H lowered to 2 for 10 min) but not heated, and saliva diluted 1 : 10 with 0.9% saline; ○ — ○ plasma exposed to silicate powder, heated to 56° C for 1 hr and treated with acid, and saliva diluted 1 : 10 with 0.9% saline; ▲ — ▲ plasma exposed to silicate powder and treated with acid, not heated, and saliva used undiluted. Plasma kinin activity developed is expressed as in Fig. 2.

dialysed against buffered saline of *p*H 7.4, its kininase activity reappeared (Fig. 6). Dialysis of plasma treated with acid had no such effect.

Action of various kinin-forming enzymes on the substrate. The final plasma substrate, whether treated with acid or with edetate, formed a large amount of plasma kinin not only on addition of saliva or urine, but also on addition of trypsin or of pancreatic kallikrein. It also developed plasma kinin activity on addition of fresh, contact-activated plasma. Addition of a small amount of fresh

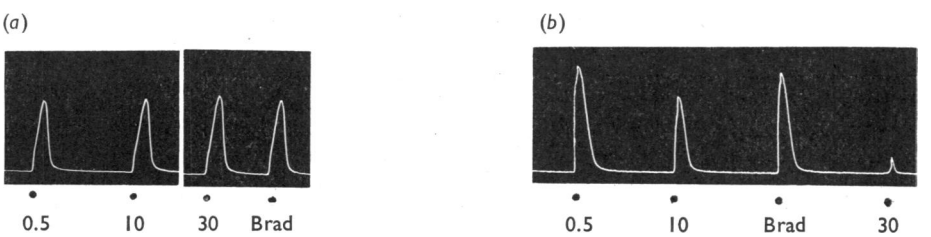

Fig. 6. Reappearance of kininase activity on dialysis of substrate plasma containing disodium edetate. Incubation of 0.9 ml. of plasma substrate with 0.1 ml. of a solution containing 5 μg/ml. of synthetic bradykinin. Contractions of rat isolated uterus on addition of 0.1 ml. of incubates. Times of incubation are given below in min. At Brad, addition to 10 ml. organ-bath of a standard dose of 50 ng of synthetic bradykinin. (*a*) Substrate plasma containing edetate; (*b*) same substrate plasma after dialysis for 24 hr against buffered saline.

plasma which had been shaken with glass beads caused a gradual development in the substrate plasma of considerable plasma kinin activity (Fig. 7). Such an addition to the substrate plasma caused generation of plasma kinin also when carried out after 1 hr storage of the contact-activated plasma. This plasma was by then itself incapable of generating plasma kinin, as shown by its incubation with saliva (Fig. 7). These findings were interpreted as showing that our substrate plasma reacted with plasma kallikrein as well.

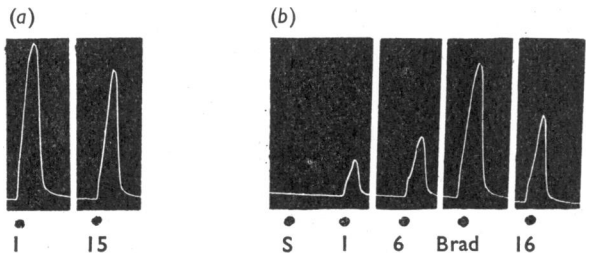

Fig. 7. Development of plasma kinin in substrate plasma on addition of contact-activated plasma. Incubation at 37° C of 0.1 ml. of contact-activated plasma (shaken for 3 min with 0.2 g of glass beads/ml. of plasma) with 1.0 ml. of final substrate plasma (exposed to silicate powder, heated to 56° C for 1 hr and *p*H lowered to 2 for 10 min). Contractions of rat isolated uterus on addition of 0.3 ml. of incubate at various intervals. Times of incubation are given below in min. (*a*) Incubation started immediately after contact-activation of the fresh plasma specimen; (*b*) incubation started 1 hr after the activation of the fresh plasma. Substrate used in incubation of (*b*) had had 0.1 ml. of a 5% solution of disodium edetate per ml. of plasma added to it. At Brad, addition to 10 ml. organ-bath of a standard dose of 25 ng of synthetic bradykinin. At S, addition to organ-bath of 0.3 ml. of a mixture of 1 ml. of the contact-activated plasma (taken 1 hr after its contact-activation) with 0.1 ml. of saliva; this incubation was carried out at 37° C for 1 min.

The substrate plasma could be kept for many hours at room temperature without losing any of the plasma kinin precursor. Even after 72 hr the loss of precursor was small.

DISCUSSION

Armstrong, Keele, Jepson & Stewart (1954) and Armstrong *et al.* (1955, 1957) showed that when blood is brought in contact with glass, plasma kinin is formed. Margolis (1958) demonstrated that blood deficient in Hageman factor will not produce plasma kinin when brought in contact with glass, and that activated Hageman factor is a common denominator for initiation of the so called " intrinsic " blood clotting system and for a plasma kinin generating system. As shown by Margolis (1958, 1963), Hageman factor and also other plasma components participating in blood coagulation and kinin formation may be removed from plasma by exposure to a large contact surface. The factors will adhere to the particles of glass, quartz or other silicate powders added to plasma. Our investigations have demonstrated that a rapid passage of human plasma through a column of silicate particles will alter the plasma in such a way that it no longer develops plasma kinin on storage in a glass tube at 37° C or on dilution with saline. It is reasonable to believe that one or more factors which are needed for the generation of plasma

kinin initiated by contact with glass or by dilution has been removed by the exposure of the plasma to the large surface of the silicate particles.

The large amount of plasma kinin precursor available in human plasma did not apparently diminish by such a rapid passage through a silicate column. The amounts of plasma kinin formed as a result of the passage must therefore have been relatively small.

Kraut, Frey & Bauer (1928) and Werle (1934) demonstrated that plasma contains inhibitors of kallikrein, and that these inhibitors could be destroyed by heating. Our findings agree with this. These inhibitors may be competitive to the plasma kinin-forming enzymes of saliva as their effect can be overcome by increasing the amount of enzymes in the incubate.

Several investigators have shown that there are powerful kininases present in plasma. Horton (1959) demonstrated that these kininases can be destroyed in dog plasma by lowering the *p*H to 2 for 10 min at 37° C. The present investigations show that such treatment with acid also renders our contact-exposed and heated human plasma completely free from kininase activity.

The plasma kinin-inactivating capacity of plasma is thought to be due mainly to a carboxypeptidase which inactivates bradykinin by splitting off a molecule of arginine rendering an inactive octapeptide. Erdös & Sloane (1962) have investigated this carboxypeptidase thoroughly and found numerous inhibitors of it. Among these we have tried disodium edetate which in our studies prevented the breakdown of formed plasma kinin or of added synthetic bradykinin for at least 4.5 hr. Edetate probably acts as an inhibitor of kininase activity by its binding of some metal ion necessary for the function of the enzyme (or enzymes). It is interesting to note that the kininases are not irreversibly destroyed by the presence of edetate, as kininase activity reappears on removal of this compound by dialysis.

Our studies furthermore show that if there are more than one kininase present in plasma, heating and subsequent addition of edetate inhibit and/or inactivate them all. The plasma kinin activity induced in a plasma by edetate can probably be explained by the kininase inhibiting effect of the compound. Even in plasmas which do not show " spontaneous " plasma kinin activity on storage in a glass tube, plasma kinin is probably being formed and broken down continuously. This contention is supported by the fact that such plasmas will be exhausted of their plasma kinin precursor simply by storage in the same test-tube at room temperature for some hours. Furthermore, addition of edetate to such plasmas while they still contain plasma kinin precursor induced detectable plasma kinin activity, whereas no such activity was registered after addition of edetate to a plasma which had previously been exposed to massive contact.

Large amounts of plasma kinin may be produced in human plasma when the inhibitors of kinin-forming enzymes and the kininase activity have been eliminated as described. Our final plasma could develop plasma-kinin activity corresponding to between 1 and 4 μg of synthetic bradykinin/ml. Since this plasma has been somewhat diluted by addition of citrate, hydrochloric acid and sodium hydroxide—or of edetate—the content of plasma kinin precursor in undiluted plasma would

correspond to between 1.5 and 6 μg of synthetic bradykinin. This is somewhat less than the 10.6 μg/ml. reported as the mean content of human plasma as estimated after treatment with trypsin by Diniz, Carvalho, Ryan & Rocha e Silva (1961). There are no indications that we have lost large amounts of precursor during the various steps of preparation. We might have lost some precursor however in the interval between collection of the blood and exposure of its plasma to silicate powder. Diniz *et al.* (1961) worked with heparinized blood whereas we have used citrated blood, a difference which might be important.

The final plasma obtained provides a very good substrate for testing and evaluating plasma kinin-forming enzymes in biological fluids. It reacts to the kinin-forming enzymes but shows no tendency to "spontaneous" kinin formation. In addition it is stable and can be kept for many hours at room temperature without losing any of its content of plasma kinin precursor. It may therefore be useful also for studies of the kinetics of plasma kinin formation.

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