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INFLUENCE OF METALS ON THE ACTIVITY OF A BRADYKININ-DESTROYING ENZYME KININASE

BY

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Kininase prepared from guinea-pig serum was inhibited by cysteine at *p*H 7.4 to 7.6. A similar effect was found with edetic acid. The enzyme blocked by edetic acid was reactivated immediately by addition of Mn++, Co++ and Zn++. These findings indicate that kininase is a metal-activated enzyme.

Rocha e Silva, Beraldo & Rosenfeld (1949) found in their experiments on formation of bradykinin by trypsin and snake venoms that the activity was rapidly destroyed on further incubation ; this destruction could be prevented by cysteine hydrochloride (Frey, Kraut & Werle, 1950 ; Rocha e Silva, 1955). It was suggested that cysteine hydrochloride inhibits the kininase of serum and kidney. Lewis (1960) reported that cysteine also potentiates the smooth muscle stimulating action of plasma kinin. This was confirmed by Picarelli, Henriques & Oliveira (1962), who ascribed this potentiating effect of cysteine to inactivation of the kininase present in the test organs.

However, it is possible that cysteine hydrochloride as a strongly acid compound not only inhibits the kininase activity but also interferes with the smooth muscle stimulating action of plasma kinin by changing the *p*H of the medium. Besides, cysteine may inactivate kininase by binding divalent metal ions, for Smith (1951) found that several peptidases in crude tissue extracts are specifically inactivated by cysteine, cyanide and sulphide, compounds which strongly and specifically bind divalent metal ions. Since the last possibility seemed to us most likely, we studied the influence of neutralized solutions of cysteine hydrochloride on kininase activity, thus excluding any effect that lowering the *p*H of the medium might have on the activity of this enzyme or on smooth muscle.

METHODS

Crude kininase preparation. As Schachter (1960) stated that serum of the guinea-pig has an extremely high kininase activity, we used it as a source of kininase. The serum was poured into ten volumes of acetone at −10° C. The mixture was left in the refrigerator overnight. The precipitate was filtered by suction, washed with ether and dried *in vacuo*.

Isolated guinea-pig ileum. A 4 cm segment of terminal ileum from animals weighing about 250 g was suspended in a 10 ml. bath filled with magnesium-free Tyrode solution at 34° C ; 1 μg atropine sulphate and 2 μg mepyramine hydrochloride were added to the bath after each washing. A cycle of 4 min with 1 min contact was used.

Estimation of kininase activity. A weighed amount of the crude kininase preparation was dissolved in deionized water on the day of the experiment. The incubation mixtures were made up from a solution of 2 μg synthetic bradykinin per ml. magnesium-free Tyrode, a solution of kininase preparation 2 mg/ml. and magnesium-free Tyrode with and without metal-binding agents in the volume-ratio of 1:1:3. Cysteine hydrochloride and ethylenediamine tetra-acetic acid (edetic acid) were used as metal-binding agents. The solutions of these agents in Tyrode were neutralized with sodium hydroxide beforehand. These mixtures were incubated at 34° C and during this incubation samples of 0.2 ml. of these were alternately tested on the isolated guinea-pig ileum at 8 min intervals.

To study the effect of Mg^{++}, Mn^{++}, Fe^{++}, Cu^{++} and Zn^{++} their sulphates were used; for the study of Co^{++} $CoCl_2$ was taken. In the experiments with cysteine these salts were added to the incubation mixture simultaneously with cysteine; edetic acid was added 37 min after zero time.

Estimation of autoxidation of cysteine. The influence of some of the metals used and also of the crude kininase preparation on the autoxidation of cysteine was measured at 37° C with the Warburg technique. The gas phase was pure oxygen. At zero time 0.1 ml. of a solution of cysteine hydrochloride (2×10^{-1} M) in the side arm was tipped into the principal flask containing 2.9 ml. of a tris-(hydroxymethyl)-aminomethane buffer (5×10^{-2} M) with or without the metal ion studied. The final *p*H value after tipping was 7.4.

RESULTS

Potency of kininase preparation. It was found that 400 μg equivalent to serum 6 μl. of our crude kininase preparation, per ml. incubation mixture, was a suitable concentration for our experiments. Fig. 1 shows that with this concentration the bradykinin in the mixture was inactivated in about half an hour at 34° C. At 0° C the kininase activity was very poor. As the decrease of the bradykinin activity was dependent on both temperature and the amount of kininase preparation, we supposed that this was caused by an enzymatic process.

No loss of activity was apparent when the kininase had been stored in the refrigerator in the dry state for over six months.

The kininase-inhibitory effect of cysteine and the reactivation by metals. In accordance with Van Arman (1955) and Horton (1959) we used cysteine hydrochloride in a concentration of 1 mg/ml. of incubation mixture (6.3×10^{-3} M). The *p*H of this mixture was 7.4 to 7.6, so that inactivation of kininase by acid was excluded. The kininase activity was completely inhibited; as shown in Figs. 2 and 3, the bradykinin activity remained constant in the presence of cysteine. However, one-tenth of the concentration of cysteine used in these experiments had no effect on the kininase activity.

We supposed that the kininase-inhibitory effect of cysteine could be ascribed to the elimination of metal(s) from the enzyme system. Smith (1951) found that manganese and magnesium were involved in the activity of some peptidases. Therefore, we wondered if these metals would be able to abolish the effect of cysteine. Fig. 2 demonstrates clearly that Mn^{++}, in a final concentration of 4×10^{-4} M, added to the incubation mixture simultaneously with cysteine abolished the effect of the latter. In the same concentration, Mg^{++} was ineffective. The results of these experiments are presented graphically in Fig. 3.

Autoxidation of cysteine and the influence of metals. The effective concentration of Mn^{++} was not stoichiometrically proportional to the concentration of cysteine.

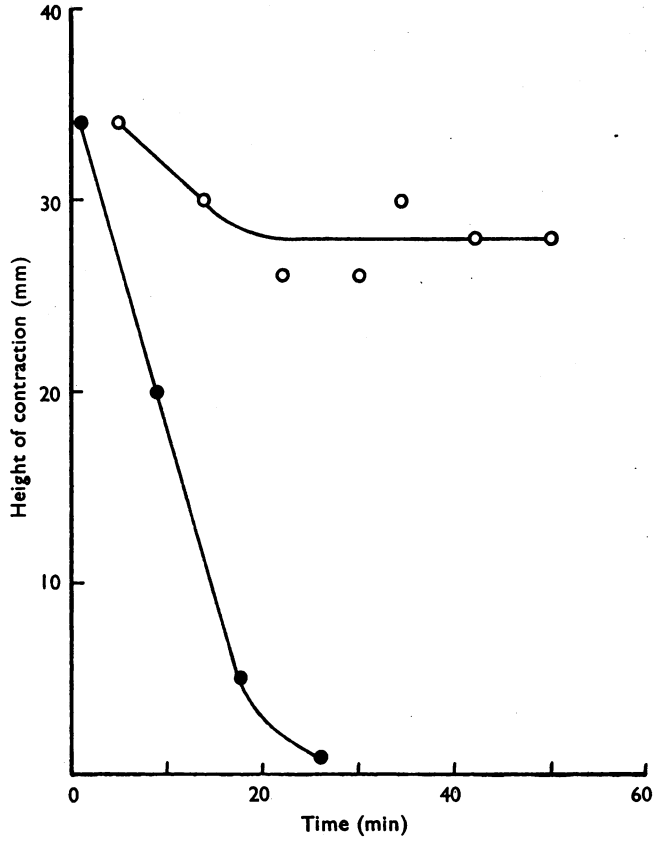

Fig. 1. Influence of temperature on kininase activity. Two mixtures of synthetic bradykinin (400 ng/ml.) with crude kininase preparation (400 μg/ml.) were incubated at different temperatures: 0° C (○—○) and 34° C (● — ●). During the incubation samples of 0.2 ml. of these mixtures were alternately tested on the same piece of isolated guinea-pig ileum at 8 min intervals. The abscissa represents time of incubation in min.

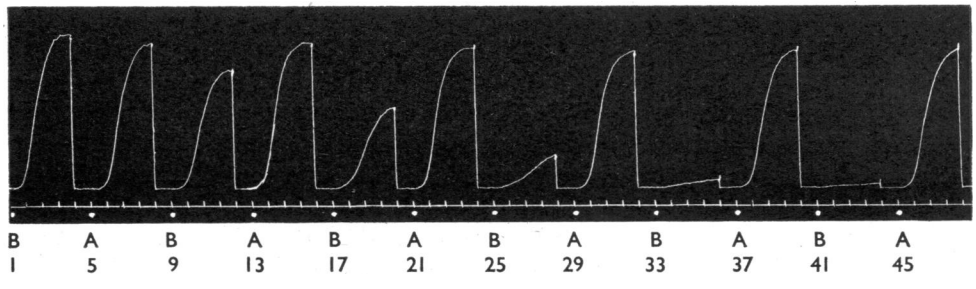

Fig. 2. Contractions of the isolated guinea-pig ileum to 0.2 ml. of an incubation mixture containing 80 ng synthetic bradykinin, 80 μg crude kininase and 200 μg cysteine hydrochloride without (A) and with (B) 4×10^{-4} M $MnSO_4$ (final concentration) in magnesium-free Tyrode. The *p*H of the mixtures was adjusted to 7.4 with additional sodium hydroxide. The numbers represent the time of incubation in min. Time in 15 sec.

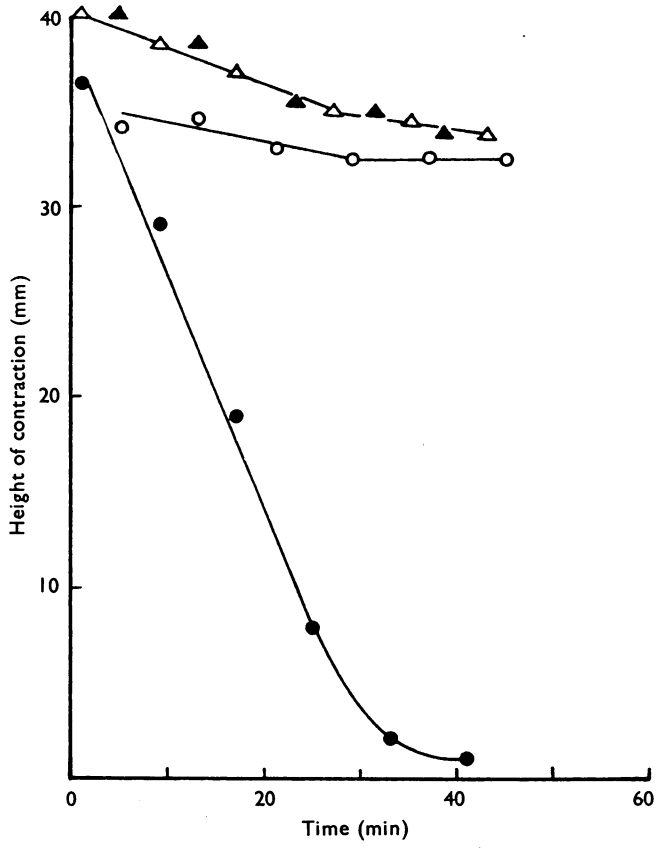

Fig. 3. Graphical presentation of the results of the experiments with $MnSO_4$ (○ ●) and $MgSO_4$ (△▲). During the incubation of 400 ng synthetic bradykinin with 400 μg of the kininase preparation the kininase activity had been inhibited by cysteine (6.3×10^{-3} M) (○—○ and △ — △). Addition of $MnSO_4$ in a final concentration of 4×10^{-4} M reactivated the kininase blocked by cysteine (● — ●) (experiment of Fig. 2), while $MgSO_4$ in the same concentration was unable to do so (▲—▲).

This might be due to elimination of cysteine during the experiment, for it is known that cysteine is autoxidized under certain circumstances (Warburg & Sakuma, 1923; Gerwe, 1931). This induced us to study the autoxidation of cysteine at the *p*H of our incubations and the influence of Mn^{++} and Mg^{++} on it. The autoxidation of cysteine was found to be enormously accelerated by Mn^{++} in a concentration of 3.3×10^{-4} M. As shown in Fig. 4, 80% of the initial amount of cysteine was oxidized in a period of 1 hr. The crude kininase preparation itself also had an accelerating effect on the autoxidation of cysteine. Mg^{++}, however, had no influence.

It was possible, therefore, that the reactivation of the cysteine-inhibited kininase by Mn^{++} was due to elimination of cysteine by autoxidation. Hence the influence on kininase activity of edetic acid as another metal-binding agent was investigated.

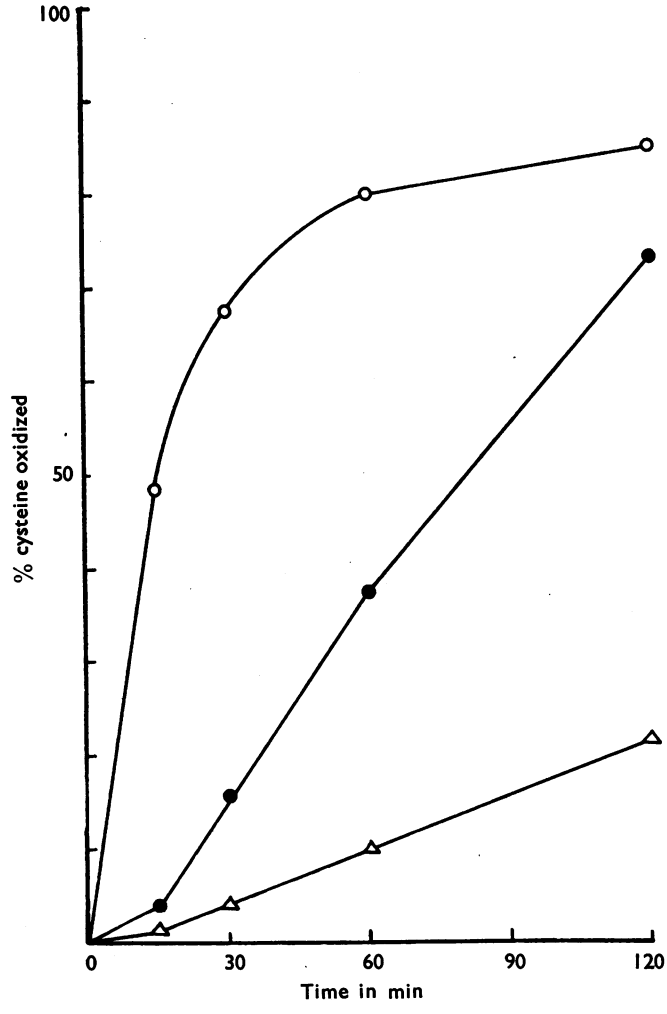

Fig. 4. Oxidation at 37° of 20 μmoles cysteine in 3 ml. of buffer solution (*p*H 7.4) alone (△—△), with 400 μg/ml. kininase (● — ●); and with 3.3×10^{-4} M $MnSO_4$ (○—○).

From the experiments of Armstrong, Jepson, Keele & Stewart (1955) it is known already that edetic acid inhibits the decay of the bradykinin-like activity in human plasma, rat and guinea-pig plasma induced by contact with glass.

Kininase-inhibitory effect of edetic acid and the reactivation by metals. Preliminary experiments showed that edetic acid in a concentration of 2.6×10^{-3} M inactivated the kininase, while one-tenth of this concentration had no influence. The quantity of edetic acid present in the samples tested on the guinea-pig ileum had no visible influence on the test organ itself; however, a small inhibitory effect of edetic acid on the bradykinin activity was seen in the beginning of the experiment in some cases. In these experiments the final concentration of edetic acid in the organ bath was 5.2×10^{-5} M.

Fig. 5 represents the results of a typical experiment, in which two samples of a bradykinin-kininase, one incubated without and one with 2.6×10^{-3} M edetic acid, were tested alternately on the same piece of guinea-pig ileum. In the incubate without edetic acid hardly any bradykinin was demonstrable after a period of 30 min, while no loss of bradykinin took place in the other sample which had been incubated in the presence of edetic acid during about 1 hr except for a small initial decrease. This initial decrease was ascribed to the inhibitory effect of edetic acid itself on the reaction to bradykinin.

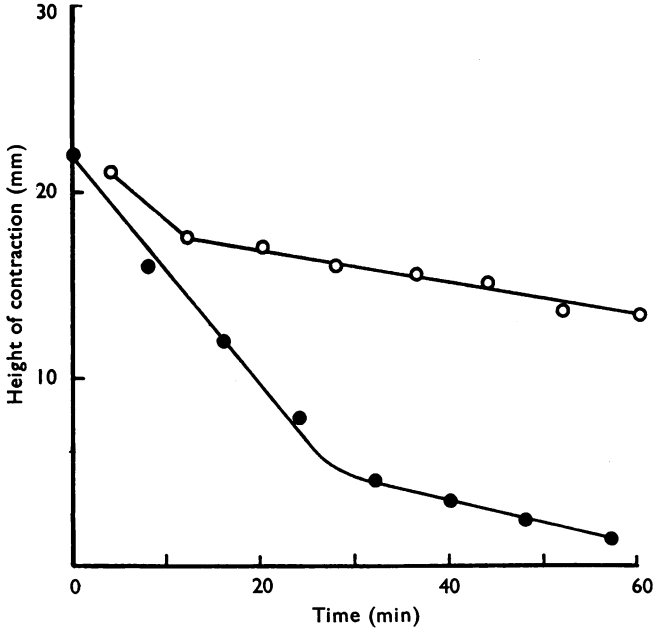

Fig. 5. Inactivation of kininase by edetic acid. Two mixtures of synthetic bradykinin (400 ng/ml.) with kininase (400 μg/ml.) were incubated in the presence of 2.6×10^{-3} M edetic acid (○—○) and without edetic acid (● — ●). During the incubation samples of 0.2 ml. were alternately tested on the same piece of guinea-pig ileum at 8 min intervals. The time of incubation is plotted in min on the abscissa.

Thus, edetic acid was able to inactivate the kininase activity, most likely by binding the metal ion or ions required for its activity. If the kininase-inhibitory effect of edetic acid was produced only by binding the metal activator or activators and so rendering them unavailable for the enzyme, it should be possible to reverse this effect by adding metal ions. The following metals were therefore examined: Mn^{++}, Mg^{++}, Fe^{++}, Cu^{++}, Zn^{++} and Co^{++}. In a concentration of 5×10^{-3} M, Mn^{++} completely abolished the kininase-inhibitory effect of edetic acid (Fig. 6A), while half this concentration was ineffective. The influence of the other metal ions on the kininase, inhibited by edetic acid, was examined using the same concentration of 5×10^{-3} M. We found that Co^{++} and Zn^{++} also abolished the kininase-inhibitory effect (Fig. 6B and C), while Mg^{++} had no discernible influence (Fig. 6D). The

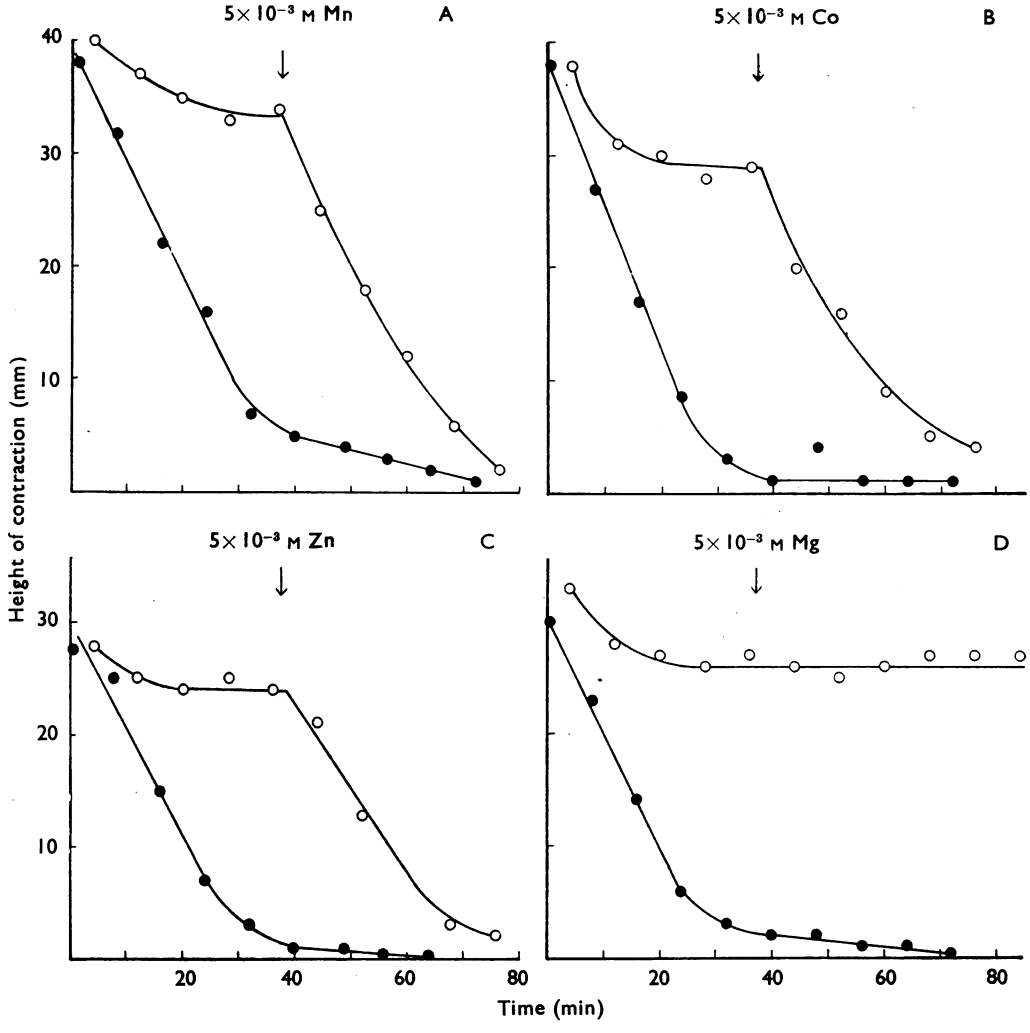

Fig. 6A, B, C and D. Influence of Mn^{++}, Co^{++}, Zn^{++} and Mg^{++} on the kininase blocked by edetic acid. Two mixtures of synthetic bradykinin (400 ng/ml.) and kininase preparation (400 μg/ml.) were incubated, in the presence of 2.6×10^{-3} M edetic acid (○—○) and without edetic acid (● — ●). Samples of 0.2 ml. were alternately tested on the guinea-pig ileum at 8 min intervals. The metals were added at ↓, 37 min after zero time. The abscissa represents the time of incubation in min.

effect of Cu^{++} on the kininase activity was difficult to assess, as the amount of Cu^{++} present in the test samples of the incubation mixture increased the tonus of the guinea-pig ileum ; even after many washings the ileum did not relax completely. Moreover, strong rhythmic contractions were provoked by Cu^{++}. However, we estimated that 47 min after the addition of Cu^{++} no demonstrable loss of bradykinin in the incubation mixture had taken place, indicating that the kininase was inactive. The instability of the Fe^{++} ion in the incubation mixture presented a complicating

factor in the study of the effect of this ion. Very soon after the addition of Fe^{++} the incubation mixture started to turn brown due to oxidation of Fe^{++}. A decrease of the concentration of bradykinin occurred after the addition of Fe^{++}. This decrease, however, was not so rapid as in the control mixture, indicating an incomplete return of kininase activity.

DISCUSSION

The crude kininase preparation used in these experiments was prepared by simple acetone treatment of serum. This method yields a preparation which can be stored for a long time without loss of activity. Another advantage of the acetone treatment is that the so-called lipid-soluble slow-reacting substances (Vogt, 1958) are eliminated.

Cysteine hydrochloride inhibited the bradykinin-destroying activity of kininase at *p*H 7.4 to 7.6. This effect could be abolished by Mn^{++}. However, the concentration of Mn^{++} used accelerates enormously the autoxidation of cysteine at this *p*H.

The inhibition of kininase by cysteine may be interpreted in two ways. Firstly the inhibitory effect may be due to complex formation with the metal ions essential for the activity of kininase. However, metal ions stimulate the autoxidation of cysteine, and therefore a second possibility remains, viz., that cysteine inhibits kininase activity directly. Hence the experiments on cysteine give no information about the question whether kininase is a metal-activated enzyme.

To answer this question we investigated the influence of edetic acid as a specific metal-binding agent on the kininase activity. Edetic acid also inactivated kininase and this inactivation could be abolished by some metals. After the completion of these experiments our attention was drawn to the paper of Erdös & Sloane (1962), who found that Zn^{++} and Co^{++} can reactivate the bradykinin-destroying activity of human plasma fraction IV–1, inhibited by chelating agents. This indicates that metals are involved in the kininase activity; hence we assume that the kininase-inhibitory effect of cysteine is probably due to metal binding.

Erdös & Sloane (1962) preincubated the enzyme with the investigated inhibitors and reactivators. However, our experiments show that both inhibition by the investigated metal-binding agents and reactivation by metal ions set in immediately after adding these to the enzyme. We feel this indicates that kininase is a metal-activated enzyme rather than a metallo-enzyme, as postulated by Erdös & Sloane (1962) for the bradykinin-destroying enzyme in human blood plasma.

Kininase blocked by edetic acid was reactivated by Mn^{++}, Zn^{++}, Co^{++} and probably also by Fe^{++}, while Mg^{++} and Cu^{++} were inactive. It should be noted that after the addition of Mn^{++}, Zn^{++} and Co^{++} to the incubation mixture in which the kininase was blocked by edetic acid the bradykinin disappeared with the same velocity as in the control mixture (see Fig. 6A, B and C). These results seem to conflict with some of those obtained by Erdös & Sloane (1962). They also found that Zn^{++} and Co^{++} reactivate the bradykinin-destroying activity in human plasma fraction IV–1 inhibited by metal-binding agents. In their experiments, however, Mn^{++} was a good inhibitor of this enzyme, whereas Co^{++} accelerated its activity.

The possibility cannot be excluded that the bradykinin-destroying enzymes in human plasma and in guinea-pig serum have different properties. Another explanation for this apparent discrepancy between our results and those of Erdös and Sloane (1962) is that the two enzyme preparations may have differed in their degree of purity. It is known (Dixon & Webb, 1958) that the effect of activating ions may vary with the purity of the enzyme.

The results of our experiments give no evidence that the reactivating metals found are " specific " activators of kininase. It is known that most enzymes are activated by two or three metals, and that Mn^{++}, Co^{++} and sometimes Zn^{++} are interchangeable for some of the peptidases and related enzymes.

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