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THE IN VITRO INACTIVATION AND FORMATION OF PLASMA KININS BY SPLEEN CATHEPSINS*

BY

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Lewis (1963, 1964) has suggested that plasma kinins may play a role in producing inflammation. Evidence in support of this hypothesis is the ability of bradykinin and kallidin (lysyl-bradykinin) to mimic the inflammatory response in producing pain, increased capillary permeability, vasodilatation and the migration of leucocytes. As far as the formation of kinins is concerned, Werle (1955) and Pierce and Webster (1961) have demonstrated that kallikrein enzymes cleave the plasma protein bradykininogen (kallidinogen) to release bradykinin or kallidin. Erdös has demonstrated that destruction of kinins in blood is catalysed mainly through the action of a kininase known as carboxypeptidase-N (Erdös & Sloane, 1962). The amount or activity of this enzyme has been shown to vary in different species (Fasciolo & Halvorsen, 1964).

After injury, tissue enzymes in addition to the plasma enzymes mentioned above might be considered to play a role in the regulation of kinin levels. The major reason for this is the release of proteases from the lysosomes of the damaged tissue. Direct evidence for participation of lysosomal hydrolases in the inflammatory reaction is still lacking (see review by Thomas, 1965) although tentative results have been obtained which suggest that plasma kinin forming factors are activated when blood or plasma comes in contact with damaged tissues (Eisen & Keele, 1964). The object of a series of investigations by this laboratory has been to determine if tissue cathepsins, known to be contained in the lysosomal fraction of the cell (De Duve, Pressman, Gianetto, Wattiaux & Appelmans, 1955), have catalytic properties which might produce kinins from bradykininogen.

In previous papers, evidence was presented that spleen catheptic carboxypeptidases degrade bradykinin to liberate free arginine and phenylalanine (Greenbaum & Yamafuji, 1965). This paper demonstrates the effect of the spleen cathepsins on the pharmacological activity of bradykinin and kallidin. In addition an observation is presented demonstrating the potential of catheptic enzymes in producing a kinin from bradykininogen.

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METHODS

The cathepsin preparation was prepared from beef spleen by procedures previously described (Greenbaum & Fruton, 1957). The preparation contained four different proteolytic activities as measured by the hydrolysis of various synthetic substrates. The enzymes and the synthetic substrates they hydrolyse are listed in Fig. 1. The protein concentration of the preparation was 9.0 mg/ml. Each ml. of the preparation contained the following units of each enzyme: catheptic carboxypeptidase A-29, catheptic carboxypeptidase B-24, cathepsin B-25, cathepsin C-8. The definition of a unit of each enzyme has been previously described (Greenbaum & Yamafuji, 1965).

Catheptic Enzymes in "Hg-ethanol" fraction	Typical Synthetic substrate	
Catheptic carboxypeptidase A	Cbz-L-Glu-L-Tyr	40U*
Catheptic carboxypeptidase B	Bz-Gly-L-Arg	25U
Cathepsin B	Bz-L-Arg-Am	25U
Cathepsin C	Gly-L-Phe-Am	10U

* (U ; % Hydrolysis/min/ml E/ml)

Fig. 1. The cathepsins present in the "Hg-ethanol" fraction of the spleen extract.

Bradykinin and kallidin were gifts of Dr R. B. Merrifield of the Rockefeller Institute and Dr R. Bircher of the Sandoz Corp. Perphenazine was a gift of the Schering Corp.

Human bradykininogen was prepared by procedures previously described for the preparation of bovine bradykininogen except that outdated ACD whole blood was used as the starting material (Greenbaum & Hosoda, 1963).

Kinin assays were carried out on the guinea pig ileum in a 5 ml. bath. The muscle was bathed in oxygenated Tyrode solution containing atropine sulphate and diphenhydramine each in concentrations of 1.0 μg/ml. The temperature of the chamber was maintained at 35° C. The muscle was placed under 1.0 g tension and used after 30 min under the same tension. The response of the muscle was recorded on a Grass polygraph by using a Grass force transducer (there was no spring load on the transducer). *P*-toluenesulphonic acid in a concentration of 0.001 M was used as the diluting solution when samples of the reaction mixture were diluted for assay on the guinea pig ileum. This agent was found to protect against adsorbtion of bradykinin to glass (Greenbaum, Yamafuji & Hosoda, 1965).

RESULTS

In order to test the cathepsin preparation for its ability to inactivate kinins, bradykinin and kallidin were incubated with the cathepsin preparation for 60 min at *p*H 5.1 in the presence and absence of thiol activators. Thiol activators are usually necessary for catheptic activity against synthetic substrates. As seen in Fig. 2, the pharmacological activity of the kinins, as measured by the guinea-pig ileum assay was greatly reduced only in the reaction mixture containing cathepsin and cysteine.

Fig. 2,*B* demonstrates that a thiol containing compound other than cysteine, e.g. mercaptoethanol can substitute as an activator for the inactivating enzymes. Since cathepsin C is not activated by mercaptoethanol (unpublished observations), this enzyme is ruled out as the degrading enzyme. The requirement for an SH activator is in keeping with

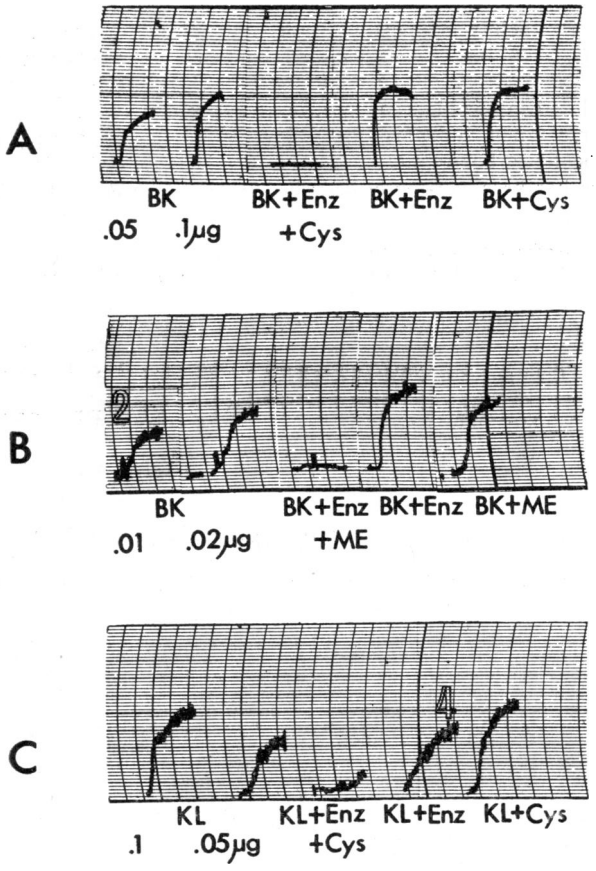

Fig. 2. Inactivation of bradykinin and kallidin by the cathepsin preparation. The complete reaction mixture consisted of 0.016 mg bradykinin, 0.2 ml. 0.05 M sodium acetate *p*H 5.1, 0.05 ml. 0.4 M cysteine-HCL at *p*H 5.1, 0.02 ml. spleen extract and water to a final volume of 0.5 ml. The solution was incubated at 37° for 60 min after which a 0.1 ml. sample was added to 1.9 ml. *p*-toluenesulphonic acid. 0.05 ml. of this solution was added to the muscle bath. *A*. BK—0.05 μg and 0.1 μg of synthetic bradykinin, BK+ENZ+CYS.—complete reaction mixture, BK+ ENZ—cysteine omitted, BK+CYS.—enzyme omitted ; *B*. As in *A* except mercaptoethanol was substituted for cysteine ; *C*. As in *A* except kallidin was substituted for bradykinin.

the properties of catheptic enzymes and differentiates the cathepsins from carboxypeptidase-N (the kininase of blood) which is actually inhibited by sulphydryl compounds (Erdös & Sloane, 1962). As further confirmation that the degrading enzyme in the spleen extract was a cathepsin, 0.001 M iodoacetic acid, a known inhibitor of many of the catheptic enzymes was used in the incubation mixture. The amount used had no effect on the ileum. Fig. 3 demonstrates that iodoacetic acid inhibits the inactivation reaction by the cathepsin preparation providing further proof of the catheptic nature of the enzyme(s) involved.

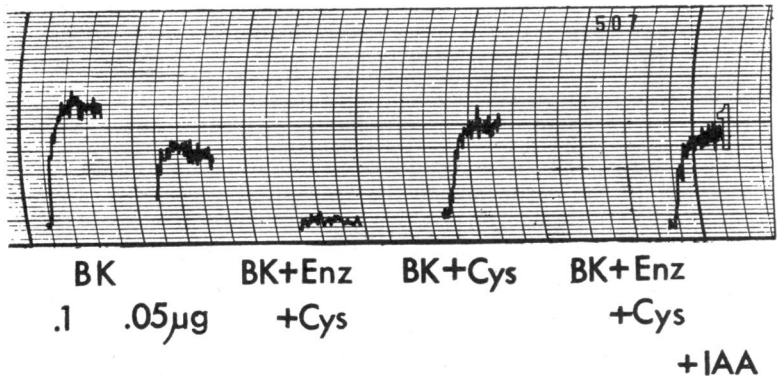

Fig. 3. Effect of iodoacetate on the inactivation of bradykinin. The reaction mixture was the same as in Fig. 2. BK—0.05 μg and 0.1 μg synthetic bradykinin. BK+E+CYS—complete reaction mixture, BK+CYS—enzyme omitted, BK+ENZ+CYS+IAA—complete system in the presence of 0.001 M iodoacetate (adjusted to *p*H 5.1).

The rate at which inactivation occurs when bradykinin and cathepsin are incubated together is seen in Fig. 4. No inactivation occurred in control samples incubated without the enzyme under the same conditions. It may be observed that, with the enzyme concentration used in the experiments reported in Fig. 2, 80% of the activity of bradykinin was destroyed in 60 min. If the enzyme concentration is increased five-fold a complete loss of activity was noted in 15 min.

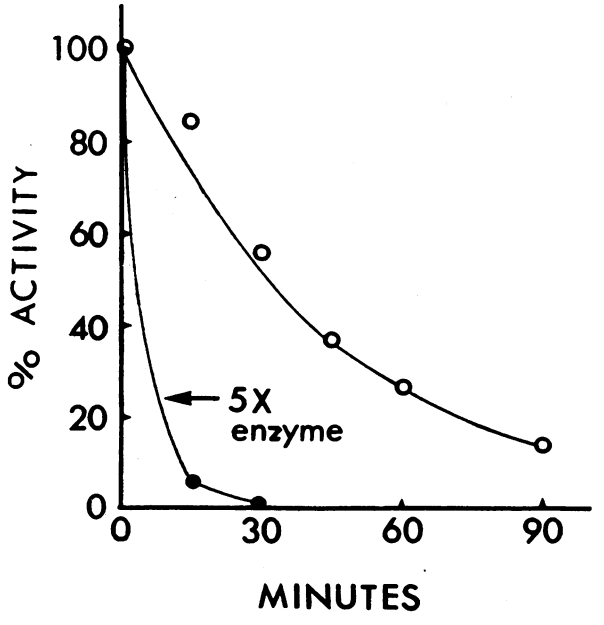

Fig. 4. Rate of inactivation of bradykinin by the spleen preparation. ○——○ reaction mixture as in Fig. 2; ●——● reaction mixture with 5X the enzyme content as in Fig. 2.

Krivoy & Kroeger (1964), have shown that phenothiazines such as perphenazine inhibit the kininase activity of brain extracts presumably by inhibiting the carboxypeptidases contained in these extracts. In order to determine if similar enzymes were involved in the preparation being used from beef spleen, pure perphenazine in concentrations up to 0.002 M was placed in the incubation mixtures of bradykinin and cathepsins. No inhibition was found. However, when a commercial preparation of perphenazine (trilafon) was used as a source of the perphenazine the catheptic inactivation of bradykinin was clearly inhibited at concentrations of 0.002 M. Since trilafon contains high concentrations of sodium bisulphite in addition to perphenazine, sodium bisulphite was suspected as being the inhibitory agent, although never previously reported as an inhibitor of intracellular proteinases. Investigation into the effects of sodium bisulphite on the catheptic inactivation of bradykinin was carried out by incubating bradykinin and cathepsin in the presence of 0.004 M sodium bisulphite. Fig. 5 clearly demonstrates that sodium bisulphite is a potent inhibitor of the catheptic inactivation of bradykinin.

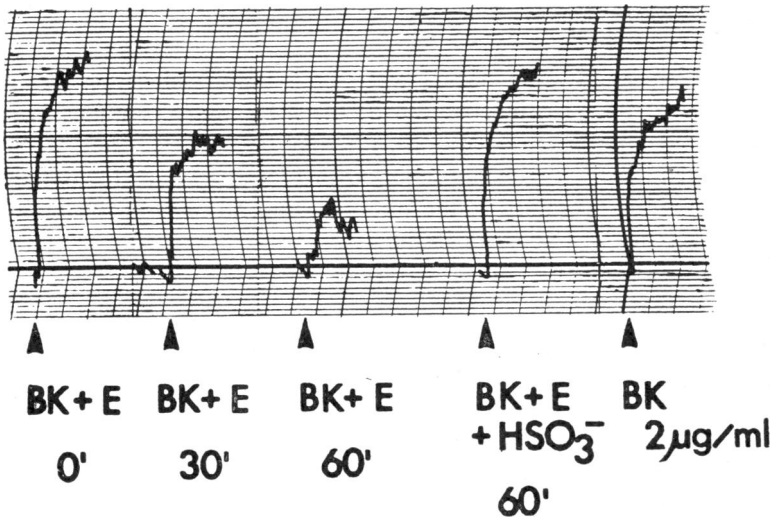

Fig. 5. Inhibition of catheptic inactivation of bradykinin by sodium bisulphite. Complete reaction mixture consisted of 0.02 µg of bradykinin in 0.025 M acetate buffer *p*H 4.0, 0.02 M mercaptoethanol and 0.02 ml. enzyme in a total volume of 0.5 ml. at 37°. At the times indicated 0.1 ml. samples were removed and diluted twenty-fold with 0.001 M *p*-toluenesulphonic acid. 0.04 ml. was added to the muscle bath. BK+E—samples of the reaction mixture removed at the times indicated. BK+E+HSO_3,—reaction mixture contained 0.004 M sodium bisulphite. BK, 2 µg/ml.—solution of synthetic bradykinin from which a 0.04 ml. sample was added to the muscle chamber.

Paper chromatography was previously used as a technique to demonstrate the formation of free arginine and phenylalanine from incubation mixtures of bradykinin and cathepsins (Greenbaum & Yamafuji, 1965). In the current experiments this technique was used to determine the optimal *p*H range at which the cathepsins degrade bradykinin. Samples from incubation mixtures of bradykinin and cathepsins carried out at neutral *p*H and at *p*H 4.0 were removed after varying times and chromatographed by descending chromatography. Fig. 6 demonstrates that at acid *p*H more arginine and phenylalanine

are formed than at neutrality thus providing evidence that degradation takes place more readily at acid *p*H. These results again differentiate the actions of the spleen enzymes from the kininase of blood. Fig. 6 also confirms the previous findings that sulphydryl compounds are required for optimal activity of the enzymes.

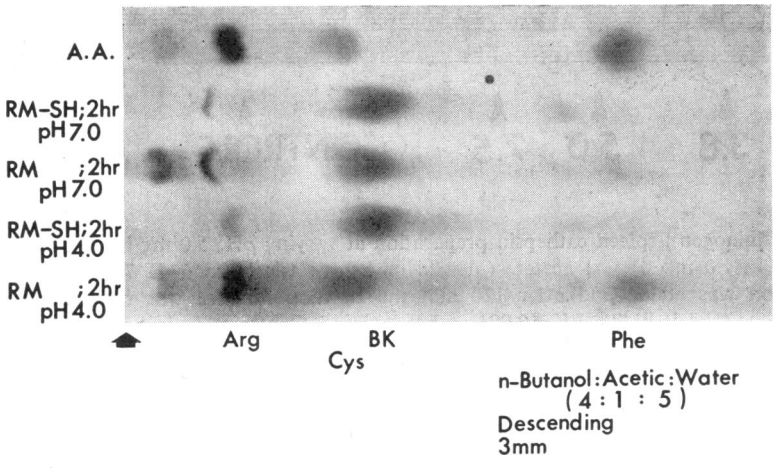

Fig. 6. Descending chromatography of the products of incubation of bradykinin and the cathepsin preparation. The final reaction mixture consisted of 1.0 mg bradykinin in 0.01 M cysteine and 0.05 M acetate buffer, *p*H 4.0 or in 0.05 M phosphate buffer *p*H 7.0, final volume 0.1 ml. 0.002 ml. catheptic preparation was added and after incubation for 2 hr at 35°, 0.01 ml. was spotted on paper. The chromatogram was run for 18 hr and developed for ninhydrin positive material as previously described (Greenbaum & Yamafuji. 1965). AA—amino acid standards, 0.05, μmole of arginine, phenylalanine, and cysteine (cysteine is oxidized partially to cystine during the chromatography—spot closest to the origin, cystine). RM—complete reaction mixture. RM—SH, reaction mixture without cysteine.

The experiments reported above provide definitive evidence that tissue cathepsins can degrade and inactivate bradykinin under specified conditions. It was of equal interest to determine if the catheptic enzymes in the spleen preparation might produce kinins by cleaving bradykininogen. Human bradykininogen and the catheptic preparation were incubated at varying *p*H for 120 min. Thiol groups were omitted from the reaction mixture so as to prevent the action of the inactivating cathepsins. Fig. 7 demonstrates that kinin-like activity is produced optimally at acid *p*H by this purified cathepsin preparation. No kininase activity is present in the cathepsin preparation at neutrality as demonstrated by the fact that the activity of bradykinin solutions were not diminished when incubated with the cathepsin preparation at neutrality. (Bradykinin solutions also maintained complete activity after incubation with the bradykininogen substrate used.) Thus the activity formed in the experiment illustrated in Fig. 7 represents an actual *p*H optimum rather than a resultant of activation and inactivation. Additional experiments not reported in detail here have demonstrated that the activity produced is destroyed by carboxypeptidase B (pancreatic) and chymotrypsin, thus indicating that the material is polypeptide in nature. The purified nature of the bradykininogen used would also lend strong evidence that the material produced is similar to bradykinin although further investigations are being pursued to confirm this.

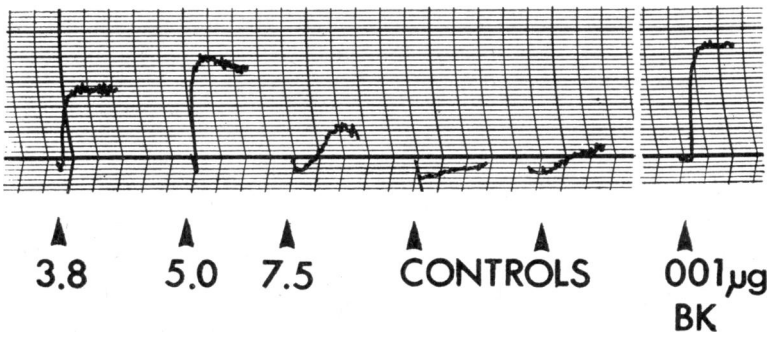

Fig. 7. Bradykininogen + spleen cathepsin preparation at varying *p*H. 5.0 mg bradykininogen (human) dissolved in 1.0 ml. 0.05 M acetate (*p*H 3.8 or 5.0) or tris buffer (*p*H 7.5). 0.05 ml. cathepsin preparation was added and after 120 min the reaction mixture was neutralized and 0.2 ml. added to the muscle bath. BK = 0.001 μg bradykinin. Controls—Left, bradykininogen in acetate buffer at *p*H 5.0 with no cathepsin. Right, cathepsin and acetate buffer at *p*H 5.0 with no bradykininogen. Similar controls were run at *p*H 3.8 and at 7.5 with no activity being formed.

DISCUSSION

Current theory as to the formation and degradation of kinins may be seen in Fig. 8. The dark arrows represent in this simplified scheme the major pathways of the formation and degradation of kinins such as bradykinin. In these reactions tissue kallikreins formed from a zymogen or prokallikrein (Frey, Kraut & Werle, 1950) catalyse the formation of kinins from the plasma protein known as bradykininogen (kallidinogen) while the plasma enzyme carboxypeptidase-N inactivates the kinins formed. Three possible reactions in which catheptic enzymes might participate are also shown in Fig. 8 and are indicated by the light lines. The results of the *in vitro* experiments presented above have shown that two of these pathways are possible, e.g., tissue cathepsins can inactivate and produce at acid *p*H bradykinin or related polypeptides.

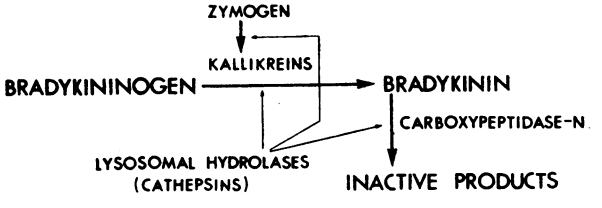

Fig. 8. Possible roles of catheptic enzymes in producing or inactivating bradykinin.

The inactivating enzymes are inhibited by iodoacetic and by sodium bisulphite and require sulphydryl compounds for activation. These findings and previous observations concerning the properties of spleen proteases (Greenbaum & Sherman, 1962) led to the conclusion that the degrading enzymes are catheptic carboxypeptidase B & A. These

intracellular enzymes remove carboxyl-terminal amino acids from synthetic peptides as illustrated in Fig. 1. The degradation of bradykinin proceeds as illustrated in Fig. 9. Catheptic carboxypeptidase B cleaves the carboxyl-terminal arginine from the polypeptide which results in its inactivation. The phenylalanine moiety is then removed from the resulting octapeptide by catheptic carboxypeptidase A. The proline moiety is probably resistant to catheptic carboxypeptidase A and prevents the degradation from continuing further. Experiments to be reported elsewhere have demonstrated that sodium bisulphite which inhibits the inactivation of bradykinin by these enzymes also inhibits their activity toward synthetic substrates. Sodium bisulphite might prove to be a valuable agent in preventing peptide degradation during extraction of kinins from tissues.

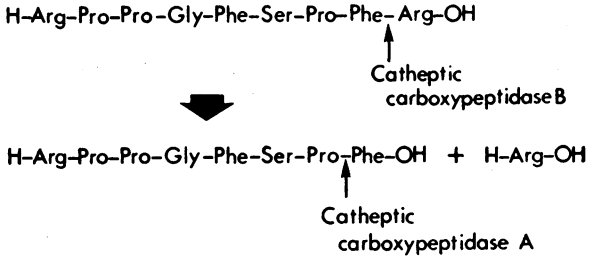

Fig. 9. Mode of degradation of bradykinin by catheptic carboxypeptidases B & A.

The ability of catheptic enzymes in the spleen to produce kinin-like material maximally at acid *p*H differentiates this activity from that of kallikreins which are maximally active at neutrality (Webster & Pierce, 1961). The results lend support to the idea that during tissue injury enzymes (lysosomal in origin) from tissues might be liberated which have the catalytic capability of producing kinins. The importance of the reaction would depend on the nature of the environmental *p*H. Acidic conditions which are supposedly present in inflammation would favour kinin formation or inactivation by catheptic enzymes. An additional regulating mechanism might be the presence of sulphydryl compounds which apparently as demonstrated from these experiments would favour inactivation of kinins. An additional possibility concerning the role of cathepsins in the " kinin system " is also outlined in Fig. 8 as the arrow demonstrating catheptic activation of the zymogen (kallikreininogen) of kallikrein. Formation of this enzyme by a tissue protease would enhance formation of kinins during injury. Cathepsin B, an enzyme which has been shown to convert the zymogen trypsinogen to trypsin (Greenbaum, Hirschkowitz & Shoichet, 1959) might be an agent that could catalyse such a reaction. A similar idea has been expressed by Eisen & Keele (1965). Experiments confirming such a hypothesis must await purification of kallikreininogen.

Thomas 1965) in a recent review of the role of lysosomal enzymes in tissues injury points out that although such hydrolases probably play a role in inducing various forms of tissue damage, direct evidence for their participation in the inflammatory reaction is lacking. The current results provide additional evidence, although still indirect, that tissue hydrolases may participate under well defined conditions in the formation and degradation of kinins thus possibly contributing to the inflammatory response.

SUMMARY

1. Beef spleen cathepsins inactivate bradykinin and kallidin *in vitro* at acid *p*H and in the presence of thiol groups.

2. Iodoacetic acid and bisulphite inhibit the inactivating enzyme. Sodium bisulphite is suggested as an agent which might prevent enzymatic hydrolysis of peptides during extraction from tissues.

3. Catheptic carboxypeptidase B and catheptic carboxypeptidase A degrade bradykinin from the carboxyl-terminal end and are presumably the enzymes in spleen which inactivate the kinin.

4. In the absence of added thiol compounds, the spleen preparation produced kinin-like material as assayed on the guinea-pig ileum. The optimal *p*H for this reaction was shown to be at *p*H 5.0.

5. The possible implications of these reactions occurring *in vivo* during tissue injury and playing a role in inflammation are discussed.

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