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THE KININ-FORMING AND KININASE ACTIVITIES OF RABBIT POLYMORPHONUCLEAR LEUCOCYTES*

BY

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The major pathway for bradykinin and kallidin formation in the extracellular compartments in the body is by cleavage of the blood protein bradykininogen by kallikreins as demonstrated by Werle (1955) and by Pierce & Webster (1961). Inactivation of these peptides is mediated by carboxypeptidases as shown by Erdös & Sloane (1962). Additional reactions leading to kinin formation and destruction have been shown by Greenbaum & Yamafuji (1966, 1966a) to be possibly mediated by tissue cathepsins.

Lewis (1963, 1964) has postulated that bradykinin and kallidin may contribute to the signs and pathological responses seen in inflammation since these peptides can cause increased capillary permeability, migration of leucocytes, vasodilatation and pain. The question remains unanswered as to the actual role of these kinins in inflammation—that is, whether they are produced during the inflammatory state. Indeed, Janoff has presented evidence that the inflammatory response may be mediated by agents which are not kinins (Janoff, Bean & Schuller, 1965) but which are basic proteins which have no enzymatic activity.

An integral part of the inflammatory response is the migration of polymorphonuclear leucocytes (PMN) to the site of injury. These cells carry on phagocytosis at which time their intracellular pH becomes quite acidic. After several hours, the PMN cells may even disintegrate, liberating their lysosomal and cytoplasmic enzymes into the inflamed area (Rogers, 1964). The current investigation was carried out to answer the question whether or not PMN cells contained enzymes that could produce kinins from the protein substrate bradykininogen. In addition, we have investigated in some detail the kininase (kinin inactivating) activity of the PMN leucocytes first described by Schwab (1962). In the current investigation, polymorphonuclear leucocytes were obtained from an inflammatory exudate produced by glycogen in the peritoneal cavity of rabbits. The cells were lysed and the lysate subjected to differential centrifugation. The lysosomal and extra-lysosomal fractions so obtained were tested for kinin forming and kinin inactivating activities. Previous communications have provided the background to this investigation (Greenbaum & Yamafuji, 1966 ; Greenbaum, Freer & Kim, 1966) as well as initially demonstrating that the contents of lysed PMN cells contain kinin-forming activity.

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METHODS

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 μg/ml. The temperature of the chamber was maintained at 35° C. The muscle was placed under 1 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 with no spring load. 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 had previously been found to aid against adsorption of bradykinin to glass (Greenbaum, Yamafuji & Hosoda, 1965). The minimal quantities of bradykinin that were used to obtain standard responses of the ileum ranged from 5 to 20 ng added to the 5 ml. bath. However, in experiments where kinin-forming activity was being measured, the ileum was treated with chymotrypsin as described by Edery (1964) in order to increase the sensitivity of the ileum to bradykinin. 0.2 mg of chymotrypsin (Worthington) was added to the muscle bath for 1 min. The chymotrypsin was washed out and the muscle used after 10 min. A two to fourfold sensitization of the muscle to bradykinin was usually obtained.

Polymorphonuclear leucocytes were obtained from peritoneal exudates of rabbits injected intraperitoneally with glycogen by procedures described by Cohn & Hirsch (1960). Three hours following the injection of glycogen, the exudates were obtained by gravity drainage after introducing heparinized saline. The exudates from five rabbits were pooled (1,500 ml.) and the suspension centrifuged at $250 \times g$ at 4° to collect the cells (2×10^9). The cells were washed twice by gentle stirring in 0.34M sucrose followed by low speed centrifugation. The cells were then lysed by vigorous pipetting after being suspended in 0.34M sucrose. The suspension was centrifuged at $400 \times g$ to remove the nuclei, cell debris and any red blood cells. The resulting 20 ml. supernatant fluid was centrifuged at $8{,}200 \times g$ for 15 min at 4°. The pellet, which is composed of the lysosomal granules, was suspended in 4 ml. of 0.34M sucrose and frozen for future use or treated with an equal volume of 0.2M acetate at pH 4 for 30 min at 2° to lyse the granules. In some cases this was followed by treatment with ammonia sulphate to 50% saturation at 2° after which the resulting precipitate was dissolved in water and dialysed against two changes of distilled water over 18 hr. Any insoluble material was centrifuged off and discarded.

Human bradykininogen was prepared from outdated ACD whole blood by procedures described previously for bovine bradykininogen (Greenbaum & Hosoda, 1963). The human bradykininogen had specific activities of 0.2 to 0.5.

Bovine bradykininogen was a gift from Dr E. Haberman of the Institut für Pharmakologie der Universitat Würzburg, Germany. Bradykinin was a gift of Dr R. Bircher of the Sandoz Corp. Trasylol (Bayer Corp.) was a gift of Dr Sue Buckingham of Columbia University.

RESULTS

Cellular location of kininase activity

The kininase activity of the lysosomal and extralysosomal fractions was first investigated. The $8200 \times g$ pellet (lysosomal fraction) and the $8200 \times g$ supernatant fluid (extralysosomal fraction) of the PMN cells were tested for their kininase activity by incubating bradykinin in the presence of these fractions and removing samples at various times and assaying them on the guinea-pig ileum. As seen in Fig. 1, bradykinin activity was reduced after incubation with the extra-lysosomal fraction (supernatant) but not after incubation with lysosomal fraction of the PMN cells (pellet). Table 1 demonstrates on a quantitative basis that the kininase acivity is located in the cytoplasmic fraction outside of the lysosomes—that is, in the extra-lysosomal fraction.

pH optimum of the kininase activity

Incubation of bradykinin with the $8200 \times g$ supernatant fraction was carried out over a

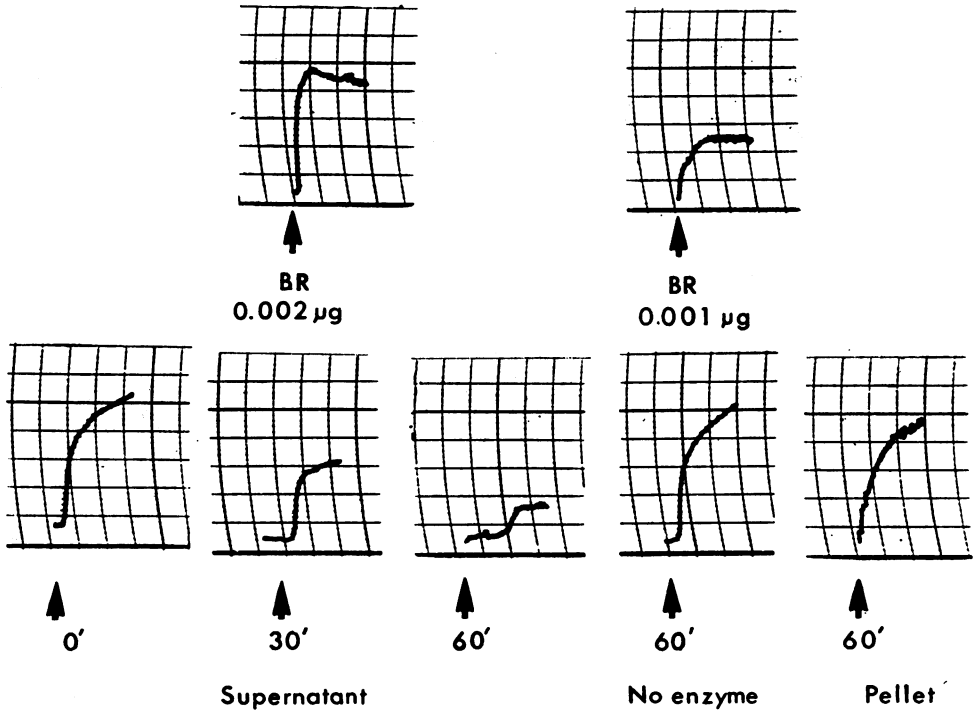

Fig. 1. Kininase activity of the lysosomal and extra-lysosomal fractions of the PMN cells. The incubation solution consisted of 0.02 mg bradykinin, 0.05 M Tris buffer pH 8.5, 0.2 ml. of the 8200 × *g* supernatant or pellet in a final volume of 0.6 ml. at 37°. At the times indicated a 0.05 ml. sample was diluted to 1.0 ml. with *p*-toluenesulphonic acid (0.001 M) and 0.01 to 0.05 ml. of this added to the muscle bath. BR = bradykinin added to the bath. Supernatant = 8200 × *g* supernatant (extra-lysosomal) and bradykinin. Pellet = 8200 × *g* pellet (lysosomes) after lysis in acetate and bradykinin. No enzyme = incubation solution minus the enzyme.

TABLE 1

KININASE ACTIVITY OF THE PMN FRACTIONS

Bradykinin was incubated with the 8200 × *g* supernatant or pellet for 60 min as described in Fig. 1

8200 × *g* fraction	Total units*	Total protein (mg)	Specific activity	Volume (ml.)
Supernatant	3,880	85·2	44·3	20
Pellet (lysosomes)	14	24·5	0·5	4

* Unit: 100 ng bradykinin destroyed/min/ml. enzyme.

varied pH range. As seen in Fig. 2, the optimum pH for the kininase activity was reached at about pH 8.5, although a considerable amount of enzyme activity is present at neutrality.

Effect of various agents on the kininase activity in the 8200 × g supernatant

In order to determine some of the properties of the kininase enzyme, its activity in destroying bradykinin was tested in the presence of various agents. In Table 2 are the

results of such experiments. Using the value of 100% for the activity of the enzyme and bradykinin alone, it may be seen that unlike the kininase in blood (Erdös, Sloane & Wohler, 1964), manganese and cobalt fail to stimulate the activity of the PMN enzyme. Copper, mercury and DFP inactivate the kininase. Zinc, sodium bisulphite and

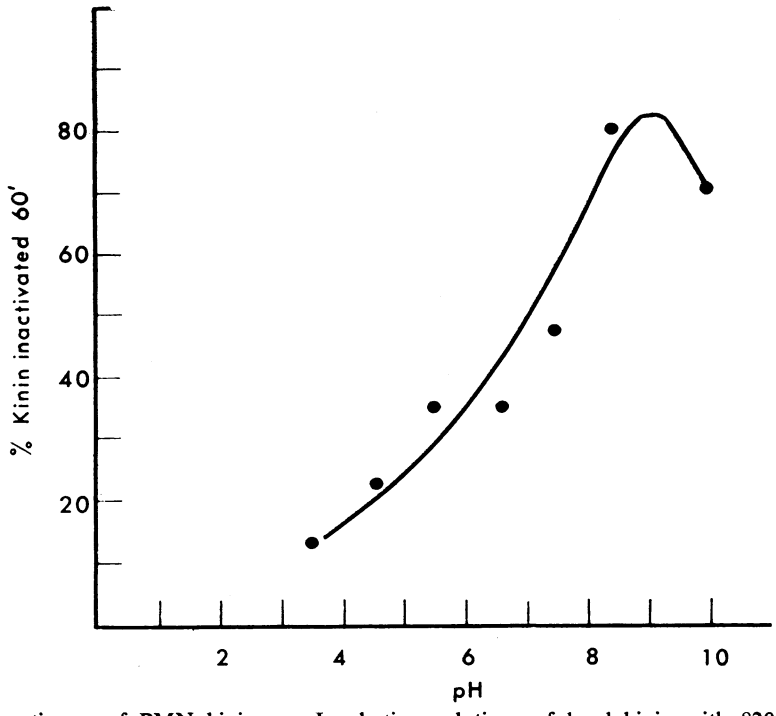

Fig. 2. pH optimum of PMN kininase. Incubation solutions of bradykinin with 8200 × *g* supernatant for 60 min as in Fig. 1 but using varying pH. 0.025 M tris buffer was used at pH values from 7.5 to 10. Lower pH experiments were carried out in the presence of 0.05 M acetate buffer.

TABLE 2

EFFECT OF VARIOUS AGENTS ON PMN KININASE

Solutions of bradykinin and 8200 × *g* supernatants were incubated for 60 min at pH 7·5 in the presence of the agents listed below under conditions given in Fig. 1

Agent (10^{-4} M)	% Kininase activity remaining
None	100
$Mn Cl_2$	
$Co (NO_3)_2$	
Trasylol (50 KIU)	
EDTA	
Cysteine	
Bisulphite	70
$Zn Cl_2$	
α-α dipyridyl	
$Cu SO_4$	0
$Hg Cl_2$	
DFP	

α-α-dipyridyl inhibit only partially. The action of the latter agents was investigated since they inhibit catheptic carboxypeptidases which also destroy bradykinin (Greenbaum & Yamafuji, 1966a). Neither trasylol nor EDTA had inhibitory activity on the kininase.

Other properties

The kininase activity has been found to be somewhat labile on standing at 2° or on freezing and thawing. It is not unusual to experience a 50% loss in activity within a short time after storage. The activity then seems to stabilize at a lower level. Whether this loss in activity is due to the physical properties of the enzyme itself or inactivation by heavy metals or other proteases is being investigated. The enzyme does not hydrolyse hippuryl-l-arginine or hippuryl-l-lysine as determined by chromatography of incubation mixtures of the synthetic substrates with the 8200 × *g* supernatant.

Kinin-forming activity

Examination of the lysosomal and extra-lysosomal fractions for kinin-forming activity was carried out by incubating a sample of these fractions with partially purified human bradykininogen. These incubations were initially carried out at acid pH, an environment found to favour the reaction since the kininase activity would be minimal. Figure 3 demonstrates that the lysosomal fraction in the presence of human bradykininogen

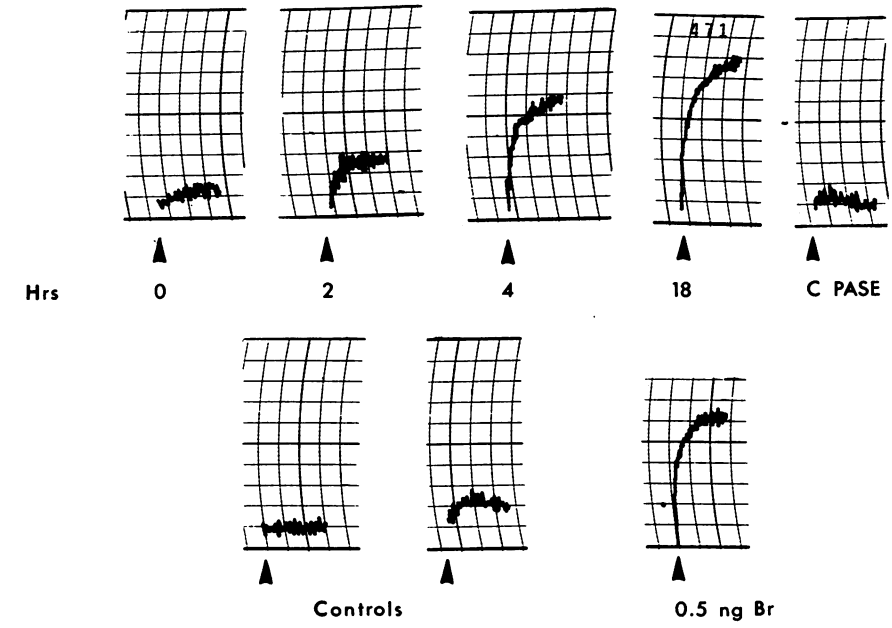

Fig. 3. Kinin formation by PMN pellet. To 25 mg of human bradykininogen in 5 ml. of 0.1 M acetate buffer at pH 3.5 was added 1 ml. of 8200×*g* fraction pellet. At the times indicated, 1 ml. samples were neutralized and centrifuged. 0.2 ml. aliquots of the resulting supernatant fluid were added to the muscle bath. The muscle had been sensitized by chymotrypsin by the procedure of Edery. Hrs = time of incubation in hours. CPASE = 15 μg of carboxypeptidase B added to a 0.5 ml. of the 18 hr sample and tested after 15 min. Controls = left, incubation solution without bradykininogen; right, incubation solution without 8200×*g* pellet. 0.5 ng Br = response of the muscle to 0.5 ng bradykinin added to the bath.

produces, over a period of time, increasing quantities of a kinin-like material as measured by the response of the guinea-pig ileum. The biological activity of this material is destroyed after incubation with carboxypeptidase B, indicating its peptide nature and the presence of a basic amino acid on the carboxyl-terminal position. Similar results are obtained when the extra-lysosomal fraction is incubated with bradykininogen—that is, kinin-like material is formed.

Cellular location of the kinin-forming activity

Table 3 demonstrates quantitatively the presence of the kinin-forming activity in the $8200 \times g$ pellet (lysosomal) and $8200 \times g$ supernatant (extra-lysosomal) fraction. The

TABLE 3
KININ-FORMING ACTIVITY OF THE PMN FRACTIONS
Human bradykininogen was incubated with the 8200 × *g* supernatant or pellet as described in Fig. 3

8200 × *g* fraction	Total units*	Total protein (mg)	Specific activity	Volume (ml.)
Supernatant	592	85·2	6·8	20
Pellet (lysosomes)	430	24·5	17·5	4

*Unit: 1 ng Bradykinin released/hr/ml. enzyme.

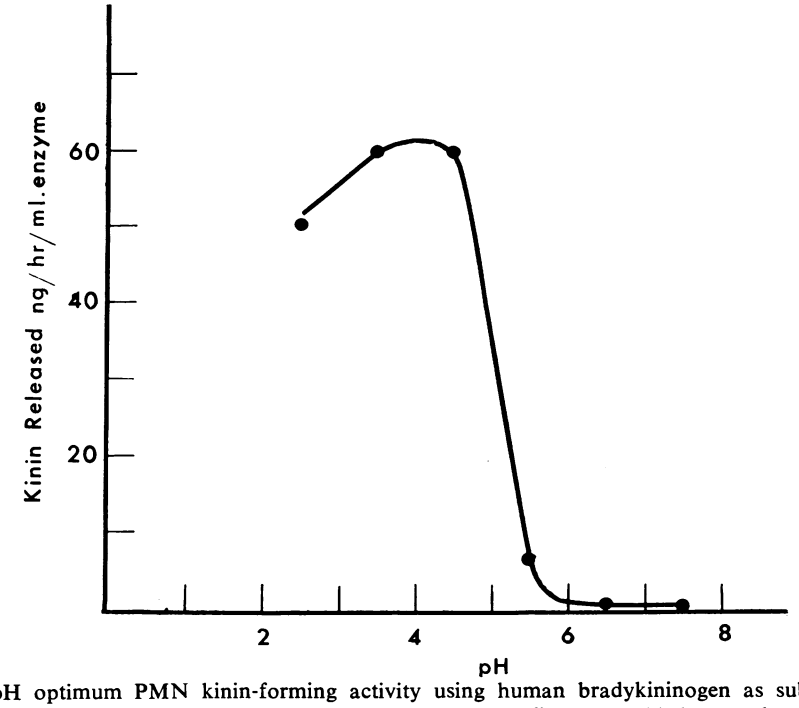

Fig. 4. pH optimum PMN kinin-forming activity using human bradykininogen as substrate. To 5 mg of human bradykininogen dissolved in 1 ml. buffer was added 0.2 ml. of the pellet suspension. After 240 min at 37° the pH of the solution was brought to neutrality and 0.2 ml. added to the muscle chamber. Bradykininogen alone and enzyme alone showed no activity. Acetate was used in the pH range below neutrality in a final concentration of 0.1 M. 0.05 M tris buffer was used in the alkaline range.

results are in sharp constrast to the demonstration that the kininase activity is present only in the extra-lysosomal fraction of the cell (see above). However, the presence of kinin-forming activity in the extra-lysosomal fraction requires further investigation since the possibility exists that leakage from the lysosomes may have occurred during fractionation. The presence of kinin-forming activity in the lysosomal fraction must be considered quite conclusive.

pH optimum of kinin-forming activity from human bradykininogen

Figure 4 demonstrates the pH optimum of the kinin-forming enzyme using human bradykininogen as a substrate. In contrast to the kininase activity, the maximal activity is produced in the acid range at about pH 5. A sharp drop in kinin formation occurs if the pH of the reaction is raised near 6. This drop is due in part to the fact that the human bradykininogen preparations used have an isoelectric point at 5.3. Thus, only small amounts are present in solution at this range. It can be noted, however, that, at a pH at 7–8 where solubility is good, little or no activity is produced by the PMN enzyme on the substrate although trypsin is very active on the substrate in this pH range (Greenbaum & Hosoda, 1963). The results would indicate that acid pH favours production of kinins by the PMN enzyme using this particular substrate preparation of human bradykininogen.

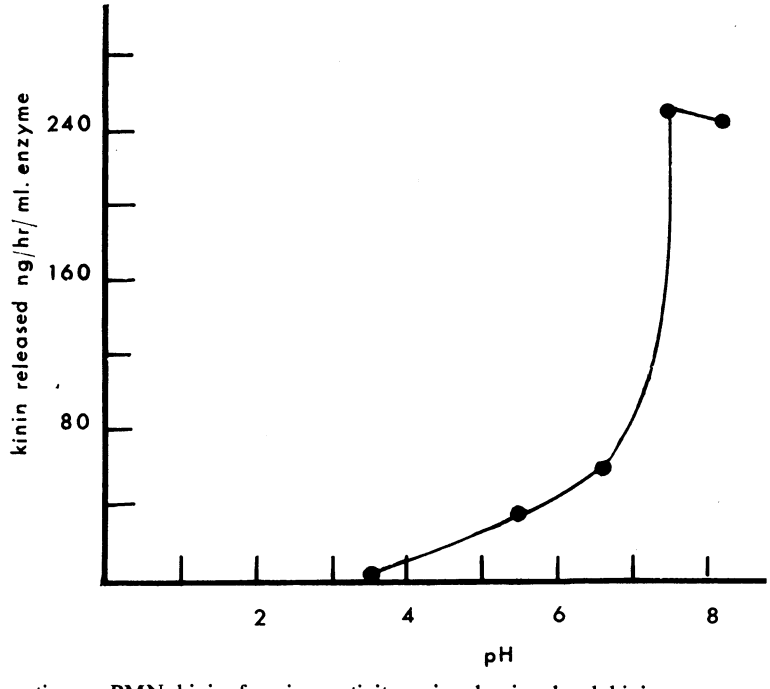

Fig. 5. pH optimum PMN kinin forming activity using bovine bradykininogen as substrate. To 1 ml. 0.1 M acetate buffer or 1 ml. 0.05 M tris buffer was added 0.4 ml. of a 1 mg/ml. solution of bovine bradykininogen and 0.2 ml. of the pellet fraction. Samples were removed for assay 4 hr after incubation at 37°.

pH optimum of kinin-forming activity using bovine bradykininogen

The availability of a small sample of highly purified bovine bradykininogen (S.A. 12.8) made possible an experiment whereby the bovine preparation (which has excellent solubility) could be used as a substrate for kinin-forming activity of the PMN cells at varying pH and thus contrast it with the human preparation. As seen in Fig. 5, kinin formation using the bovine substrate optimally occurred in the neutral range, although kinin formation in the acid range also was produced. Future experiments must be carried out to determine if the difference in pH optimum using the bovine and human substrates is one of the differences in substrate structure or if there are different kinin-forming enzymes in the PMN cell which can attack one substrate preferentially over the other.

Other properties of the kinin-forming activity

Using the human substrate, the kinin-forming activity was found to be very stable in that warming the enzyme to 50° C for 30 min did not diminish its activity. Boiling for 10 min completely destroyed the activity. When tested at pH 4, DFP had only a slight inhibitory effect at (10^{-3}M) while trasylol had no effect even at high concentrations (50 KIU/ml. of incubation). Trypsin soya bean inhibitor up to 1 mg/ml. had no effect. Heavy metal ions such as Zn^{++}, Ni^{++} and Fe^{+++} ions did reduce the kinin-forming activity of the enzyme at a concentration of 10^{-3}M.

DISCUSSION

A role in inflammation of the kinin forming and kininase enzymes in the PMN cell must be seriously considered in view of the lysis of the PMN cell during inflammation. The results obtained demonstrate conclusively that rabbit PMN cells contain an enzyme or enzymes that can directly catalyse the formation of kinin-like material from human and bovine bradykininogen. The results also confirm the findings of Schwab (1962) that kininase activity is present in these cells. The resultant effect of lysis of the cell contents into the area of injury would depend on pH and the nature of the kininogen substrate present. The PMN cells can provide a medium with a variety of pH since they are known to produce lactic acid within the cell during phagocytosis. Acidic pH and the presence of a bradykininogen substrate similar to the human substrate used in the experiments reported would favour kinin formation by the kinin-forming enzymes present. At acidic pH the kininase activity would be minimal. A shift of pH to the neutral or alkaline range would also reduce the production of kinins by allowing the kininase to act. If kinins are actually formed by enzymes from PMN cells during inflammation, the interesting possibility presents itself that such kinins might also cause the migration of more leucocytes to the inflamed area by increasing the permeability of the capillary wall, a property bradykinin and kallidin are well known to possess. The diminution of kinin formation at neutral or alkaline pH would cause the migration of the leucocytes to cease.

The formation of kinin by the kinin forming activity in the PMN cell in these experiments is quite slow (0.5 ng of bradykinin/10^6 cells/hr). Since the system used is an *in vitro* system the question as to whether this is the rate of formation which might occur *in vivo* is not answerable. However, since localized rather than systemic reactions are probably involved in inflammation, small amounts of kinin produced at localized areas

might still be very effective in producing various pharmacological effects. In addition, as pointed out above, the substrate present and the pH would be important factors in the quantity of kinin produced by the kinin forming enzyme. It should also be considered that the slow formation of kinins may indicate that they play a greater role in the late events seen in the inflammatory response rather than the early phases.

The finding that the kinin-forming activity is present in the lysosomal and extra-lysosomal fractions of the PMN cell while the kininase activity is present only in the extra-lysosomal fraction leads to the speculation that, while lysis of the PMN cell itself may cause little kinin formation at the site of lysis, lysis of the lysosomal particle, which is free of kininase activity, may be the potent factor in local kinin formation.

While the question of whether or not kinins play a role in inflammation has not been resolved by this investigation, the finding that PMN cells taken from the site of an inflammatory reaction have enzymes which can produce kinins must be taken into account when the chemical mediators of the inflammatory response are being considered. Previous findings that spleen lysosomal enzymes may produce kinins (Greenbaum & Yamafuji, 1966) are also in accord with the possible role of kinins in injury and inflammation.

SUMMARY

1. Polymorphonuclear leucocytes collected from peritoneal exudates of rabbits contain enzyme activity that can catalyse the formation of kinin-like material from purified human and bovine bradykininogen. The pH optima of the reaction varied with the substrate used.

2. The kinin forming activity was found to be present both in the lysosomal and extra-lysosomal fractions of the cell. Trasylol did not inhibit the kinin-forming activity.

3. Previous reports by other investigators on the presence of kininase activity in PMN cells have been confirmed. The activity was present in the extra-lysosomal fraction of the cell but absent in the lysosomal fraction. The pH optima of the enzyme was about 8.5 when assayed against bradykinin.

4. The kininase differs from that of the blood since it does not catalyse the hydrolysis of hippuryl-L-arginine as does the blood enzyme.

5. The properties and cellular location of the kinin-forming and kininase enzymes of PMN cells have been discussed in relation to their possible role in the inflammatory response.

REFERENCES

COHN, Z. A. & HIRSCH, J. G. (1960). The isolation and properties of the specific cytoplasmic granules of rabbit polymorphonuclear leucocytes. *J. exp. med.*, **112**, 983–1004.

EDERY, H. (1964). Potentiation of the action of bradykinin on smooth muscle by chymotrypsin, chymotrypsinogen and trypsin. *Br. J. Pharmac. Chemother.*, **22**, 371–379.

ERDÖS, E. G. & SLOANE, E. M. (1962). An enzyme in human blood plasma that inactivates bradykinin and kallidins. *Biochem. Pharmac.*, **11**, 589–592.

ERDÖS, E. G., SLOANE, E. M. & WOHLER, I. M. (1964). Carboxypeptidase in blood and other fluids. 1. Properties, distribution, and partial purification of the enzyme. *Biochem. Pharmac.*, **13**, 893–905.

GREENBAUM, L. M., FREER, R. & KIM, K. S. (1966). Kinin forming and inactivating enzymes in polymorphonuclear leucocytes. *Fedn. Proc. Fedn. Am. Soc. exp. Biol.*, **25**, 287.

GREENBAUM, L. M. & HOSODA, T. (1963). Studies on the isolation of bradykininogen. *Biochem. Pharmac.*, **12**, 325–330.

GREENBAUM, L. M. & YAMAFUJI, K. (1966). The role of cathepsins in the inactivation of plasma kinins. In Proceedings of the 3rd International symposium on *Hypotensive Peptides*, ed. ERDÖS, E. G., BACK, N. and SICUTERI, F., held Florence, Italy, 1965. Springer Verlag, New York.

GREENBAUM, L. M. & YAMAFUJI, K. (1966a). The *in vitro* inactivation and formation of plasma kinins by spleen cathepsins. *Br. J. Pharmac. Chemother.*, **27**, 230–238.

GREENBAUM, L. M., YAMAFUJI, K. & HOSODA, T. (1965). Peptic degradation of bovine bradykininogen. *Biochem. Pharmac.*, **14**, 411–420.

JANOFF, A., BEAN, M. A. & SCHULLER, E. (1965). Mediators of inflamation in leukocyte lysosomes. *Life Sci.*, **4**, 2361–2373.

LEWIS, G. P. (1963). Pharmacological actions of bradykinin and its role in physiological and pathological reactions. *Ann. N.Y. Acad. Sci.*, **104**, 236–249.

LEWIS, G. P. (1964). The role of peptides in the first stages of inflammation. In: *International Symposium on Injury, Inflammation and Immunity*, ed. THOMAS, L. UHR, J. W. & GRANT, L. H. Williams and Wilkins, Baltimore.

PIERCE, J. V. & WEBSTER, M. E. (1961). Human plasma kallidins; isolation and chemical studies. *Biochem. biophys. Res. Commun.*, **5**, 353–357.

ROGERS, D. E. (1964). Intracellular inflammation: dynamics and metabolic changes in polymorphonuclear leukocytes participating in phagocytosis. In: *International Symposium on Injury, Inflammation and Immunity*, ed. THOMAS, L., UHR, J. W. & GRANT, L. H. Williams and Wilkins, Baltimore.

SCHWAB, J. (1962). Kininases in leucocytes and other tissues. *Nature* (*Lond.*), **195**, 345–347.

WERLE, E. (1955). The chemistry and pharmacology of kallikrein and kallidin. In: *Polypeptides which Stimulate Plain Muscle*, 20–27, ed. GADDUM, J. H. Livingstone, London.

NOTE ADDED IN PROOF

Since the initial findings of this work were presented (Greenbaum & Yamafuji, 1966; Greenbaum, Freer & Kim, 1966) preliminary evidence concerning various aspects of kinin-forming and kininase enzymes in *human* leucocytes has appeared (Cline, M. J. & Melmon, K. (1966). *Science*, **153**, 1135; Zachariae, H., Malmquist, J. & Oates, J. A. (1966). *Life Sciences*, **5**, 2347).

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