Biochemical Pharmacology, 1966, Vol. 15, pp. 905–914. Pergamon Press Ltd., Printed in Great Britain.

FORMATION OF ANAPHYLATOXIN IN RAT PLASMA, A SPECIFIC ENZYMIC PROCESS

W. VOGT and G. SCHMIDT

Department of Pharmacology, Max Planck-Institut für experimentelle Medizin, Göttingen, Germany

(*Received* 29 *November* 1965; *accepted* 13 *January* 1966)

Abstract—Evidence is presented that anaphylatoxin is formed in rat plasma by the action of a specific enzyme on its substrate, anaphylatoxinogen. The enzyme is present in rat plasma as an inactive precursor. It is activated by contact with Sephadex or other contact agents known to induce anaphylatoxin formation in rat plasma. The preparation of enzyme and substrate fractions is described and some properties of the enzyme have been studied. There is no indication that the anaphylatoxin-forming enzyme belongs to the complement group. It is a metallo-protein with essential carbonyl groups. A similar enzyme is present in cobra venom.

ANAPHYLATOXIN, originally, was postulated to be a toxic compound cleaved from antigens in the presence of antibody by the action of complement. The symptoms of anaphylactic shock were ascribed to its effects.[1] Later, it was found to be a potent histamine liberator formed from serum or plasma proteins after contact with various agents.[2, 3] No uniform view has developed concerning the mechanism of formation of anaphylatoxin. An enzymic mechanism has been proposed by some investigators, but it has not been accepted by others. Friedberger thought complement to be involved. Recently, Osler *et al.*[4] have given new support to this theory. Participation of complement would mean an enzymic process. On the other hand, Giertz and Hahn[5] as well as Rothschild *et al.*[6] have presented evidence against complement being involved. Ungar found that serum acquires increased proteolytic activity when induced to form anaphylatoxin.[7] According to other hypotheses anaphylatoxin is a compound formed as the result of non-enzymic physical alterations of plasma proteins—denaturation, aggregation or complex formation of two components—which alterations are brought about by contact of the proteins involved with the surfaces of substances known as anaphylatoxin forming agents. A comprehensive review of this problem has been given by Giertz and Hahn.[8]

One fact that has been taken as indicating a physical mechanism rather than a chemical one is that various polysaccharides will induce the formation of anaphylatoxin when brought into serum as a solid but not or much less so when added in solution. However, while contact with foreign surfaces may primarily produce physical alterations of proteins, this may activate enzymes, i.e. trigger chemical processes.

As the effect of contact agents is ambiguous, compounds and methods have been looked for in the present experiments by which anaphylatoxin is formed without contact of plasma with active surfaces. The results obtained demonstrate that the

anaphylatoxin forming system can be resolved into an enzyme and a substrate. The enzyme is present, in rat plasma, in an inactive state and is activated by contact with compounds known as anaphylatoxin forming agents. A similar enzyme is present in cobra venom, in an already active state. Some of the results have been published previously, as short communications.[9-11]

METHODS

The formation of anaphylatoxin was studied in rat plasma. Blood was obtained from rats by cutting their neck, after a blow on the head. The blood was collected in polythene vessels containing 0·01 ml of 1% heparin solution per ml of blood expected. After centrifugation the plasma was used immediately or stored in polythene bottles, at −20°.

Except for special experiments, anaphylatoxin formation was induced by adding about 10 mg of fine dry Sephadex G 75 (not in beads but as ground material) per ml of rat plasma and incubating the mixture for 30 min at 37°.

EDTA-plasma contained 0·02 ml 10% $Na_2H_2EDTA.2H_2O$ per ml. For recalcification 0·06 ml 1% $CaCl_2$ were added.

In the earlier gel filtration experiments, Sephadex was used in the ground form which was distributed by the producer before the bead form became available. Fractions obtained by chromatographic procedures were concentrated (and washed if necessary) by ultrafiltration through collodium membranes (Membranfilter-Gesellschaft, Göttingen).

Anaphylatoxin was assayed on isolated strips of guinea pig ileum, in a bath containing 10 ml of Tyrode solution at 35°. Because of tachyphylaxis, intervals were intercalated in between the tests of anaphylatoxin, either by applying two to four doses of acetylcholine or by allowing the muscle to rest for 10 to 15 min. Usually, all-or-none responses were obtained by anaphylatoxin in varying doses. Only rarely, submaximal contractions were observed. These have been marked as (+) in the tables. When two anaphylatoxin preparations had to be matched the one expected to be the more active one was given last. It was considered to be at least *n* times stronger than the reference preparation when it produced a stronger contraction in a dose *n* times smaller than the reference, given before.

RESULTS

Formation of anaphylatoxin by cobra venom

Friedberger *et al.*[12] found that cobra venom on incubation with guinea pig serum gave rise to the formation of anaphylatoxic activity. Cobra venom promised to be a useful tool for studying the mechanism of anaphylatoxin formation because it acts in solution. Production of anaphylatoxin in a system devoid of active surfaces would render a purely physical mechanism unlikely.

When incubated with rat plasma at 37° cobra venom, in a final concentration of from 10^{-6} to 10^{-5}, produces anaphylatoxin in about 5 to 10 min. The mechanism of the action is different from that of contact agents. This is clear from the following finding. Samples of rat plasma which had been passed through columns of Sephadex, in the cold, did not form anaphylatoxin with any contact agent, including immune precipitates, (see below). However, these samples still developed anaphylatoxic activity when incubated with cobra venom. The essential principle is present in a fraction of

the venom obtainable by ion exchange chromatography and gel filtration.[11] It is devoid of toxic effects in rats or guinea pigs, in doses corresponding to 5 mg crude venom/kg and has no haemolytic, casein-splitting nor phospholipase A activity. For most experiments this fraction of cobra venom was used instead of whole venom.

It seemed reasonable to assume that rat plasma lost, by gel filtration, the precursor of an anaphylatoxin forming factor which could be activated by contact agents, normally. This missing factor could be replaced by a similar one present in cobra venom. To prove the enzymic nature of venom-induced anaphylatoxin formation, samples of gel-filtrated rat plasma and cobra factor were incubated in varying proportions. From Table 1 is seen that the amount of anaphylatoxin formed is dependent on the amount of the rat plasma fraction present in the mixture, but not on the venom fraction, once the threshold concentration of the latter is exceeded. Thus the cobra-venom fraction acts as a catalyst, i.e. as an enzyme whereas the rat-plasma fraction contains the substrate determining the amount of product—anaphylatoxin—formed.

TABLE 1*

Cobra venom fraction (% of total volume of incubated mixture)	Rat plasma fraction	Effect
9	18	0
27	18	+
82	18	+
27	54	>++

* Formation of anaphylatoxin by incubation of the active fraction of cobra venom (solution equivalent to 46 μg/ml crude venom) with the substrate fraction of rat plasma (four times concentrated with respect to original plasma volume), in various proportions. All samples were incubated for 30 min at 37°. The amount of anaphylatoxin formed increases with increasing proportions of the rat plasma fraction, but not when only the venom fraction is increased. The activity was assayed on the isolated guinea pig ileum.

>++: This sample was more than twice as active as the second and the third samples.

The following experiments were performed to check whether the product of the action of cobra venom on gel-filtrated rat plasma is the result of the same reaction as that induced by contact agents in whole plasma. Rat plasma (1 ml) was incubated with 10 mg Sephadex G 75, for 30 min at 37° and afterwards, for a further 15 min with the purified venom enzyme (equivalent to 100 μg venom/ml.) Another sample of 1-ml plasma was treated with the cobra enzyme only. It showed as strong an anaphylatoxin activity as the sample that had been incubated twice. This indicates that the same substrate is used for production of the smooth muscle stimulating activity in both processes.

The same result was obtained by another procedure. Two rats were injected twice intravenously with the purified enzyme fraction of cobra venom (each injection equivalent to 1 mg/kg whole venom), with an interval of 2 hr in between the injections. Plasma was collected from the animals, 30 min after the second injection.

These specimens of plasma did not form anaphylatoxin on incubation with Sephadex, nor with cobra venom, *in vitro*. Apparently, their substrate had been exhausted, *in vivo*, by the previous treatment with the venom enzyme.

Both anaphylatoxin formed by cobra venom and by contact agents produce the same type of contraction in the guinea pig ileum, which is subject to tachyphylaxis and inhibited by antihistaminics. Moreover, there is cross-desensitization between the two. It is concluded, therefore, that the enzyme of cobra venom produces the same anaphylatoxin in rat plasma which otherwise can be formed by an endogenous enzyme.

Separation of enzyme and substrate of anaphylatoxin formation in rat plasma

(1) *Substrate preparations.* Various procedures have been tried to separate the anaphylatoxin forming enzyme assumed to be present in rat plasma from its substrate. To test for the presence of substrate (anaphylatoxinogen), fractions obtained were concentrated by ultrafiltration, to the original volume of plasma. The concentrate was incubated with one tenth of its volume of purified cobra enzyme solution containing the equivalent of 100–1000 μg crude venom per ml. It was then assayed for anaphylatoxin activity.

Gel filtration. In the first experiments, 1–4 ml rat plasma were passed through Sephadex G 75 medium (1·6 × 40 cm) with 0·9% NaCl as eluent, at 0–4°. The effluent from 18–24 ml contained a protein fraction which produced anaphylatoxin when incubated with cobra venom but not after incubation with contact agents. This fraction apparently contained anaphylatoxinogen free of enzyme. Gel filtration has later been used for large scale preparation of anaphylatoxinogen.[13]

When these experiments were repeated more recently with Sephadex G 100, the substrate fraction contained some anaphylatoxin forming enzyme as well. Even by gel filtration through an old preparation of powdered G 75 medium, we did not succeed in obtaining the anaphylatoxinogen entirely free of enzyme, although the activity was reduced, being recognizable only after concentration of the fractions. The reason for these differences is not clear.

R_4-plasma. Osler *et al.*[4] found that treatment of rat serum with hydrazine, according to the procedure used for preparing R_4-serum abolished its anaphylatoxin forming capacity. The same result is obtained when rat plasma is incubated with 0·25 ml/ml 0·15 N NH_3, for 90 min, at 37°. Plasma treated with these agents, is however, still able to develop anaphylatoxin activity when it is incubated with the cobra enzyme. The treatment thus leaves the substrate intact, destroying selectively the supposed plasma enzyme.

(2) *Enzyme preparations.* As a test for the anaphylatoxin forming (pro-) enzyme, fractions obtained were concentrated by ultrafiltration and then incubated with an anaphylatoxinogen preparation and Sephadex as contact agent, for 30–60 min at 37°. The mixtures were assayed on the guinea pig ileum for anaphylatoxin activity.

Attempts to purify the enzyme by ion-exchange chromatography have, so far, not given reproducible results. More success was obtained with gel filtration. As mentioned above, in recent experiments anaphylatoxin forming activity was recovered in the effluent when rat plasma was passed through Sephadex G 75 or G 100, unlike earlier results. The enzymic activity was, however, not well separated from anaphylatoxinogen.

In another series 10–30 ml rat plasma were passed through columns of Biogel P 100 (4·5 × 30 cm) equilibrated with 0·067 M phosphate buffer, pH 6·0, and developed with the same buffer (Fig. 1). Whereas the substrate was eluted with the main peak of plasma proteins, the enzyme came off the column later, showing some overlapping with anaphylatoxinogen and considerable tailing. The same result was obtained when the rat plasma was dialyzed against the buffer, before gel filtration.

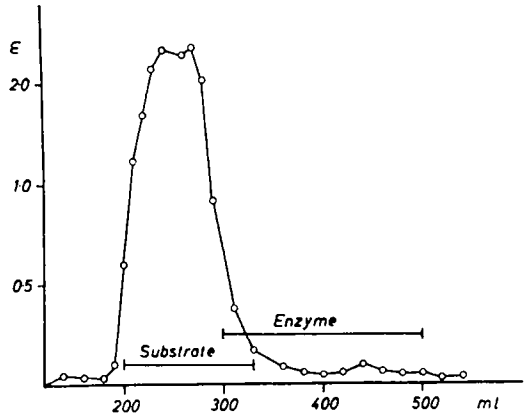

FIG. 1. Gel filtration of 10 ml rat plasma on Biogel P-100, with 0·067 M phosphate buffer, pH 6·0. Ordinate—absorbancy at 590 mμ after modified Folin-Lowry reaction.[15]

During the passage through Biogel the enzyme became activated. This was clear from the fact that the enzyme containing fractions (after concentration and washing with 0·9% NaCl solution) formed anaphylatoxin when incubated with anaphylatoxin preparations, without any addition of contact agents. The active fractions were turbid or became so on standing or after concentration. When the turbid solution was centrifuged at 30,000 *g*, a precipitate separated which contained the anaphylatoxin forming activity, the clear supernatant being inactive. It was not possible to redissolve the precipitate in water, 0·9% NaCl solution or buffers between pH 4 and 9.

By mixing the purified enzyme suspension and substrate solution in varying proportions it was possible to demonstrate the catalytic nature of the enzyme action, in a similar manner as had been done before with the cobra enzyme (Table 2).

Resolution of anaphylatoxin formation into a two step process. Attempts of earlier investigators to split the process of anaphylatoxin formation into one of activation followed by one of product formation had been unsuccessful. The formation of anaphylatoxin had been found to be possible only when contact agent and two factors of plasma were present simultaneously.[5] By the following procedure, we succeeded in demonstrating a two-step process. To fresh rat plasma was added 5·4 mM EDTA (0·02 ml of a 10% neutralized solution of $Na_2H_2EDTA.2\ H_2O$ per ml plasma) and, afterwards, about 10 mg/ml Sephadex G 75. The mixture was incubated for 3–18 hr at 37°, centrifuged and the clear supernatant transferred to a polythene tube. Care was taken not to take along any particle of Sephadex and sometimes the incubated and centrifuged plasma was filtered, in addition. After this treatment the plasma

did not show anaphylatoxin activity because of the inhibitory action of EDTA on its formation (see later).

(When the samples were incubated for more than 6–8 hr they produced a contraction of guinea pig ileum strips as a consequence of kinin formation. This activity disappeared on recalcification or dialysis.)

TABLE 2*

Volume (in % of total) of Enzyme solution	Substrate solution	Dose tested (ml)	Effect
29	14	0·4	—
29	14	0·6	(+)
57	14	0·4	0
57	14	0·6	0
57	43	0·3	++
57	43	0·3	+

* Catalytic action of the rat plasma enzyme fraction obtained by Biogel filtration. Once the threshold concentration of the enzyme necessary for measurable formation of anaphylatoxin (30 min, 37°) is reached the amount of anaphylatoxin formed depends on the amount of substrate present. No increase in enzyme concentration is necessary in order to obtain higher amounts of product.

When sufficient Ca^{2+} was added to the incubated plasma (0·06 ml 1% $CaCl_2$/ml) to bind the EDTA present, anaphylatoxin formed on further incubation for 30 min at 37°, without any addition of contact agents. Apparently, the plasma enzyme had been activated during the first incubation but was then prevented from acting on its substrate because of the presence of EDTA.

Inhibitors of anaphylatoxin formation

Chelating agents. *o*-Phenanthroline (20mM), dithizone (0·8 mM) and 8-hydroxyquinoline (0·1 mM) did not inhibit anaphylatoxin formation in rat plasma induced by contact with Sephadex. Also cyanide (1·5 mM) was ineffective. EDTA (5·4 mM) completely suppressed the formation, confirming the findings of Osler *et al.*[4] Even contact with Sephadex for 24 hr did not produce anaphylatoxin when EDTA was present. The threshold for the inhibiting action of EDTA was found to be between 2·7 and 5·4 mM, in a sample of rat plasma that contained a total concentration of 3·3 mM Ca^{2+}. Ca-EDTA (5·4 mM) had no inhibitory effect.

An attempt was made to deplete rat plasma from free divalent cations by ultrafiltration through collodium membranes, in the presence of 5·4 mM EDTA. The retentate was washed by further ultrafiltration with one volume of 5·4 mM EDTA solution in 0·9% NaCl and then with three successive portions of pure 0·9% NaCl. It was expected that plasma treated in this way would not form anaphylatoxin and that the effect of adding various divalent cations could be studied. Surprisingly, no addition of cations was necessary but the retentate formed anaphylatoxin when incubated with Sephadex, in the normal manner. (The sample of Sephadex used for contact activation had been washed with EDTA, too).

The cobra enzyme is also inhibited by EDTA, though not completely. In the presence of 5·4 mM EDTA a concentration of cobra enzyme equivalent to 100 μg venom/

ml rat plasma produced about one third to one half of the amount of anaphylatoxin that was formed in the absence of the EDTA, in 15 min at 37°.

Carbonyl reagents. As already mentioned, the suppression of anaphylatoxin formation in whole rat-plasma by treatment with hydrazine or ammonia is due to a selective blocking reaction with the plasma enzyme. Also the carbonyl reagent hydroxylamine (0·06 M) blocks the enzyme, leaving the substrate unaffected. The reaction with ammonia is reversible. By acidifying ammonia-treated plasma with dilute hydrochloric acid to pH 5 and then bringing it back to pH 6–7 the enzyme is reactivated. This is apparent from the finding that anaphylatoxin formation can be induced in such plasma by contact.

The cobra enzyme is blocked by aniline (1 vol of aniline added to 10 vol of enzyme solution equivalent to whole venom 10^{-3}) and by hydrazine (about 0·16 M). We did not succeed, however, in inactivating it by ammonia or regularly by hydroxylamine, in the concentrations used for rat plasma.

Pyridoxal-5-phosphate (P-5-P). From the inhibitory effect of EDTA and of carbonyl reagents on the anaphylatoxin forming enzyme, it was concluded that their action depends on the presence of protein-bound metal and on certain carbonyl groups. These are features of P-5-P containing enzymes. No direct proof of the presence of P-5-P has been obtained. Rats which were fed a diet free of vitamin B_6 and containing 6 mg desoxypyridoxine-HCl per kg food, for 7 weeks and 12 mg for further 6 weeks, were found to have in their plasma the complete anaphylatoxin forming system.

The following finding may give an indirect hint that pyridoxal-phosphate participates as a coenzyme in the formation of anaphylatoxin. No anaphylatoxin was formed in rat plasma on incubation with Sephadex, for 30 min, when free pyridoxal-phosphate had been added to give the high concentration of 3·8 mM. As indicated in Table 3, the same inhibition is seen when an equimolar amount of $CaCl_2$ is added with the P-5-P.* Addition of the $CaCl_2$ alone, or of substances related to P-5-P such as pyridoxal, pyridoxamine-phosphate, pyridoxim phosphate does not interfere with the anaphylatoxin formation in a comparable manner. The effect thus is specific for

TABLE 3*.

Compound added	Concentration (mM)	Formation of anaphylatoxin
Pyridoxal-5-phosphate	3·8	0
Pyridoxamine-phosphate	4·0	+
Pyridoxim-phosphate	3·8	+
Pyridoxal	4·9	+
Pyridoxal-5-phosphate + $CaCl_2$	3·8 4·0	0
$CaCl_2$	4·0	+

* Formation of anaphylatoxan in rat plasma. Plasma incubated with Sephadex for 30 min at 37°C, in the presence of various pyridoxine derivatives, and $CaCl_2$.

* This experiment had been kindly suggested by Dr. Osler, as P-5-P might inhibit simply by chelating some essential metal, in a similar way as EDTA.

P-5-P. It may be explained tentatively, by the assumption that free P-5-P competes with the enzyme for the substrate because of a similar affinity.

Di-isopropyl-fluorophosphate (DFP). When rat plasma is treated with DFP (0·065 mM), it still gives rise to anaphylatoxin formation on subsequent incubation with Sephadex. This confirms the results of Becker.[14] It is, however, no clear proof that the anaphylatoxin forming enzyme is not susceptible to a blocking reaction with DFP. As an inactive proenzyme in plasma, it may be protected against phosphorylation, like other proenzymes are, e.g. kallikreinogen or trypsinogen. After activation of the enzyme, the production of anaphylatoxin proceeds in the course of several minutes, so that DFP may react too slowly to interfere with the enzyme action.

Therefore, rat plasma was first activated with Sephadex, in the presence of EDTA, for 18 hr at 37°. Then DFP was added to a final concentration of 0·065 mM, the mixture was incubated for 2 hr at 37° or for 4 hr at room temperature and was then recalcified. On subsequent incubation—without addition of contact factors—for 30 min at 37°, it formed anaphylatoxin in the same way as did a control sample which differed only in that no DFP had been added.

The purified active enzyme obtained by gel filtration of rat plasma on Biogel P 100 and the cobra enzyme are similarly not blocked by pretreatment with DFP in the same concentration.

Hypertonic solution of NaCl. When the concentration of NaCl in plasma is raised to 0·35 M, no anaphylatoxin is formed by contact with Sephadex. The cobra enzyme remains still active at this concentration. It is blocked only when 1·1 M NaCl is present.

Stability of the anaphylatoxin forming enzyme. In several experiments, the anaphylatoxin forming enzyme seemed to decay once it had been activated in whole rat plasma. To test this, the following experiment was carried out. Three samples each containing 1 ml of the same rat plasma were prepared for anaphylatoxin formation. To the first one 1 ml of 0·9% NaCl and 20 mg Sephadex, were added, to the second one, 1 ml of anaphylatoxinogen solution (equivalent to 3 ml rat plasma) and Sephadex. To the third sample Sephadex alone was added. All three mixtures were incubated for 1 hr at 37°. Then 1 ml anaphylatoxinogen solution was added to the third sample and this was incubated for another hour. The second and the third sample contained definitely more anaphylatoxin than the first one which had not been supplemented with additional substrate. The third was, however, less active than the second one, indicating that some enzyme activity had been destroyed during the first incubation period, so that the additional substrate added afterwards was not fully converted to anaphylatoxin (Fig. 2). After 24 hr at 37° Sephadex-activated rat plasma was no longer able to form anaphylatoxin from newly added substrate.

Freezing and thawing do not impair the ability of untreated rat plasma to form anaphylatoxin on subsequent incubation with contact factors. However, the active enzyme obtained by gel filtration on Biogel loses activity when frozen and thawed. It is rather stable when kept in suspension in 0·9% NaCl solution, at 0–4°.

When rat plasma is acidified with HCl to a pH below 3, its substrate as well as its enzyme is destroyed, even when it is neutralized again within a few minutes. Heating rat plasma to 56° for 30 min destroys anaphylatoxinogen and a considerable proportion of the enzyme. At 80° the enzyme is completely destroyed in 30 min. The purified active plasma enzyme shows the same heat sensitivity.

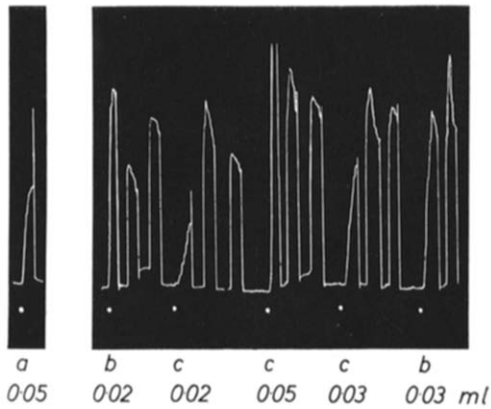

FIG. 2. Guinea-pig ileum. Action of samples of rat plasma, incubated with or without additional anaphylatoxinogen, in the presence of Sephadex. a = plasma + 0·9% NaCl solution (1 + 1 ml), 60 min 37°. b = plasma + anaphylatoxinogen solution (1 + 1 ml), 60 min 37°. c = plasma (1 ml) 60 min 37°, then addition of 1 ml anaphylatoxinogen solution and incubation for further 60 min. Contractions not specified are responses to acetylcholine (0·03 μg/10 ml). Taking the decreasing sensitivity into consideration it is evident that b and c contain more anaphylatoxin than a, b being slightly stronger than c.

facing page 912

The cobra enzyme is stable in rat plasma. After injection into rats *in vivo*, it was recovered from the plasma even after 24 hr. It is inactivated in the course of seconds at a pH below 3.[11] Freezing and thawing also diminish its activity, but it seems to be more stable to heat than the rat plasma enzyme. After heating to 56° for 30 min it does not show loss of activity. It does not resist heating to 100°, however.

DISCUSSION

The results obtained indicate that anaphylatoxin is formed in rat plasma by a specific enzymic process. Two essential factors have been separated, both showing properties of a protein, being non-dialyzable and heat labile. One of these behaves as an enzyme acting catalytically on the other factor which determines the yield of anaphylatoxin. Thus the second factor serves as substrate, anaphylatoxinogen.

This finding is different from earlier observations of Giertz and Hahn.[5] They also obtained two essential fractions from rat serum but none of these seemed to act catalytically. The formation of anaphylatoxin was rather considered to be the result of a stoechiometric reaction between the two components. Whether this can be explained by our finding that the enzyme decays in plasma, once activated, is open to question. According to our experiments the decay does not proceed very quickly but it may, at low concentrations of enzyme, be sufficient to lower the activity rapidly below threshold.

When purified, the active plasma enzyme is rather stable in aqueous suspension. This indicates that its decay in whole plasma is brought about by other plasma constituents.

Further evidence for an enzymic mechanism is given by the successful resolution of the formation of anaphylatoxin into two steps. In the first reaction, the enzyme is activated by contact with suitable surfaces, e.g. Sephadex, or by passage through Biogel in phosphate buffer at pH 6·0. In the second step the enzyme acts on anaphylatoxinogen. For this reaction no contact agents are necessary.

The anaphylatoxin forming enzyme bears no relation to any factor of complement. Unlike the C'1 esterase it is stable to prolonged exposure to EDTA solution or to DFP and it is active in the absence of free divalent cations. Unlike C'2 and C'3 it is blocked by carbonyl reagents and unlike C'4 it can be reactivated after it had been blocked by treatment with ammonia. Rat plasma was found (unpublished observation) to lose its complement activity, as measured by immune hemolysis of sensitized sheep erythrocytes, irreversibly by treatment with ammonia.

The fact that EDTA blocks anaphylatoxin formation and that this block can be reversed simply by dialysis, without adding any divalent cations, indicates that (1) some divalent metal capable of chelation with EDTA is essential for the enzymic activity, (2) this metal is not present as free ion being unable to leave the dialysis bag and (3) it dissociates easily from EDTA on dilution of the latter. These seemingly contradictory conclusions can be reconciled by assuming that the metal is bound to the enzyme, i.e. the anaphylatoxin forming enzyme of rat plasma is a metallo-protein. EDTA probably combines with it blocking an essential site, but this ternary complex is much less strong than chelates with free cations, dissociating on dialysis and thus regenerating the activity of the enzyme.

Both the anaphylatoxin forming enzyme of rat plasma and that of cobra venom are similar but not identical in their properties. Whereas no destruction of the cobra

enzyme was observed after heating to 56° for 30 min, the plasma enzyme loses activity, though only partially, when treated in the same way. Further, the cobra enzyme is only partially inhibited by EDTA and not by 1 M NaCl, whereas the plasma enzyme is blocked under the same conditions. The similarity is apparent from the fact that they act on the same substrate and it is likely that they form the same product. It cannot be excluded that minor differences exist between the two anaphylatoxins. So far, no gross chemical nor qualitative biological differences have been found. There is an indication that the enzyme may contain pyridoxal-phosphate as coenzyme, but no direct proof has been obtained. Rats deficient in vitamin B_6 nevertheless showed anaphylatoxin forming activity in their plasma. A partial reduction would, however, not have been detected. The nature of the reaction which the anaphylatoxin forming enzymes catalyze, is unknown. The cobra enzyme does not split casein, TAME, nor LME or ATEE,[11] and is probably not a conventional protease. The same may be the case for the plasma enzyme as it acts on the same substrate and forms the same active product. The possible participation of pyridoxal-phosphate suggests—though there is no experimental support—a decarboxylating action, which would be in accord with the finding that anaphylatoxin is more basic than its precursor.[13]

REFERENCES

1. E. FRIEDBERGER, *Z. ImmunForsch. exp. Ther.* **4**, 636 (1910).
2. F. HAHN and A. OBERDORF, *Z. ImmunForsch. exp. Ther.* **107**, 528 (1950).
3. M. ROCHA E SILVA and M. ARONSON, *Br. J. exp. Path.*, **33**, 577 (1952).
4. A. G. OSLER, H. G. RANDALL, B. M. HILL and Z. OVARY, *J. exp. Med.* **110**, 311 (1959).
5. H. GIERTZ and F. HAHN, *Int. Archs Allergy appl. Immun.*, **19**, 94 (1961).
6. A. M. ROTHSCHILD and M. ROCHA E SILVA, *Br. J. exp. Path.*, **35**, 507 (1954).
7. G. UNGAR and H. HAYASHI, *Ann. Allergy, S. Paul* **16**, 542 (1958).
8. H. GIERTZ and F. HAHN, In *Heffters Hdbch. der Pharmakologie, Ergänzungsband* (1966).
9. W. VOGT, *Naunyn-Schmiedebergs Archs exp. Path. Pharmak.* **246**, 31 (1963).
10. W. VOGT, *Naunyn-Schmiedebergs Archs exp. Path. Pharmak.* **247**, 327 (1964).
11. W. VOGT and G. SCHMIDT, *Experientia* **20**, 207 (1964).
12. E. FRIEDBERGER, S. MITA and T. KUMAGAI, *Z. Immun Forsch. exp. Ther.* **17**, 506 (1913).
13. H. STEGEMANN, W. VOGT and K. D. FRIEDBERG, *Hoppe-Seyler's Z. physiol. Chem* **337**, 269 (1964).
14. E. L. BECKER, *Symposium, Mechanisms of Hypersensitivity*, Little Brown, Boston (1959).
15. H. STEGEMANN, *Hoppe-Seylers Z. physiol. Chem.* **319**, 64 (1960).