HISTAMINE RELEASE FROM THE PERFUSED LUNG OF THE GUINEA PIG BY SEROTOXIN (ANAPHYLATOXIN).*

M. ROCHA E SILVA AND M. ARONSON.†

From the Department of Biochemistry and Pharmacodynamics, Instituto Biologico, São Paulo, Brazil.

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THERE are two theories of the mechanism of anaphylaxis, one cellular and the other humoral. The first was formulated by Dale (1913, 1920, 1929), and held that the cells were the site of liberation of the anaphylactic poison (histamine). The humoral theory, developed by Friedberger (1909, 1911), Bordet (1913), Keysser and Wassermann (1911) and Nathan (1913) pointed to the blood as the source of a toxic principle, the anaphylatoxin, which was generated by contact with a specific precipitate or substances such as kaolin, barium sulphate, agar, starch, or inulin. Since anaphylatoxin prepared by treating serum with agar did not contain enough histamine to kill a guinea-pig, and did not regularly produce a stimulating effect on the isolated uterus, Dale and Kellaway (1922) decided that it could not be a true anaphylactic poison.

Recently the histamine theory has been preferred to the anaphylatoxin theory because of the experiments of Bartosch, Feldberg and Nagel (1932), Daly, Peat and Schild (1935), Ungar and Parrot (1936) and Schild (1939) in which it was conclusively shown that direct contact of the antigen with washed guinea-pig lungs and several other tissues was enough to result in the release of histamine. However, the amounts of histamine that can be liberated by perfusion of the guinea-pig lung with Tyrode solution containing antigen have always been very small, 0·4–4·0 μg. in the experiments of Bartosch *et al.* (1932) and 0·5–12·0 μg. in those of Daly *et al.* (1935).

Recently Hahn and Oberdorf (1950) showed that antihistaminics prevented the shock produced in the guinea-pig by anaphylatoxin which had been prepared by incubation of normal guinea-pig serum with inulin. This led us to study the mechanism by which anaphylatoxin produces its effects. We want to suggest that it is a powerful histamine-releasing agent and hope thus to combine the cellular and the humoral theories of anaphylaxis. Since the final mediator of the shock (histamine) comes from the tissues and the intermediate releasing agent (anaphylatoxin) is a blood-borne principle, a general theory of anaphylaxis might embody both views as parts of the same phenomenon (Rocha e Silva, Aronson and Bier, 1951 ; Rocha e Silva, 1952).

MATERIALS AND METHODS.

Serotoxin or " activated serum " was prepared according to Bordet (1913) by incubating guinea-pig serum with 0·2 ml. of a 0·5 per cent suspension of agar

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† Present address : Weizmann Institute, Rehovoth, Israel.

per ml. serum. After 1 hr. at 38° the material was centrifuged for 15 min. to remove most of the agar in suspension. That part of the agar which remained in the centrifugate precipitated when left in the ice box for a day. No significance can be attached to these traces of agar in the " activated serum " ; even if a few ml. of the original agar suspension were injected into the guinea-pig lung no pharmacological effect could be observed. The agar used in these experiments was a fairly pure specimen, containing only 0·2 mg. N per 100 g. This is the N content given by Bordet and Zunz (1915) for purified agar. The method of

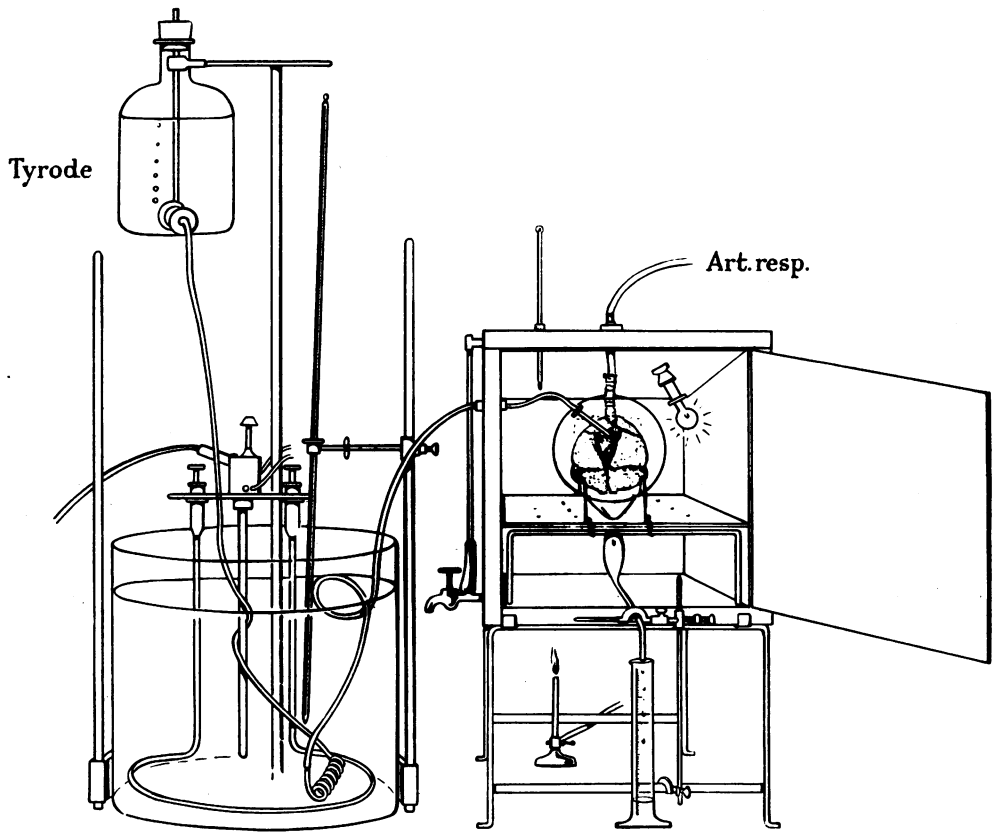

FIG. 1.—Apparatus used for perfusion of the guinea-pig's lung.

Bordet and Zunz to reduce N in agar was applied but failed to lower the N content any further.

Perfusion of the lungs was performed as follows : Guinea-pigs weighing 450–600 g. were anaesthetised with ether. The trachea was cannulated and connected with an artificial respiration pump (10 to 12 strokes per minute) and the thorax was opened. Just before inserting the perfusing cannula into the pulmonary artery the animal was bled by cutting through the abdominal vessels. After tying the cannula and washing the lung free of blood as completely as possible, both lungs were transferred to a watch-glass (12 cm. diam.), which was maintained over a funnel inside a cabinet at 37°–38°, as indicated in Fig. 1. The Tyrode

solution passed through a coil in a waterbath at 38–40° from a Mariotte's flask kept at a height of 80 cm. which secured a perfusion rate of 15 to 25 ml./min. With the fast rate of perfusion the lung was washed out rapidly and different injections could be made at a few minutes' interval. The perfusates were tested until no traces of histamine could be detected. They were collected every minute and the rate of perfusion checked all the time. Rhythmic inflations of the lungs with air at the rate of 4 to 6 per min. helped to maintain the perfusion flow. Four ml. of the activated serum were then injected and the histamine content

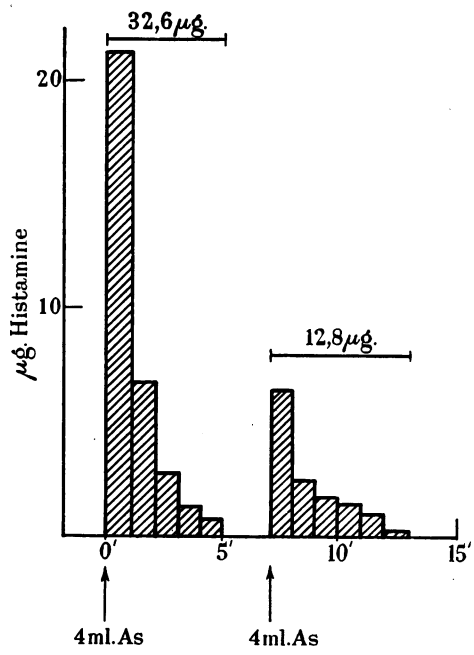

FIG. 2.—Histamine release from a guinea-pig lung by two injections into the cannula of the same amount of normal guinea-pig serum incubated with agar for 30 min. at 38°. Abscissae : time in minutes.

measured until only traces of histamine could be detected in the perfusates. The injection of 4 ml. of activated serum was then repeated and the release of histamine followed up until no more histamine could be found in the perfusates. Usually 4–6 min. was the interval necessary to collect the total histamine released after each application of activated serum.

The estimation of histamine in the perfusates was done on the guinea-pig ileum suspended in a 10 ml. chamber in a Dale's apparatus, using as a standard a histamine dihydrochloride dilution of $0{\cdot}5 \times 10^{-6}$ of the base. All histamine estimations were calculated as the base. The perfusates were assayed directly and the histamine was not specially extracted. The histamine in the activated serum was estimated separately and never attained levels sufficient to change the significance of the results. Thus in 4 ml. of guinea-pig serum no more than 0·2 to 0·5 μg. were usually found. In ox blood only negligible amounts of histamine were present.

RESULTS.

Release of histamine by activated guinea-pig serum.

As shown in Table I, the normal serum of the guinea-pig activated by incubation with agar for 1 hr. releases considerable quantities of histamine when perfused through the isolated lung of the guinea-pig. Fig. 2 shows the output of histamine following two injections of 4 ml. activated serum (Exp. 2). In the first minute of perfusion 21·3 μg. of histamine could be found in the collected

TABLE I.—*Histamine Release from the Guinea-pig Lung by Perfusion with Agar-Anaphylotoxin.*

	1st injection (4 ml. serum).	Histamine released (μg.).	2nd injection (4 ml. serum).	Histamine released (μg.).
1.	Normal (non-activated)	0	Activated	21·4+
2.	Activated	32·6	Same	12·8
3.	Dialysed (24 hr.), then activated	15·1	„	8·9
4.	Activated	25·6	—	—
5.	Activated, then dialysed	10·4	Dialysed, then activated	23·9
6.	Heated (30 min. at 56°), then activated	22·8*	„ „ „	103·3+
7.	Heated (60 min. at 60°), then activated	Traces	—	—
8.	Same serum as 7, activated (same lung as 7)	14·3	Same	9·7

" Activated " = serum treated with agar.
" Dialysed, then activated " = serum submitted to dialysis before treatment with agar.
" Activated, then dialysed " = incubated with agar for 24 hr. before dialysis.
" Normal " = untreated guinea-pig serum.
" + " = perfusion slow (about 5 ml./min.).
" * " = „ very slow (about 2–3 ml./min.).

perfusate. In 2–6 min. of perfusion the histamine in the perfusates diminished sharply, and before the second injection the perfusates were practically histamine-free. As a rule the second injection of exactly the same amount yielded about one-half of the total histamine released on the first occasion. Table II shows a comparison of the means of total histamine released in three experiments in which the same material was injected twice into the lung.

TABLE II.—*Two Successive Injections of Identical Materials in 3 Different Guinea-pig Lungs.*

Guinea-pig.	Material injected (4 ml.).	1st injection (μg. histamine).	2nd injection (μg. histamine).	Differences (μg. histamine).
1.	Activated serum	32·6	12·8	19·8
2.	Dialysed activated serum	15·1	8·9	6·2
3.	Activated serum	14·3	9·7	4·6
Averages		20·7	10·4	10·2

Dialysis of the serum performed before activation did not alter the result. In one experiment (Table I, Exp. 6) dialysis for 24 hr. before activation appeared to increase the resulting output; not less than 103·3 μg. histamine were released in the course of 10 min.; however, in other experiments (Table I, Exp. 3 and 5) previous dialysis did not produce any appreciable effect on the course of activation. Dialysis *after* activation, as performed in Table I, Exp. 5 (second injection),

appeared to reduce the activity, but this activated serum was left for 24 hr. at 5° in the cold room and spontaneous inactivation might have occurred.

Effect of temperature on the activation of serum by agar.—As shown in Table I, heating the serum for 60 min. at 60° *before* the activation with agar completely destroyed its capacity to be activated; however this was not so after heating at 56° for 30 min. When heated *after* activation the activated principle was somewhat more resistant. A temperature of 60°–69° for 90–120 min. reduced to less than one-half the releasing activity but did not destroy it completely. Table III shows 5 pairs of experiments in 10 different guinea-pigs in which the

TABLE III.—*Effect of Heating Activated Serum on Histamine Release.*

Guinea-pig.	Unheated activated serum (μg. histamine).	Guinea-pig.	Serum activated then heated (μg. histamine).	Guinea-pig.	2nd injection: activated unheated serum (μg. histamine).
5.	21·4	12	9·0 (1 hr. at 60°)	12	13·0
8.	32·6	13	14·1 (2 „ „)	13	19·1
2.	25·6	14	3·7 (1½ „ 65°)	14	8·0
11.	14·3	15	11·6 (1½ „ „)	15	9·4
16.	26·6	17	11·8 (1½ „ 69°)	17	15·6
Averages	$\bar{x}_1 = 24·1$	$\bar{x}_2 = 10·04$			13·0
Dif.	$\bar{x}_1 - \bar{x}_2 = 14·06$	$sd = \sqrt{11·37} = 3·37$; $t = 14·06/3·37 = 4·17$; $d.f. = 8$; $P < 0·01$ HS.			

release of histamine by activated serum is compared with serum that was activated and heated. As shown in the Table, the average for the first injections (total output) dropped from 24·1 μg. to 10·04 μg. histamine. The difference was found to be highly significant ($P < 0·01$).

In the experiment presented in Table III we did not go beyond 69°, to avoid coagulation of the serum proteins. In two separate experiments, however, after activation the serum was treated with 2 vol. ethyl alcohol heated at 65° for 15 min., and the filtrate was dried *in vacuo* and extracted with water. On passage through the lungs this material was completely inactive, showing that the treatment either destroyed the releasing agent or that this was not soluble in hot ethyl alcohol.

Effect of citrate on activation of serum by agar.

If instead of serum citrated plasma was used, no activation could be observed after incubation with agar for 1 hr. In the experiments shown in Fig. 3, 4, 5 and 6 ox instead of guinea-pig blood was used; ox blood yields a less active material, but clear-cut results could be obtained by comparing blood samples collected with 20 per cent of a 3·8 per cent solution of trisodium citrate with samples that were left to clot spontaneously. Both citrated plasma and serum were incubated for exactly the same time and under identical conditions. In some experiments citrate was added to the serum before addition of agar and also after activation and removal of agar. As shown in Fig. 3, 4 and 5, citrate hindered activation when added to serum before its activation by agar; added afterwards citrate did not prevent the release of histamine from guinea-pig lungs.

In order to check whether the effect of citrate could be due to removal of

$Ca^{\cdot\cdot}$, the citrated blood or plasma or serum were recalcified after 12 to 15 min. contact with the citrate. The concentration of citrate used was about 0·025 M and a slight excess of $CaCl_2$ (0·04 M) was added. After coagulation of the plasma

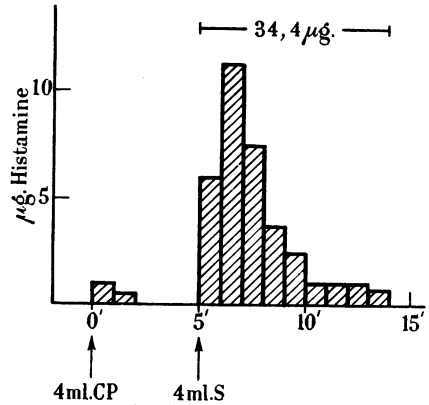

FIG. 3.—Histamine released from a guinea-pig lung by perfusion with citrated plasma (CP) and serum (S) both incubated with agar for 30 min. at 38°.

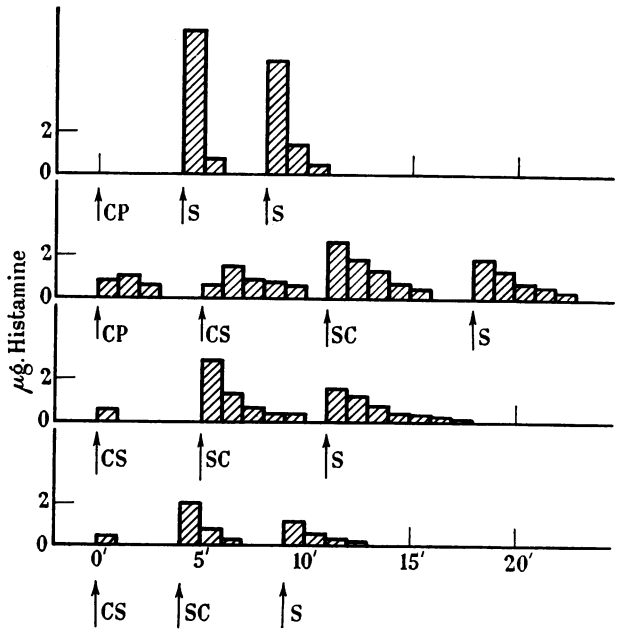

FIG. 4.—Histamine released from the lungs of 4 guinea-pigs by perfusion with citrated plasma (CP), citrated serum (CS), serum citrated after incubation (SC) and normal serum (S): all incubated with agar for 1 hr. at 38°. Abscissae: time in minutes.

the clot was removed by centrifugation and the recalcified material incubated with agar in the usual way. The results are shown in Fig. 6. If citrate is added to blood and the plasma, obtained by centrifugation, is submitted to the action of agar, it will not show any capacity of releasing histamine from the perfused

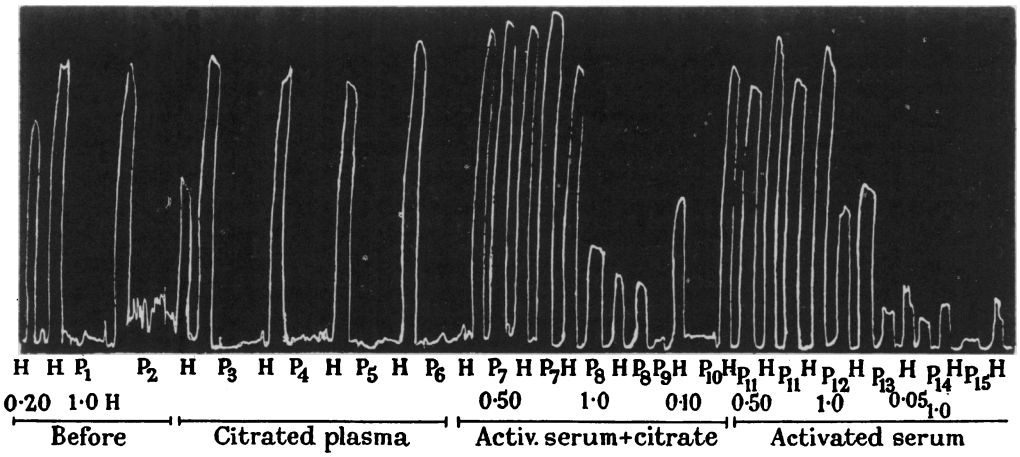

FIG. 5.—Histamine release from a perfused guinea-pig lung, as assayed upon the guinea-pig gut after passage of citrated plasma incubated with agar for 30 min. at 38° (P_3 to P_6): activated serum with citrate added after incubation with agar (P_7 to P_{10}) and same serum without citrate (P_{11} to P_{15}). (H = histamine).

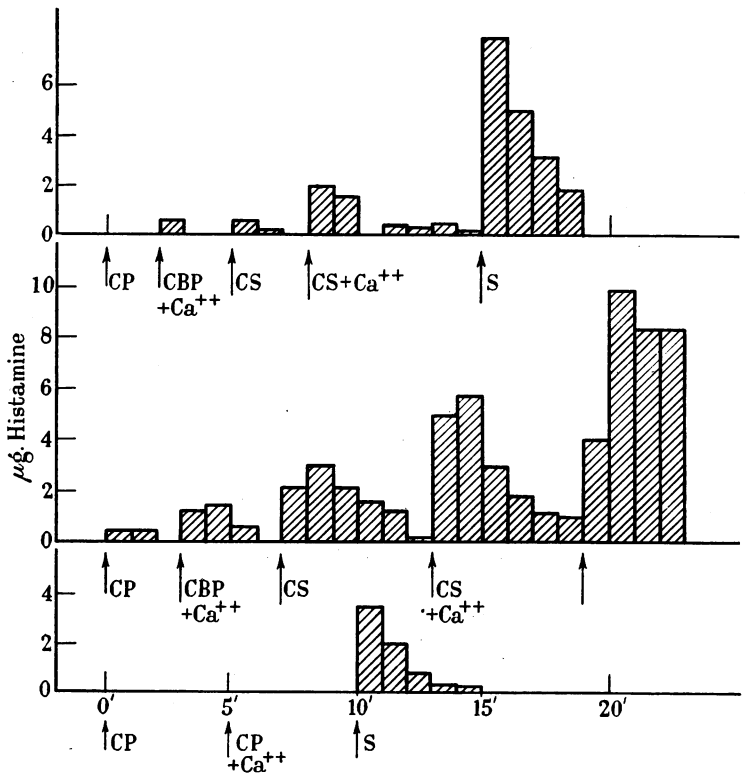

FIG. 6.—Histamine release from 3 guinea-pigs' lungs by perfusion with citrated plasma (CP), recalcified citrated plasma (CP + Ca^{++}), serum obtained by recalcification of citrated blood (CBP + Ca^{++}); citrated serum (CS); recalcified citrated serum (CS + Ca^{++}) and serum (S); all activated with agar for 1 hr. at 38°. Abscissae: time in minutes.

guinea-pig lung whether it is recalcified or not. If the $CaCl_2$ is added to the citrated blood and then the serum incubated with agar some very slight activation takes place. By using citrated serum more histamine was released than by using the corresponding citrated plasma. Recalcification of this citrated serum improved considerably its capacity of being activated by agar.

Interpretation of these experiments is not easy; it appears probable that the effect of agar needs an activating factor or co-factor that is given up by the cells when coagulation takes place. Citrate prevents the release of this factor completely and at least 90 per cent irreversibly when added to the total blood before clotting takes place. When added to serum, citrate reduces its capacity of being activated by agar but the effect is less strong than that on total blood. Some activator seems to escape the action of citrate after spontaneous clotting, although when the plasma is collected from citrated blood and then recalcified, or when recalcification is applied to total citrated blood, the effect of recalcification is slight or *nil*.

DISCUSSION.

The experiments presented in this paper show that normal blood contains a factor that can be activated to produce a conspicuous discharge of histamine from the perfused isolated guinea-pig lung. The relationship between the blood constituents and the discharge of histamine from dog's liver was postulated by Rocha e Silva and Graña (1946) on the basis that anticoagulants such as citrate and heparin reduced or abolished the release of histamine from dog's liver perfused with shock-producing agents such as peptone, ascaris extracts and so forth. Since platelets appeared to disintegrate in the liver and this disintegration was obviously connected with the intensity of the discharge of histamine and heparin from dog's liver either *in vivo* or *in vitro*, we have assumed (Rocha e Silva and Grãna, 1946; Rocha e Silva, Scroggie, Fidlar and Jaques, 1947) that the breakdown of platelets occurring in this kind of shock might play a major part in the discharge of the active substances from dog's liver. Dragstedt, Wells and Rocha e Silva (1942) showed that an excess of heparin partially inhibited the release of histamine from cells to plasma when samples of rabbit blood were put into contact with the shock-producing agents. More recently McIntire, Roth and Richards (1949), using samples of blood from sensitised rabbits, studied the shift of histamine from cells to plasma following the addition of the sensitising antigen. Addition of several anticoagulants, such as heparin, citrate, oxalate, large doses of $Ca^{\cdot\cdot}$ or $Mg^{\cdot\cdot}$, partially or totally inhibited the discharge of histamine. Soya bean trypsin inhibitor, however, decreased very slightly the discharge of histamine by the antigen but could completely stop the liberation of histamine by trypsin (McIntire, Roth and Sproull, 1950). The factors which participate in the clotting of the blood could be discarded as possible agents for the release of histamine, since the dose of anticoagulant to hinder the discharge of histamine was substantially different from those preventing blood-clotting (more heparin and less citrate or oxalate). Furthermore, the inhibition by citrate was 50 to 70 per cent irreversible after recalcification of the blood.

In the experiments on histamine release by anaphylatoxin we have seen a similar situation. When the blood is collected with 20 per cent of sodium citrate (3·8 per cent solution) the activation of anaphylatoxin by agar is completely

inhibited. Recalcification of the citrated blood or plasma results in no or very slight increase in its capacity to be activated by agar. If the citrate is added to the serum obtained by spontaneous clotting the process of activation is also considerably reduced, and recalcification definitely increased the effect of agar. According to the hypothesis that platelets might contribute with an activator or co-factor to the process of activation, one might explain this phenomenon by assuming that some intact platelets are still present in the serum and become protected by citrate and only partially disintegrate following recalcification. We hope to publish in the future experiments showing that heparin does not interfere with the activating process.

The mechanism by which serum becomes activated by treatment with agar may well be enzymic in nature. Heating the serum at 60° before the addition of agar completely destroys its capacity to be activated, as would be expected from work by Bordet (1913) on anaphylatoxin assayed on the intact guinea-pig, and by Novy and de Kruif (1917), who showed that at 70° only half of the anaphylatoxin activity was lost, but that heating at 100° completely destroyed its activity. We can assume therefore that the process depends on an activator that is destroyed at 60° for 1 hr., and that the active material (releasing factor) is somewhat more resistant to heating. As suggested by Novy and de Kruif, one might suppose that heat alters the precursor (matrix) of the anaphylatoxin in its molecular arrangement or denatures it.

In the earliest days of anaphylatoxin it became an accepted belief that the effect of agar, kaolin or barium sulphate on serum is to activate plasma protease by removal of the so-called anti-tryptic power of the blood. In a recent paper Bronfenbrenner (1944) discusses this idea on the basis of old and new experiments. There seems to be no doubt that in anaphylactic and peptone shock plasma protease is activated as indicated by the fibrinolysis that can be observed using the " protamine fibrinolytic test " (Rocha e Silva, Andrade and Teixeira, 1946). All attempts in our laboratory to show activation of a proteolytic enzyme after incubation with agar have been unsuccessful. Estimations of non-protein N or decreased viscosity of a gelatin solution entirely failed to show proteolytic activity in the serum activated with agar. Estimations of free α-amino-N in the serum submitted to incubation with agar have shown slight and possibly significant increases. However, the injection of a powerful preparation of fibrinolysin resulted in no release of histamine in lung perfusion experiments.

The histamine liberator—anaphylatoxin—released by incubation of normal serum with agar is probably not related to simple bases such as tubo-curarine, diamidines and so forth that are capable of releasing histamine (Alam, Anrep Barsoum, Talaat and Weininger, 1939; Rocha e Silva and Schild, 1949; MacIntosh and Paton, 1949; Reid, 1950), since the anaphylatoxin principle is destroyed by boiling and not extracted by hot alcohol. The complexity of the process of histamine release has often been overlooked. It is likely that several processes might be involved. Trypsin and other proteolytic enzymes displace histamine probably by hydrolysing a peptide linkage (Rocha e Silva, 1944); ammonia and simple tertiary or quaternary bases might release histamine by displacing it from its cell connections. Which mechanism underlies the releasing action of the histamine liberator released from serum by the action of agar and other polysaccharides is unknown. Its investigation might clarify the mechanism of liberation of histamine in anaphylaxis and inflammation.

SUMMARY.

Serotoxin (anaphylatoxin) prepared according to Bordet by incubating guinea-pig or ox serum with agar is capable of releasing considerable quantities of histamine when perfused through the isolated lung of the guinea-pig. The effect of temperature on the activation of serum has been studied.

Trisodium citrate added to whole blood before separation of the plasma, or to the serum after clotting of the blood, inhibited the formation of anaphylatoxin.

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