ACTIVATION OF A HISTAMINE-RELEASING AGENT (ANAPHYLATOXIN) IN NORMAL RAT PLASMA.*

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IN previous reports from this laboratory (Rocha e Silva, Aronson and Bier, 1951 ; Rocha e Silva, 1952 ; Rocha e Silva and Aronson, 1952) it was shown that anaphylatoxin or serotoxin, prepared according to the method described by Bordet (1913), is able to release considerable quantities of histamine when perfused through the isolated guinea-pig lung. This histamine-releasing activity of normal rat plasma treated with agar can also be demonstrated by simply adding the " activated plasma " to the chamber containing a piece of guinea-pig ileum, in a Dale's apparatus. Rat plasma incubated for a few minutes with agar acquires the property of eliciting a strong contraction of the isolated guinea-pig ileum. That this contraction is the result of the release of histamine from the gut itself could be demonstrated since : (*a*) this stimulating effect can be completely blocked by anti-histaminics and not by atropine ; (*b*) de-sensitization (tachyphylaxis) follows a number of additions of the same dose of the " activated " plasma or serum ; (*c*) this tachyphylaxis is most probably a result of exhaustion of the store of bound histamine since the gut's sensitivity to histamine remains unimpaired. The present work is a study of the mechanism by which this histamine-releasing agent becomes activated in rat plasma or serum under varying experimental conditions.

MATERIALS AND METHODS.

Preparation of anaphylatoxin (activated plasma).

Blood was collected from the lightly (ether) anaesthetized rat by heart puncture, into a syringe containing 1 mg. heparin/10 ml. blood. Several animals were bled successively and the plasma of the pooled samples collected by centrifugation. The clear plasma was mixed at once with the indicated amounts of polysaccharides and incubated for 10–30 min. at 37°, unless otherwise stated. Usually 0·05–0·10 ml. of a diluted (1/2) and fully activated plasma produced a maximal contraction of the gut. In many experiments plasma was activated by dilution to 1/10 or less with distilled water and incubated for 10 min. at 37°. Activation could also be obtained by dialysis of plasma against distilled water for 2 hr. at room temp. Most of the experiments were done with fresh plasma or plasma kept for 1 or 2 days in the frozen state.

Assay on the guinea-pig ileum.

The method of detecting activation of anaphylatoxin by its effect on the isolated guinea-pig ileum has been employed as a routine assay. The chamber containing the piece of gut had the capacity of 4·0 ml., and the conditions of the assay are the same as those described in previous papers from this laboratory. In most of the experiments a frontal writing lever

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of the type described by Schild (1944) has been used. Tyrode solution at pH 7·4 was used as perfusing fluid. The standard histamine solution had a concentration of 0·05 mg./100 ml. calculated as the base. Additions to the chamber were made after a 1 min. interval after washing out the gut with fresh Tyrode solution. Pieces of ileum from a freshly killed guinea-pig were always used, since it was found that the responsiveness of the gut to activated plasma decreased fairly rapidly when the gut was kept in Tyrode solution at room temp. or in the refrigerator for more than a few hours, probably as a result of diffusion or destruction of the bound histamine.

Sulphation of the polysaccharides.

Agar, dextran and starch were sulphated as described by Karrer, Koenig and Usteri (1943), using chlorosulphonic acid in pyridine below 0° in a dry atmosphere. After slowly adding the dried polysaccharide the mixture was heated for several hours at 60–80°, and the sulphated polysaccharide extracted with cold water and precipitated with ethanol. The precipitated material was collected by filtration, washed with ethanol, suspended in water and re-dissolved after alkalinization with NaOH. The solution was dialysed for 48 hr. against running tap water and re-precipitated 1 or 2 times in the same manner, using ethanol. The final precipitate was washed with acetone and dried *in vacuo*. The brownish or yellowish loose powder thus obtained gave a strong metachromatic colour with toluidine blue.

Fractionation of the hydrolysates from starch.

Acid hydrolysis of a commercial potato starch (defatted by continuous extraction with ethyl ether) was obtained by submitting 40 g. of the raw material, suspended in 800 ml. 0·1 N-HCl, to 10 min. heating at 100°. The solution was neutralized with NaOH and the different fractions obtained by fractional precipitation with increasing amounts of ethanol. Each fraction was collected by centrifugation and dried over alcohol and acetone. The coefficient " Total glucose/reducing glucose " for each fraction was obtained by estimating the total hydrolysable glucose and the terminal " free " reducing glucose by the iodine titration method according to Cajori (1922).

Decalcification of rat plasma.

Rat plasma was decalcified by passing it through a column of the cationic ion-exchange resin Dowex 50, which had been previously washed with HCl, NaOH, concentrated NaCl and saline. The plasma so treated no longer gave any reaction with sodium oxalate.

Effect of salts on the activation of rat plasma by agar.

An amount of each salt calculated to give the desired concentration in the final mixture was dissolved in 0·5 ml. saline and added to 0·5 ml. of the agar solution (0·01 per cent) in saline and 0·5 ml. rat plasma. The mixtures were incubated for 10 min. at 37° and when not assayed immediately, were kept in crushed ice until tested. All incubations were at pH 7–7·4. The anaphylatoxin assay was performed by adding 0·05 or 0·10 ml. samples to the perfusion bath ; with such amounts, only rarely did the controls containing salt and plasma alone have any effect on the gut. The mixtures containing the highest salt concentrations (*i.e.*, affording the greatest protection from activation) were tested first, until the concentration affording threshold activation had been reached ; samples containing agar and different levels of sodium chloride as inhibitor were tested as controls, at the end of the experiment.

Agar.—The stock solution of agar (Difco), was routinely made by suspending the weighed material in saline and heating to the boiling point for a few minutes ; usually a 0·25 per cent solution of agar thus made was stored in the ice box for a few weeks. Before using the solution it was again gently heated to facilitate pipetting. The *soluble starch* (Mallinkrodt Chemical Works) had, according to our method of determination (alkaline iodine titration), an average total of 35 glucose units per reducing group. Previous to this titration, the material had been incubated for 16 hr. with yeast to eliminate glucose and maltose.

Two kinds of *dextran* were employed : " crude dextran " was prepared from a culture of *Leuconostoc mesenteroides* by deproteinization and alcohol precipitation and kindly supplied by Dr. H. Moussatché, of the Oswaldo Cruz Institute in Rio de Janeiro ; " partially hydrolysed dextran " (batch TDR 221) was kindly supplied by Pharmacia from Uppsala, Sweden.

Alginic acid and its sulphated derivative (*Paritol*) were kindly supplied by the Wyeth Institute of Applied Biochemistry, Philadelphia, U.S.A.

Three different kinds of bacterial polysaccharides from *Bacillus anthracis* and from the somatic antigen of *Salmonella typhi* and *Salm. paratyphi* were kindly provided by Dr. Anne Marie Staub, from the Institut Pasteur, Paris.

Inulin.—A clinical brand of the type employed for renal clearance tests was used.

Glycogen.—Dog's liver glycogen was prepared in our laboratory by precipitation with alcohol from a trichloracetic filtrate of the macerated organ.

Kollidon (poly-vinyl-pyrrolidone).—Three preparations of different molecular weights of the order of 500,000 (K_1), 47,000 (K_2) and 11,000 (K_3) were assayed. These materials were kindly supplied by Prof. H. Weese, Bayer A. G., Elberfeld.

Peptone.—Bacto peptone (Difco) was used.

Heparin.—A commercial preparation (Vitrum) containing 50 mg./ml. was generously supplied by Apo Vitrum Comercial of S. Paulo, Brazil.

Ovo-mucoid.—A preparation obtained in our laboratory according to the method described by Meyer (1945) was used.

Soy-bean trypsin inhibitor.—A 5-times recrystallized preparation was kindly supplied by Dr. M. Kunitz of the Rockefeller Institute for Medical Research, Princeton, N.J.

Specific precipitate from rabbit serum immunized to egg albumin.—The washed serum-free suspension was kindly supplied by Dr. Maria Siqueira from the Department of Immunology of the Instituto Biologico, S. Paulo, Brazil.

Sulphated polysaccharides.—The sulphated products of cellulose glycol ether, chondroitin sulphuric acid and of pectin, were kindly supplied by Prof. P. Karrer of Zürich Polytechnic Institute, Switzerland.

Salts.—The inorganic and organic salts used in the salt inhibition experiments were of analytical grade, and were dried to constant weight before use, unless a hydrated form of known formula was used.

RESULTS.

Effect of Rat Anaphylatoxin on the Guinea-pig Ileum.

Heparinized normal rat plasma added to the bath containing a piece of guinea-pig ileum elicits a small contraction when added in doses of 0·3–0·5 ml. Usually 0·2 ml. or less produces only a slight change of tonus accompanied by rhythmic contractions. When the same plasma is incubated for a few min. with agar, it will elicit a powerful tonic contraction, similar to that produced by histamine, as shown in Fig. 1. Successive additions of the same dose of " activated plasma " (anaphylatoxin) will produce a lesser and lesser effect and finally the muscle is de-sensitized or tachyphylactic to it. That this tachyphylaxis is due to a de-sensitization effect and not to a destruction of the spasmogenic agent by further contact with the plasma, could be demonstrated by changing the piece of ileum. The new piece will react with the same dose of anaphylatoxin, again with a strong contraction followed by de-sensitization. Since anaphylatoxin prepared by incubation of normal rat plasma with agar is very potent as a histamine releaser when perfused through the isolated lung of the guinea-pig, it was assumed that this effect on the guinea-pig gut is also due to a release of histamine. Atropine in concentrations which completely block acetylcholine had no effect on the contraction produced by anaphylatoxin, and anti-histaminics (Antistin or Neo-antergan) in concentrations that had no effect on the spasmogenic action of acetylcholine, completely blocked the contraction produced by threshold doses of anaphylatoxin, as shown in Fig. 2. In a few cases, however, a slow contraction of the gut could be obtained even in the presence of Neo-antergan. As de-sensitization proceeds, the latent period, which is very short after a first addition of anaphylatoxin, becomes longer and longer, until the muscle becomes completely unresponsive.

The constancy of the stimulating effect of rat anaphylatoxin on the guinea-pig ileum contrasts with the irregular and inconstant results obtained with

anaphylatoxins prepared from guinea-pig serum (Dale and Kellaway, 1922), or from the serum of horse, rabbit or pig (Adant, 1950). In a long series of experiments only occasionally did the agar-activated plasma fail to produce a strong contraction. However, usually this failure was due to the unresponsiveness

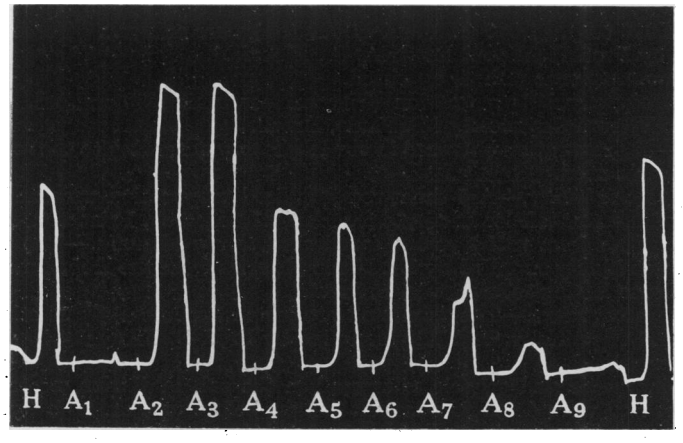

FIG. 1.—A_1 means a sample (0·15 ml.) taken from the mixture of rat plasma with 0·1 mg. of agar per ml., after incubation at 37° for 20 sec. A_2, A_3 etc. indicate additions of equal samples taken every 3 or 4 min. from the same incubating mixture. Note progressive desensitization of the gut toward anaphylatoxin. H, 0·05 μg. histamine (base).

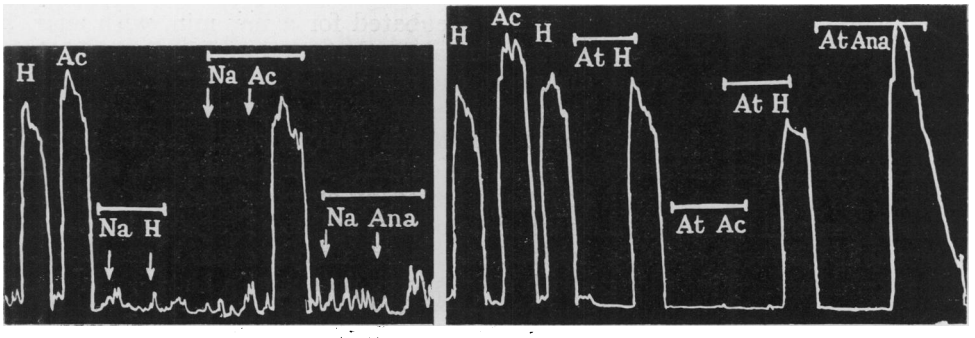

FIG. 2.—Effect of Neo-antergan and atropine on the action of anaphylatoxin on the guinea-pig ileum. H, 0·1 μg. histamine (base). Ac, 0·08 μg. acetylcholine. NA, 2·0 μg. Neo-antergan (anthisan maleate). At, 2·0 μg. atropine. Ana, 0·25 ml. rat plasma activated by incubation with 0·1 mg./ml. agar.

of the gut rather than to the absence of activity in the plasma, since by taking the piece of ileum from a new guinea-pig, the response to the activated material was usually obtained.

In spite of the de-sensitization effect, semi-quantitative results could be obtained in the same piece of gut in many cases. For instance, in the experiment presented in Fig. 3, the same rat plasma was incubated with increasing doses of starch. As little as 100 μg. produced a threshold effect, although full activity was

only obtained with 10 mg. starch. Under similar conditions agar was about 10 times more potent, affording full activation with 1 mg./ml.; but doses as small as 5 to 10 μg. agar produced a detectable effect when incubated with 1 ml. rat plasma.

Many different substances were tested for capacity to activate rat plasma (Table I). As an approximate indication of their relative activating potencies their effects are compared to those given by adding an equivalent amount of agar-activated plasma to the same gut.

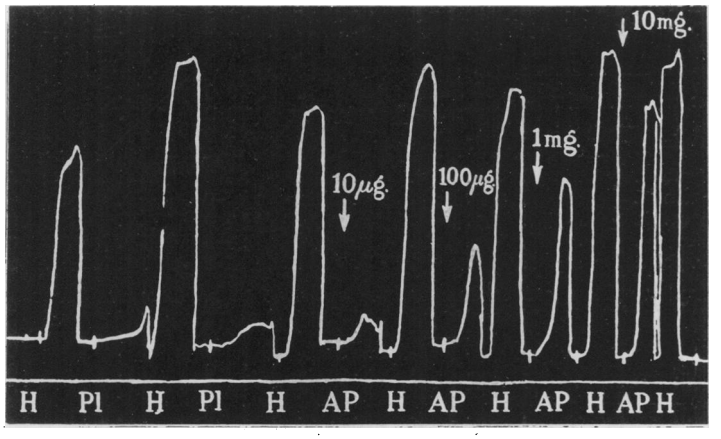

FIG. 3.—Effect of the dose of activator (potato starch) on the degree of activation of rat plasma. H, 0·05 μg. histamine (base). Pl, 0·2 ml. rat plasma diluted 1/2 with saline. AP, 0·1 ml. rat plasma incubated for 10 min. at 37° with varying amounts of starch. The amounts shown are wt. starch/ml. plasma.

The ability of starch to produce activation of rat plasma depends on the degree of polymerization. Non-hydrolysed starch was assayed in comparison with 5 different fractions of an acid hydrolysate of starch obtained by fractional precipitation with increasing concentrations of ethanol (Fig. 4). It is clear that the fractions containing the longest chains of split products (precipitated at lower concentrations of ethanol) are the most effective in producing activation. To have an idea of the relative chain lengths of these fractions, their reducing powers were estimated by the alkaline iodine titration method as described, and the ratio "total glucose/reducing glucose" expressed as shown in Table II. The colours produced by iodine with the different fractions are also stated.

Effect of Temperature on the Activation of Plasma by Polysaccharides.

As shown before (Rocha e Silva and Aronson, 1952) heating normal plasma for 30 min. at 56°–60° completely destroys its capacity for activation by polysaccharides, although the already active anaphylatoxin is somewhat more resistant to heat, since 1 hr. at 69°–70° caused only a partial inactivation of pre-formed anaphylatoxin. Concerning the effect of temperature on the activating process, the results presented below definitely indicate that a chemical or enzymic reaction is going on as a consequence of the interactions of the polysaccharide with the plasma proteins. As shown in Fig. 5, at 0°–2° incubation with 1 mg. agar/ml.

TABLE I.—*Compounds Tested as Activators of Rat Plasma Anaphylatoxin.*

	Dose (mg./ml. plasma).	Effect.
Agar-agar	0·05	+++
Starch (soluble)	0·1	++
„ (potato)	0·1	++
Dextran (crude)	0·1	+++
„ (partially hydrolysed)	1·0	+
Inulin	1·0	+
Specific precipitate from rabbit anti-ovo-albumin serum and ovo-albumin	1·0	++
Sodium alginate (Manucol)	2·0	+
Polyvinyl pyrrolidone (Kollidon)	2·0	+
Glycogen (dog's liver)	5·0	+
Polysaccharides from :		
(*a*) Somatic antigen of *Salm. typhi*	20·0	++
(*b*) „ „ *Salm. paratyphi*	20·0	+
(*c*) *B. anthracis*	20·0	++
Peptone	20·0	0
Heparin	25·0	0
Ovomucoid	20·0	0
Kaolin	20·0	0
Silica-gel	5·0	0
Sulphated products from :		
(*a*) Agar-agar	20·0	0
(*b*) Dextran	20·0	0
(*c*) Starch	20·0	0
(*d*) Alginic acid	20·0	0
(*e*) Chondroitin sulphuric acid	20·0	0
(*d*) Pectin	20·0	0

For each test, weighed amounts of each compound were dissolved in 0·5 ml. saline, mixed with 0·5 ml. plasma and incubated at 37° for 30 min.

0, no activation ; + weak activation ; ++, moderate activation ; +++, maximum activation.

TABLE II.—*Properties of Potato Starch Hydrolysates.*

Fraction.	Vol. of alcohol needed for precipitation (per unit vol. of hydrolysate).	Colour with iodine.	$\frac{\text{Total glucose}}{\text{Reducing glucose.}}$
I	1	Blue	37·0
II	2	Violet	33·4
III	3·5	Red	15·9
IV	5·5	Brown	11·9
V	7·5	None	9·1

plasma failed to produce any detectable activation even after 4 hr. At 10° approximately 60 min. and at 20° 20–30 min. were required to produce a threshold activation effect. At 27° activation was somewhat slower than at 37°, when it was complete in 3–5 min. At 47° activation of this plasma sample by agar was almost instantaneous, since maximal activity was observed after only 1 min. incubation. These results were obtained with one sample of pooled plasma, variations in the time-temperature relationship having to be expected between one plasma and another. The high temperature coefficient apparent for this process suggests that a chemical reaction probably of enzymic nature is involved.

As mentioned before, activation of rat plasma could also be obtained by dilution to 1/10 or less with distilled water, without addition of any activator. In Table III the influence of temperature on activation by dilution is shown. In this

case the influence of temperature is much smaller, although still perceptible at 0°–2°. However, at a dilution of 1/3 the influence of temperature on activation is more evident. Thus, at the lowest level, no full activation was observed even after 5 hr. incubation; at 18° the process is still very slow, complete activation requiring about 1 hr. These time intervals should be compared with that at 37°, where full activation was obtained in 7–10 min.

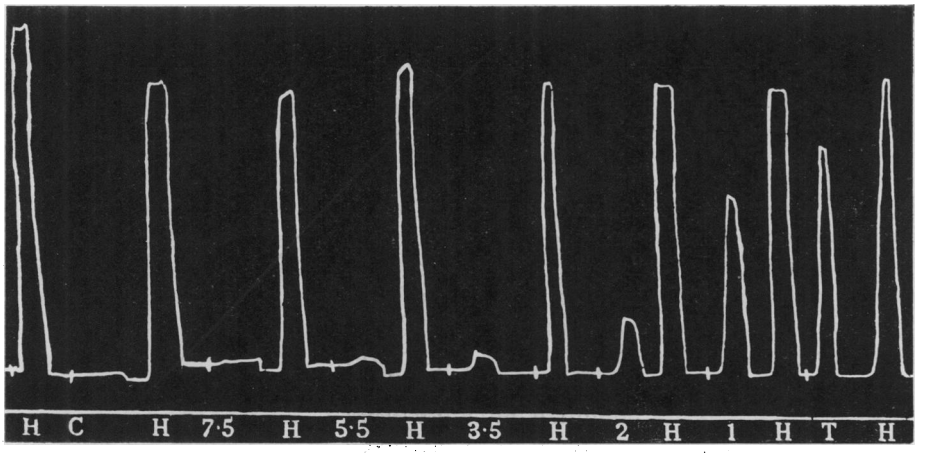

FIG. 4.—Effect of chain length on the activation of rat plasma by various fractions obtained from a potato starch hydrolysate. C, 0·1 ml. rat plasma diluted 1/2 with saline. 7·5 to 1–0·05 ml. rat plasma incubated for 30 min. at 37° with fractions of the hydrolysate precipitated by 7·5, 5·5, 3·5, 2·0 and 1·0 volumes of alcohol respectively. In each case the plasma was incubated with 10 mg./ml. of the activator dissolved in 1 ml. saline. T, incubated with total non-hydrolysed starch. H, 0·05 μg. histamine (base).

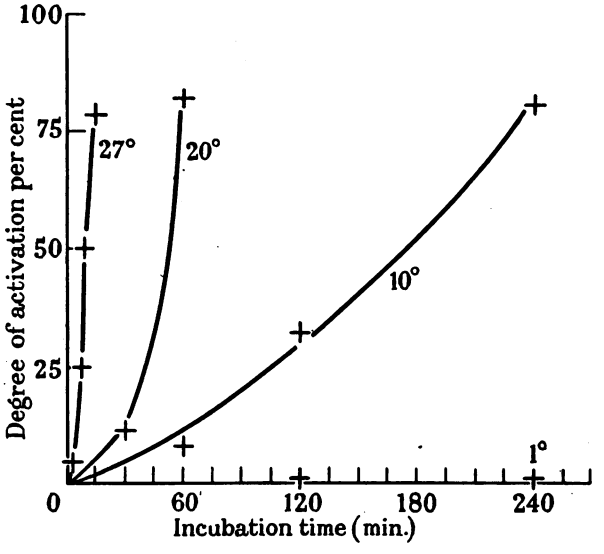

FIG. 5.—Effect of temperature on the rate of activation of rat plasma by agar. Ordinates represent the relation between the activation of plasma incubated with 1 mg./ml. agar at different temperatures and the maximal activation obtained with agar.

TABLE III.—*Influence of Temperature on the Spontaneous Activation of Rat Plasma after Dilution with Distilled Water.*

Incubation (min.).	Dilution 1/10. Temperature				Dilution 1/3. Temperature		
	1°	18°	27°	37°	1°	18°	27°
3	0	+	++	+++	0	0	++
5	0	+++	+++	+++	0	0	+++
10	0	+++	+++	+++	0	0	+++
15	0	+++	+++	+++	0	+	+++
35	+++	+++	+++	+++	0	+	+++
60	+++	+++	+++	+++	0	+	+++
300	+++	+++	+++	+++	+	+++	+++

0, no activation ; +, threshold activation ; ++, moderate activation ; +++, maximal activation.

It thus appears that activation by dilution is, like activation by agar, not an instantaneous process, but a chemical, probably enzymic, effect, with a high activation energy. In order to explain the quicker effect of the 1/10 dilution compared with that at lesser dilutions, it would be reasonable to assume that the activator is liberated in greater amounts at this dilution, than at 1/3. It seems unnecessary to postulate the existence of two different thermolabile activators, one for agar and another for activation by dilution, since in both cases heating the plasma for 56°–60° for 30 min. completely destroys its capacity to be activated by either agent. However, the process involved in activation by agar might depend on another step, *i.e.*, the action of agar itself releasing the activator, which will in turn act enzymically on the precursor of anaphylatoxin, setting anaphylatoxin free. The shape of the curves in Fig. 5, with the concavity turned upwards, could not be reproduced with material diluted 1/3. The effect of temperature followed in this case a more straightforward pattern, the tendency of the curve being to bend downwards.

Activation of Anaphylatoxin by Dialysis.

The mechanism by which plasma is activated by dilution was studied further, by submitting it to dialysis against different ionic strength solutions. Dialysis for 2 hr. against distilled water at room temperature caused definite activation. No such effect was obtained by dialysis against saline (ionic strength 0·15). Maximal activation was obtained after 12 hr. dialysis against distilled water at 8°. Under such conditions dialysis against saline (ionic strength 0·15) produced only a small activation which could be greatly increased by diluting an aliquot of the dialysed plasma with distilled water to 1/10. No such increase could be obtained by diluting plasma which had been dialysed against distilled water. It was further observed that no activation could be produced by diluting plasma with saline (1/10), followed by incubation at 37° for up to 30 min. ; under similar conditions dilution with 3 vol. distilled water produced a high degree of activation within 30 min.

Effect of Ions on the Activation of Anaphylatoxin by Agar.

The above experiments strongly suggested that activation of anaphylatoxin depends on the ionic strength of the medium. This idea was corroborated by the finding that in a medium of approximately twice the ionic strength of saline (0·30–0·35) activation of rat plasma by agar was always prevented. Subsequent

dilution to normal saline level (ionic strength 0·15) allowed activation to proceed normally. A study of a series of cations and anions which proved to be effective as inhibitors of the process was therefore made. As shown in Table IV the anions

TABLE IV.—*Effect of Anions on the Activation of Anaphylatoxin by Agar.*

Anion.	Average " threshold " concentration.	Average " threshold " ionic strength.
SO_4^{++}	0·12-N	0·18
Cl^+	0·12-N	0·12
I^+	0·06-N	0·06
NO_3^+	0·06-N	0·06
SCN^+	<0·06-N	<0·06
$Salicylate^+$	<0·06-N	<0·06
$Oxalate^{++}$	0·04-N	0·06
$Citrate^{+++}$	<0·04-N	<0·12
	>0·02-N	>0·06

Each result is the average of two or more determinations of the salt concentrations just sufficient to allow for a beginning activation of rat plasma by agar (0·1 mg./ml.). All salts were assayed as sodium or potassium compounds.

could be arranged in the following order: citrate $\geqslant$ oxalate $>$ salicylate $>$ thiocyanate $>$ nitrate $=$ iodide $>$ chloride $\geqslant$ sulphate, when the concentrations allowing threshold activation are presented in normalities. However, if the ionic strengths of these solutions are compared, the differences between citrate, oxalate and chloride are much less pronounced and the effect of sulphate becomes significantly smaller than that of chloride. Still the differences between salicylate, iodide, thiocyanate and chloride will not be reduced so that in the case of the monovalent ions, the size of the charged particles appears to constitute a factor in the inhibition of the activation process by electrolytes. In separate experiments more direct comparisons between different anions were made. For instance, thiocyanate was always more potent than iodide and nitrate, although nitrate and iodide did not show substantial differences. These additional data permitted a better localization of the different ions in the series. As regards cations, comparisons were made with only a few, the results, as well as those using urea and glucose being given in Table V. There was no significant difference between the monovalent cations (Na^+, K^+ and Li^+). In the divalent series, however, Ca^{++} was definitely stronger than Mg^{++} and about as active as Sr^{++}. Other cations such as Ba^{++}, Zn^{++} or Al^{+++}, could not be properly assayed because of the effects on the gut of such doses as might prevent activation.

Action of Sulphated Polysaccharides on Rat Plasma.

In view of the increasing importance of the sulphated polysaccharides as heparin substitutes, the action on rat plasma of starch, dextran and agar sulphates was studied. As it can be seen from Table I, these three most active polysaccharides entirely lost their anaphylatoxin-activating capacity after being sulphated. Even doses twenty times higher than those of the non-sulphated products giving maximum contraction failed to produce any effect. Heparin, also a sulphated derivative of a polysaccharide, was equally ineffective when assayed in doses of 5, 10 and 25 mg./ml. plasma. However, when assayed in connection with agar, heparin showed an inhibitory action, by completely preventing agar activation of plasma when present in the plasma previous to the addition of the agar, at a final concentration of 15 mg. heparin per ml. At the 10 mg./ml. level, this inhi-

TABLE V.—*Effect of Cations and Neutral Compounds on the Activation of Rat Plasma by Agar.*

Cation.	Average " threshold " concentration.	Average " threshold " ionic strength.
Na^+	0·12-N	0·12
K^+	0·12-N	0·12
Li^+	0·12-N	0·12
Mg^{++}	0·06-N	0·09
Ca^{++}	0·02-N	0·03
Sr^{++}	0·02-N	0·03
Glucose	>0·50-M	
Urea	0·25-M	

The determinations were carried out as described for Table IV. All salts were assayed as chlorides.

bition was only partial, while at the 5 mg./ml. level, activation was indistinguishable from that of the controls containing the usual concentration, enough to prevent coagulation.

DISCUSSION.

The striking stimulating effect of rat anaphylatoxin on the isolated ileum of the guinea-pig afforded a simple and reliable technique to study the conditions under which activation of the histamine-releasing principle takes place, under varying experimental conditions.

Although a slight activation of normal rat plasma was observed even when the plasma was left for several hours at 37° or at room temperature, the process could greatly be accelerated by adding minute amounts of polysaccharides, or by diluting plasma or dialysing it against distilled water. Amounts of agar of the order of 0·02 mg./ml. plasma produced a perceptible effect, and dilution with an equal volume of distilled water, followed by a 6 minutes' incubation at 37°, also conferred definite histamine-releasing activity. Although no conclusive evidence is offered of an identity of the mechanisms of production of the polysaccharide-activated anaphylatoxin and the histamine-releasing principle appearing after dilution or dialysis, several facts strongly point in this direction, both processes being highly dependent upon temperature, inhibited at higher ionic strengths and by the previous heating of plasma to 56°.

In an effort to shed some light upon the nature of the interaction between the activating polysaccharide and the plasma proteins, some structural differences between active and non-active polysaccharides were analysed. The chain lengths of the different fractions of hydrolysed starch greatly affect their capacities to activate anaphylatoxin, the shorter the chain length, the smaller being this effect. Possibly the observations of Ingelman (1949) and others regarding the direct relationship between chain length and toxicity of dextran could be explained on such a basis, since dextran is an efficient activator of rat plasma anaphylatoxin. The presence of strongly charged negative substituents on the polysaccharide chain could be shown to be another factor capable of reducing or even abolishing its anaphylatoxin-activating capacity. Thus, products obtained by sulphation of agar, dextran or starch, and the carboxyl-containing alginates had no or very slight activity. Moreover, it was noted that in stronger concentrations the sulphated polysaccharides could antagonize activation of rat plasma by agar. This effect, also observed with heparin, might be explained as being due to a block resulting from a salt-like combination between the negative sulphate groups of the

polysaccharides and some positive grouping of the plasma proteins, which are involved in the activating process either as precursors or as enzymic activators of anaphylatoxin. As shown by Jaques (1943), heparin is capable of reacting at physiological pH with several plasma proteins.

The activation of rat plasma by agar is greatly dependent on the ionic strength of the medium. In the systematic study of the threshold concentrations of several salts which permitted activation by agar, it was observed, however, that besides ionic strength, more specific effects could be observed. Thus anions could be arranged in this order of decreasing effectiveness : citrate $\geqslant$ oxalate $>$ salicylate $>$ SCN $>$ I $=$ NO_3 $>$ Cl $>$ SO_4, while in the cationic series the sequence Ca $>$ Mg $>$ 2Na was found. Except for citrate and oxalate, the position of the anions in this series resembles closely that of anions of the lyotropic series. A similar sequence has been shown by Marrack (1934) to hold for the relative inhibiting power of anions on the formation of the specific precipitate.

Gordon and Thompson (1933) have shown a similar effect of anions on the inhibition of complement in the haemolysis of sensitized ox red blood cells by guinea-pig serum. Edsall and Lever (1951) also demonstrated such an anionic sequence in the inhibition of the clotting of fibrinogen by thrombin. That Ca^{++} ions are more effective than Na^{+} in the prevention of this process has also been shown to hold true by Edsall, although it is not yet clear whether this action is truly an inhibition of the proteolytic action of thrombin upon fibrinogen or merely an inhibition of the polymerization of " activated " fibrinogen into fibrin.

In a previous publication (Rocha e Silva and Aronson, 1952) it had been assumed that sodium citrate inhibited the activation of anaphylatoxin either by removing Ca^{++} ions from the medium or by preventing the disintegration of platelets, and it was thought possible that platelet disintegration could furnish the activator or a co-factor for activation of anaphylatoxin. More recent results have shown, however, that the presence of free Ca^{++} ions is not needed for anaphylatoxin activation, since decalcified plasma obtained by passage through a Dowex 50 resin column can still be normally activated by agar. As far as the second hypothesis is concerned it would appear that sodium citrate, as well as the other anionic inhibitors tested would rather act on plasma proteins blocking the precursor or activator of anaphylatoxin. The observation that salt inhibition proved equally effective either when tested upon plasma or upon serum points towards this view. However, the fact that citrate and oxalate may not act solely by means of ionic strength effects cannot be excluded.

McIntire, Roth and Richards (1949) showed that relatively high doses of heparin as well as citrate, oxalate, Ca^{++} and Mg^{++} were able to inhibit or reduce the *in vitro* shift of histamine from blood cells to plasma of sensitized rabbits when antigen was added. Comparing the doses required in McIntire's experiments with those needed to inhibit anaphylatoxin, a rather close similarity can be seen in nearly all cases. These facts, as well as the incapacity of soy-bean trypsin inhibitor to affect either the activation of anaphylatoxin by agar or the anaphylactic release of histamine in rabbit blood, strongly suggest that a similar mechanism is involved in both reactions.

As regards the relationship between the facts presented in this paper and the intimate mechanism of anaphylaxis, a general discussion has been presented before (Rocha e Silva, 1953). It is clear that anaphylatoxin can be activated under such conditions as those existing in anaphylactic shock. The specific

precipitate obtained by the interaction of ovo-albumin, for instance, and the serum of rabbits immunized to it, can replace the polysaccharides in the process of activation of anaphylatoxin. This fact, known for a long time, has been confirmed in our laboratory using the isolated ileum of the guinea-pig and rat plasma, to show activation of anaphylatoxin by the specific precipitate. However, recent experiments by Schachter (1953) showing that sensitized rabbit organs perfused with antigen in Locke solution in the absence of blood are able to release histamine, might indicate that some direct interaction of the antigen with the cells is a sufficient stimulus for the release of histamine. However, the impossibility of removing all traces of blood from a perfusion system, as well as the probable presence of anaphylatoxin in the interstitial fluid (lymph), might be factors in histamine release under the conditions of Schachter's experiments.

SUMMARY.

Rat plasma, incubated with agar, starch, dextran and several other polysaccharides, acquires the capacity to release histamine from the guinea-pig ileum, as shown by the powerful contraction elicited *in vitro* followed by desensitization. Dilution or dialysis of the plasma against distilled water followed by a brief incubation at 37° has a similar effect.

Activation both by agar and by dilution are strongly dependent on temperature, the former having a higher temperature coefficient than the latter.

The length of the polysaccharide chains constitutes an important factor, the products having a shorter chain being less efficient activators than those with longer chains.

Sulphation of the polysaccharides suppresses their activating capacity. Relatively small increases in ionic strength of plasma inhibit its activation by agar. A systematic study of the relative inhibitory powers of several anions showed that their relative efficiencies paralleled their positions in the lyotropic series. Among the cations studied, Ca^{++} proved to be much more effective as inhibitor than Mg^{++}, Na^{+}, K^{+}, or Li^{+}.

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