PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Anaphylaxis and Anaphylatoxins

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VI.—*Anaphylaxis and Anaphylatoxins.*

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In the study of the phenomena of anaphylaxis there are certain points on which some measure of agreement seems to have been attained. In the case of anaphylaxis to soluble proteins, with which alone we are directly concerned in this paper, the majority of investigators probably accept the view that the condition is due to the formation of an antibody of the precipitin type. Concerning the method, however, by which the presence of this antibody causes the specific sensitiveness, the means by which its interaction with the antibody produces the anaphylactic shock, there is a wide divergence of conception. Two main currents of speculation can be discerned.

One view, historically rather the earlier, and first put forward by Besredka (1) attributes the anaphylactic condition to the location of the antibody in the body cells. There is not complete unanimity among adherents of this view as to the nature of the antibody concerned, or as to the class of cells containing it which are primarily affected in the anaphylactic shock. Besredka (2) himself has apparently not accepted the identification of the anaphylactic antibody with a precipitin, but regards it as belonging to a special class (sensibilisine). He also regards the cells of the central nervous system as those primarily involved in the anaphylactic shock in the guinea-pig. Others, including one of us (3), have found no adequate reason for rejecting the strong evidence in favour of the precipitin nature of the anaphylactic antibody, produced by Doerr and Russ (4), Weil (5), and others, and have accepted and confirmed the description of the rapid anaphylactic death in the guinea-pig as due to a direct stimulation of the plain-muscle fibres surrounding the bronchioles, causing valve-like obstruction of the lumen, and leading to asphyxia, with the characteristic fixed distension of the lungs, as first described by Auer and Lewis (6), and almost simultaneously by Biedl and Kraus (7). But the fundamental conception of anaphylaxis as due to cellular location of an antibody, and of the reaction as due to the union of antigen and antibody taking place in the protoplasm, is common to a number of workers who thus differ on details.

The central conception of the other widely prevalent type of theory is the "anaphylatoxin." This is a poisonous substance, or condition, which is supposed to be produced whenever an antigen is injected into an animal rendered anaphylactic to it, and which is regarded as the immediate cause of the symptoms. Vaughan (8) was apparently the first observer to postulate this formation of a non-specific poison as the first stage of the anaphylactic reaction. He regarded it as a product of

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proteolytic cleavage of the antigen by a specific ferment, appearing in the blood as the result of the preparatory injection. A later development of this view regarded the first stage of the reaction as the union of the antigen with a specific non-proteolytic antibody, this complex being then subjected to non-specific proteolysis by some ferment present in the blood plasma. This form of the theory originated with Friedemann (9), and has been vigorously defended in a long series of communications by Friedberger (10), who identifies the non-specific proteolytic ferment with the constituent of the serum known, in connection with phenomena which are not obviously proteolytic, as "complement." Another ingenious modification of this theory of an "anaphylatoxin" formed by proteolysis seems to have been reached independently by Jobling and Petersen (11), and by Bronfenbrenner (12). These observers picture the union of antigen and antibody in the blood as somehow removing from the sphere of action the natural antitryptic constituent of the blood plasma, so that the protease, which it normally holds in check, becomes effective, digesting the proteins of the plasma itself and liberating toxic products of cleavage. This conception of the anaphylactic shock as due to the liberation in the blood of a poisonous cleavage product arose from the close similarity between the symptoms of the shock, with their pronounced superficial differences in different species, and the symptoms produced in the same species by various products of proteolysis (Biedl and Kraus (13), Vaughan (8)). Later, a large volume of evidence was produced as to the methods by which normal guinea-pig's serum could be made to yield "anaphylatoxin," *i.e.*, could be endowed with such toxicity that a few cubic centimetres of it injected into a guinea-pig would produce death, with the supposedly characteristic symptoms. When this was effected by digesting the serum with protein substances, such as specific precipitates or bacteria, a proteolytic action was assumed, though there was little direct evidence for it. Later, when it was found that non-nitrogenous colloids, such as sols of agar, Bordet (14), starch or inulin, Nathan (15), were effective in the same way, some observers adopted the view that the toxicity was caused by a physical rather than a chemical change.

A large volume of literature and many ingenious experiments have been devoted to the controversy between the adherents of one or other of the minor variants of this anaphylatoxin theory, and there seems to have been a tendency to lose sight of the main issue, namely, of the question whether the anaphylatoxin phenomena have any direct bearing on the problem of anaphylaxis. In this paper we produce, in the first place, an additional item of evidence which seems to us decisive in favour of the view that anaphylaxis is due to location of the antibody, probably a precipitin, in the cells. In the second place, we describe a series of experiments made with the object of discovering what is the nature of the change taking place in fresh serum when it acquires the toxic properties attributed to the presence of anaphylatoxin, and what is the relation of the reaction it produces in the guinea-pig to the true anaphylactic shock in the same species.

I. Anaphylaxis and Immunity to a Foreign Protein Demonstrated *in vitro*.

Weil (16) rendered guinea-pigs sensitive to an antigen by a small injection, given a day or two previously, of precipitating serum from a rabbit immunised to the same antigen, and found that a further large dose of the precipitating serum, given intravenously, protected them from the effect of an otherwise fatal dose of the antigen injected immediately afterwards. On the other hand, experiments on isolated muscle made by Schultz (17) and by Dale (18) showed that the tissues from an actively immunised guinea-pig are as sensitive to the antigen as those of one which is anaphylactic. Manwaring and Kusana (19), again, in experiments on the reaction of the perfused lungs of the anaphylactic guinea-pig, found that addition of serum from an immunised animal to the perfusion fluid protected against the action of the antigen.

Such experiments appear to be clearly in favour of the theory which regards the antibody as sensitising when predominantly located in the cells, and as protective when it is in excess in the blood. The anaphylatoxin theory provides no explanation for such facts, but its persistence seems to show that they have not been accepted as decisive. It seemed desirable, therefore, to devise an experiment in which the conditions had a diagrammatic simplicity, so that the effects on sensitiveness of cellular and humoral antibody could be isolated from other possible factors.

The antigen chosen was pure, crystallised albumin from the hen's egg. The antibody was obtained by immunising rabbits with a series of injections. For this immunisation a filtered dilution of crude egg-white was used, as sufficient of the crystalline albumin was not available. The serum of the immune rabbits, however, exhibited a highly developed precipitating action with the pure albumin. When a sufficiently high titre had been attained, the rabbits were bled out under ether, and the serum was separated and centrifuged till perfectly clear. Since rabbit's serum, like other normal sera, has of itself a stimulating effect on guinea-pig's plain muscle isolated in Ringer's solution, and since this effect would be an undesirable complication in the experiment, the antibody was purified, and at the same time concentrated by the following procedure :—

30 c.c. of the serum were diluted to 150 c.c. with distilled water and 150 c.c. of neutral, saturated solution of ammonium sulphate were added. The resulting precipitate of globulins was separated by the centrifuge, and the supernatant fluid sucked off sharply and rejected. The precipitate was redissolved by adding distilled water, the solution being made up to 130 c.c., and an equal volume of saturated ammonium sulphate was again added. The precipitate was again separated, and was then washed into a collodion dialysing thimble with a small quantity of water. The thimble, which was attached air-tight to a rubber cork pierced by a glass tube, was lowered into a large vessel containing 0·9 per cent. salt solution, and the glass tube was attached to a rubber tube leading from a strong

glass vessel, in which pressure could be produced by means of a bicycle pump. The pressure in the vessel, and therefore in the dialysing sac, was slowly raised to 12 lbs. per square inch. At this pressure, dialysis and ultrafiltration proceeded together, so that when, after several changes of the external saline, the fluid in the sac ceased to give off ammonium sulphate its volume had been reduced to 11 c.c., containing, in 0·9 per cent. saline solution, all the globulins from the 30 c.c. of original serum. This solution, when diluted ten times, formed a definite precipitate with crystallised egg-albumin in dilutions up to 1 in 320,000, and showed distinct turbidity with even higher dilutions.

The globulins were separated from a normal rabbit's serum by an exactly similar procedure, and their solution reduced by dialysis under pressure to the same concentration, in relation to the volume of original serum. The solution, tested with crystallised egg-albumin, showed no trace of precipitin reaction. We had, therefore :—

1. A solution of globulins from precipitating anti-egg-albumin serum = P.
2. A solution of globulins from normal rabbit's serum = N.

Four guinea-pigs, weighing from 200 to 250 grm. received each an intraperitoneal injection of 0·2 c.c. of the concentrated precipitating globulin P, and two days later they were used for the following experiments.

Two of them were virgin females, and their uteri were used for the tests *in vitro*, the other two animals for tests *in vivo*.

1. Tests *in vivo*, injections being made into the jugular vein, exposed under local anæsthesia with cocaine.

Guinea-pig, 220 grm. 0·3 c.c. solution P injected intravenously. 3 minutes later 0·1 mgrm. egg-albumin intravenously. Animal shivers slightly, but shows no other symptoms of any kind.

Guinea-pig, 250 grm. 0·3 c.c. solution N intravenously. 3 minutes later 0·1 mgrm. egg-albumin intravenously. Typical anaphylactic symptoms, with death in 4 minutes from the time of the injection of egg-albumin.

This result is a confirmation of WEIL's earlier observation of the protective effect of excess of antibody injected into the vein of the sensitive animal immediately before an otherwise fatal dose of the antigen. We endeavoured to exclude possibilities of alternative interpretation by freeing the antibody as far as possible from non-specific constituents of the serum, and by using as a control the similarly prepared globulin from normal rabbit's serum. The result seems to be perfectly clear. A second injection of the sensitising antibody, given just before the antigen, protects, but a similar injection of normal serum globulin does not protect. The protective action of the second injection, therefore, can only be attributed to its antibody property, *i.e.*, to the property which enables the same preparation to produce sensitiveness, when allowed time to become fixed to the tissue cells.

2. Tests *in vitro*.—The contrast can be presented more graphically by taking unstriped muscle from the sensitive guinea-pigs and suspending it in Ringer's solution, to which is added, in the one case, the solution of precipitating globulin by which sensitiveness was conferred, in the other case normal serum globulin. The details of the method are in all points similar to those described by one of us (18) in earlier publications. The guinea-pigs were young virgin females, and the uteri, before excision, were perfused with warm Ringer's solution, till all visible traces of blood were removed. In each experiment one horn of the uterus was suspended in 100 c.c. of Ringer's solution, and solution P added in successive doses of 0·25 c.c. till six doses (1·5 c.c.) had been added. The gradual addition minimised any non-specific stimulant action on the washed plain muscle, which even the purified globulin exhibits in some degree if applied suddenly in large dose. The antigen was then added to the bath, in dose sufficient to produce a maximal contraction of the unprotected sensitive muscle. It was without effect. After this had been demonstrated the bath was emptied and refilled several times with fresh Ringer's solution. The plain muscle, now in pure Ringer's solution, was tested again with the same dose of antigen. It responded with the typical contraction. The presence of the excess of antibody in the bath, at the first application of antigen, had protected the plain muscle not only from stimulation by the antigen, but from even partial desensitisation. The other horn of the same uterus was then suspended in fresh Ringer's solution, and the procedure was exactly repeated, with the exception that solution N was substituted for solution P. The normal globulin had no trace of protective action; the uterus in its presence responded typically to the first dose of antigen, and was therewith desensitised, so that, when subsequently tested in pure Ringer's solution, it gave no further response to the antigen.

Figures 1 and 2 reproduce the graphic records of one of these experiments.

The meaning again seems to be perfectly clear. A precipitating antibody, when so firmly attached to the plain muscle that it cannot be removed by prolonged perfusion, renders the muscle specifically sensitive to the antigen; the same antibody present in excess in the fluid bathing the sensitive muscle protects it, while the corresponding normal globulin does not protect. These results are exactly analogous to those observed in the whole animal. Neither set of observations can be reconciled with the theory attributing the anaphylactic reaction to the formation of "anaphylatoxin" by union of antigen and antibody in the blood; both, on the other hand, are completely in accordance with the theory which attributes the anaphylactic condition to cellular location of the antibody, and postulates a protective action for antibody circulating in the body fluids.

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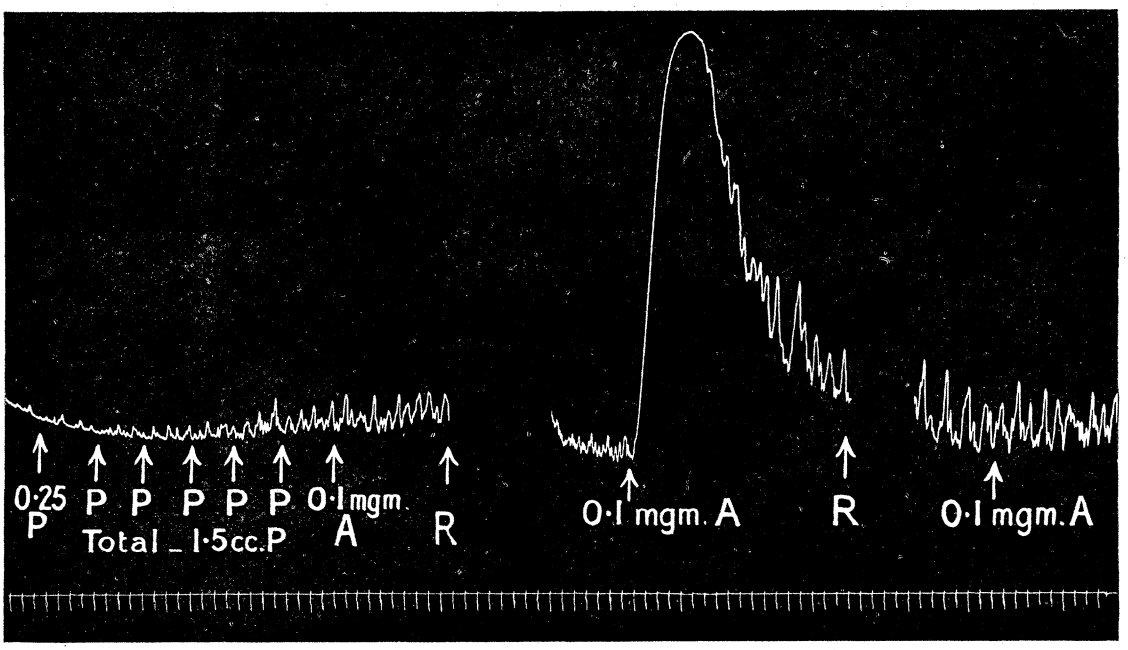

FIG. 1.—Horn of the uterus of guinea-pig, previously sensitised to egg-albumin by 0·2 c.c. of concentrated rabbit precipitin for egg-albumin (= P). Perfused and suspended in 100 c.c. Locke-Ringer solution; 6 doses, each of 0·25 c.c., added to the bath. Then 0·1 mgrm. cryst. egg-albumin (A). R = change of Locke-Ringer.

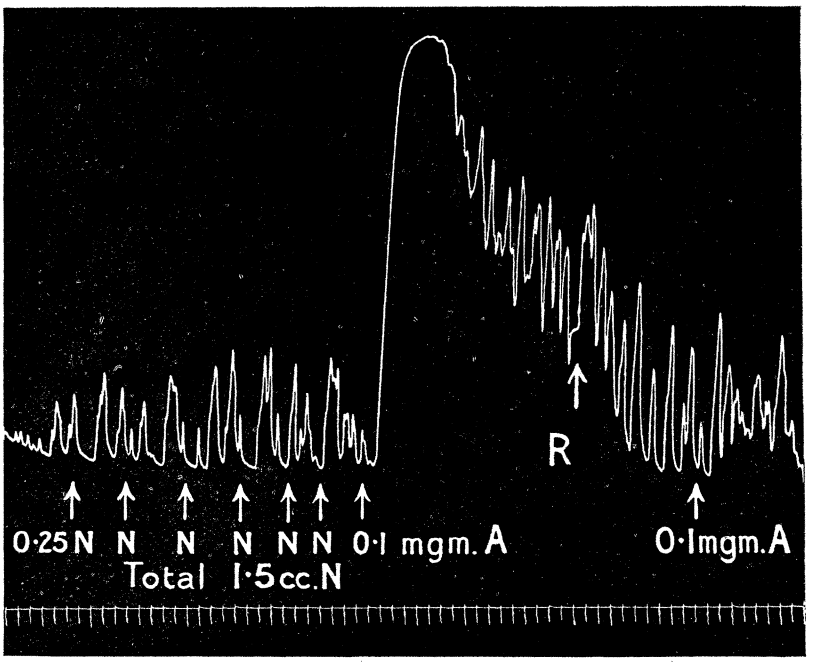

FIG. 2. Other horn from same uterus as fig. 1. Concentrated *normal* rabbit serum-globulin (N) added to bath instead of P.

II. Experiments with "Anaphylatoxin."

The Preparations used.

We have made experiments with guinea-pig's serum rendered toxic by four of the methods which different authors have described—by incubation with starch paste (Nathan (15)), with a weak agar gel (Bordet (14)), with bacteria (Friedberger (20)) and after emulsification with chloroform (Jobling (11)).

The details of preparation were uniform in each case, except when the rate of appearance of toxicity was under investigation, and may here be given once for all.

Starch Anaphylatoxin.—A freshly prepared 10 per cent. sol of "soluble starch" was added to fresh guinea-pig serum in the proportion of 1 c.c. to 5 c.c. and thoroughly mixed therewith. The mixture was incubated at 37° C. for 3 hours. The flocculent precipitate which had formed was removed by centrifugation for about 20 minutes at 4,000 r.p.m. and the clear fluid carefully pipetted off.

Agar Anaphylatoxin.—Agar was dissolved in boiling water in the proportion of 0·25 grm. per 100 c.c., and the sol was filtered hot, distributed in test-tubes, and sterilised in the autoclave. For each preparation of the anaphylatoxin a tube was opened, and the weak gel was added to fresh guinea-pig serum in the proportion of 1 c.c. to 5 c.c. The mixture was incubated for 2 hours, centrifugated and pipetted off as for the starch preparation.

Bacterial Anaphylatoxin.—Two 24-hour slope cultures on nutrient agar of *Bacillus prodigiosus* were taken and the bacilli carefully removed and emulsified in 3 c.c. of saline solution. The emulsion was added to fresh guinea-pig serum in the proportion of 1 c.c. to 10 c.c., the mixture incubated at 37° C. for $1\frac{1}{2}$ hours, allowed to stand for 18 hours at room temperature, centrifugated for 20 minutes at 4,000 r.p.m. and pipetted off.

Chloroform Anaphylatoxin.—Fresh guinea-pig serum was thoroughly emulsified by shaking with twice its volume of chloroform, and the emulsion incubated for 24 hours at 37° C. It was then centrifugated till separation was as complete as possible. The clear upper layer of serum was then removed and subjected to a current of air till it retained no trace of the odour of chloroform.

By each of these methods we succeeded without difficulty in imparting to guinea-pig's serum a toxicity of the order which the original describer of the method had indicated. Our experiments have dealt with (1) the nature of the change accompanying the appearance of toxicity, (2) the nature of the symptoms produced by the different kinds of anaphylatoxin, (3) the production of tolerance for anaphylatoxin, and its relation to "antianaphylaxis."

The Nature of the Change Accompanying the Appearance of Toxicity.

We cannot attempt here a review of the enormous mass of largely controversial literature on the meaning of the appearance of toxicity in serum treated according to the different methods which yield an "anaphylatoxin." Admirable critical summaries

are available, and reference may be made to the very full and recent review by DOERR (21). There are two main conceptions to be considered. (1) The chemical, which regards the toxicity as due to the appearance of cleavage products of proteins. In the original conception of FRIEDBERGER (22) these were regarded as produced by the digestion of the antigen or protein in the foreign substance added. This conception was obviously inapplicable to the cases where the serum was rendered toxic by incubation with protein-free colloids; but the possibility remained of a self-digestion of the serum, initiated by a removal or inactivation of the normal anti-tryptic substance, on the lines indicated by JOBLING (11). BORDET and ZUNZ (23), indeed, described experiments showing an increase of coagulable nitrogen in serum digested with agar, though DE KRUIF and GERMAN (24) and TEALE and BACH (37) failed to confirm this observation. (2) The physical conception which attributes the toxicity to a physical change in the serum. This view takes various forms. FRIEDBERGER (25) dismisses the preparations made with starch, etc., claiming that their action is simply due to imperfect removal of the particles of the added matter by centrifugation; according to him adequate centrifugation, or simple filtration through paper, removes the toxicity. KOPACZEWSKI (26) points out that the substances imparting the toxicity to serum are all electronegative colloids, and attributes their action to a raising of the surface tension and alteration of the electric charge of the globulin particles of the serum. He finds that previous addition to the serum of substances reducing the surface tension prevents the appearance of toxicity on incubation with colloids which otherwise produce it. Prominent among the writers who have attributed the toxicity to a physical change of some kind in the serum is H. SACHS (27).

As a preliminary to an examination of the mode of action of the anaphylatoxins, and a comparison of their effects with those constituting the true anaphylactic reaction, we made experiments on a few points, which seemed still to be in doubt, as to the nature of the changes accompanying the appearance of toxicity.

When serum is mixed and incubated with starch or agar there is early formation of a fine, flocculent precipitate. The total amount is small, and it is almost entirely formed in about half-an-hour, long before the full development of the toxicity. The nature of this precipitate has been left uncertain. Many writers seem to take it for granted that it represents a flocculation of the added colloid, FRIEDBERGER (25) even attributing the toxicity to its incomplete removal. The questions which we put to the test of experiment were the following:—

1. Is the appearance of toxicity accompanied by any protein cleavage in the serum?

2. Does the complete removal of suspended particles by centrifugation impair the toxicity?

3. Is the added non-protein colloid removed by the precipitate which forms, or does it remain in the clear centrifugated serum?

4. Can any obvious physical change be detected in the mixture corresponding to the appearance of toxicity?

Does Protein Cleavage Accompany the Appearance of Toxicity?

Method.—Bordet and Zunz (23) precipitated with alcohol fresh, untreated serum, and the same serum after treatment with agar, and estimated the amino-nitrogen in the filtrates. The filtrate from the agar-serum showed a small excess over that from the normal serum. The method is not altogether above criticism. Any slight modification of the conditions of such an alcoholic precipitation is liable to affect the small content of nitrogen in the filtrate, and a very small difference looks large in relation to the small total. It cannot be assumed that admixture and incubation with agar will not affect the precipitation; indeed, it is highly probable that it would. An increase of nitrogen in the filtrate, therefore, cannot safely be attributed to proteolytic change. For these reasons we considered it better to estimate the free amino-groups in the whole serum, before and after development of the toxicity, by Van Slyke's method, avoiding all procedures designed to separate coagulable from non-coagulable protein. If the toxicity were really due to proteolysis, it might well be due, in large measure, to early products of cleavage, still coagulable by alcohol, and the appearance of these could only be detected if alcohol precipitation were avoided.

i. *Serum rendered Toxic by Incubation with Chloroform* (Jobling).

There is no doubt as to the occurrence of proteolysis under these conditions, and the experiment was made as a control of the method of detecting such cleavage.

Fresh guinea-pig's serum, the pooled yield from a number of guinea-pigs, was taken and divided into three portions. One (I) was placed in the refrigerator as a control, and to each of the others was added twice its volume of chloroform, with which thorough emulsification was secured by shaking. One of these mixtures was then immediately placed in the centrifuge and spun until the maximum separation was effected. The supernatant serum and residual layer of emulsion and precipitate were pipetted off, only the perfectly clear layer of chloroform being rejected. The chloroform was removed from the serum by blowing air over it until no more odour was perceptible, and the partially precipitated material was all in clear solution. This serum formed sample II, being serum which had been emulsified with chloroform, but separated again before incubation. The third sample, after emulsification with chloroform, was incubated in a sealed vessel at 37° C. for twenty-four hours, and the separation was then effected precisely as for II, the time needed for removing the chloroform being practically identical in the two cases. The serum separated after incubation with chloroform formed sample III.

In carrying out the estimates of free amino-groups with Van Slyke's apparatus, the sample of serum was mixed with the nitrous acid and caprylic alcohol by a

preliminary shaking, the mixture was allowed to stand for thirty minutes, and the desaminating bulb was then again shaken for exactly five minutes in each determination. A blank determination was made with the reagents only, under identical conditions. The following results were obtained:—

Material determined.	No. of c.c. of nitrogen.	Temperature.
		°
Blank (reagents)	0·7	17
3 c.c. I	4·7	17
3 c.c. II	5·1	18
3 c.c. III	5·8	19

Correcting for the blank determination and for temperature and pressure, the results give the following yields of amino-nitrogen:—

3 c.c. I . . . . . = 2·24 mgrm.
3 c.c. II . . . . . = 2·45 mgrm.
3 c.c. III . . . . . = 2·83 mgrm.

No great significance can be attributed to the difference between I and II, since II had been subjected to the process of blowing off the chloroform. Even if this procedure were held responsible for the whole difference between these two samples, it must play a much smaller part in the difference between II and III. which were both subjected to it for approximately equal times. Ideally, the total nitrogen should have been determined in each case and the results expressed as a ratio of free amino-nitrogen to total nitrogen. The fact, however, that III showed an excess over II, nearly twice as great as that of II over I, was sufficient indication that the method as used by us would detect proteolysis if it occurred. Probably the difference between II and I is mainly due to commencing cleavage.

ii. *Serum rendered Toxic by Incubation with Agar* (Bordet).

30 c.c. of pooled fresh serum from normal guinea-pigs were thoroughly mixed with 6 c.c. of 0·25 per cent. agar gel.

12 c.c. of the mixture were taken immediately, and of this 5 c.c. were run into the Van Slyke apparatus, and the remainder was used for testing toxicity.

The remaining 18 c.c. were transferred to the incubator and kept at 37° for two hours. The mixture was then cooled to room temperature, 5 c.c. run into the Van Slyke apparatus, and the remainder rapidly centrifugated for the toxicity test. Injections were made, as usual intravenously.

Toxicity Tests.

Weight of guinea-pig in grm.	Dose.	Result.
1. 220	5 c.c. unincubated mixture . . .	Slight symptoms. Recovery.
2. 200	3 c.c. incubated ,, . . .	† $3\frac{1}{2}$ minutes.
3. 210	2·5 c.c. ,, ,, . . .	† $2\frac{1}{2}$ minutes.
4. 200	2·0 c.c. ,, ,, . . .	Very severe symptoms. Just recovered.

Incubation, therefore, had produced the characteristic toxicity, with normal intensity.

Determination of Free Amino-Groups.

Material for determination.	Nitrogen in c.c.
5 c.c. of serum-agar mixture, unincubated . . .	5·6
5 c.c. ,, ,, incubated	5·6
Blank (reagents)	0·7

The temperature throughout the determinations was constant at 22° C. and the barometer at 735 mm. The yield in milligrammes of nitrogen, after correction, is 2·67 mgrm. from 5 c.c. in both cases. There is no trace of proteolysis to be detected.

iii. *Serum rendered Toxic by Incubation with Starch* (NATHAN).

45 c.c. of pooled fresh guinea-pig's serum was thoroughly mixed with 9 c.c. of a freshly prepared 10 per cent. sol of soluble starch. 15 c.c. were taken immediately for toxicity test and determination of amino-groups. The mixture for toxicity test was thoroughly centrifugated, and the sample for analysis was drawn from the mixture without allowing any sedimentation. The remaining 30 c.c. were incubated in a stoppered vessel for three hours at 37° C., and then, after cooling to room temperature, 10 c.c. of the thoroughly mixed suspension were used for two analyses and the remainder centrifugated for toxicity tests.

Toxicity Tests.

Weight of guinea-pig in grm.	Dose.	Result.
1. 200	5 c.c. unincubated	No symptoms.
2. 210	4·5 c.c. incubated	† 4 minutes.
3. 190	3·5 c.c. ,, ...	Extremely severe symptoms, with convulsions, collapse, and respiratory obstruction. Just recovered.
4. 220	3·0 c.c. ,, ...	Severe symptoms. No convulsions. Recovered.

Again, there is a normal development of the characteristic toxicity.

Determination of Free Amino-Groups.

Material determined.	Nitrogen in c.c.	Temperature.	Barometer.	Corrected determination in mgrm. of nitrogen per 5 c.c.
		°	mm.	
5·2 c.c. unincubated mixture .	6·25	21	756·2	3·06
5·1 c.c. incubated mixture . .	5·95	22	756·2	2·94
5·0 c.c. incubated mixture . .	5·85	19	751·0	2·97

There is no evidence of even a trace of proteolysis.

Refractive Index.

A change of refractive index should afford a delicate test for the occurrence of protein cleavage. The experiments were made only on the production of anaphylatoxin by starch. In an initial experiment it was thought that a slight increase of refractive index of the mixture accompanied the appearance of toxicity.

Repetition, however, with adequate precaution against loss of water during incubation and centrifugation, the latter being necessary to obtain a clear solution for refractometry, failed to show even the slightest change by this very delicate method of observation. Fresh guinea-pig serum was taken, thoroughly centrifugated, a sample reserved, and the main bulk mixed with one-fifth of its volume of a 10 per cent. sol of soluble starch. The mixture was distributed into stoppered tubes, which were placed in the incubator, one being removed and the contents centrifugated at hourly intervals up to three hours. It was found necessary to cover the centrifuge tube with a paper disc during this procedure, so as to avoid confusion of the results by evaporation.

Control experiments had shown a steady increase of toxicity during this period of incubation, and in this case only the activity of the three-hour sample was determined, as a guarantee that the toxicity had developed normally.

Weight of guinea-pig in grm.	Dose of 3-hour starch-serum.	Result.
220	4 c.c.	† in 2½ minutes.
220	3 c.c.	Severe symptoms. Dyspnœa. Collapse. Recovery after 5 minutes.

Refractometer Readings.—Zeiss's dipping refractometer, with the accessory prism, was used, with immersion in a bath kept constantly at 17·5°, ten minutes being allowed for attainment of constant temperature before the reading was taken in each case.

Material.	Reading.	N_D.
1. Pure serum	46 3	1 34511
2. Serum + starch, unincubated	47·3	1·34548
3. Serum + starch, incubated 1 hour	47·3	1·34548
4. Serum + starch, incubated 2 hours	47·3	1·34548
5. Serum + starch, incubated 3 hours	47·3	1·34548

It will be noted that, not only is there no evidence of any proteolytic change sufficient to disturb the reading, but the amount of starch or protein removed by centrifugation shows no sufficient increase with incubation to affect the refractive index.

We may conclude, then, that the appearance of toxicity in the mixtures of serum with star or agar is not accompanied by cleavage of the serum proteins, early or late.

Is the Toxicity due to Suspended Particles of Starch, etc.?

In recent papers, FRIEDBERGER (28) has urged that the toxic serum produced by incubation with starch is not a true anaphylatoxin, but owes its action to the presence of small particles of starch gel agglomerated with protein and removable by more thorough centrifugation. He has further stated that injection of the starch sol, unmixed with serum, will produce symptoms similar to those produced by the starch-anaphylatoxin. These statements we have entirely failed to confirm.

1 c.c. of the 10 per cent. starch sol was diluted with 5 c.c. of 0·9 per cent. tap-water saline, to produce a dilution of starch corresponding to that in the serum mixture. Of this dilution 5 c.c., injected into the vein of a guinea-pig of 240 grms. weight, produced no trace of symptoms of any kind.

A 1 : 5 starch serum mixture was made in the ordinary way and incubated for $2\frac{3}{4}$ hours. It was then centrifugated for 6 minutes at 4,000 r.p.m. 7·5 c.c. of the supernatant fluid was drawn off and tested as follows :—

Weight of guinea-pig in grm.	Dose in c.c.	Result.
220	4·5	† in 3 minutes.
250	2·25	Moderate symptoms. Recovers after 4 minutes.

The remainder was then again centrifugated for an hour at 4,000 revolutions. No significant amount of further deposit could be detected, but the clear fluid was carefully drawn off, without agitation of the deepest layer, and again tested.

Weight of guinea-pig in grm.	Dose in c.c.	Result.
200	4·5	† $3\frac{1}{4}$ minutes.
230	3·5	† $2\frac{3}{4}$ „
250	2·25	† $2\frac{3}{4}$ „

The toxicity has apparently undergone a further development during the prolonged centrifugation; certainly it showed no diminution.

As a complement to this observation we collected, in another experiment, the material thrown down by the centrifuge from a starch-serum mixture. The clear serum showed a normal toxicity, killing small guinea-pigs in doses of 4–5 c.c. The deposit corresponding to 20 c.c. was shaken up into a uniform suspension in 2 c.c. of saline and injected intravenously into another guinea-pig. It produced no symptoms of any kind.

It has been stated, again, that the starch-serum preparation loses its toxicity if filtered through paper. In the one experiment of this kind which we made, the material available did not permit an exact evaluation of the toxicity, before and after filtration, but the result was sufficient to prove that no serious impairment was produced, and was quite compatible with none. The filter paper used was of an ordinary grade known as "Whatman No. 1."

Filtration of Starch-Anaphylatoxin through Paper.

Weight of guinea-pig in grm.	Material.	Dose in c.c.	Result.
225	Unfiltered	4·5	† $3\frac{1}{2}$ minutes.
220	Unfiltered	3·0	† 4 „
220	Filtered (paper) . .	4·0	† $3\frac{3}{4}$ „

On the other hand, experience confirms the observation that the toxicity is stopped by a Kieselguhr candle (Berkfeld), as the following two records show.

Filtration of Starch-Anaphylatoxin through a Berkfeld Candle.

Weight of guinea-pig in grm.	Material.	Dose in c.c.	Result.
I. 240	Unfiltered . . .	4·5	† in $4\frac{1}{2}$ minutes.
260	Unfiltered . . .	3·5	Very severe symptoms. Recovery.
270	Filtered . . .	5·0	No symptoms.
II. 240	Unfiltered . . .	5·0	† in 5 minutes.
250	Unfiltered . . .	4·5	Severe symptoms. Recovery.
250	Filtered . . .	5·0	No symptoms.

Through the kindness of our colleague, Mr. J. E. BARNARD, we had the opportunity of observing that this reduction of toxicity with filtration is accompanied by a great reduction in the number of the larger aggregates visible in the clear toxic serum with the ultramicroscope.

In the case of an agar-anaphylatoxin the toxicity was less easily affected by filtration, even through a Kieselguhr candle. Two experiments were made, with filters of different grades, the second one used being of great fineness, and showing signs of increasing obstruction during the filtration. The filter used in the first experiment corresponded to that used in the experiments on starch anaphylatoxin, and it will be seen that it allowed the toxicity of the agar-preparation to pass with but little loss.

Effect of Filtration through Kieselguhr Candles on Agar-Anaphylatoxin.

Weight of guinea-pig in grm.		Dose.	Result.
I.	220	2·5 c.c., unfiltered . . .	† in 3 minutes.
	210	2·0 c.c., unfiltered . . .	Severe symptoms. Recovery.
	220	2·5 c.c., filtered . . .	No symptoms.
	210	3·0 c.c., filtered . . .	† in 3 minutes.
	200	3·5 c.c., filtered . . .	† in 2 minutes.
II.	230	2·5 c.c., unfiltered . . .	† in $4\frac{1}{4}$ minutes.
(finer filter)	230	1·5 c.c., unfiltered . . .	† in $4\frac{1}{4}$ minutes.
	230	2 c.c., filtered	Slight symptoms. Recovery.
	230	3 c.c., filtered	Slight symptoms. Recovery.

It will be seen that, in the first experiment, the lethal dose is raised only from 2·5 c.c. to 3 c.c.—a difference of no great moment. In the second, with a finer filter, an unusually toxic preparation, with a lethal dose of 1·5 c.c. or less, fails after filtration to produce more than slight symptoms in a dose of 3 c.c.; the toxicity has largely disappeared. The reaction of isolated plain muscle to these preparations will be described in a later section.

These results with centrifugation and filtration give a definite negative to FRIEDBERGER'S suggestion, that the toxicity is due to the presence of such gross particles as can be removed by the centrifuge or filter-paper. On the other hand, they show that the toxicity is not due to the constituents of the clear mixture which easily pass a Kieselguhr filter, as would be expected of protein cleavage products; it is rather associated with the colloidal aggregates larger than those occurring in the normal serum, readily visible with the ultramicroscope, and stopped to a large extent by the finer grades of filter-candle.

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Does the added, Non-protein Colloid fall out in the Precipitate, or remain dispersed in the Toxin Mixture?

We were only able to examine this point in the case of starch, which could be estimated after conversion into reducing sugar, no similarly suitable method for measuring small quantities of agar being available. An attempt to estimate agar as furfurol was not successful.

To 50 c.c. of fresh guinea-pig serum was added 10 c.c. of a freshly prepared sol of soluble starch (approximately 5 per cent.), and the mixture was incubated for 3 hours at 37°. At the end of this period the mixture was thoroughly centrifugated.

The clear fluid was fatal to a guinea-pig weighing 240 grm., when injected intravenously in a dose of 5 c.c.; 4·5 c.c. produced very severe but not fatal symptoms in a guinea-pig of 250 grm.

The deposit was thoroughly washed with water, then hydrolysed by boiling with N/20 HCl for an hour. The resulting fluid was neutralised, and the reducing sugar estimated by BERTRAND'S method. The total yield was 20·8 mgrm. of copper as cuprous oxide, corresponding to 10·2 mgrm. of dextrose.

A determination was next made of the amount of dextrose produced by hydrolysis of a volume of the starch sol equal to that used in the mixture. A sample was diluted with water to five times its volume, and 10 c.c. of the dilution made up to N/20 normal with HCl. The mixture was boiled under a reflux condenser for an hour. It was then cooled, neutralised, made up accurately to 20 c.c., and 2 c.c. of this taken for estimation by BERTRAND'S method. The yield was 21·8 mgrm. of copper as cuprous oxide, corresponding to $21{\cdot}8 \times 50 = 1{\cdot}09$ grm. of copper, or 0·525 grm. of dextrose from the 10 c.c. of starch sol.

For determining the starch content of the serum-starch mixture it was not practicable to use hydrolysis to dextrose with acid. We employed instead a diastatic conversion to maltose by use of Taka-diastase. This preparation itself contained maltose, which had to be allowed for, and when it was digested with normal serum a small additional quantity of maltose was obtained. To 5 c.c. of the normal serum was added 4 c.c. of a 1 per cent. solution of Taka-diastase. After 4 hours' incubation at 37° and 1 hour at 50° C. the solution was precipitated with metaphosphoric acid, made up to exact volume, and the reducing sugar determined in aliquot portions of the filtrate (GYE'S* modification of BERTRAND'S method).

Normal Serum.

5 c.c. normal serum + 4 c.c. 1 per cent. diastase, yielded	27·9 mgrm. copper.
4 c.c. 1 per cent. diastase, yielded	23·8 mgrm. copper.
Difference	4·1 mgrm.

* Personal communication.

5 c.c. samples of the starch-serum anaphylatoxin, after thorough centrifugation, were then digested in exactly the same way, with the same amount of diastase.

5 c.c. starch-anaphylatoxin + 4 c.c. 1 per cent. diastase, yielded . .	83·2 mgrm. copper.
4 c.c. 1 per cent. diastase, yielded . .	23·8 mgrm. copper.
Difference	59·4 mgrm.

The whole quantity of material taken consisted of 50 c.c. serum + 10 c.c. of the starch sol.

Reduction by the whole 60 c.c.	= 59·4 × 12 = 713 mgrm. copper.
Of this, 50 c.c. of serum alone would account for . .	4 1 × 10 = 41 mgrm. copper.
Difference	672 mgrm.

This difference represents the reduction due to maltose produced from the 10 c.c. of added starch sol which has remained in the 60 c.c. of finished anaphylatoxin. For comparison, we have the reduction produced by the dextrose from 10 c.c. of the same starch sol, obtained by hydrolysis with HCl. We must, therefore, convert the reduction by maltose into that which would be produced by the equivalent of dextrose. This gives us $0{\cdot}672 \times 100/62 = 1{\cdot}084$ grm. So that we have :—

Dextrose from hydrolysis of 10 c.c. of starch sol reduces	1·09 grm. copper.
Dextrose equivalent to the starch remaining in 60 c.c. of anaphylatoxin, in production of which 10 c.c. of starch sol were used, reduces . . .	1·084 grm. copper.
Dextrose from precipitate, formed in production of 60 c.c. of anaphylatoxin, reduced	0·021 grm. copper.

For practical purposes, it can be said that the whole of the starch remains dispersed in the finished anaphylatoxin, and that the quantity removed by centrifugation is negligible. By analogy, it may be regarded as practically certain that the same is true of agar.

Other Physical Properties.

In addition to the refractometer readings already mentioned, we made determinations of the viscosity and the tension at an air-surface of the serum-starch mixture immediately after preparation, at an early stage of incubation, and when toxicity was fully developed. No significant change in either could be detected.

1. Of a starch mixture after 1 hour's incubation 4·5 c.c. caused only slight dyspnœa when injected intravenously into a 200 grm. guinea-pig. After 3 hours' incubation 2·25 c.c. of the same mixture killed a similar guinea-pig in $3\frac{1}{2}$ minutes with the usual symptoms. The corresponding viscosimeter readings at 17·3° C. were :

45 minutes' incubation	90·8 seconds.
3 hours' incubation	91·1 seconds.

Of a similar mixture 5 c.c. before incubation produced no symptoms; after 3 hours' incubation 3·5 c.c. killed in 5 minutes. The viscosimeter readings at 20° C. were:

Before incubation	84·6 seconds.
3 hours' incubation	84·4 seconds.

2. The same mixture as that used in the second experiment on viscosity described above was used for determining the surface-tension at the serum-air surface by means of a stalagnometer. The readings were:

Before incubation	200 drops in 12 c.c.
3 hours' incubation	200 drops in 12 c.c.

Conclusions.

The toxicity acquired by starch-serum and agar-serum mixtures during incubation is not due to proteolysis, for the occurrence of which no evidence was found. It is not due to the imperfect removal of suspended particles by the centrifuge, nor is its appearance accompanied by any detectable changes in the general physical properties of the mixture. The added non-protein colloid is not carried down by the precipitate which forms, but remains dispersed in some way in the clear serum. The toxicity appears to be associated with the presence of larger aggregates, visible with the ultramicroscope, than those seen in normal serum; it can be greatly reduced by passage through a sufficiently fine filter.

The Symptoms Produced by the Different Anaphylatoxins.

Our object was to compare, in detail, the symptoms produced by the various anaphylatoxins with those constituting the true, acute anaphylactic shock, as produced by the intravenous injection of a soluble antigen. It is obviously with this type of shock that comparison should be made; if its appearance is due to the formation of anaphylatoxin, then the ready-formed anaphylatoxin, injected directly into a vein, should produce the same symptoms, with even greater rapidity, owing to the elimination of the time needed for its formation. We may compare, in the first instance, the effects detected by simple, naked-eye observation in the different cases.

General Symptoms.

When a guinea-pig, actively or passively sensitised to a certain protein, receives an intravenous injection of an effective dose of that protein, symptoms appear with a variable delay. If the dose is large, signs of respiratory obstruction are frequently visible before the injection has been finished. With a dose nearer the effective minimum, the animal usually exhibits practically no symptoms for 20–30 seconds after the injection, so that it appears normal for a brief period after release. The onset of symptoms is then clearly seen. The animal gives a slight chattering cough,

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the hair on the head and back is erected, and the fore paws make cleaning movements over the nostrils, as if to brush away an obstruction. The chest is then raised from the table by extension of the fore limbs, and the hind limbs are frequently extended at knee and ankle, and the back arched upwards, so that chest and belly are lifted. Meanwhile, inspiration has rapidly become obstructed, and the familiar sequence of convulsive, ineffective inspiratory efforts, continued for some time after the animal has fallen unconscious, passage of urine and fæces, and the terminal twitching of the accessory muscles of respiration, lead to death within 5 minutes. The *post-mortem* appearance is well known.

Turning to the anaphylatoxins, we may dismiss at once from the comparison the preparation produced by autolysis of the serum after shaking with chloroform (JOBLING). This preparation is often highly toxic, but the symptoms have hardly any point of similarity with those seen in the anaphylactic shock. The animal becomes profoundly collapsed, stops breathing altogether for 10–20 seconds, but later shows resumption of weak breathing, with some signs of obstruction. The time to death is very variable. If death occurs rapidly, the right heart and large veins are found full of clot; if death is delayed, the blood is generally incoagulable *post-mortem*. The lungs show hæmorrhage, but are little, if at all, distended. The picture is that of poisoning by a thrombokinase, and suggests that the primary incidence of the effect is on the circulation; there is nothing resembling the true, obstructive asphyxia seen in the anaphylactic shock.

The action of the anaphylatoxins made with starch, agar, or bacterial suspensions has many more points of similarity with the true anaphylactic shock, though it is usually not difficult for an observer who is familiar with both types of reaction to distinguish the one from the other. In the symptoms produced by any of the anaphylatoxins, symptoms of collapse play a much more prominent part than in the true anaphylactic shock. The respiratory obstruction seen in the anaphylactic reaction also appears in the action of the anaphylatoxins, but it is usually by no means so predominant in the picture.

Starch-anaphylatoxin.—When a lethal dose of starch-anaphylatoxin has been injected into a vein, the animal, on release, usually sprawls helplessly, or falls on its side. Symptoms of respiratory obstruction soon appear, and therewith convulsions, but the latter are less clearly the result of the asphyxiating obstruction. The terminal stages of the poisoning are very similar to those of the anaphylactic shock, in which the animal lies flaccid and unconscious, and a succession of increasingly feeble respiratory efforts end in failure of the respiration, with the heart still beating. The effects of different batches of the starch-anaphylatoxin, which we prepared, were not uniform. In the action of a few collapse was so predominant that the guinea-pig died without convulsions, or even vigorous respiratory efforts; with others the respiratory obstruction was much more predominant, and its onset could be detected before the injection was completed.

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Agar-anaphylatoxin.—The action of our preparations of agar-anaphylatoxin was more uniform, and conformed, on the whole, the most closely of any to the anaphylactic reaction. Respiratory obstruction was always present, but was associated with much more primary collapse and weakness than is seen in the true anaphylactic shock of the guinea-pig.

Bacterial Anaphylatoxin.—We made fewer experiments with this preparation, but our observation of its effects leads us to regard it as intermediate in its type of action between the agar and starch preparations. Collapse was less pronounced from the outset of its action than in the case of the starch serum, more so than in that of the agar serum.

Action of the Anaphylatoxins on Anaphylactic Animals.

We have dealt hitherto with the effects of these preparations on normal guinea-pigs, in which they are supposed to produce symptoms identical with those of the anaphylactic shock, though our observation leads us to regard the similarity as being very far short of identity. There is an observation, however, which we made incidentally, in the course of experiments to be dealt with in a later section, which should be mentioned here.

We have observed it most clearly in the case of the starch anaphylatoxin, and not with all batches of this. Certain batches of the starch anaphylatoxin, which, for another purpose, were injected in graded doses not only into normal guinea-pigs, but also into others which had been made anaphylactic to egg-albumin, exhibited a quite definitely greater toxicity for the anaphylactic than for normal guinea-pigs. Not only was a smaller dose lethal, but its action on the anaphylactic animals showed a very much closer symptomatic correspondence to the true anaphylactic shock than did the effect on the normal animals. We have seen that the effect of the starch anaphylatoxin, in particular, on normal animals, has many points of difference from the true anaphylactic shock ; it would, on the contrary, be impossible, from inspection of the symptoms produced by some of the same preparations on anaphylactic animals, to discover that the animal was not dying of the true anaphylactic shock, produced by injecting the specific antigen.

The interpretation of the phenomenon is difficult, and is complicated by its appearance only with certain batches of the anaphylatoxin. Other batches exhibit a toxicity which is somewhat greater for normal than for anaphylactic animals, if it differs at all. In the case of the one batch of bacterial anaphylatoxin with which the comparison was made, this appeared to be the case. We have no record of complete comparisons with the agar serum, but the behaviour of anaphylactic guinea-pigs to intravenous injections of agar sol makes it seem probable that they would usually prove more sensitive than normal guinea-pigs to the ready-formed agar-anaphylatoxin.

Examples of both kinds of difference are shown in the following records :—

Starch Anaphylatoxin.

First Preparation.

Normal Guinea-Pigs.

Weight of guinea-pig.	Dose.	Result.
250	4 c.c.	Severe dyspnœa. Recovery.
255	4·5 c.c.	Very severe collapse, respiratory obstruction, convulsions. Recovery.
240	5 c.c.	† 5 minutes. M.L.D = 5 c.c.

Guinea-Pigs made Anaphylactic by 1 mgrm. of Egg-albumin, injected 34 days previously.

Weight of guinea-pig.	Dose.	Result.
270	2·5 c.c.	Obstructive dyspnœa. Recovered.
300	3 c.c.	Very severe symptoms. Just recovered after 5 minutes.
260	3·5 c.c.	† 4 minutes. Symptoms very like anaphylactic shock.
240	4·5 c.c.	† 3½ minutes. Symptoms very like anaphylactic shock. M.L.D. = 3·5 c.c.

Second Preparation.

Normal Guinea-Pigs.

Weight of guinea-pig.	Dose.	Result.
240	4·5 c.c.	† 4½ minutes.
260	3·5 c.c.	Very severe symptoms. Recovery.

Guinea-Pigs Anaphylactic to Egg-albumin (1 mgrm. 39 days previously

Weight of guinea-pig.	Dose.	Result.
250	3 c.c.	Mild symptoms. Recovery.
280	4·5 c.c.	Severe symptoms. Recovery.

There is no enhancement of sensitiveness by anaphylaxis in this case. So far as the evidence goes it points to a smaller sensitiveness of the anaphylactic animals.

BACTERIAL ANAPHYLATOXIN.

Normal Guinea-Pigs.

Weight of guinea-pig.	Dose.	Result.
220	0·5 c.c.	Moderate symptoms. Recovers.
220	0·5 c.c.	† 4 minutes.
220	0·5 c.c.	Moderate symptoms. Recovers.
230	1·0 c.c.	† $3\frac{3}{4}$ minutes.
220	1·5 c.c.	† $3\frac{1}{2}$ minutes.

The preparation apparently kills in doses down to 1 c.c., and occasionally in doses of 0·5 c.c.

Anaphylactic Guinea-Pigs. (Sensitised to egg-albumin by injection of rabbit precipitin for that protein.)

Weight of guinea-pig.	Dose.	Result.
210	1 c.c.	Moderate symptoms. Recovered.
230	1 c.c.	Moderate symptoms. Recovered.
210	1·5 c.c.	† $3\frac{1}{2}$ minutes.

The lethal dose for these is more than 1 c.c. The anaphylactic animals, if they differ at all, are less sensitive than the normal to this preparation.

Injection of Agar-Sol intravenously.

BORDET (14), in his earlier observations, found that the agar-sol with which he rendered guinea-pig serum toxic *in vitro* was harmless when it was itself injected intravenously. NOVY and DE KRUIF (29) recorded certain experiments in which the injection of a small dose of agar-sol into the vein of a guinea-pig produced fatal symptoms, resembling those produced by the preformed anaphylatoxin. Recently BORDET (30) himself has also recorded somewhat similar observations, and has described the effect of a first sub-fatal dose of the agar-sol as rendering the guinea-pig tolerant for the time being of further normally fatal injections of the same preparation. He found, further, that serum from a guinea-pig thus rendered tolerant to agar injections would not yield an anaphylatoxin when incubated with agar *in vitro*.

These observations, with which we were acquainted only through abstracts, were for some time puzzling to us. We had repeatedly injected doses of agar-sol, of the same dimensions as those used by BORDET, into small guinea-pigs, weighing 200–250 grm., such as are normally used for experiments on specific anaphylaxis or

on anaphylatoxins, without producing definite symptoms of any kind. When we obtained access to BORDET'S original paper, we noticed that, departing from the almost invariable practice in experiments on anaphylatoxin, he had used adult guinea-pigs, weighing 500–600 grm., for these experiments with agar-sol. Using guinea-pigs of this type we observed his phenomenon. In addition to this, we found that anaphylactic guinea-pigs, even if of lower weight, not only responded to intravenous injections of agar-sol with severe or fatal symptoms, but that they exhibited, in place of the delayed and protracted reaction described by BORDET, symptoms appearing within a minute following the injection, and presenting, in their type and in the rapidity of their fatal issue, a much closer resemblance to those of the true anaphylactic shock.

Normal Guinea-Pigs.

Weight of guinea-pig.	Dose of 0·25 per cent. agar-sol.	Result.
260	1 c.c.	Doubtful slight depression.
260	2 c.c.	Slight evanescent dyspnœa.
285	1 c.c.	Pronounced dyspnœa. Recovery after 15 minutes.
570	1 c.c.	Profound collapse. Heart severely inhibited. Recovery after 1 hour.
590	1 c.c.	Dyspnœa, collapse, convulsions. † 6½ minutes.

Guinea-Pigs Anaphylactic to Egg-albumin (59 days).

Weight of guinea-pig.	Dose of 0·25 per cent. agar-sol.	Result.
310	1 c.c.	Symptoms like anaphylactic shock. † 4 minutes.
350	1 c.c.	Similar symptoms. † 3 minutes.

We must postpone, for the moment, a full discussion of the meaning of these puzzling results. At first sight they might seem to lend some support to the view that the anaphylatoxins, or at least some samples of them, contain the true anaphylactic poison, liberated in the shock. It is clear that the tissues of the anaphylactic guinea-pig, in addition to their highly specific sensitiveness for a particular protein, have a more general instability to toxic influences producing a similar type of action; and it might be thought that the differences between the symptoms produced by anaphylatoxins in the normal animal and those produced by the specific antigen in the anaphylactic animal could be thus explained. We shall see, however, that there is no evidence of such greater sensitiveness of the *tissues* of the anaphylactic animal to the anaphylatoxins themselves. Nor would such a supposition explain the appearance of the difference only with certain batches of anaphylatoxin, or the surprising

fact that an injection of agar-sol will sometimes kill the anaphylactic guinea-pig as rapidly as does the ready-formed anaphylatoxin.

Post-mortem *Appearances.*

The permanent distension of the lungs, first described by Auer and Lewis (6), has long been regarded as the most characteristic result of death from anaphylactic shock in the guinea-pig. It is so, in the sense that it is the expression of the bronchial spasm which is the cause of anaphylactic death in that species. The failure of the lungs to collapse, when the chest is opened after death, cannot by itself, however, be regarded as indicating any connection between the cause of death and anaphylaxis. The guinea-pig is peculiarly liable to death from obstruction of the bronchial tubes, and obstruction by blood, secretion or œdema-fluid, produces a picture which is easily mistaken for that of pure bronchial spasm. There are substances, moreover, such as histamine, which have no obvious relation to the phenomena of anaphylaxis, but produce the typical, fatal inspiratory distension of the lungs, due, as in the anaphylactic reaction, to simple constriction of the plain muscle of the bronchioles. All the three anaphylatoxins, with which we are specially concerned, produce distension of the lungs in guinea-pigs killed by them. The resemblance to the lungs of a guinea-pig killed by anaphylactic shock or by histamine is not always complete. The similarity is greatest and most constant in the effects of agar-anaphylatoxin. In the lungs of guinea-pigs killed by this preparation, there is usually no definite evidence of œdema, and hæmorrhagic patches are comparatively rare. These other features are commoner in the lungs from guinea-pigs killed by bacterial anaphylatoxin, and still more prominent in the effects of starch-anaphylatoxin. This last preparation seems, again, in its effects seen *post mortem* as in the symptoms it produces, to be more variable in action than the other two. One particular sample caused a hæmorrhagic œdema of the lungs so profuse, and so early in its onset, that a red froth appeared, some time before death, in the nostrils of all the guinea-pigs which received a fatal dose of it; and the *post-mortem* finding in these cases was, naturally, widely different from that seen when the cause of death was mainly a muscular spasm of the bronchioles. We shall refer to other differences in dealing with the finer analysis of the symptoms.

Changes in the Blood.

Coagulation.—Delayed coagulation of the blood, when taken from the vessels after death, seems to have been regarded by many observers as a second cardinal sign of the anaphylactic nature of a reaction. Such delay is seen in the blood of guinea-pigs dying from anaphylactic shock, or from injections of any of the three anaphylatoxins. The delay is variable and never very great; a normal coagulation-time of 3–5 minutes at room temperature may be lengthened to 10 minutes or

rather more, but we have never observed in the guinea-pig the practically permanent loss of coagulability which the anaphylactic reaction produces in the dog's blood. In this respect, however, there is no obvious discrepancy between the effects of the anaphylatoxins and the true anaphylactic reaction in the guinea-pig. We have already mentioned the fact that the serum digested with chloroform has a very different effect, causing widespread intravascular clotting.

Leucocytes.—A leucopenia, affecting chiefly the polymorphonuclear cells, is seen in guinea-pigs killed with any of the anaphylatoxins, as in those dying of the true anaphylactic shock. There does not seem to be any great significance, however, in this correspondence, since a quite similar leucopenia is seen in the blood of guinea-pigs killed with histamine, and probably with many other poisons.

Platelets.—The effect on the blood-platelets is much more important, in that it shows a definite discrepancy between the effects of the anaphylatoxins and those of the specific antigen acting on the anaphylactic animal. For observation of the abundance and condition of the platelets, blood was taken from the heart, or, in the case of normal controls, sometimes from a punctured vein in the ear. It was drawn immediately in a blood-counting dilution pipette, and therein rapidly diluted with ten times its volume of physiological saline, containing 1 per cent. of sodium citrate. The mixture was then ejected into a small test-tube. The normal, control mixture, with blood drawn from the heart by a syringe armed with a fine needle, or in some cases from an ear vein, was set aside until the experiment was finished. The guinea-pig then received the intravenous injection of anaphylatoxin, specific antigen or histamine, according to the nature of the experiment. Immediately after death the chest was opened, and blood taken from the still beating right ventricle with a clean glass capillary pipette, and diluted under exactly the same conditions as the control sample. The control sample was lightly shaken, to remove the effects of incipient sedimentation, and both samples were then allowed to stand side by side, until the red corpuscles had subsided sufficiently, or, more usually, were subjected together to a very brief centrifugation. It was sufficient to place the tubes in the centrifuge, to start the turbine until a speed of not more than 500 revolutions was attained, and then to allow the rotor to come naturally to rest. This produced a sufficient surface layer, practically free from red corpuscles, to enable a drop to be taken conveniently for examination, without producing any significant sedimentation of the platelets of the normal sample.

We made a number of attempts to obtain quantitative comparisons by counting the platelets, and to facilitate this by staining. We soon reached the conclusion, however, that numerical records gave a purely fictitious appearance of accuracy to a comparison between normal samples, in which the platelets were separate and evenly distributed, and abnormal samples, in which the few remaining in suspension in the plasma were agglutinated into clumps. We found, however, that the use of the

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Thoma counting-chamber produced a layer of convenient thickness, while the constant depth facilitated comparison.

Even without the use of the microscope the change in the abundance of platelets in the plasma, following death or severe symptoms produced by any of the anaphylatoxins, was sufficiently obvious. The diluted and citrated plasma left above the sedimenting red corpuscles, always showed a slight but distinct turbidity when the blood was from a normal animal or from one in which death had been caused by histamine, or by acute anaphylactic shock. This turbidity, which was sometimes, but not always, perceptibly less in the post-anaphylactic plasma than in the normal, was due to the suspended platelets. The plasma of blood taken from guinea-pigs killed, or severely poisoned, by any of the anaphylatoxins usually looked, by contrast, perfectly clear. The microscopic appearances entirely confirmed these naked-eye impressions. In specimens of the citrated plasma from animals which were normal, or killed by histamine, the platelets were abundant, almost entirely discrete and evenly distributed. In samples taken after anaphylactic death they were usually definitely fewer than in the corresponding normals, and a few clumps of agglutinated platelets were mingled with many which were still discrete. In samples from animals killed by the anaphylatoxins it was difficult to find platelets at all, and the few remaining were almost entirely agglutinated into clumps consisting of fifteen to twenty or more.

This difference between the blood of animals dying in anaphylactic shock and that of animals killed by the anaphylatoxins came as a surprise to us. Agglutination of the platelets has been described by earlier observers as caused by the anaphylatoxins (Aynaud, Bedson (38)); but it has also been described as characteristic of the true anaphylactic shock, and has even been regarded by Behring (31), as the cause of the symptoms, which he attributed to embolism of the cerebral capillaries. We are certain, however, of the validity of the distinction which we have indicated. Agglutination of the platelets occurs in the true anaphylactic shock to a variable but usually small extent; in many cases of typically acute shock we could hardly detect any, and certainly not more than is caused by intravenous injection of a few cubic centimetres of normal guinea-pig serum, causing no symptoms. In the shock produced by the anaphylatoxins a large proportion of the platelets seems to disappear entirely; they are not removed by sedimentation, since they are not much more abundant in the plasma immediately above the red-cell layer, but the few remaining are practically all agglutinated.

Conclusions.

By observations of the symptoms, by post-mortem examination, and by examination of the blood, certain differences can be detected between the effects produced by fatal doses of any of the anaphylatoxins and those which constitute the true anaphylactic shock. Effects on the blood, especially the platelets, and on the endothelium of the capillaries, seem to be more prominent in the action of the anaphylatoxins than in the true anaphylactic shock, in which the effect on plain

muscle is so conspicuous as to overshadow others. The differences so far noted, however, in any case only warrant a conclusion that the anaphylatoxins do not represent physiologically pure preparations of an anaphylactic poison; the effects on platelets, etc., might be due to adventitious substances. This criticism is strengthened by the curious observation that anaphylactic guinea-pigs react to certain batches of anaphylatoxin with symptoms much more nearly resembling the true anaphylactic reaction. If, however, these preparations contain a poison which is concerned as the direct agent in the anaphylactic shock, they must exhibit a quite extraordinary direct stimulant action on the guinea-pig's plain muscle. This was put to the test of experiment.

Direct Action on Plain Muscle.

The plain muscle used was, in most cases, that of a horn of the uterus of a virgin guinea-pig. When this has been thoroughly washed and suspended in Ringer's solution it exhibits a definite sensitiveness of reaction to small doses even of fresh guinea-pig serum. In testing the anaphylatoxin preparations, therefore, we kept in every case a sample of the serum used in the preparation, and compared the action of this on the plain muscle with that of the anaphylatoxin prepared from it. This was additionally important, in view of the fact that different samples of plain muscle exhibit a widely differing sensitiveness to the action of serum.

Starch Anaphylatoxin.—We made the experiment with several batches of this preparation, and failed in every case to detect any difference between its action on the isolated plain muscle and that of the normal serum from which it was prepared. One experiment may be quoted in detail.

30 c.c. of normal guinea-pig serum were mixed with 6 c.c. of starch sol and incubated for 3 hours. It was then centrifugated and compared for toxicity, by intravenous injection, with a sample of the normal serum from which it was prepared.

Weight of guinea-pig in gramme.	Dose.	Result.
150	5 c.c. normal serum	Slight shivering. No other symptoms.
210	2 c.c. starch-anaphylatoxin . . .	Moderate symptoms. Severe dyspnœa. Recovery.
180	4 c.c. starch-anaphylatoxin . . .	† 4 minutes.

The preparation, therefore, was normally toxic, and the serum from which it was made was normally free from toxicity. Fig. 3 shows the effect, on the isolated uterus of a normal guinea-pig, of equal doses of the normal serum (N) and the starch anaphylatoxin (S) prepared from it. It is clear that the latter has no more stimulant action on the plain muscle than the normal serum. So that, whatever may be the mode of action of the starch anaphylatoxin in producing death of the guinea-pig, it

does not act directly on the plain muscle fibre, as the antigen does on that of the anaphylactic guinea-pig.

Bacterial anaphylatoxin.—Two 24-hour agar slopes of *B. prodigiosus* were centrifugated in 3 c.c. of physiological saline, and the whole was added to 33 c.c. of fresh guinea-pig serum. The mixture was kept at 37° for $1\frac{1}{2}$ hours, allowed to stand at 18° for 18 hours, and then thoroughly centrifugated. It was tested as follows :—

Weight of guinea-pig.	Dose.	Result.
220	0·5 c.c.	† 4 minutes.
210	1 c.c.	Moderate symptoms. Recovered.
230	1 c.c.	† $3\frac{3}{4}$ minutes.
230	1 c.c.	Slight symptoms. Recovered.
210	1·5 c.c.	† $3\frac{1}{2}$ minutes.
220	1·5 c.c.	† $3\frac{1}{2}$ minutes.

1·5 c.c. seems to be the certainly lethal dose, but as little as 0·5 c.c. occasionally kills. The preparation has a high toxicity for the intact animal. It was then tested on normal isolated plain muscle of the uterus, the serum from which it was made being used as a control. It will be clear from fig. 4 that the bacterial anaphylatoxin (F) has definitely greater stimulant action on the plain muscle than the normal serum (N).

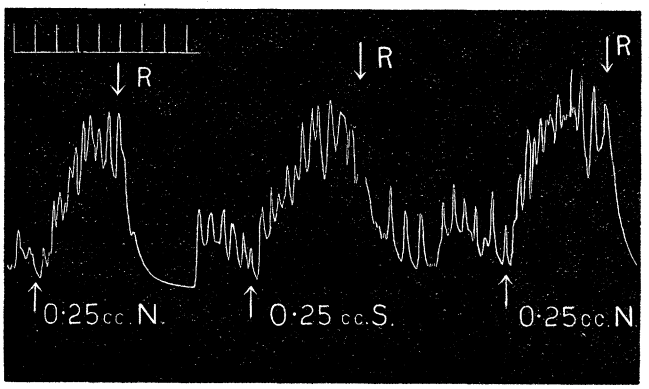

FIG. 3.

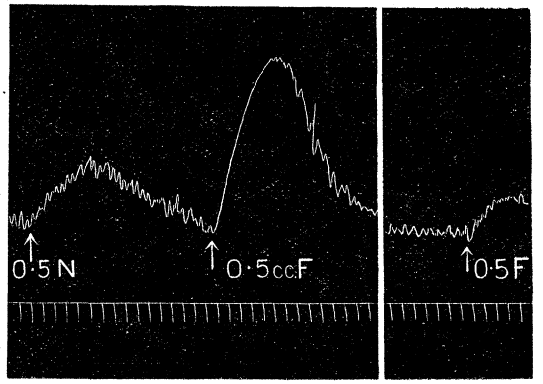

FIG. 4.

FIG. 3.—Normal guinea-pig uterus. N = normal guinea-pig uterus. S = starch anaphylatoxin prepared from N.

FIG. 4.—As fig. 3. N = normal guinea-pig serum. F = bacterial anaphylatoxin prepared from N.

The ratio between the two activities could not be measured, since, as the figure shows, a subsequent dose has a very greatly reduced effect. It can be said, however, quite definitely, that the discrepancy between the activities on the isolated plain muscle is not of such a nature as to offer any explanation of the difference between the effects on the entire animal, which the anaphylatoxin kills, in some instances, in a dose as little as 0·5 c.c., while the normal serum produces practically no symptoms in ten

times that dose. The activity of the anaphylatoxin on the plain muscle may be twice or thrice that of the normal serum ; certainly it is not more.

While the action of this preparation on plain muscle is under discussion, we may recall the fact that FRIEDBERGER (32) himself has tested it in this way. He found that it stopped the activity of an isolated slip of intestinal muscle, and argued, somewhat surprisingly, that this represents the true anaphylactic reaction, while the contraction of the isolated anaphylactic uterus in response to its antigen has not this significance, because the muscle is stimulated and not killed. We have failed to confirm FRIEDBERGER'S observation. In our experiments the bacterial anaphylatoxin had a moderate, though not in our opinion very significant, stimulating action on intestinal as on uterine muscle (see fig. 5). We entirely fail to understand his argument. It is the animal, not its plain muscle, which dies in anaphylactic shock, and contraction of plain muscle is, in the guinea-pig, the cause of death. Our records of effects on plain muscle with the anaphylatoxin are less incompatible with its supposed connection with anaphylaxis than are FRIEDBERGER'S own ; but for quantitative reasons we conclude that this direct action on the plain muscle does not materially contribute to its lethal action on the whole animal.

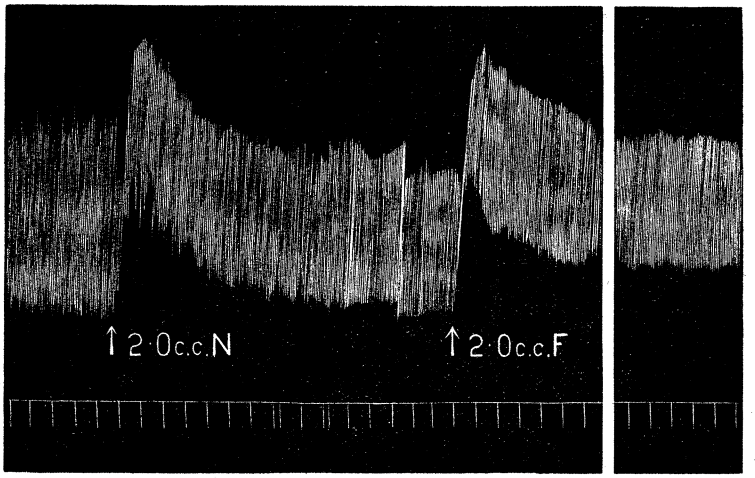

FIG. 5.—Loop of normal rabbit's small intestine. N and F as in fig. 4. Gap in tracing = interval of 10 minutes.

Agar-anaphylatoxin.—This, as a rule, shows a more definitely superior activity on isolated plain muscle, as compared with normal serum, than is exhibited by the other two. Fig. 6 shows a record in which excess of directly stimulant action in an agar-anaphylatoxin is unusually well marked. If it stood by itself we should have some hesitation in regarding this activity as altogether without significance for the effect on the whole animal. Other experiments, however, seemed to exclude quite definitely any connection between the two kinds of action.

A preparation of agar-anaphylatoxin was made in the ordinary way. Part of it was filtered through a moderately fine Kieselguhr candle, the remainder being kept as

a control. The toxicity of the two samples was first compared by intravenous injection into normal guinea-pigs.

Weight of guinea-pig.	Dose of agar-anaphylatoxin.	Result.
190	1·5 c.c., unfiltered	Symptoms. Recovery.
210	2 c.c., unfiltered	Symptoms. Recovery.
220	2·5 c.c., unfiltered	† in 3 minutes.
220	2·5 c.c., filtered	Nil.
210	3 c.c., filtered	† in 3 minutes.
200	3·5 c.c., filtered	† in 2 minutes.

It seems probable that a slight loss of toxicity has occurred; but 3 c.c. filtered = 2·5 c.c. unfiltered.

Fig. 7 shows the result of testing these preparations on the isolated uterus, in comparison with the normal serum from which they were prepared. The unfiltered

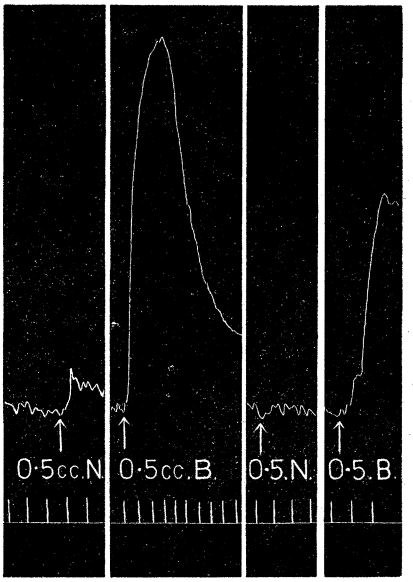

Fig. 6.

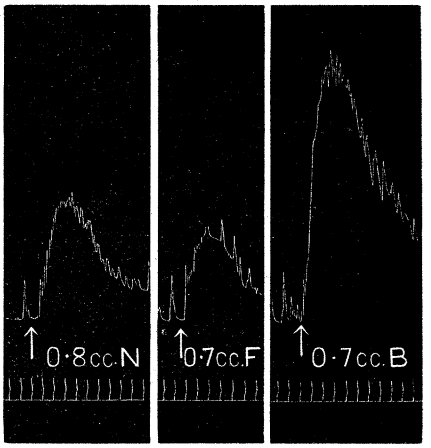

Fig. 7.

Fig. 6.—As fig. 3. N = normal guinea-pig serum. B = agar anaphylatoxin from N.
Fig. 7.—As fig. 3. N and B as in fig. 6. F = B filtered through Kieselguhr candle.

preparation (B) shows enhanced activity, though not of an order corresponding to its high toxicity for the whole guinea-pig; the filtered preparation (F) has no greater activity than the normal serum (N), though its toxicity for the whole animal was very little inferior to that of the unfiltered one. Such excess of activity as the latter shows on the plain muscle cannot, therefore, play any serious part in the symptoms produced by intravenous injection. This removal of the plain muscle stimulant, by a filtration which scarcely affects the toxicity for the entire animal, is surprising; it can only be attributed to a selective adsorbent action of the Kieselguhr.

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Action on Anaphylactic Plain Muscle.—We mentioned above the observation that some batches of anaphylatoxin appear to have a preferential toxicity for guinea-pigs which have been rendered anaphylactic to a foreign protein. If their toxicity represented the effect of a poison, which is the directly effective agent in the anaphylactic shock, the plain muscle of the anaphylactic guinea-pig might be expected to show an enhanced sensitiveness to its action. We have been unable to find any indication of such enhanced sensitiveness; the plain muscle of the anaphylactic guinea-pig gives a response to an anaphylatoxin which is neither less nor greater than that exhibited by the normal plain muscle. Fig. 8 shows the response of anaphylactic plain muscle to bacterial anaphylatoxin (F). With successive doses the muscle acquires a partial tolerance to this, which does not in any way affect its subsequent response to 0·1 mgrm. of its specific antigen (A). To this point we shall return later. The point here to be noted is that the response to the anaphylatoxin is very similar to and certainly not greater than that of normal plain muscle, as shown in fig. 4.

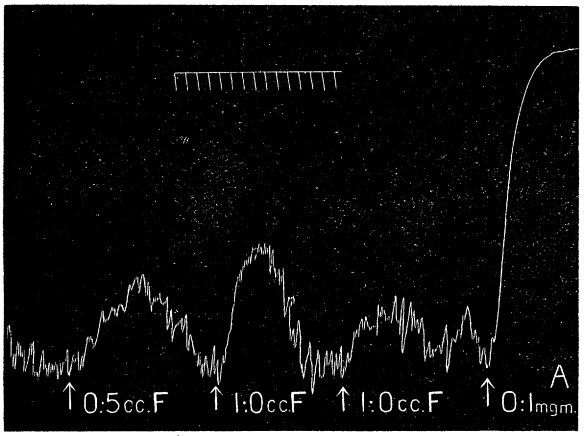

FIG. 8.—Horn of uterus from guinea-pig actively sensitised to egg-albumin. F = bacterial anaphylatoxin, as in figs. 4 and 5. A = cryst. egg-albumin.

We reach the conclusion, therefore, that though the anaphylatoxins exhibit, irregularly, some stimulant action on isolated plain muscle in excess of that shown by normal serum, this excess is quite inadequate to account for their effects *in vivo*. The contrast between their inadequacy in this direction and the intense, direct stimulant effect shown by traces of a specific antigen on the plain muscle from an animal rendered anaphylactic to it, cannot in any way be reconciled with the view that these anaphylatoxins represent the direct agent in the anaphylactic shock. The evidence points to a precisely opposite conclusion; it is the antigen which acts directly on the anaphylactic cells, while some intermediate mechanism is needed to account for the action *in vivo* of the anaphylatoxins.

Further Analysis of the Action.

The effect of the anaphylatoxins *in vivo* not being due to immediate action on the plain muscle, it was necessary to enquire through what immediate reaction their effect is brought about. For this purpose it was necessary to administer them under conditions permitting of operative interference, but not involving the use of a chemical anæsthetic. We found that this could easily be achieved by pithing the brain of the guinea-pig with a sharp bradawl, which, if the head is well flexed, can be pushed instantaneously through the skin, muscle and *Foramen magnum* into the brain, which is thoroughly destroyed. The animal is immediately turned over and tied out, the trachea is laid bare and opened, a cannula with adjustable side-opening is tied into it, and connected to a small bellows worked by a motor. The whole operation can be completed in less than half a minute, and the heart continues then to beat vigorously. A cannula is tied into a jugular vein for injections and the chest is opened so that the rhythmical expansion and collapse of the lungs can be watched. If a fatal dose of one of the anaphylatoxins is now injected, either into the vein or, with a needle, directly into the right ventricle, in a few seconds the effect of the bellows on the lung-volume begins to be reduced, and the impairment rapidly progresses until the lungs become stationary. The effect is very similar to that seen in the anaphylactic animal when a dose of antigen is given under the same conditions. There is, however, this difference. In the case of the immobilisation of the lungs produced by the true anaphylactic reaction, when once the obstruction has fully developed, no increased volume or vigour of the artificial respiration will force the block. The volume and force of the pump-stroke can be made sufficiently forcible to blow the cannula out of the trachea without any air being forced past the bronchial obstruction. In the case of the immobility produced by any of the anaphylatoxins, even a moderate increase of the stroke of the bellows sufficed to force the obstruction. When once it had been passed, the lungs soon began again to expand and contract with the rhythm of the bellows, the heart was rapidly relieved from the asphyxial distension of its right chambers, and the circulation was restored. The preparation was now apparently in the same condition as before injection; the only difference was that a further intravenous or intracardiac injection of anaphylatoxin was wholly without effect—a point to which we shall return in the next section.

This difference in the behaviour of the lungs seemed to us to be qualitative, rather than merely quantitative. In the anaphylactic animal the smallest dose of antigen sufficient to occlude the bronchioles completely when the artificial ventilation was moderate, seemed to close them firmly against a much greater pressure; while the impediment produced by the largest dose of any of the anaphylatoxins was forced with comparative ease. The contrast naturally raises the question whether the block is produced by the same mechanism. There is hardly room for doubt that the obstruction in the anaphylactic reaction is due almost entirely to plain muscle

contraction; histamine, the effect of which on the guinea-pig seems to be very purely an action on plain muscle, produces an impassable bronchiolar block like that seen in the anaphylactic reaction. The suspicion arises that the obstruction produced by the anaphylatoxins may be largely due to action of another kind—to swelling of the mucous membrane lining the tubes and excessive secretion, possibly combined with a contraction of the muscular coats, which would be inadequate by itself to produce occlusion but, in combination with other factors, can produce a closure which resists a low pressure, yet is broken through with relative ease by a higher. We made some attempts to test this possibility by histological comparison of lungs from guinea-pigs killed by anaphylatoxin and by anaphylactic shock respectively, but without any success. Even in lungs from normal guinea-pigs, killed by decapitation, we found many bronchioles contracted almost to the point of complete closure, and it seemed clear that we had no method of fixation which could be trusted to preserve the natural condition. We must rely, therefore, on the physiological evidence for the difference between the two types of reaction.

There remained the question whether any other organ was concerned as an intermediary in the action of the anaphylatoxins. We made a series of eliminating experiments with all three. In pithed guinea-pigs, prepared as above, impediment to artificial respiration being taken as the sign and measure of the effect, we found that neither section of the vagi, nor complete ligature of the arteries to the head, nor ligature of the whole blood supply to the stomach, intestines and liver, including the portal vein, nor of the aorta and vena cava at the level of the diaphragm, made any difference to the effect on the lungs. So that we can exclude action on the medullary centres, if they escape destruction in pithing the brain, on the liver or alimentary canal, and on the main muscular mass of the body, as having no part in the production of the effects of the anaphylatoxins on the lungs. We are left practically with the lungs themselves, their blood vessels, the blood circulating through them, and the heart which propels it. In this limited mechanism all that the anaphylatoxins can effect is the bronchial obstruction to which their lethal action is mainly due; on the plain muscle isolated from the body they have no action adequate to account for it. Effects on the heart can be excluded. Provided the ventilation of the lungs can be kept going, the action of the heart is not adversely affected by any of these preparations. There remain the blood, and the endothelium of the blood vessels. It is here that we must seek the clue to the action of these preparations, and we have some evidence of the nature of the action in the extensive destruction of the platelets which follows the injection of any of them. We made some attempts to discover whether their effect on the platelets is produced outside the blood vessels, but without success. When the blood, with its normal content of ionised calcium, is brought into contact with glass or other foreign substance the platelets are agglutinated in any case; when sufficient citrate is added to prevent this, addition of anaphylatoxin to the citrated plasma causes

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no agglutination of the platelets. The question must, therefore, be left open, as to whether the anaphylatoxins produce their action on the platelets *in vivo* wholly by direct action, or whether the effect on these structures is to any extent secondary to an action on the endothelium. In any case it would appear that the action of the anaphylatoxins, when they are injected into the circulation, corresponds to that which would be produced by the exposure of the whole blood to a large extent of foreign surface. Numerous observers have drawn attention to the fact that in the process of clotting, and particularly in the early stages of the process preceding the actual formation of a clot, blood acquires a toxic action of the type with which we are here concerned. Mills, Raap and Jackson (33), for example, in a recent paper, demonstrate a constriction of the bronchioles, contraction of the uterus, and fall of arterial blood-pressure, occurring in the dog when any substance is injected which causes intravascular coagulation, and fully developed before actual coagulation begins. When the substance injected is ready-formed thrombokinase (cytozyme) in adequate dose, the picture is soon obscured by clotting in the vessels, as in the action of sera rendered toxic by digestion with chloroform. In the effect of the anaphylatoxins, the process, in our view, would have a more gradual development, the kinase being slowly liberated as the platelets are disintegrated; and in that case the clotting is prevented by the formation of antagonistic substances, so that the blood is found fluid after death, and clots slowly after removal from the body. Whether the endothelium is directly injured by the contact with the "foreign" substance in the blood, or wholly by poisonous substances liberated in the "precoagulation" changes, cannot be determined; it is clearly to the latter, however, that we must attribute the action on plain muscle.

We have found no support for the view held by some, which attributes the action of the anaphylatoxin to embolism of the lung capillaries, produced by large colloidal aggregates, agglutinated platelets, agglutinated red cells or small thrombi, though these may all play a part in some of the phenomena which have loosely been described as "anaphylactic" or "anaphylactoid" (*cf.* Hanzlik and Karsner (34)). In the phenomena with which we are dealing there is no evidence of serious impediment to the pulmonary circulation. A bronchiolar block occurs, and, if this is relieved, the right side of the heart empties itself normally and the circulation is resumed.

Desensitisation to Anaphylatoxins and to Specific Antigen.

It has been recorded by several observers (35) that injection of a sub-lethal dose of an anaphylatoxin is followed by the appearance of a condition which renders the guinea-pig, for the time being, insensitive to further injections of doses which are lethal for the normal guinea-pig. We have repeatedly confirmed this observation. The refractory condition appears very rapidly, being apparently fully developed by the time any symptoms, which the sub-lethal dose produces, have passed off. It has

greatly diminished by the following day, the normal sensitiveness being largely or completely re-established. Two examples will suffice.

Starch-anaphylatoxin.

Lethal dose of preparation.	First injection.	Result.	Interval.	Second injection.	Result.
3·5 c.c.	3 c.c.	Very severe symptoms	12 mins.	5 c.c.	Nil.

Agar-anaphylatoxin.

Lethal dose of preparation.	First injection.	Result.	Interval.	Second injection.	Result.
3·5 c.c.	2 c.c.	Very severe symptoms	15 mins.	4 c.c.	Nil.

Besredka (36) made experiments to discover whether this acquired insensitiveness to anaphylatoxin was accompanied, in the anaphylactic animal, by loss of sensitiveness to the specific antigen, and stated that this was not the case. We made a number of experiments of this kind, with results which seemed to show a curious irregularity. In our earliest experiment, made with starch anaphylatoxin, we found no evidence of diminished sensitiveness to the specific antigen in anaphylactic guinea-pigs, which were rendered insensitive to the starch anaphylatoxin, by preliminary injections of this preparation. Later, with agar-anaphylatoxin, we observed in some cases a similar retention of the specific sensitiveness in guinea-pigs rendered tolerant to the agar-serum, but in many others the sensitiveness to the antigen was very much reduced. Examples of both may be quoted:—

1. *Starch anaphylatoxin.*

Guinea-pigs sensitised by 1 *mgrm. Crystallised Egg-Albumin,* 25 *Days previously.*

Estimations of Sensitiveness.

Weight.	Dose of egg-albumin intravenously.	Result.
250	0·2 mgrm.	† 3½ minutes.
235	0·1 mgrm.	Slight symptoms. Recovered.

Toxicity of Starch-anaphylatoxin.

Weight.	Dose.	Result.
250	4·5 c.c.	† 3½ minutes.
250	3·5 c.c.	† 4½ minutes.
270	3·0 c.c.	† 5 minutes.

An anaphylactic guinea-pig of the same series then received injections as follows :

Weight.	Dose of anaphylatoxin.	Result.	Interval.	Second injection.	Result.
260	2·5 c.c.	Severe symptoms	18 mins.	0·2 mgrm. egg-albumin.	† 5 mins.

There is no evidence of any serious degree of protection, though the death is not quite so prompt.

2. *Agar-anaphylatoxin.*

Guinea-pigs passively sensitised to egg-albumin by injection 48 hours previously of 0·2 c.c. concentrated rabbit precipitin for egg-albumin.

Lethal Dose of Egg-albumin.

Weight.	Dose.	Result.
250	0·1 mgrm.	† 3¼ minutes.
255	0·05 mgrm.	Nil.

Lethal Dose of Agar-anaphylatoxin.

Weight.	Dose.	Result.
220	2 c.c.	† 3 minutes.
220	1·5 c.c.	† 3 minutes.
215	1·5 c.c.	Severe symptoms. Recovered.

Test of Desensitisation.

Weight.	First dose of anaphylatoxin.	Result.	Interval.	Second dose of anaphylatoxin.	Result.	Interval.	Dose of egg-albumin.	Result.
220	0·75 c.c.	Symptoms	10 mins.	2 c.c.	Nil	25 mins.	0·2 mgrm.	† 5 mins.

Here again there is an indication of only a small degree of protection, if any.

In these cases, and others like them, it will be seen that a relatively short interval elapsed between the desensitisation to the anaphylatoxin and the test for sensitiveness to the specific antigen. In a number of other experiments we found indications of a far greater loss of specific sensitiveness, and it was only a subsequent comparison of

our records which brought to our notice the fact that we had left a much longer interval between the desensitisation to anaphylatoxin and the subsequent test for specific sensitiveness to the antigen. If the interval was somewhat longer than the above, a partial protection became obvious, as in the following :—

Guinea-pigs actively sensitive to egg-albumin, 17th day.
Lethal dose of egg-albumin—0·2 mgrm.

Lethal dose of agar-anaphylatoxin—between 1 and 2 c.c.

Weight.	First dose of anaphylatoxin.	Result.	Interval.	Second dose of anaphylatoxin.	Result.	Interval.	Dose of egg-albumin.	Result.
230	1 c.c.	Slight symptoms	21 mins.	2 c.c.	Nil	31 mins.	0·2 mgrm.	—*

* Very severe symptoms. Just recovered.

When a much longer interval was allowed a much more decided loss of sensitiveness was observed, as in the following series :—

Guinea-pigs actively sensitised to egg-albumin, 26th day.
Sensitiveness to egg-albumin—0·05 mgrm. kills regularly; 0·03 mgrm. does not kill.

Lethal dose of agar-anaphylatoxin—3 c.c. In every case in the series the first dose of anaphylatoxin caused symptoms of severity varying with the dose, the second dose caused no symptoms.

Weight.	First dose of anaphylatoxin.	Interval.	Second dose of anaphylatoxin.	Interval.	Dose of egg-albumin.	Result.
270	1·5 c.c.	50 mins.	3·5 c.c.	2¼ hours	0·05 mgrm.	Nil.
250	2·5 c.c.	50 mins.	3·0 c.c.	2¼ hours	0·1 mgrm.	Nil.
250	2·0 c.c.	55 mins.	3·25 c.c.	2¼ hours	0·2 mgrm.	Nil.
240	1·5 c.c.	—	—	2½ hours	0·5 mgrm.	† 3 mins.

We could cite many other examples of a similar nature, and it is evident that, when a long interval is allowed to elapse between the completion of desensitisation to anaphylatoxin and the testing of sensitiveness to the specific antigen, the latter is found to be greatly reduced. This delayed appearance of partial non-specific desensitisation in the anaphylactic animal, in contrast to the immediate desensitisation to anaphylatoxin, makes it clear that the phenomena are not identical. The lack of identity is further emphasised by experiments which we made on the passive transfer of the resistance. When serum from guinea-pigs which have been rendered tempo-

rarily tolerant to anaphylatoxin is injected intravenously into a normal guinea-pig, the tolerance is immediately transferred to the latter. If the same serum is injected into an anaphylactic guinea-pig, the sensitiveness of the latter to its specific antigen is not perceptibly changed, as the following record shows.

Serum was obtained from guinea-pigs which had been rendered completely tolerant to agar-anaphylatoxin.

Titration of Anaphylatoxin on Normal Guinea-Pigs.

Weight.	Dose.	Result.
250 260	2·5 c.c. 1·5 c.c.	† 3¼ minutes. Very severe symptoms.

6 c.c. of the serum from tolerant guinea-pigs were injected intravenously into a third normal guinea-pig, weighing 270 grms. Three minutes later 2·5 c.c. of the agar-anaphylatoxin were injected, and trifling, doubtful symptoms resulted.

A series of guinea-pigs actively anaphylactic to egg-albumin (26th day) was then taken. The lethal dose of egg-albumin was determined as 0·05 mgrm. Two of the series received immunising doses of agar-anaphylatoxin, and, after 1½ hours, were bled out and their serum separated from the clot and mixed. Another of the series was then given 6 c.c. of this serum intravenously, and, 5 minutes later, 0·05 mgrm. of egg-albumin, which caused typical anaphylactic death in 3½ minutes. There was no trace of protection.

This makes it clear that the tolerance for anaphylatoxin, exhibited by a guinea-pig which has survived a sub-lethal dose of the same, is due to the presence in the blood of some antagonistic substance, which can transfer the protection to another animal. On the other hand, the mere presence of this antagonistic substance in the circulation of an anaphylactic animal does not afford any protection against the specific antigen.

We found, however, that if, instead of examining for protective effect immediately after injection of a large dose of serum from tolerant animals, the anaphylactic guinea-pig was tested an hour or so later, a definite desensitisation was perceptible. The same effect, however, was produced by injecting a similar dose of *normal* guinea-pig serum into the vein of an anaphylactic guinea-pig, and testing its sensitiveness after an hour's interval; in this case also a partial desensitisation had been produced, as the following experiment shows:—

A series of guinea-pigs were taken which had been made actively anaphylactic to egg-albumin (36th day). The lethal dose of egg-albumin was found to be 2 mgrm.; 1·5 mgrm. was not regularly fatal.

Four of the guinea-pigs were rendered tolerant to agar-anaphylatoxin as in previous

experiments; an hour later they were killed, their blood was collected, and the sera drawn off and mixed, 15 c.c. of serum being obtained. Normal serum was similarly obtained from two normal guinea-pigs.

Three of the remaining guinea-pigs of the anaphylactic series were then tested as follows :—

Weight.	Dose of serum intravenously.	Interval.	Dose of egg-albumin.	Result.
1. 260	6 c.c. (tolerant) . .	5 minutes	2 mgrm.	† 3½ minutes.
2. 240	6 c.c. (tolerant) . .	55 minutes	2 mgrm.	Moderate symptoms.
3. 250	6 c.c. (normal) . .	70 minutes	2 mgrm.	Slight symptoms.

This late weakening of the sensitiveness of anaphylactic guinea-pigs, therefore, is not due to the transfer of protective substance from those which have been made tolerant to anaphylatoxin. It is simply an expression of a disturbance in the relation between the circulating fluids and the sensitive tissue, which follows any large injection of serum, even of normal serum; and it is obvious that the desensitisation to the specific antigen, which appears in the anaphylactic animal at a relatively long interval after the injection of one or more doses of anaphylatoxin, can only be interpreted in the same way. It has no real connection with the immediate tolerance to anaphylatoxin itself which such injections produce, but must be due to a disturbance of the delicate balance of distribution of the antibody between tissue cells and circulating fluid, on the maintenance of which the anaphylactic condition depends.

An anaphylactic guinea-pig, then, can be rendered insensitive to anaphylatoxin, without losing its specific sensitiveness to the antigen. The converse also holds. When an anaphylactic guinea-pig is specifically desensitised, its sensitiveness to anaphylatoxin is very slightly, if at all, affected.

Two guinea-pigs, rendered passively sensitive to egg-albumin (lethal dose 0·125 mgrm.), were desensitised, one by a subcutaneous injection of 1 mgrm., the other by fractional intravenous doses. On the following day both were given intravenous injections of 0·25 mgrm. of egg-albumin, without perceptible reaction. The sample of agar-anaphylatoxin used was tested on *normal* guinea-pigs with the following results :—

Weight.	Dose.	Result.
230	2 c.c.	Moderate symptoms.
220	2 c.c.	Moderate symptoms.
220	2·5 c.c.	† 3¼ minutes.

The desensitised anaphylactic guinea-pigs reacted as follows :—

Weight.	Dose.	Result.
230	2·5 c.c.	Extremely severe symptoms. Just recovered.
220	3·0 c.c.	† $3\frac{3}{4}$ minutes.

The number of animals is insufficient to warrant the conclusion that any difference in reaction exists between these two sets of animals, the normal, and the anaphylactic which have been specifically desensitised. Clearly, if there is any difference at all, it is of a trifling nature ; so that it may be stated definitely that specific desensitisation produces no significant tolerance for anaphylatoxin.

Similar results were obtained with starch anaphylatoxin.

The experiments in both directions lead to the same conclusion. Since an anaphylactic animal can be made highly tolerant to anaphylatoxin without significantly affecting its specific sensitiveness, and can be specifically desensitised without increasing its tolerance to anaphylatoxin, poisoning by anaphylatoxin has no direct relation to the anaphylactic reaction, and the anaphylatoxins cannot represent substances produced in the anaphylactic reaction and responsible for the symptoms.

General Conclusions.

The main results of the experiments and observations above described may be summarised as follows :—

1. Direct evidence is presented in favour of the view that anaphylaxis is produced by antibody in cells of the tissues, the same antibody, when free in the circulating blood or in the fluid surrounding isolated plain muscle, having a protective function. The theory attributing the anaphylactic symptoms to formation of an "anaphylatoxin" in the blood is therefore untenable.

2. An attempt has been made to discover whether the so-called "anaphylatoxins" have any relation to the phenomena of anaphylaxis, and the following conclusions have been reached :—

(*a*) The formation of anaphylatoxins by contact of serum with non-protein colloids is not accompanied by proteolysis.

(*b*) The action of such preparations is not due to embolism by suspended particles.

(*c*) The non-protein colloid remains dispersed in the toxic serum.

(*d*) No macroscopic physical change takes place in the serum during the development of toxicity.

(*e*) The symptoms, *post-mortem* appearances and blood changes produced by the anaphylatoxins differ from those of the true anaphylactic shock, injury of vascular endothelium and lysis and agglutination of platelets being much more conspicuous in the action of all of them.

(*f*) The anaphylatoxins do not owe their action to *direct* stimulation of plain muscle, as they should on any theory connecting them with anaphylaxis. The presence of the circulating blood is necessary for the development of their toxic effects.

(*g*) Acquired tolerance to anaphylatoxin does not involve desensitisation of the anaphylactic animal, nor does specific desensitisation involve tolerance to anaphylatoxin.

We are left with no complete conception of the manner in which the anaphylatoxin is developed. If attention were confined to the preparations made with non-protein sols, it would be possible to attribute the action to the formation, by their adsorption on the colloidal aggregates of the serum, of a system in which the foreign colloid presented a maximum extent of foreign surface to the blood when injected. It would be more difficult, without further evidence, to apply this conception to the development of toxicity by contact of serum with suspensions, such as kaolin and barium sulphate, which can subsequently be removed completely by the centrifuge. Possibly the earlier conception, attributing the toxicity to the removal from some serum constituent, *e.g.*, from the globulin, of a "protective" substance, without which it may act like a "foreign" body, is the true one. Whatever the precise mechanism, however, we regard it as certain that the so-called anaphylatoxins produce their effects not as direct poisons to the tissues, but indirectly by their action on the blood, on which their action is similar to that which would be expected from its sudden exposure to a large extent of mildly injurious foreign surface. To the theory connecting them with the anaphylactic reaction of the guinea-pig to a soluble antigen, this seems to be definitely fatal. On that theory the specific antigen should exhibit its action on the tissue cells of the anaphylactic animal only in the presence of blood, while the anaphylatoxins should act directly and in its absence. The facts are the direct converse of this expectation.

While these considerations appear to us to make it impossible to give any general significance to the anaphylatoxins in the explanation of anaphylaxis, we are not concerned to deny that their effects may furnish analogies for some of the heterogeneous assemblage of phenomena to which this name has been applied. If the term is to be allowed to cover any of the shock-like phenomena which may result from the sudden formation of precipitates or agglutination of corpuscles in the circulating blood, there will obviously be many points of possible analogy between some of the phenomena called "anaphylactic" and the action of the "anaphylatoxins." In our own view this extension and vagueness of nomenclature is regrettable, as being

detrimental to clearness of conception. We have confined our attention to the condition to which the name anaphylaxis was first applied, and for the explanation of which the formation of anaphylatoxin was first put forward—the condition of abnormal sensitiveness to the injection of a soluble foreign protein. We have found no evidence for, and much against the supposition that a process analogous to the formation of anaphylatoxin plays any part in the reaction of an animal, in this condition, to the protein to which it has been rendered sensitive.

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