THE CLOTTING OF PLASMA THROUGH STAPHYLOCOCCI AND THEIR PRODUCTS

By H. D. WALSTON

From the Department of Pathology, University of Cambridge, and the Department of Bacteriology, Harvard University

(With 1 Chart in the Text)

Introduction

Loeb (1903) was the first to report the power of Staphylococci to clot plasma, a power which according to him was shared by *B. pyocyaneus*, *B. prodigiosus*, and *B. coli*. His work was done with normal goose plasma, and it was not until Much's paper (1908) that it was realised that Staphylococci could also clot plasma where clotting had been prevented by citrate, hirudin, and other substances: this property, he thought, was peculiar to Staphylococci, and only certain Staphylococci.

This paper does not seem to have aroused much interest, and the next contribution of any importance came from v. Gonzenbach and Uemura (1916), who confirmed and slightly extended Much's original observations. Since then many papers have appeared, the most important being those of Gratia and of Gross, which have added considerably to our information on this problem. But a great deal of uncertainty and contradiction still remain. The work here recorded was undertaken in the hope that it might contribute by further experimental observations to an understanding of the complex problems involved.

Methods

Throughout the experiments, except where otherwise mentioned, the following procedure was adopted. Blood was obtained from a normal rabbit by heart puncture with a sterile syringe which already contained a small amount of sterile 4 per cent. sodium citrate in saline. The blood was then centrifuged in a hard-glass tube after sufficient citrate had been added to bring the total concentration to 2 per cent., and the supernatant citrated plasma was pipetted off with the usual precautions against infection.

Dilutions of the material to be tested for clotting powers were made in sterile broth or saline, the total volume being 0·5 c.c., and to each dilution was added 0·5 c.c. of the citrated plasma.

The mixtures were then shaken and placed in an incubator at 37° C. and observed at varying intervals, the time of clotting being taken as that when a definite, though not necessarily a complete, clot had formed.

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The action of whole cultures of Staphylococci

It was first considered advisable to confirm the statements that whole cultures of Staphylococci clot various citrated plasmas: and to correlate this property with the haemolytic, proteolytic, and various fermentative reactions of some of the strains which were investigated.

The clotting test was made by suspending roughly one loopful of an 18-hour agar-slope culture in 1·0 c.c. sterile distilled water, and adding 0·5 c.c. of this suspension to 0·5 c.c. citrated plasma.

Table I

Strain	Haemo-lysin	Man-nite	Gelatin	L.M.	Clotting: Sheep	Human	Rab-bit	Dog	Guinea-pig	Colour	Origin
15	+	.	.	.	.	.	+	.	.	Aureus	Boils
16	–	+2	+4	.	.	.	–	.	.	Aureus	Boils
17	+	+2	+5	.	.	.	+	.	.	Aureus	Tonsil
18	+	+2	+4	.	.	.	+	.	.	Aureus	Sycosis
21	.	.	.	.	.	.	+	.	.	Aureus	Antrum
22	+	+5	+2	A.C.	.	.	+	.	.	Aureus	Air
23	.	–	–	.	.	.	–	.	.	Pale aureus	Air
24	.	–	+3	.	.	.	+	.	.	Aureus	Pus
34	+	+3	+1	.	.	.	+	.	.	Aureus	Pus
37	+	+1	+1	A.C.	–	.	+	.	.	Pale yellow	Carbuncle
39	+	+2	+2	A.C.	–	+	+	–	–	Aureus	Pus orchitis
40	+	+2	+3	.	.	.	+	.	.	Aureus	Sheep mastitis
41	–	–	+2	A.C.	.	.	–	.	.	Aureus	Sheep mastitis
42	+	+2	+6	A.	+	+	+	+	–	Aureus	Sheep mastitis

The figure after the + shows the time in days taken for the reaction to appear.

All that could be gathered from this was that certain strains possessed the power to clot the plasmas of certain animals, and that all those strains which showed clotting powers were also haemolytic (Table I). Incidentally, different strains clotted the plasmas of different animals with varying degrees of ease: for example, strain 39 clotted human plasma more readily than it did rabbit plasma; dog plasma it did not clot at all; while strain 42 clotted dog plasma more easily than it did rabbit plasma; and rabbit plasma more easily than human plasma. These observations explain the discordant statements of Gross (1928*a*) and of Gratia (1921), the former saying that rabbit plasma was the most easily clotted, while the latter maintained that dog plasma was clotted best.

The action of staphylococcal filtrates

The next point to establish was whether bacteria-free filtrates had power to clot, Kleinschmidt (1909) having denied it, and Gross (1928*b*) having claimed that they had.

Broth cultures and agar-plate washings, from 5 to 14 days old, the media being adjusted both to *p*H 7·2 and 6·0, were filtered through Seitz E.K. pads,

Table II

Strain	Medium	Filter	Time of growth days	*p*H	Highest dilution to clot after 48 hours
39	Agar plate	Seitz	5	6·0	—
42	,,	,,	5	6·0	—
39	,,	,,	5	7·2	—
42	,,	,,	5	7·2	—
40	Broth—Vaillant peptone	,,	14	7·2	—
40	,,	,,	10	7·2	—
15	Agar plate	,,	5	6·0	—
17	,,	,,	5	6·0	—
18	,,	,,	5	6·0	—
22	,,	,,	5	6·0	—
40	Broth—Vaillant peptone	Berkefeld	5	7·2	>1/320
40	,,	,,	3	7·2	1/320
40	,,	,,	5	7·2	>1/320
40	Broth—Difco peptone	,,	7	6·5	1/20
40	,,	,,	7	7·2	1/40
40	,,	,,	7	7·6	1/20
40	,,	,,	7	7·8	1/160
40	Broth—Parke-Davis peptone	,,	7	7·8	>1/320

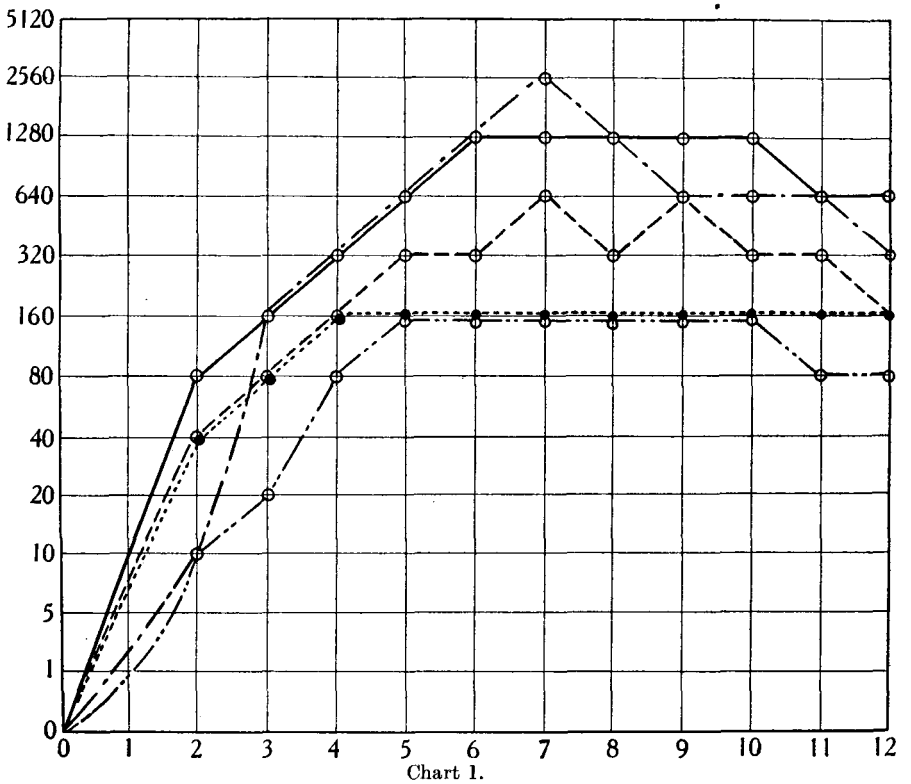

Chart 1.

Parke-Davis infusion broth —o— Parke-Davis low-meat broth —·—o—·—
Neo-peptone infusion broth — —o— — Neo-peptone low-meat broth ···—···o···—···
Infusion broth ···•···

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and although ten different strains were tried, of which eight were able to clot before filtration, no active filtrate was obtained. However, Berkefeld V filtrates of 2–14-day cultures were found to be active, and, except for one or two unexplained failures to obtain a clotting filtrate by these means, consistent results were obtained (Table II). Both the age of the culture and the type of peptone used, however, exerted a considerable effect on the strength of the filtrate. The best results, as may be seen from the table, were obtained with a 5 to 7-day growth on a low-meat medium containing 2 per cent. Parke-Davis peptone adjusted to about *p*H 7·3: the final reaction of this medium, after filtration, was in the neighbourhood of *p*H 8·0 (Chart 1).

Attempts at isolation of the clotting principle

Various attempts have been made to isolate the active principle, but with only partial success.

The clotting factor comes down in the precipitate formed by the addition of alcohol, 10 volumes of 96 per cent. giving the best result: the precipitate can be dissolved in distilled water. The factor is precipitated by acetic acid and re-dissolved when brought to neutrality in distilled water: each of these procedures destroys a small amount of the clotting principle, but both together destroy far more (Table III).

Table III. *Highest clotting dilution: reading* 48 *hours*

Filtrate	1/640
Residue of filtrate after dialysis	1/1280
Alcoholic precipitate	1/320
Residue of alcoholic precipitate after dialysis ...	—
Acetic acid precipitate	1/320
Acetic acid precipitate of alcoholic precipitate ...	1/80
Half-saturation with $(NH_4)_2SO_4$	1/80

The precipitate formed by half-saturation with ammonium sulphate is active, but not all of it goes into solution in distilled water.

When present in the crude filtrate the clotting principle is not dialysable through cellophane against distilled water, though after alcoholic precipitation it becomes dialysable. This appears to contradict the findings of Gross (1928*b*) that rabbit plasma in a cellophane bag suspended in a Staphylococcus culture clots. He failed, however, to give any control to this experiment, so the clotting may have been due to something other than the coagulase. The coagulase is inactive after trypsin digestion.

Effect of heat

Staphylococci suspended in distilled water lost their clotting power after heating in a water-bath for 15 min. at 100° C., most of the power being lost at even shorter exposures. But when a whole broth culture is heated for 15 min. at 100° C. the power is not lost, thus confirming v. Gonzenbach and Uemura (1916). No titration with living cultures was carried out, it was not found

to give reliable results where living organisms were used, since, if any organisms at all were present they would, after 24–48 hours, have grown out sufficiently to give complete clotting, and the end-point obtained would merely indicate that dilution of organisms at which growth still occurred.

As regards the heat stability of filtrates, titrations showed that even after ½ hour at 100° C. the activity was only slightly less than that of the unheated filtrate, thus confirming the findings of Gross (1933).

The acetic acid precipitate is less resistant to heat and storage than is the untreated filtrate, but still retains some activity after both procedures (Table IV).

Table IV. *Highest clotting dilution: reading* 48 *hours*

	Unheated	100° C. for 15 min.
Culture	—	1/640
Filtrate	1/640	1/320
Alcoholic precipitate	1/320	1/160
Acetic acid precipitate	1/320	1/160
Control	—	—

ANTIGENICITY AND DIFFERENTIATION OF CLOTTING PRINCIPLE FROM HAEMOLYSIN

Since a number of the filtrates which have failed to show any clotting power have been strongly haemolytic, it is clear that the two properties cannot be the same, although they generally occur in the same strain. Experiments have been made to confirm this diversity of haemolysin and clotting principle.

A filtrate of known clotting activity was allowed to act on susceptible red cells overnight and was then tested for clotting power: this was found to be exactly the same as before the haemolysis. In other words, although some, if not all, of the haemolysin had been absorbed by the red cells, the clotting principle was not affected.[1]

In a second experiment serial dilutions of an antihaemolytic (antitoxic) serum were mixed with an equal volume (0·5 c.c.) of staphylococcal filtrate: after 1 hour's incubation 0·5 c.c. of a suspension of rabbit's washed red cells were added to each tube: a reading taken after 18 hours showed that no haemolysis had taken place in the tubes containing the higher concentration of antiserum, while in those containing very low concentrations haemolysis was complete, thus indicating that some of the haemolysin in the filtrate had been neutralised. This experiment was repeated, using, instead of the red cells, 0·5 c.c. citrated rabbit plasma and halving the volumes of filtrate and antiserum (this was done so that the plasma would not be too diluted to show a firm clot): all tubes clotted at the same time, as did a control tube in which there was broth instead of antiserum, thus showing that the antihaemolysin in the serum had no effect on the clotting principle.

There are occasional sera in which a neutralisation of the clotting principle is apparent. Since such sera may also contain antihaemolysin, it follows that

[1] Since after haemolysis there was no difference whatsoever in the clotting power, it was not considered necessary to repeat this experiment after absorption of the haemolysin in the cold.

antihaemolytic sera may sometimes show anticoagulant properties, but it should be remembered that since this property does not occur in sera containing antihaemolysin only it is not as a consequence of that substance that clotting is inhibited. This may explain the results of Gross (1933) and Kemkes (1928) the latter finding that antistaphylococcal serum has no anticoagulating properties, while the former reported that certain sera from both sick and healthy persons inhibited coagulation, the anticoagulant power usually corresponding with the antitoxin titre: he was careful, however, to add that the identity of the anticlotting antibody with antihaemolysin was by no means certain.

So far the writer has been unable to produce any resistance to clotting in two rabbits injected intravenously and intraperitoneally with an active clotting filtrate. The antigenic properties of the coagulating substance are therefore still uncertain, though the undoubted fact that resistant sera are found suggests strongly that the "coagulase" is antigenic.

Since we have carried out only two immunising experiments up to the present time, the negative results obtained cannot be regarded as conclusive.

Not only is the clotting substance distinct from haemolysin, but experiments made with Dr Menkin (Menkin and Walston (1935)) and published elsewhere show that it is entirely distinct from the substance responsible for local inflammatory fixation.

Mode of action

The mode of action of the clotting principle was next investigated. The contradictory findings of Gratia (1919) who said that pure fibrinogen was clotted by the "coagulase", and of Much (1908) and Nolf (1919) who denied this, have left these aspects of the problem in an unsatisfactory condition.

There is so much confusion, chiefly of terms, in the literature of blood clotting, that it may be advisable to outline the hypothesis followed in this paper. It is assumed that when blood is shed, the prothrombin normally present in the blood (thrombogen, serozyme) reacts in the presence of calcium ions with the thrombokinase (cytozyme), liberated from the platelets and the damaged tissues or vessel walls. This reaction leads to the formation of thrombin, which, in its turn, reacts with the blood fibrinogen to form fibrin.

In the citrated plasma, which was used in the earlier experiments, the factor essential to clotting which was lacking was calcium. It was therefore conceivable that the Staphylococcus was acting merely by supplying the calcium, though this was unlikely since there was an excess of citrate, and a strong filtrate was able to clot 0·5 c.c. of the citrated plasma even when less than 0·001 c.c. of the filtrate was present. Since this same plasma requires 0·5 c.c. of a 0·5 per cent. $CaCl_2$ solution to clot it, it follows that the filtrate would have to contain as much calcium as a 250 per cent. solution of $CaCl_2$ in order to bring about clotting merely by the addition of calcium. Setting this argument aside, however, and assuming that the clotting is due merely to a supply of the necessary calcium (or some substance which takes the place of calcium), the

clotting process would then proceed along the normal lines set out above, and thrombin would be formed: in this case heparin would inhibit the reaction, acting as it does as an antithrombin (Bancroft, 1935).[1] That this is not so is shown in Table V.

Table V. *Time in hours taken to clot plasma*

Dilution of filtrate	1/1	1/5	1/10	1/20
Heparinised plasma	1¼	2	20	20
Oxalated plasma	1¼	3½	20	20

Since, then, clotting was not caused by the addition of calcium, and since the clotting principle did not take the place of either prothrombin or thrombokinase (both these factors being normally present in shed blood), it may be assumed that the clotting principle either was thrombin (or acted in a manner similar to thrombin) or else was capable of activating thrombin. To answer this question pure fibrinogen was prepared and its susceptibility to coagulase studied. Three methods were used—those of Gratia (1919), of Tillett and Garner (1933), and of Florkin (1930). Fibrinogen solution was not considered pure unless it was clotted by thrombin (fresh serum), but not by calcium and thrombokinase (tissue extract), nor by calcium and prothrombin (inactivated serum). The former procedure showed the absence of prothrombin, and the latter of thrombokinase. The activity of the prothrombin and thrombokinase was checked by adding them both together, and in the presence of calcium, to the fibrinogen solution, which then clotted. The first two methods had the advantage of simplicity, but in our hands failed to give consistently pure fibrinogen. Florkin's method consistently yielded a pure fibrinogen, but was more difficult to obtain in stable solution.

Gratia used citrated plasma, removed the thrombokinase by Berkefeld filtration, and the prothrombin by adsorption with tricalcium phosphate. Gratia was able to clot fibrinogen prepared by this method, but Nolf (1919) failed to confirm his results, believing that Gratia had not used enough phosphate completely to adsorb the prothrombin. However, Gratia's results are confirmed by the experiments recorded in Table VI where samples of plasma were tested after the first, second, and third adsorption.

Table VI. *Clotting time in hours*

Plasma	¼
Fibrin prepared according to Gratia:	
1st adsorption	½
2nd ,,	½
3rd ,,	½
Tillett and Garner	½
Florkin	1

[1] According to Howell and Holt (1918) heparin does not itself act as an antithrombin but, in plasma, causes the appearance of antithrombin: therefore, for the purposes of this argument, where whole plasma and not purified fibrinogen is being used, the heparin may be considered as antithrombin.

Since writing this, a paper by Fischer (1935) has appeared which suggests an entirely new mechanism for the action of heparin: we have had no time, so far, to confirm the results given therein.

The second method consists in precipitating oxalated plasma by one-third saturation with $(NH_4)_2SO_4$ and dissolving the precipitate in $M/100$ phosphate buffered normal saline of *p*H 7·4. Table VI shows that fibrinogen prepared by this method was likewise clotted.

The third method is probably the best, and the fibrinogen solution obtained by it has been shown by Florkin's studies on its solubility to be chemically pure. For our purposes it was considered that two precipitations were sufficient, while Florkin precipitated his solution three times. Citrated plasma, adjusted to *p*H 6·0, is treated with equal volumes of saturated NaCl of *p*H 6·0, and the precipitate dissolved in 2 per cent. NaCl also of *p*H 6·0, to bring the volume back to that of the original citrated plasma. The process is repeated once, the whole being carried out as near 0° C. as possible, otherwise the solution is said to become unstable.

Although taking longer to clot than did the unaltered plasma, fibrinogen prepared by all of these methods was clotted by a staphylococcal filtrate, thus showing the clotting agent is either thrombin or a thrombin-like substance (Table VI).

To decide between these alternatives the effect of heparin was investigated, as mentioned above. Heparin is an antithrombin, reacting quantitatively with thrombin to neutralise it. It was first ascertained that plasma treated with 1 mg. of heparin per c.c. was clotted in the same manner as was citrated or oxalated plasma. Then 0·25 c.c. oxalated plasma was mixed with falling dilutions of 0·25 c.c. heparin solution and a constant amount of staphylococcal filtrate added. To a control series of tubes thrombin (fresh serum) was added. The results shown in Table VII indicate that the clotting principle is not

Table VII. *Time in hours taken to clot plasma*

Heparin dilution	1/5	1/10	1/20	1/40
0·5 c.c. filtrate	1½	1½	1½	1½
0·25 c.c. serum	—	3½	1½	1

thrombin in that it is not affected in any way by heparin. That the clot formed by the addition of "coagulase" to plasma really is fibrin is shown by an experiment conducted with Dr E. W. Dennis, where some streptococcal fibrinolysin was mixed with citrated rabbit plasma and coagulase. The clot which formed was later lysed, thus showing that it was true fibrin.

Additional observations

During the course of this work various random observations have been made.

Cobra venom, which is itself an anticoagulant, fails to inhibit staphylococcal clotting, as does chlorazol-fast pink, although in this case clotting is not so definite as with other anticoagulants. As the mode of action of these two substances is at present unknown, these observations throw no light on the mechanism of staphylococcal clotting.

Hydroquinone does not interfere with this form of clotting, and filtrates are able to act also when mixed with plasma in the absence of oxygen. The reaction proceeds most rapidly at 37° C., slower at 22° C. and very slowly indeed at 2° C.

RESULTS

The results given in this paper show reasons for the apparent contradictions in the reports of earlier workers: there are also some new observations on the properties and conditions of production of the clotting principle, and on its mode of action.

CONCLUSIONS

Certain staphylococcal strains under suitable environmental conditions produce a heat-stable filterable substance capable of clotting pure fibrinogen. This substance is active in a semi-purified state in high dilutions. The activity of this substance may be inhibited by certain human sera but by no other substance so far observed. All human beings suffer from trivial staphylococcal infections, and it is impossible to make certain by inquiry that any individual has not been subject to a moderately severe infection. Consequently, one cannot say whether the properties of a so-called normal serum are the result of natural resistance or acquired immunity.

SUMMARY

1. Whole cultures, suspensions in distilled water, and cell-free filtrates of Staphylococci clot citrated and other plasmas.

2. Whole cultures and filtrates are comparatively thermostable, while suspensions are less so. Partially purified filtrates are likewise less stable than the crude filtrates.

3. The clotting principle is precipitable by alcohol, acetic acid, and ammonium sulphate.

4. The clotting principle is dialysable through cellophane after alcoholic precipitation but not before.

5. The clotting principle is distinct from haemolysin.

6. Sera can be obtained which have a neutralising action on the clotting principle.

7. Pure fibrinogen can be clotted by a staphylococcal filtrate.

8. The clotting principle is distinct from the toxin responsible for local inflammation and fixation.

9. The clot formed by the addition of "coagulase" to plasma is composed of true fibrin.

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