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Factors Influencing the *in vitro* Production of Staphylococcal Coagulase

BY G. E. DAVIES

*Imperial Chemical Industries Ltd., Biological Laboratories,
Hexagon House, Blackley, Manchester*

SUMMARY: The amount of coagulase formed *in vitro* by coagulase-positive staphylococci depends on: the age of the bacterial cells, coagulase being produced only during the latter part of the lag-phase of growth; the period of incubation of the bacterial cells under ideal conditions, the optimum period depending upon the age of the cells when incubation starts; the initial concentration of bacteria. It also depends on the presence in the medium of a fermentable sugar and a factor found in brain-heart infusion and partially replaceable by peptone; the salt concentration in the medium; the initial pH value of the medium; the temperature of incubation; and upon substances present in rabbit serum and, to a lesser extent, horse serum, human serum and egg yolk, egg white and commercial nucleic acid not showing this effect. The activity of rabbit serum is destroyed by treatment with pepsin. Provided that the requisite conditions are met, cell-free coagulase which clots rabbit plasma within a few minutes, can be prepared in less than one hour.

Since the demonstration that coagulase is an important factor in determining the pathogenicity of staphylococci, many papers on this substance have appeared (e.g. Christie & Keogh, 1940; Fairbrother, 1940; Fisher, 1936; Spink & Vivino, 1942; Walston, 1935). Following the lead of Smith & Hale (1944), most workers have attempted to elucidate the mechanism of the reaction of coagulase with fibrinogen (Kaplan & Spink, 1948; Miale, 1949; Tager, 1948*a*; Tager & Hales, 1948). Attempts have also been made to purify coagulase (Tager, 1948*b*; Walker, Derow & Schaffer, 1948) but little attention has been given to the way in which the staphylococci produce coagulase. With a view to investigating the contribution of coagulase to the virulence of staphylococci we have studied the factors which govern its production *in vitro*.

The terminology of the coagulase phenomenon has become confused. We have chosen the simplest names which are in common use. The active principle produced by the organisms in broth is referred to as 'pro-coagulase'. This, together with a substance found in certain sera 'coagulase-reacting factor', forms 'coagulase' which transforms fibrinogen into fibrin.

EXPERIMENTAL METHODS

Inoculum. A 24-hr. culture of *Staphylococcus aureus* (various strains were used) in Wright's broth (Wright, 1933) was centrifuged and the cells suspended in sterile 0.85 % saline. Large clumps of cells were broken up by shaking with glass beads and the strength of the suspension determined by comparison with standard opacity tubes.

Production of coagulase. The inoculum was added to the production-medium, both having been previously warmed to 37°. The mixture was incubated at 37° for the desired period and then thiomersalate was added to give a final concentration of 0.01 %. Coagulase, if present, was contained in the supernatant formed after centrifuging at 4,000 r.p.m. for 10 min. This procedure provided a clear liquid from which over 99 % of the bacterial cells had been removed.

Assay of coagulase. One-tenth ml. of the supernatant was mixed in 3 in. × 3/8 in. tubes with 0.9 ml. of 10 % (v/v) rabbit plasma in saline. The tubes were incubated in a thermostatically controlled water-bath at 37°. The degree of clotting was recorded at intervals as follows: 0=No clot, liquid clear; 1=trace clot, liquid clear; 2=50 % clot, liquid slightly turbid; 3=almost complete clot; 4=complete clot.

An arbitrary unit of coagulase activity was derived from the expression $Kt=100$, where t is the time in min. for a '2' clot to form under the conditions defined above.

RESULTS

In the usual method of testing for coagulase production by staphylococci a portion of broth culture is added to diluted plasma (Fisk, 1940; Williams & Harper, 1946). When the supernatant from this culture is used instead of the whole culture it can be shown that it contains only a small proportion of the total pro-coagulase. A little more pro-coagulase is obtained in the first saline-washing of the cells but none in subsequent washings. The washed cells, however, clot the plasma at about the same titre as the original culture (Table 1).

Table 1. *Production of pro-coagulase by washed staphylococci*

Source of pro-coagulase	Highest dilution giving '2' clot		
	1 hr. reading	2 hr. reading	3 hr. reading
Whole culture	1/24	1/48	1/192
Supernatant	1/6	1/12	1/12
1st washing	— No coagulase at 1/12 —		
2nd washing	— No coagulase at 1/12 —		
Washed cells	1/24	1/24	1/192

We found that thiomersalate (0.01 %) inhibited the production of pro-coagulase by the washed cells but had no action on the pro-coagulase in the supernatant or on the coagulase formed from this pro-coagulase in the presence of plasma. Thiomersalate was, therefore, used in subsequent experiments whenever we wanted to stop the production of pro-coagulase without affecting its action or that of coagulase. It was apparent from the experiments shown in Table 1 that, under the conditions described, the staphylococci were producing pro-coagulase within a very short time, possibly before the cells started to divide. The rapid production of pro-coagulase was proved by the

following experiment. A concentrated suspension of staphylococcal cells (strain BB) was prepared by suspending the washed cells from ten 10 ml. 24-hr. broth cultures in 7 ml. of saline. One ml. of this suspension was added to each of 9 sterile 50 ml. conical Pyrex flasks: 3 containing 10 ml. of Difco brain-heart infusion, 3 containing 9 ml. of this infusion and 1 ml. of rabbit plasma, and 3 containing 1 ml. of the plasma and 9 ml. of saline buffered to pH 7.6. After 1, 2 and 2½ hr. incubation at 37°, one flask of each set was withdrawn from the incubator, the contents centrifuged and thiomersalate added to the supernatants. The coagulase titre of the supernatants against 10 % rabbit plasma in saline was then determined.

The results showed that both broth and plasma must be present for rapid production of coagulase. The supernatant from the complete medium, which had been incubated for only 1 hr., clotted the plasma in 2 hr. at a dilution of 1/192, supernatants from the media lacking either broth or plasma, and incubated for 2½ hr., failed to clot the plasma in 2 hr. at a dilution of 1/6. The active supernatants contained coagulase rather than pro-coagulase since they clotted both plasma and solutions of pure fibrinogen at about the same rate. Living cultures did not clot the fibrinogen solution. The rabbit plasma in the above medium could be replaced by rabbit serum but not by bovine fibrinogen. These experiments indicated that there are two essential factors or groups of factors for the production of coagulase. One is present in rabbit serum and the other in brain-heart infusion.

Experiments on the serum-factor necessary for coagulase production

In order to investigate the effect of serum on bacteria of varying age, the experiment summarized in the following diagram was performed:

We thus had three supernatants from each flask: A, containing pro-coagulase produced in the absence of serum; B, containing coagulase formed

by the action of serum on the cell-free pro-coagulase in A; C, containing coagulase formed by the action of serum on the living cells.

Table 2 and Fig. 1 show that pro-coagulase was first detectable in supernatant A when the cocci had been incubated for 60 min. The amount of pro-coagulase was small (less than 10 units/ml.) and increased to only 45 units/ml. after incubation for a further 300 min. When 10 % of rabbit serum was added at the start of incubation, a large amount (143 units/ml.) of coagulase was produced within 30 min. The results were similar when serum was added to cocci which had been incubated for 30 min. However, cocci

Fig. 1. Influence of rabbit serum on the production of coagulase by staphylococci.

Organism incubated for varying periods in brain-heart infusion; $\odot$ — $\odot$, rabbit serum added and mixture incubated 30 min. and then centrifuged; $\square$ — $\square$, culture centrifuged and supernatant incubated 30 min.; $\triangle$ — $\triangle$, culture centrifuged, rabbit serum added to supernatant and mixture incubated for 30 min.; $\times$ — $\times$, concentration of bacteria as determined by opacity tubes; $\circ$ - - $\circ$, pH of culture in brain-heart infusion.

incubated for 60 min. before the addition of serum produced only 84 units coagulase/ml. in the subsequent 30 min. and those incubated for 120 min. produced only 29 units/ml., an amount corresponding to the pro-coagulase formed in the same time by cocci which had not been acted on by serum (column C, Table 2). Contact between serum and living bacteria was essential for coagulase production; serum added to the cell-free supernatant for 30 min. produced coagulase in amount corresponding to the pro-coagulase already present (column B). Measurements of pH values and determinations of cell concentrations indicated that the period during which serum can stimulate the bacteria to produce coagulase corresponds to the lag-phase of growth.

Rabbit serum exerts its maximal effect with great rapidity provided that the bacteria are at the appropriate stage of growth (i.e. towards the end of the lag-phase; Table 3).

Table 2. *Influence of rabbit serum on the production of coagulase by staphylococci; effect of adding serum to cells of varying age*

Strain: E.B.1.; 0.4 mg. dry wt. cells/ml. Medium: brain-heart infusion, pH 7.6. Incubation temperature: 37°.

Age of cells (min.)	Conc. of cells (mg./ml.)	Supernatant from serum with cells for 30 min. (C)		Serum with supernatant from cells for 30 min. (B)		Supernatant from cells (A)	
		pH	Coagulase units/ml.	pH	Coagulase units/ml.	pH	Coagulase units/ml.
0	0.16	7.23	143	7.21	<10	7.37	<10
30	0.16	7.07	143	7.11	<10	7.26	<10
60	0.19	6.78	84	6.84	12	7.07	25
90	0.19	6.63	45	6.65	25	6.83	29
120	0.23	6.60	29	6.52	25	6.73	29
180	0.32	6.57	33	6.46	33	6.66	25
240	0.38	6.55	25	6.43	29	6.63	27
300	0.42	6.63	29	6.42	29	6.64	40
360	0.56	6.50	29	6.42	33	6.64	45

See Fig. 1.

Table 3. *Influence of rabbit serum on the production of coagulase by staphylococci: effect of varying the age of cells and the time of their contact with serum*

Strain: E.B.1; 0.3 mg. dry wt. cells/ml. Medium: brain-heart infusion+10 % rabbit serum, pH 7.3. Incubation temperature, 37°.

Period of incubation of cells in broth before addition of serum (min.)	Coagulase units/ml. formed after serum has been in contact for				
	5 min.	10 min.	20 min.	30 min.	60 min.
0	<10	<10	<10	15	30
10	<10	<10	<10	15	15
20	15	15	15	20	40
30	40	60	50	50	50
40	60	60	50	60	100
60	100	100	100	80	80

Horse serum, horse plasma and sheep serum are less effective than rabbit serum. With each of these the highest titre of coagulase is reached at about the same time as with rabbit serum and further incubation does not cause the titre to decline. Egg yolk is about as active as horse serum. Egg white, ribo- and deoxyribonucleic acids are inactive. Rabbit serum when treated with pepsin loses its power to promote the formation of coagulase, indicating that the active portion is either a protein or is associated with a protein. Increase in the concentration of rabbit serum above 10 % does not result in

increased production of coagulase; the optimal concentration is in the neighbourhood of 10 %. A large excess (80 %) of serum decreases the yield of coagulase, probably because of the presence of an inhibitor in the serum.

Experiments on the broth-factors necessary for coagulase production

All the foregoing experiments were done using Difco brain-heart infusion as the source of 'broth-factors'. Our efforts were then directed to find a medium simpler than this complex infusion. We found that a medium containing peptone, sodium chloride, glucose and phosphate buffer at pH 8.0 could partially replace the infusion, as shown in Table 4. In these and in all subsequent experiments in this section, 10 % rabbit serum was used as source of serum-factor. The amount of coagulase produced was largely independent of the concentration of peptone between 0.5 and 5.0 % and of the concentration of glucose, also between 0.5 and 5.0 %. The ratio of activity between brain-heart infusion and the optimal glucose-peptone medium varied slightly in different experiments but on the whole the infusion was rather more efficient.

Table 4. *Effect of varying the concentration of glucose and peptone on the production of coagulase by staphylococci*

Strain: B.B.; 0.35 mg. dry wt. cells/ml. Medium: Evans Peptone; 0.5 % glucose; NaCl; 0.25 % Na_2HPO_4 ; 10 % rabbit serum; pH 7.9. Incubation temperature 37°.

Peptone (%)	Glucose (%)	Coagulase units/ml. formed in 1 hr.
5.0	0.5	130
2.0	0.5	170
1.0	0.5	140
0.5	0.5	130
1.0	0	70
1.0	1.0	140
1.0	2.0	160
1.0	5.0	130
Brain-heart infusion, pH 7.3:		140

Ether-extraction of the brain-heart infusion did not alter its capacity to serve as a medium for coagulase production and addition of the extracted lipids to the peptone-glucose solution did not improve the latter. Acid-hydrolysed casein was about half as efficient as peptone when both were prepared in 0.5 % glucose buffer. The yield of coagulase was independent of the concentration of casein hydrolysate between 0.5 and 5.0 %. We considered it possible that peptone and casein hydrolysate were less efficient than the infusion because they were poorer as growth media, i.e. they would prolong the lag-phase so that the period of maximal coagulase production would be reached later. However with three concentrations of peptone the yield of coagulase after incubation for 2 hr. was the same as that after 1 hr.

Influence of pH on the production of coagulase

It can be seen from Table 2 that, concurrent with the production of coagulase in the complete medium, there is a rapid fall in the pH value of the medium. When the pH of the medium was kept between 7·3 and 7·6, the maximum yield of coagulase was reached rather earlier but was not higher than that produced when the pH value was allowed to fall (Table 5). In these experiments, again, the results indicated that coagulase was produced during the lag-phase of growth.

Table 5. *Influence of pH value on production of coagulase by staphylococci; comparison of neutralized with non-neutralized medium*

Strain: B.B.; 0·47 mg. dry wt. cells/ml. Medium: brain-heart infusion + 10 % rabbit serum. Incubation temperature 37°.

Period of incubation (min.)	pH not controlled			pH kept at between 7·3—7·6		
	pH	Cell conc. (mg./ml.)	Coagulase units/ml.	pH	Cell conc. (mg./ml.)	Coagulase units/ml.
0	7·30	0·47	0	7·30	0·47	0
10	7·20	0·47	35	7·30	0·47	<10
20	7·16	0·47	120	7·38	0·47	10
30	6·99	0·59	170	7·40	0·47	145
40	6·89	0·71	120	7·34	0·59	200
50	6·89	0·71	170	7·60	0·59	250
60	6·73	0·75	200	7·60	0·85	200
90	6·53	0·85	290	7·45	0·85	200
120	6·47	0·94	290	7·34	0·85	200
180	6·42	0·94	290	7·30	0·85	200
360	6·34	0·94	200	—	—	—

The yield of coagulase does, however, depend upon the initial pH value of the medium as is shown in Table 6 and Fig. 2. With an initial pH of 6·1 no coagulase was produced. Increase in initial pH resulted in increased yield of coagulase which reached a maximum with initial pH values between 8·4 and 9·3. With an initial pH value of 9·3 the rate of coagulase production was slower but the final yield was greater (Fig. 2). Fig. 3 shows that coagulase is produced when the actual pH of the medium is between about 7 and 8·5 with a maximum yield at pH 7·6.

Influence of aeration on the production of coagulase

When the medium was gently stirred, by directing a stream of air on to the surface, more coagulase (660 units/ml.) was produced than in a control non-aerated flask (330 units/ml.). Incubation beneath a vaseline seal resulted in a lower yield of coagulase (100 units/ml.). No attempt was made to separate the effects of aeration and agitation.

Table 6. *Influence of the initial pH of the medium on the production of coagulase by staphylococci*

Strain: E.E.; 0.4 mg. dry wt. cells/ml. Medium: 1% proteose peptone; 0.5% NaCl; 0.25% Na₂HPO₄; 10% rabbit serum. Incubation temperature 37°.

Period of incubation							
0 min.		30 min.		60 min.		90 min.	
pH	units/ml.	pH	units/ml.	pH	units/ml.	pH	units/ml.
6.15	<10	5.85	<10	5.62	<10	5.56	<10
6.92	<10	6.67	29	6.50	33	6.46	40
7.62	<10	7.21	67	6.95	84	6.89	84
8.39	<10	7.66	84	7.26	84	7.24	100
9.30	<10	8.40	29	7.67	84	7.61	120
9.95	<10	10.03	<10	9.93	<10	9.74	<10

See Fig. 2.

Fig. 2. *Influence of the initial pH of the medium on the production of coagulase by staphylococci in glucose-peptone medium containing rabbit serum.*

Influence of cell concentration

For each strain of organism there is an optimum concentration of staphylococci for the production of coagulase. For instance, *Staph. aureus* strain BB

in brain-heart infusion with 10 % rabbit serum produced 200 coagulase units/ml. when the cell-concentration was between 3.5 and 7.0 mg. dry wt./ml.; at 35 mg./ml. only 50 units coagulase/ml. were produced.

Fig. 3. Relationship between actual pH of medium at time of sampling and the concentration of coagulase contained in the sample. Results from experiment which is also illustrated in Fig. 2. ○ — ○, 30 min. incubation; △ — △, 60 min. incubation; □ — □, 90 min. incubation.

Influence of temperature

Coagulase is produced at temperatures between 20 and 40°, with a maximum yield at about 35°. An experiment in which the staphylococci were incubated in brain-heart infusion with 10 % rabbit serum at various temperatures gave the following results: 2°, <5 units coagulase/ml.; 20°, 40 units/ml.; 30°, 200 units/ml.; 35°, 250 units/ml.; 40°, 140 units/ml.; 45° <5 units/ml.

Influence of concentration of salt

The total salt concentration influences the yield of coagulase. An optimal salt concentration was reached at 0.5 % NaCl with 0.25 % Na_2HPO_4 . With concentrations of NaCl greater than 5 % no coagulase was produced.

DISCUSSION

At a particular phase of their growth, corresponding to the latter part of the lag-phase, staphylococci, in defined conditions of cell-concentration, pH, temperature and composition of medium, and in the presence of a protein in rabbit serum, produced a large amount of coagulase within a few minutes. In the absence of serum under the same conditions only a small amount of pro-coagulase was produced. These observations may be interpreted in two ways: (1) The cells form pro-coagulase in large amounts on their surface during the lag-phase. Normally this pro-coagulase is mainly bound to the surface and only a small proportion is shed into the medium. Serum-factor disrupts this bond with the consequent liberation of pro-coagulase and the formation of coagulase. As the age of the cells increases, during the phase of active cell-division, the pro-coagulase at the cell-surface, but not that in the surrounding medium, is destroyed and the subsequent addition of serum is without effect. (2) The cells form pro-coagulase in small amounts during the lag-phase and shed it in the surrounding medium. Serum-factor accelerates this formation of pro-coagulase. When cell-division starts, pro-coagulase is no longer produced.

We have seen from the experiment shown in Table 3 that serum exerts its effect with great rapidity since, when the cells are 60 min. old, serum can cause the formation of a maximal amount of coagulase in less than 5 min. The speed of this reaction is more in accordance with the hypothesis that already-formed pro-coagulase is liberated, than that which assumes the production of fresh coagulase. This transient production of a substance by young cells is reminiscent of capsule formation (Lyons, 1937). The possible identity of the serum-factor with the reaction-factor of Smith & Hale (1944) remains to be considered. The broth-factors appear to function simply as nutrients, i.e. they assist the cells to start their growth-cycle.

If these results can be applied to the more complex conditions obtaining *in vivo*, they enable us to put forward the following picture of the sequence of events taking place in the formation of a staphylococcal lesion. When bacteria gain access to the tissues, there is a time-lag before cell-division starts. During this lag-period the defence mechanisms of the host are being mobilized with great speed and the organism must protect itself with even greater speed if it is to establish itself. We have seen from the experiments described above that staphylococci can produce coagulase within a few minutes. Hale & Smith (1945) have shown that coagulase forms a barrier of fibrin round the bacteria and protects them from phagocytosis. The production of too much coagulase would be a liability to the organism, since an impermeable barrier of fibrin would prevent diffusion of nutrient material, and would also be a mechanical hindrance to colonial expansion. But we have shown that coagulase is produced only during the lag-phase, and furthermore, certain strains of staphylococci can produce a fibrinolysin (Christie & Wilson, 1941) which will destroy the already-formed barrier. According to this view, therefore, the establishment of a staphylococcal lesion is an orderly sequence of

events: the initial inoculum is protected from phagocytosis by a barrier of fibrin; later this barrier is removed and by this time the cells have elaborated a series of toxic principles (haemolysins, leucocidins, hyaluronidase, etc.) to assist them in their conflict with the phagocytes and to further the development of the lesion.

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