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Extraction of Bound Coagulase from *Staphylococcal* cells

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SUMMARY

With the use of bound coagulase positive and free coagulase negative *Staphylococci*, attempts were made to extract bound coagulase by the two methods of grinding extraction and phenol extraction. As a result, the following properties of the extracts were clarified. With both the ground extract and phenol extract, properties considered to be specific to bound coagulase were seen, such as its ability absorb agglutinin, the clumping inhibiting antibody in immune sera and fibrinogen adsorbing activity. The fibrinogen adsorbing substance obtained from the ground extract was non-dialyzable, while the adsorbing substance obtained by phenol extraction was dialyzable. While the former could be observed in the ground extracts from bound coagulase negative cells and trypsin treated bound coagulase positive cells, the latter could be seen only in bound coagulase positive cells. From this, normal cells have been proved to contain two different fibrinogen adsorbing substances. The phenol extract was divided into two fractions by acetone fractionation. The precipitated fraction was non-dialyzable, antigenic, and could absorb clumping inhibiting antibody in antiserum. The supernatant fraction adsorbed fibrinogen and was dialyzable. Thus, these two substances were different entities.

INTRODUCTION

Bound coagulase of *Staphylococcus* may be readily identified because it is clumped when added to fibrinogen on a slide. For this, no plasma activator is required, unlike the case with free coagulase (Tager *et al.*, 1948). The coagulase is presumed to be on the cell surface and is stable at an acidic pH. It is stable at 100°C, but destroyed by autoclaving and by proteolytic enzymes, a fact which is considered to be one of its characteristic properties. Duthie (1954), utilizing the property of bound coagulase that it may be released into the medium during autolysis, undertook the extraction of bound coagulase from a cell autolysate. The substance extracted neutralized antibodies found in staphylo-antiserum which inhibit the clumping of *Staphylococcal* cells. Furthermore, it produced specific antibodies which inhibited the clumping of living *Staphylococcal* cells in rabbits. Therefore he supposed that it contained bound coagulase. However, the substance extracted did not show any direct reaction with fibrinogen. Again in 1955 he presented evidence that adsorption of fibrinogen and cell clumping occur at a different pH range and Na ion concentration. Furthermore he reported that cells coated with fibrinogen could absorb the antibody in serum, while cells coated with antiserum could not adsorb fibrinogen. In spite of this difference, both activities were simultaneously lost with trypsin. Therefore he presumed that a

single substance was responsible for both activities but the reaction was caused by a two-stage mechanism with different sites of action. However, evidence in support of this hypothesis is as yet rather incomplete (Duthie, 1953).

The selection of a suitable strain and the establishment of a method of extraction seem to be major factors in solving this problem. We therefore attempted to extract bound coagulase by using a free coagulase negative and bound coagulase positive D_2 culture, which was derived from the Newman strain of *Staphylococcus aureus*, by grinding extraction and by extraction with 90 per cent phenol.

Extracts thus obtained showed properties considered to be specific to bound coagulase, such as ability to absorb both agglutinin and clumping inhibiting antibody in serum and ability to adsorb fibrinogen. The phenol extract was fractionated with acetone. One fraction had antigenic properties and the other had fibrinogen adsorbing properties.

MATERIALS AND METHODS

1. Strains used

D_2 : Free coagulase negative and bound coagulase positive strain.

D_3 : Free and bound coagulase negative strain.

Both D_2 and D_3 strains are variants of the Newman strain of *Staphylococcus aureus*, and have been selected and maintained in the Laboratory of Department of Bacteriology, Osaka University Medical School (Kashiba *et al.*, 1959).

2. Fibrinogen

Difco bovine fibrinogen dissolved in physiological saline was used.

3. Bound coagulase activity of D_2 cells

Cultures grown in brain heart infusion broth for 18 hours were washed with saline and resuspended in saline to a concentration of 5×10^9 cells/ml. This concentration was used throughout the present studies as the standard density of cells.

0.2 ml of a D_2 cell suspension of standard density caused distinct clumping when mixed with 0.2 ml of 0.0005 per cent freshly prepared fibrinogen solution and shaken for five minutes. The degree of clumping of D_2 cells was checked in each experiment and cells giving little clumping were discarded.

4. Trypsin

Difco trypsin was dissolved to 0.2 per cent in physiological saline, and was added to an equal quantity of substrate. It was then reacted for 2 hours at 37°C. Then the trypsin was inactivated by heating at 100°C for 15 minutes.

5. Antisera

Antisera to D_2 strain and to D_3 strain were obtained as follows.

Cultures grown in a brain heart infusion broth for 18 hours were washed with saline and resuspended at the standard density in saline. They were killed by heating at 100°C for 5 minutes. One ml of cell suspension was injected intravenously into rabbits four times at weekly intervals. One week after the last injection the rabbits were bled. Sera were inactivated at 60°C for 30 minutes, and preserved with merthiolate (1:10000).

Anti- D_2 serum showed agglutination against D_2 cells at a dilution of 1:14000. Anti- D_2 serum was absorbed by D_2 cells treated with trypsin to remove antibody common to bound coagulase negative *Staphylococci*. The absorbed anti- D_2 serum, referred to as D_2 serum hereafter, did not even agglutinate D_3 cells but still retained agglutinin against homologous D_2 cells at a dilution of 1:400. D_2 serum was used throughout the present studies.

6. *Agglutinin absorbing test*

0.1 ml of the samples to be tested was added to 0.1 ml of increasing dilutions of D_2 serum in saline in 5×0.8 cm tubes and shaken for 30 minutes, at 37°C . Then 0.2 ml of a D_2 cell suspension of the standard density was added to 0.2 ml of each of the series of dilutions (centrifugal supernatant in the case of cell suspension), and after being shaken again for 30 minutes at 37°C agglutination was examined. The agglutinin absorbing titre was defined as the highest dilution at which no agglutination was seen. When a D_2 cell suspension of standard density was used as the absorbing agent, it absorbed 4 units of agglutinin.

7. *Titration of clumping inhibiting antibody in sera*

0.2 ml of D_2 cell suspension of standard density was added to 0.2 ml of increasing dilutions of D_2 serum in saline in 5×0.8 cm tubes and after being shaken for 20 minutes at room temperature, 0.1 ml of 0.2 per cent fibrinogen solution was added to each tube, the tubes were reshaken for 5 minutes at room temperature, and clumping of D_2 cells was examined. The highest dilution of serum causing inhibition of cell clumping was taken as the clumping inhibiting titre of the serum. The clumping inhibiting titre of D_2 serum was 1:400. No inhibiting antibody was observed in D_2 serum.

8. *Absorption test of clumping inhibiting antibody in antisera*

0.2 ml of the samples to be tested was added to 0.2 ml of increasing dilutions of D_2 serum in saline in 5×0.8 cm tubes, and was shaken for 20 minutes at room temperature. Then, 0.2 ml of D_2 cell suspension of standard density was added to 0.2 ml of each of the series of dilutions (centrifugal supernatant in the case of cell suspensions), and after being shaken for 20 minutes at room temperature, 0.1 ml of 0.2 per cent fibrinogen solution was added, and shaken vigorously for a further 5 minutes. Thus, by means of the clumping of the D_2 cells the ability of the test samples to absorb clumping inhibiting antibody was examined. When D_2 cells of standard density were used as absorbing agent, they completely absorbed 4 clumping inhibiting units in D_2 serum.

9. *Test of fibrinogen adsorption*

0.1 ml of samples to be tested was added to 0.1 ml of graded dilutions of fibrinogen (0.0005~0.004 per cent) in 5×0.8 cm tubes. The mixture was violently shaken for 5 minutes, and then 0.2 ml of D_2 cells of standard density was added (in the case of cell suspensions, a centrifugal supernatant). The mixture was vigorously shaken for 5 minutes to test for the presence or absence of clumping. When D_2 cells of standard density were used as test samples, the minimum concentration of fibrinogen giving positive clumping was 0.004 per cent.

10. *Precipitation*

Extracts to be tested were layered onto antisera in precipitation tubes 0.3 cm in diameter and the precipitation ring was examined after 1 hour and 3 hours, at room temperature.

In examining the precipitation in agar gel Ouchterlony's method was followed. 0.7 per cent Difco Bacto-agar in Petri dishes, adjusted to pH 7.1 with M/30 phosphate buffer, and contained 1/10000 merthiolate was used. In the test, agar holes were made in the agar plate, and 0.2 ml of serum or 0.2 ml of the extracts to be tested were placed in the holes and observed during 12 days incubation at 37°C .

RESULTS

1. *Extraction by ground cells*

A. *Method of extraction:*

Extraction of bound coagulase from ground D_2 cells was attempted. The details

of the method are shown in Table 1.

Table 1. Method of grinding extraction

5g wet cells + equal volume of glass powder (150 mesh) grinding in mortar 60 min.	500 mg Extract A + 10 ml dist. water
15 ml saline	0.25 sat. (NH ₄) ₂ SO ₄
centrif. 3000 rpm 5 min.	centrif. 14000 rpm 5 min.
sup. ppt.	sup. ppt.
centrif. 7000 rpm 20 min.	0.75 sat. (NH ₄) ₂ SO ₄
sup. ppt.	centrif. 14000 rpm 20 min.
centrif. 16000 rpm 40 min.	ppt. sup.
sup. ppt.	10 ml dist. water
100°C 10 min.	centrif. 14000 rpm 20 min.
centrif. 14000 rpm 20 min.	sup. ppt.
sup. ppt.	dialyze against tap water
pH 3.0 with 1-N HCl	0.6 sat. (NH ₄) ₂ SO ₄
centrif. 14000 rpm 20 min.	centrif. 14000 rpm 20 min.
sup. ppt.	ppt. sup.
adjust to pH 7.0	5 ml dist. water
dialyze against tap water	centrif. 14000 rpm 20 min.
lyophilize 500 mg	sup. ppt.
Extract A	dialyze against tap water
	lyophilize 70 mg
	Extract B

Extract A is a crude extract from which extract B was obtained by salting out twice and dialysis. A 5 per cent solution of extract A was of light yellowish, transparent and non-viscous, while a 1 per cent solution of extract B had no colour, was transparent and non-viscous. Both gave positive biuret and ninhydrin reactions, while the Molisch sugar reaction was negative. As controls, extracts of D₃ cells and D₂ cells treated with trypsin were prepared with same procedure as used for D₂ cells.

B. Properties of ground extracts :

The extract of D₂ cells thus obtained did not show any clumping when mixed with fibrinogen. Therefore, its properties were examined as follows.

- i. Reaction to antibody specific to bound coagulase in sera
- ii. Reaction to bovine fibrinogen
- iii. Inactivation of the extract by autoclaving and trypsin

1) *Test for agglutinin absorption:*

As already mentioned, D₂ serum, fully absorbed with D₂ cells treated with trypsin (termed D₂-T cells), still readily agglutinated D₂ cells which were positive for bound coagulase, but had no agglutinin to the bound coagulase negative variant D₃ cells derived from D₂ strain. Tests for activity to absorb agglutinin in D₂ serum were performed with extracts A and B. The results are shown in Table 2. These extracts had the ability to absorb agglutinin specific for bound coagulase positive cells, but this activity was completely inactivated by trypsin treatment and autoclaving. The extracts from D₃ and D₂-T cells, obtained by the same process, did not absorb agglutinin.

Table 2. Agglutinin absorption of ground extracts

D ₂ serum Agglutininogen	Agglutinin units			
	4	2	1	0
Standard D ₂ cell suspension				
5% Extract A (D ₂ *)	—	—	—	—
1% Extract A (D ₂)	++	±	—	—
1% Extract B (D ₂)	—	—	—	—
0.2% Extract B (D ₂)	++	±	—	—
Trypsin treated				
5% Extract A (D ₂)	++	++	++	—
Trypsin treated				
1% Extract B (D ₂)	++	++	++	—
Autoclaved				
5% Extract A (D ₂)	++	++	++	—
5% Extract A (D ₃)	++	++	++	—
5% Extract A (D ₂ -T)	++	++	++	—
D ₂ cells	±	—	—	—
D ₂ -T cells	++	++	++	—
Autoclaved D ₂ cells	++	++	++	—
D ₃ cells	++	++	++	—
Saline	++	++	++	—

++, +, ±, —, Indicates degree of agglutination.

* () Indicates cells from which extracts were obtained.

2) *Test for absorption of clumping inhibiting antibody:*

D₂ serum has an antibody which inhibits the clumping of D₂ cells in the presence of fibrinogen (it is not recognized in D₃ serum). This antibody was found to be absorbed by D₂ cells. Extracts A and B were both tested for their ability to absorb the clumping inhibitory antibody in D₂ serum. As shown in Table 3, these extracts could absorb this antibody, the activity of which was completely lost by trypsin treatment or autoclaving.

These facts, together with those shown in the foregoing chapter, presumably indicate that the extracts tested contained bound coagulase.

Table 3. Absorption of clumping inhibiting antibody of ground extracts

D ₂ serum	Units of clumping inhibiting antibody			
	4	2	1	0
Clumping material	Standard D ₂ cell suspension 0.2% bovine fibrinogen			
1% Extract B (D ₂ *)	++	++	++	++
0.2% Extract B (D ₂)	—	±	++	++
Trypsin treated				
1% Extract B (D ₂)	—	—	—	++
Autoclaved				
1% Extract B (D ₂)	—	—	—	++
5% Extract A (D ₂)	++	++	++	++
5% Extract A (D ₂ -T)	—	—	—	++
5% Extract A (D ₃)	—	—	—	++
D ₂ cells	±	++	++	++
D ₂ -T cells	—	—	—	++
D ₃ cells	—	—	—	++
Saline	—	—	—	++

++, +, ±, —, Indicates degree of clumping.

* Same as in Table 2.

3). Precipitation test :

To see whether the inhibition of clumping by antiserum is indeed a specific antigen-antibody reaction, a study was made of the precipitation of extracts obtained in D₂ serum. Neither in tubes nor in agar gels, was any evidence of precipitation recognized.

Table 4. Test for fibrinogen adsorption of ground extracts

Clumping material	Fibrinogen per cent			
	0.002	0.001	0.0005	0
	Standard D ₂ cell suspension			
1% Extract B (D ₂ *)	—	—	—	—
0.4% Extract B (D ₂)	++	±	—	—
2% Extract A (D ₂)	+	—	—	—
Trypsin treated				
2% Extract A (D ₂)	+	—	—	—
Autoclaved				
2% Extract A (D ₂)	±	—	—	—
2% Extract A (D ₂ -T)	+	—	—	—
2% Extract A (D ₃)	±	—	—	—
D ₂ cells	±	—	—	—
D ₂ -T cells	++	++	++	—
D ₃ cells	++	++	++	—
Bouillon	++	++	++	—
Saline	++	++	++	—

++, +, ±, —, Indicates degree of clumping.

* Same as in Table 2.

4). *Test for fibrinogen adsorption:*

The extracts obtained did not cause any noticeable clumping when mixed with fibrinogen, so tests were made of whether the extracts could adsorb bovine fibrinogen. Results shown in Table 4 may be summarized as follows.

i) Fibrinogen adsorbing activity may be seen in intact D_2 cells but not in intact D_3 cells and D_2 -T cells.

ii) Both extracts A and B from D_2 cells can adsorb fibrinogen, and these activities are not inactivated by trypsin treatment or autoclaving.

iii) Extracts from D_2 -T and D_3 cells, unlike intact cells, adsorb fibrinogen.

The fibrinogen adsorbing activity, which is considered to be specific only for bound coagulase positive cells(i), is also present in extracts of D_2 cells, but the fact that this activity is resistant to trypsin and autoclaving(ii), together with the observation (iii), should be studied further.

5). *Fibrinogen adsorbing activity of trypsin digested fluid:*

As mentioned above, the fibrinogen adsorbing activity of D_2 cells may be lost by trypsin treatment. To explain why the fibrinogen adsorbing activity was detected in extracts of trypsin treated cells the fibrinogen adsorbing activity of the trypsin digested fluid of D_2 cells was studied. The results are as shown in Table 5. Substances responsible for fibrinogen adsorbing activity were found to be released into the digestion fluid retaining their resistance to trypsin, and the fibrinogen adsorbing substance seen in the digested fluid dialyzed through a cellophane membrane, while the fibrinogen adsorbing substance of the extracts did not dialyzed.

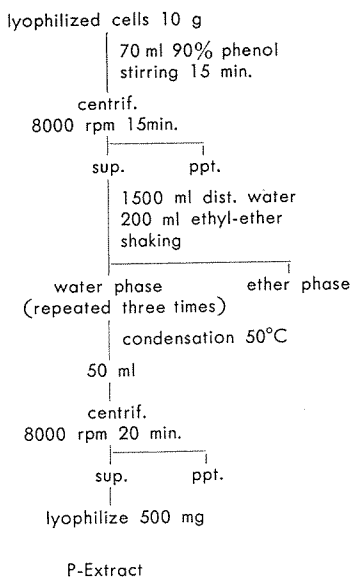
Table 5. Fibrinogen adsorbing activity of trypsin digested fluid and ground extracts of D_2 cells

	Absorption of agglutinin in D_2 serum	Absorption of clumping inhibiting antibody in D_2 serum	Adsorption of fibrinogen
D_2 cells	+	+	+
Trypsin treated D_2 cells	—	—	—
Extract A (D_2)	+	+	+
Trypsin treated Extract A (D_2)	—	—	+
Trypsin digested fluid (D_2)	—	—	+
Extract A (D_2 -T)	—	—	(dialyzable) +
			(non dialyzable)

2. *Extraction with phenol*A. *Method of phenol extraction:*

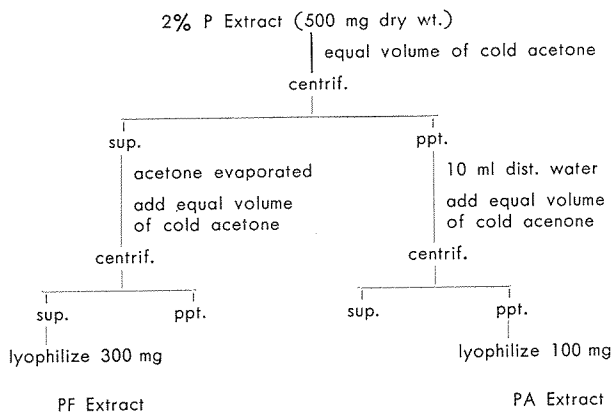
Bound coagulase was extracted with 90 per cent phenol. The details of the method are shown in Table 6.

Table 6. Method of phenol extraction



The phenol extract (termed P-extract) did not show any noticeable sedimentation on heating for 10 minutes at 100°C or at pH 3.0. It was light yellowish, transparent and non-viscous. To purify the P-extract further, it was fractionated with cold acetone.

Table 7. Acetone fractionation of phenol extracts



A 50 per cent acetone precipitate and supernatant fluid were obtained. The 50 per cent acetone precipitate was called PA and the supernatant fluid PF (Table 7). PA had no colour and was transparent and non-viscous, while PF was light yellowish, transparent and non-viscous. Extracts were also obtained from trypsin treated D_2 cells by the same process, and were used as controls.

B. Properties of phenol extracts :

The phenol extracts were tested in the same way as the ground extracts. The results obtained are summarized in Table 8.

Table 8. Antibody absorption and fibrinogen adsorption of phenol extracts

	Agglutinin units			Clumping inhibiting antibody units			Fibrinogen per cent		
	4	2	0	4	2	0	0.002	0.0005	0
1% P Extract (D_2 *)	—	—	—	++	++	++	—	—	—
0.1% P Extract (D_2)	+	—	—	—	++	++	+	—	—
Trypsin treated 1% P Extract (D_2)	++	++	—	—	—	++	—	—	—
Autoclaved 1% P Extract (D_2)	++	++	—	—	—	++	—	—	—
1% P Extract (D_2 -T)	++	++	—	—	—	++	++	++	—
0.1% PA (D_2)	—	—	—	++	++	++	++	++	—
Trypsin treated 0.1% PA (D_2)	++	++	—	—	—	++	++	++	—
Autoclaved 0.1% PA (D_2)	++	++	—	—	—	++	++	++	—
0.1% PF (D_2)	++	++	—	—	—	++	—	—	—
Trypsin treated 0.1% PF (D_2)	++	++	—	—	—	++	—	—	—
Autoclaved 0.1% PF (D_2)	++	++	—	—	—	++	—	—	—
Phenol treated D_2 cells	++	++	—	—	—	++	++	++	—
Extract A (Phenol treated D_2 cells)	++	++	—	—	—	++	—	—	—
D_2 cells	±	—	—	±	++	++	—	—	—
D_3 cells	++	++	—	—	—	++	++	++	—
Saline	++	++	—	—	—	++	++	++	—

++, +, ±, —, *, Same as in Table 2~4.

The properties of the phenol extracts of D_2 cells were closely similar to those of the ground extracts mentioned above, in respect to their ability to absorb agglutinin, the clumping inhibiting antibody in D_2 serum and the fibrinogen adsorbing activity. These properties could not be retained by the cell residues after phenol extraction, so that they must have been extracted from the cells by the

phenol. In fact, D₂ cells treated with phenol did not cause clumping even when mixed with sufficient amounts of bovine fibrinogen. However, the ground extracts of the cell residues after phenol extraction, like the ground extracts of D₂ trypsin treated cells, retained their fibrinogen adsorbing activity. Phenol extracts from D₂ cells already treated with trypsin showed no evidence of adsorbing fibrinogen.

Phenol extracts PA and PF were tested for their precipitation reaction in D₂ and D₃ sera, in tubes and in agar gels. There was a negative reaction.

Extracts PA and PF were tested for properties specific to bound coagulase as well as for dialyzability. The results thus obtained, shown in Table 9, may be summarized as follows.

Table 9. Properties of PA and PF extracts

	Absorption of agglutinin	Absorption of clumping inhibiting antibody	Adsorption of fibrinogen
PA	+	+	—
PA dialyzed solution	+	+	—
Trypsin treated PA	—	—	—
PF	—	—	+
PF dialyzed solution	—	—	—
PF dialyzate	—	—	+
Trypsin treated PF	—	—	+

The crude phenol extract of D₂ cells, which contains a substance with ability to absorb agglutinin and clumping inhibiting antibody in D₂ serum and to adsorb fibrinogen, may be separated by acetone fractionation. The fraction, precipitated by 50 per cent acetone, has the ability to absorb antibody but not fibrinogen. This is non-dialyzable. The fraction, which is not precipitated by 50 per cent acetone, has only the fibrinogen adsorbing activity and is dialyzable. The precipitate fraction loses activity with trypsin, while the fibrinogen adsorbing activity of supernatant fraction is resistant to trypsin.

DISCUSSION

Serum used for the serological verification of bound coagulase was anti-D₂ serum from which antibodies reacting with D₂ trypsin treated cells had been completely removed. It could no longer agglutinate even D₃ cells, a bound coagulase negative variant of D₂ cells, but it retained its activity to inhibit clumping of D₂ cells in the presence of fibrinogen. As for D₃ serum, it neither agglutinated D₂ cells, nor inhibited D₂ cell clumping.

The extracts of D₂ cells, obtained either after grinding or directly by phenol extraction, could neutralize the clumping inhibiting antibody in D₂ serum even at as high a dilution as 1:1000. Furthermore these properties of the extracts were destroyed by trypsin treatment or autoclaving. These data will afford a good proof that they contained bound coagulase. However, in the precipitation reaction both in tubes and in agar gel, they showed no reaction to D₂ serum. This

might be because of a too low titre of the serum antibody.

With regard to the fibrinogen adsorbing activity of the extracts, it was found that, while neither extracts noticeably clumped even when mixed with fibrinogen, fibrinogen adsorbed by the extracts did not cause clumping even when mixed with D₂ cells. The extracts may therefore have a peculiar adsorbing activity against fibrinogen.

Since the fibrinogen adsorbing activity was seen in D₂ cells but not in D₃ cells, this activity may be considered to constitute one of the characteristic properties of bound coagulase positive cells. In view of this, the ability of extracts to adsorb fibrinogen may be taken as another proof of bound coagulase in the extracts. However, even after trypsin treatment and autoclaving both extracts retained fibrinogen adsorbing activity. This should be especially noted, as compared with the fact that intact D₂ cells did lose their ability to adsorb fibrinogen following treatment with trypsin or autoclaving.

The fibrinogen adsorbing substance in the ground extract was non-dialyzable and trypsin resistant. However, fibrinogen adsorbing substance extracted from the same cells by phenol was dialyzable and trypsin resistant. So it may be presumed that there are two kinds of fibrinogen adsorbing substance in intact D₂ cells. By grinding, the fibrinogen adsorbing substances is also extracted from D₂-T and D₃ cell residues after phenol extraction, a fact favoring the assumption that reactive substance in question was derived from the inner parts of cells. On the contrary, the fibrinogen adsorbing substance obtained from D₂ cells by phenol extraction showed the same behavior in its dialyzability as the adsorbing substance seen in the trypsin digested fluid. Moreover, it was not present in a phenol extract from trypsin treated D₂ cells. Therefore it may be inferred that this substance is bound on the cell surface. Since the fibrinogen adsorbing substance, which is resistant to trypsin, is present in the trypsin digested fluid and phenol extract of D₂ cells, it seems that, when the antigenic substance of bound coagulase is destroyed by trypsin, the fibrinogen adsorbing substance is peeled off the cell surface. Perhaps, for this reason that the bound coagulase activity is often lost on storage of D₂ cell suspension in an ice room. The extraction of bound coagulase from an autolysate by Duthie was also assumed to be due to peeling off.

The phenol extract was divided into a precipitate and supernatant fraction in 50 per cent cold acetone. The precipitate fraction could absorb the clumping inhibiting antibody but had no fibrinogen adsorbing activity. It was non-dialyzable and inactivated by trypsin. The supernatant fraction did not absorb clumping inhibiting antibody but could adsorb fibrinogen. It was dialyzable and was not inhibited by trypsin.

Thus the antigenic substance of bound coagulase and the fibrinogen adsorbing substance have been separated by acetone fractionation of the phenol extracts. Duthie, from experiments made with intact cells, concluded that clumping and fibrinogen adsorption are caused by a single substance, in a two step reaction. The present extraction of two separate substances may throw some light on the nature of bound coagulase.

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