FACTORS AFFECTING THE PRODUCTION OF STAPHYLOCOAGULASE IN A CHEMICALLY DEFINED MEDIUM

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The ability to produce coagulase provides a characteristic reaction for the separation of pathogenic staphylococci from saprophytic organisms of similar morphology and biological behavior. All pathogenic staphylococci produce two types of coagulase, a free and a bound form (Cadness-Graves et al., 1943). This was later confirmed by Duthie (1954) who noted a bound form associated with the cell and a cell-free form that is liberated into the culture medium.

There is generally no difficulty in demonstrating the presence of coagulase in high concentrations when *Staphylococcus aureus* is grown in infusion or simple digest broth (Elek, 1959). Staphylococci could also be grown in chemically defined media (Fildes et al., 1936; Gladstone, 1937; Gale, 1949; Lominski et al., 1950; Ramsey and Pardon, 1954; Boniece, 1956; Jacherts, 1957; Szeto and Halick, 1958) with low yields of coagulase. Fahlberg and Marston (1960) reported coagulase production in chemically defined media with maximum titers of 1:512.

The present investigations were undertaken to try to elucidate factors which affect the production of staphylococcal coagulase in a chemically defined medium.

MATERIALS AND METHODS

Media

The chemically defined medium used for both maintenance and growth was a modification of the medium of Lominski, O'Hea, Goudie, and Porter (1950). The medium contained the following per 100 ml: 0.025 g each of L-cystine, L-histidine, L-leucine, L-phenylalanine, L-proline, L-tryptophan, and L-tyrosine; 0.05 g each of L-arginine, glycine, DL-isoleucine, L-lysine, DL-methionine, DL-serine, DL-threonine, and DL-valine; 0.125 g of both DL-aspartic acid and L-glutamic acid; 0.05 mcg of biotin; 0.125 g each of $MgSO_4 \cdot 7H_2O$, dipotassium hydrogen phosphate, sodium chloride; 0.0125 gm $FeCl_3 \cdot 6H_2O$; nicotinamide, pyridoxine, and thiamine each at a concentration of 0.0005M. All constituents were dissolved separately as stock solutions. The amino acids (with the exception of cystine and tyrosine) were dissolved in Sorensen's buffer, pH 7.3; cystine and tyrosine were dissolved in 3 N hydrochloric acid and afterwards diluted with distilled water; salts and vitamins, in distilled water. All solutions were sterilized by autoclaving. The medium, made up by aseptically mixing the stock solutions, had a final pH of 7.0. This medium was referred to throughout as "chemically defined medium."

The medium used in comparing coagulase production in shaken and unshaken cultures during the growth phase of *Staphylococcus aureus* #104 was Brain Heart Infusion Broth (Difco).

Culture

The culture of *S. aureus* #104 for this work was received from Dr. Morris Tager. This strain has been reported to be a potent coagulase producer (Tager, 1956).

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In the experiments involving chemically defined media, *S. aureus* was grown in screw cap test tubes containing 7 ml of the chemically defined medium. The test tubes were inoculated with 0.05 ml of a 24-hr culture of *S. aureus* grown in the chemically defined medium. The latter culture was the last of six daily transfers in the chemically defined medium starting with an original 24-hr culture grown in brain heart infusion broth. The tubes were incubated in a water bath at 37 C and those tubes that were shaken were attached to a Burrell Shaker. Turbidity measurements were made in a Spectronic 20 spectrophotometer at 620 mμ against blanks of the test medium.

In the experiments comparing coagulase production in shaken and unshaken cultures, *S. aureus* was grown in brain heart infusion broth in 300-ml Erlenmeyer flasks with side arm cuvettes. The flasks were inoculated with 0.05 ml of a 24-hr culture of *S. aureus* grown in brain heart infusion broth. The latter was the last of three daily transfers in brain heart infusion broth. The flasks were incubated in a water bath at 37 C. In all experiments samples for coagulase determinations were aseptically removed and immediately frozen.

Plasma

The plasma used was Bacto Coagulase Plasma (Difco).

Coagulase Titrations

The method of Yotis and Ekstedt (1959) was used. Serial twofold dilutions were made of the *S. aureus* culture in 2 per cent peptone-saline containing 1:5000 merthiolate, and to each tube containing 0.25 ml of dilution, 0.1 ml of reconstituted Bacto Coagulase Plasma was added. A control consisting of 0.25 ml diluent and 0.1 ml plasma was also included. The tubes were stoppered and incubated in a water bath at 37 C for 4 hr, removed, and allowed to stand at room temperature overnight before reading. The end point was taken as the highest dilution showing any trace of clot or fibrin thread.

Biochemicals

The biochemicals used in the experiments presented here were purchased from Nutritional Biochemicals Corporation and California Corporation for Biochemical Research.

RESULTS

The effects of varying cultural conditions on the growth and coagulase production of *S. aureus* #104 in brain heart infusion broth are given in figures 1 and 2. The results indicate that in shaken cultures, the organism maintained a normal growth curve and coagulase production followed a similar curve, that is, it increased during lag log phase and levelled off during the stationary growth phase. In the unshaken cultures a similar picture was obtained during the lag and log phases but coagulase production continued in the stationary phase.

Table 1 indicates the effect on coagulase yield of successive transfers in the modified chemically defined medium starting with a 24-hr culture of *S. aureus* #104 grown in brain heart infusion broth. Successive transfers resulted in a drop in titer of coagulase from 1:10, 240 to 1:10. In subsequent experiments, the cultures were transferred six times in order to obtain a culture with a low initial coagulase titer.

The effect of pH on coagulase production in brain heart infusion broth is shown in table 2. The highest coagulase yield was obtained at pH 5.4.

The effect on coagulase production in the chemically defined medium of carbohydrates other than glucose is indicated in table 3. In media containing sodium citrate, essentially the same coagulase titers resulted as with glucose. However,

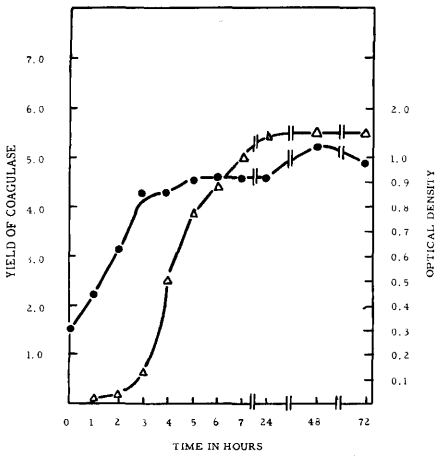

FIGURE 1. The production of coagulase and growth of *S. aureus* in shaken cultures. The culture medium was brain heart infusion broth and yield of coagulase is expressed as the log of the highest dilution giving a positive reaction. Closed circles represent coagulase production; triangles represent growth.

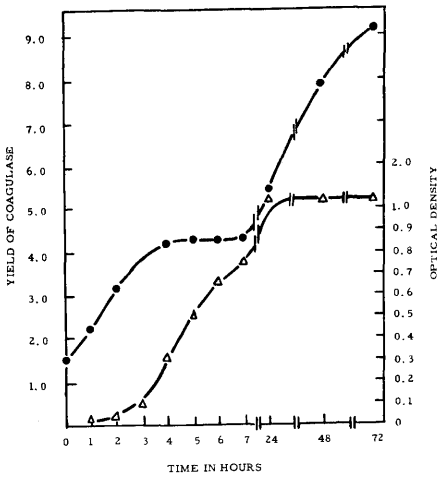

FIGURE 2. The production of coagulase and growth of *S. aureus* in unshaken cultures. The culture medium was brain heart infusion broth and yield of coagulase is expressed as the log of the highest dilution giving a positive reaction. Closed circles represent coagulase production; triangles represent growth.

TABLE 1

The effect of successive transfers in chemically defined media on coagulase production starting with a 24-hr culture grown in brain heart infusion broth

	Number of transfers												
	I*	1		2		3		4		5		6	
		A**	B***	A	B	A	B	A	B	A	B	A	B
Coagulase titer	1:163,840	1:20	1:10,240	1:5	1:320	0	1:160	0	1:80	0	1:10	0	1:10

*Original 24-hr brain heart infusion broth culture incubated at 37C.
**Immediately after inoculation but before incubation.
***After 24 hr incubation at 37C.

TABLE 2

The effect of pH on coagulase production in brain heart infusion broth

pH	Growth in 72 hr (Optical density)	Coagulase titer
5.4	1.06	1:163,840
6.1	1.00	1:640
7.4	1.00	1:2560
8.4	1.05	1:2560
9.0	0.96	1:2560

TABLE 3

The effect of various carbon sources on coagulase production in chemically defined medium

Carbon sources*	Growth in 24 hr (Optical density)		Coagulase titer	
	shaken	unshaken	shaken	unshaken
D-mannitol	0.98	0.53	1:40	1:80
D-mannose	0.84	0.69	1:10	1:160
D-lactose	0.87	0.65	1:20	1:160
maltose	0.87	0.79	1:40	1:160
sucrose	0.84	0.74	1:10	1:160
D-levulose	0.81	0.75	1:10	1:80
D-galactose	0.90	0.315	1:40	1:160
sodium citrate	0.63	0.165	1:20	1:20
D-glucose (control)	0.88	0.70	1:40	1:20

*The concentration of carbon sources in the final medium was 1 per cent.

all the other carbon sources used caused a higher coagulase yield than with glucose as the carbon source.

The effect of various concentrations of disodium versenate on coagulase production in both the chemically defined medium and brain heart infusion broth is given in table 4. It appears the 0.5 per cent concentration of disodium versenate was inhibitory to coagulase production, but not to growth in the chemically defined medium. A concentration of 0.1 per cent resulted in a coagulase titer of 1:10. However, in brain heart infusion broth, concentrations of 0.5, 1 and 2 per cent of disodium versenate inhibited growth, but 0.1 per cent was not inhibitory to growth or coagulase production, and the coagulase titer was 1:80.

Increasing the amount of biotin present in the chemically defined medium

TABLE 4

The effect of disodium versenate on coagulase production in chemically defined medium and brain heart infusion broth

Medium	Concentration of versenate in final medium (%)	Growth in 24 hr (Optical density)		Coagulase titer	
		shaken	unshaken	shaken	unshaken
Chemically defined with 1% glucose	0	0.80	0.54	1:10	1:10
Brain heart infusion broth	0	1.16	1.16	1:320	1:5120
Chemically defined with 1% glucose	0.1	0.87	0.50	1:10	1:10
	0.5	0.76	0.295	0	0
	1	0.27	0.17	0	0
	2	0.03	0.04	0	0
Brain heart infusion broth	0.1	1.05	1.05	1:20	1:80
	0.5	0.03	0.03	0	0
	1	0.03	0.025	0	0
	2	0.035	0.025	0	0

(table 5) stimulated growth and coagulase production. In media without added biotin or the usual concentration of biotin, coagulase titers of 1:10 were obtained. By increasing the amount of biotin five times that usually used (i.e., to 0.25 mcg/100 ml) a coagulase titer of 1:80 was obtained.

Table 6 reveals the effect of hematoporphyrin, protoporphyrin, and cytochrome C on coagulase production in the chemically defined medium. Both hematoporphyrin and protoporphyrin inhibited growth to some extent and completely inhibited coagulase production. Cytochrome C did not affect growth but was stimulatory to coagulase production.

TABLE 5

The effect of biotin concentration on coagulase production in chemically defined medium

Medium	Concentration of biotin added	Growth in 24 hr (Optical density)		Coagulase titer	
		shaken	unshaken	shaken	unshaken
Chemically defined with 1% glucose	0	0.81	0.85	1:10	1:10
	0.125 mcg/100 ml	0.85	0.51	1:40	1:40
	0.25 mcg/100 ml	0.72	0.53	1:80	1:80
Brain heart infusion broth	0	1.16	1.10	1:320	1:1280

TABLE 6

The effects of Hematoporphyrin, Protoporphyrin, and Cytochrome C on coagulase production in chemically defined medium

Medium	Addition	Growth in 24 hr (Optical density)		Coagulase titer	
		shaken	unshaken	shaken	unshaken
Chemically defined with 1% glucose	0	0.82	0.52	1:10	1:10
	Hematoporphyrin				
	70 mcg/ml	0.42	.45	1:5	1:5
	140 mcg/ml	0.10	.25	1:5	1:5
	350 mcg/ml	*	*	0	0
	Protoporphyrin				
	35 mcg/ml	0.25	0.37	0	0
	70 mcg/ml	0.175	0.10	0	0
	Cytochrome C				
	140 mcg/ml	0.95	0.80	1:40	1:40
	350 mcg/ml	1.05	0.76	1:40	1:80
Brain heart infusion broth	0	1.16	1.10	1:320	1:1280

*Growth could not be measured due to opacity of the medium.

The effects of increasing the Fe^{+++} concentration in the chemically defined medium are indicated in table 7. The control, the chemically defined medium, had a coagulase titer of 1:20. Upon increasing the Fe^{+++} concentration to 5, 10, and 20 times the usual amount in the medium, coagulase titers of 1:160, 1:320, and 1:80, respectively, were obtained.

Table 8 shows the effects of various imidazole derivatives on coagulase production in the chemically defined medium. The addition of imidazole, benzimidazole, and methyl-4-nitroimidazole-5-carboxylate inhibited growth and coagulase production. The remaining derivatives supported growth and 4-hydroxy-methyl imidazole HCl and dihydrourocanic acid stimulated coagulase production to

TABLE 7

The effect of Fe^{+++} concentration on coagulase production and growth in chemically defined medium

Medium	Growth in 24 hr (Optical density)		Coagulase titer	
	shaken	unshaken	shaken	unshaken
Chemically defined	0.52	0.15	1:10	1:10
Chemically defined with 1% glucose	0.89	0.72	1:20	1:20
Chemically defined with 1% glucose:				
plus 0.625 g $FeCl_3$/liter	0.86	0.62	1:80	1:160
plus 1.25 g $FeCl_3$/liter	0.87	0.66	1:40	1:320
plus 2.50 g $FeCl_3$/liter	0.83	0.62	1:40	1:80
Brain heart infusion broth	1.05	1.10	1:640	1:640

TABLE 8

The effect of imidazole derivatives on coagulase production in chemically defined medium

Medium	*Addition*	Growth in 24 hr (Optical density)		Coagulase titer	
		shaken	unshaken	shaken	unshaken
Chemically defined	0	0.72	0.15	1:5	1:5
Chemically defined with 1% glucose	0	0.86	0.71	1:10	1:10
Chemically defined with 1% glucose	4-hydroxymethyl imidazole HCl	0.90	0.70	1:40	1:320
	Imidazole acetic acid HCl	0.36	0.22	1:40	1:20
	Imidazole	0.04	0.04	0	0
	4-amino-5-imidazole carboxamide HCl	0.54	0.33	1:10	1:10
	Benzimidazole	0.02	0.03	0	0
	Methy-4-nitroimidazole-5-carboxylate	0.025	0.025	0	0
	Dihydrourocanic acid	0.92	0.73	1:160	1:160
Brain heart infusion broth	0	1.22	1.10	1:320	1:1280
	Imidazole	1.05	0.95	1:40	1:320

*The concentration of the imidazole derivatives was equal to the amount of histidine present in the chemically defined medium.

titers of 1:320 and 1:160, respectively. Imidazole, when added to brain heart infusion broth, inhibited growth slightly as compared to the control. The coagulase titer was 1:320 compared to the brain heart infusion broth control of 1:1280.

DISCUSSION

The results represented in figures 1 and 2 suggest that coagulase production, under certain cultural conditions, follows closely the growth curve pattern for the organism. However, in unshaken cultures, upon reaching the stationary phase

of growth, coagulase production continued to a much higher level than in shaken cultures. In similar experiments reported by Rogers (1954) in which the production of other staphylococcal enzymes was determined in relation to growth, it was found that after the initial lag the rate of production of hyaluronidase was higher than that of bacterial growth, whereas coagulase was produced more slowly but without initial lag.

Leitner and Cohen (1962) reported the production of penicillinase in *S. aureus* was markedly increased by acidifying a culture medium of heart infusion to pH 5.07. This is in accord with findings presented in table 2 regarding coagulase production by *S. aureus* in brain heart infusion broth.

Pardee (1959) explains the increased synthesis of certain enzymes under conditions highly unfavorable for general protoplasmic synthesis by the assumption that the inhibitory environmental factors depress preferentially the synthesis or the activity of compounds acting as repressors of the enzyme in question, while still permitting some degree of protein synthesis.

Upon addition of an energy source such as glucose, maximum growth was obtained but coagulase production was of a low titer, e.g., 1:10. Other sugars in the place of glucose in the medium gave much higher coagulase titers than did glucose (table 3). Citrate, which proved to be a poor carbon source for this organism, depressed coagulase production. These findings are in accord with Kaminski et al. (1959) who noted that in a defined medium, maltose or certain other carbon sources caused an increase in the production of penicillinase over that observed with glucose. Kaminski (1962) also noted the same stimulatory effect of maltose on penicillinase production by *S. aureus*, strain #6797 PNR. It would seem that the carbohydrates which are stimulatory to coagulase production are either utilized more efficiently for energy production or are more efficiently metabolized to a compound(s) inducing coagulase production.

The observation that an increase in the concentration of biotin in the medium causes an increase in coagulase production suggests that biotin has a role in the synthesis of coagulase.

Another observation which deserves further investigation is the effect of Fe^{+++} on coagulase production. Fe^{+++} itself was found to be stimulatory to coagulase production and in the form of a prophyrin complex, cytochrome C, it was stimulatory in much lower concentrations (table 6). In experiments not described above, it was found that hemin and hematin were without effect on coagulase production in the chemically defined medium but hemoglobin was stimulatory.

It is interesting to note that iron-free porphyrins such as hematoporphyrin and protoporphyrin depressed both growth and coagulase production, perhaps by binding iron. Disodium versenate, a potent chelating agent also inhibited both growth and coagulase production (table 4) presumably by tying up metals. It would be of interest to determine whether the stimulatory effects of Fe^{+++} on coagulase production are specific or more or less general with metals.

The observation that certain metals are stimulatory to the production of certain enzymes is in accordance with the work of Merrill and Clark (1928) who studied the production of gelatinase by *Proteus* and found that calcium and magnesium salts were required for the production of gelatinase in a synthetic medium containing lactate. No enzyme was found in cultures in which calcium or magnesium was lacking. Other examples of a requirement for metals for enzyme production are provided by Pollock (1950) who noted that Mg^{++} was stimulatory to the production of penicillianse in *B. cereus* and Mandels and Reese (1957) who noted that cellulase, an adaptive enzyme produced by *Trichoderma viride*, required calcium and certain trace elements for its production. The trace element requirement could be supplied by cobalt alone, but best cellulase production occurred if iron, manganese, zinc, or cobalt are also added.

Leitner and Cohen (1962) noted that penicillinase production in *S. aureus* was stimulated by the addition of certain inorganic ions Fe^{++}, Fe^{+++}, Co^{++}, and to a lesser extent with Mn^{++}. Ni^{++} had little or no effect on penicillinase production. They reported a penicillinase production of 310 units/mg protein with the ferrous salt and 100 units/mg protein with the ferric salt. The ferrous salt stimulated penicillinase production most strikingly when the latter had been inhibited previously by depressive cations, such as Na^{+} or Ca^{++}.

Fahlberg and Marston (1960) stated that L-histidine together with L-glutamic acid and L-lysine were stimulatory to coagulase production in the two chemically defined media they used. One of these amino acids, L-glutamic acid, is required for growth (Kaminski et al., 1959). While the above three amino acids were present in the chemically defined medium used in the work described in this paper, coagulase titers rarely exceeded 1:10 or 1:20. However it seemed to be of interest to determine whether compounds related to histidine were stimulatory to coagulase production. Of the imidazole derivatives, 4-hydroxymethyl imidazole HCl and dihydrourocanic acid, when added to the chemically defined medium stimulated coagulase titers to 1:320 and 1:160 respectively. Apparently, compounds related to histidine in structure are capable of stimulating coagulase production. It remains to be determined whether iron and imidazole derivatives together would cause even further stimulation of coagulase production.

The observation that coagulase production is so much greater in brain heart infusion broth than in chemically defined media certainly indicates the presence of a substance(s) present in the complex medium which is highly stimulatory to coagulase production. The elucidation of the chemical nature of the enzyme inducer(s) involved and the mechanism of action in the stimulation of enzyme production would certainly constitute valuable information.

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