

STUDIES ON STAPHYLOCOCCI

I. EFFECT OF SERUM AND COAGULASE ON THE METABOLISM OF COAGULASE POSITIVE AND COAGULASE NEGATIVE STRAINS¹

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Coagulase has been shown in previous reports (Ekstedt and Nungester, 1955; Ekstedt, 1956a, b) to be involved in the resistance of strains of staphylococci to a normal human serum antibacterial factor. Coagulase negative staphylococci are in general inhibited markedly in their rate of growth by normal human serum, whereas coagulase positive staphylococci are able to grow luxuriantly under the same conditions. If coagulase is incorporated into an actively bacteriostatic serum the inhibitory activity of the serum is neutralized and coagulase negative strains will also grow luxuriantly.

Results to be presented here will show that the bacteriostatic activity of the serum can be demonstrated by conventional manometric techniques. Further studies of the mechanism of the inhibition of coagulase negative staphylococci by serum and the role of coagulase in this system will be described.

MATERIALS AND METHODS

Cultures. One hundred cultures of staphylococci were obtained from the Veterans Administration Research Hospital, Chicago, and represented recent isolates during routine bacteriological examination of clinical specimens. They were received in the laboratory on agar plates used for determining antibiotic sensitivity by the disk method. Each culture was immediately subcultured to 10 ml brain heart infusion broth (Difco), incubated at 37 C for 16 hr, centrifuged, resuspended in 1 ml fresh rabbit blood and desiccated rapidly on sterile white sand under vacuum according to the method of Frobisher *et al.* (1947). The dried cultures were stored

at 4 C, and cultures for daily use were prepared by subculturing from the desiccated samples to 100 ml brain heart infusion broth. After incubation at 37 C for 16 to 20 hr 10-ml samples were frozen and stored at -70 C. These frozen stock cultures were prepared weekly from the desiccated cultures.

Serum. The human serum used in these studies was obtained from volunteers or professional donors. Blood was drawn into 250-ml Baxter Plasma-Vac bottles without anticoagulant and allowed to clot at room temperature for 1 to 2 hr. The clot was broken up and the serum separated by centrifugation in a refrigerated centrifuge at 3000 rpm for 30 min. The serum was dispensed into screw-capped tubes in a volume of 3 to 5 ml each and stored at -70 C.

Metabolic studies. Cells for the Warburg experiments were prepared by inoculating 300 ml brain heart infusion broth in a 1-L flask with 10 ml of frozen stock culture and incubating at 37 C for 16 hr. The cells were harvested by centrifugation, washed twice with distilled water, resuspended in 10 ml of distilled water, and a smooth suspension prepared with a Teflon homogenizer. The suspension was then adjusted to an optical density of 1.0 at a wave length of 660 m μ in a Coleman spectrophotometer. This suspension contained from 0.1 to 0.15 mg bacterial N per ml as determined by Kjeldahl analysis.

In a typical experiment each Warburg flask contained 1.0 ml of cell suspension, 0.5 ml of 0.1 M phosphate buffer pH 7.0, and 0.5 ml of normal human serum. Two-tenths ml of 40 per cent KOH was placed in the center wells. Any additional substances were dissolved in the buffer and replaced, in part or completely, the buffer in the control flasks. Rates of oxygen uptake in air were determined by conventional manometric techniques at 37 C for 6 hr. Q(O₂)N values were calculated from the data obtained during the

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first 2 hr of linear activity, and are expressed as $\mu\text{L O}_2$ consumed per mg bacterial N per hr.

Coagulase titration. Coagulase activity of the 100 strains of staphylococci was titrated by diluting serially the clear culture supernatant after centrifugation of an 18-hr culture of the strain grown in brain heart infusion broth at 37 C. Two-fold serial dilutions were made in 2 per cent peptone (Difco)-saline containing 1:5000 merthiolate in a volume of 0.5 ml. To each tube in the series was added 0.5 ml of a 1:5 dilution of fresh rabbit plasma, and the tubes incubated at 37 C for 4 hr. They were then removed and allowed to stand at room temperature overnight. The end point was read as the last tube showing any trace of fibrin. When the purified coagulase preparations were titrated, decimal dilutions of a 1 mg per ml solution of the preparation in the 2 per cent peptone-saline were made, 1:5 rabbit plasma added, and the tubes incubated and read as described above.

Growth on serum agar. The strains of staphylococci were screened for their ability to grow on human serum in the following manner. Twenty-five ml of fresh pooled normal human serum were added to 75 ml of 2 per cent agar (Difco) dissolved in 0.1 M phosphate buffer at pH 7.0. Plates were poured, allowed to solidify, and spot inoculated with 0.05 ml of a standardized suspension of the strains to be tested. After incubation at 37 C for 16 to 20 hr, growth was estimated on a 0 to 4+ scale. Zero indicated no growth and 4+ indicated confluent growth.

Preparation of purified coagulase. Coagulase was purified by the method described by Tager (1948). The strain of staphylococcus used for these preparations was obtained from Dr. Morris Tager, and unconcentrated culture supernatant from 20 hr growth in brain heart infusion broth at 37 C had a coagulase titer of 1:640 by our titration technique. The preparation used in these studies had an activity such that 0.005 μg produced an easily visible clot in 1:5 fresh rabbit plasma. It was also shown to possess tributyrinase activity (Drummond and Tager, 1958). These determinations were made manometrically using 1 mg of the coagulase and 0.2 ml of tributyrin (purified grade, California Foundation for Biochemical Research) as substrate. Sixty-nine μL of carbon dioxide were released from 0.04 M bicarbonate at pH 7.9 in 10 min in an atmosphere of 95 per cent N and 5 per cent CO_2 . An equivalent amount of lipase (Nutritional Biochemical

Corp.) acting on the same substrate released 262 μL of CO_2 in the same time.

RESULTS

Survey of strains. The 100 strains of staphylococci recently isolated from clinical materials were studied with regard to (a) coagulase activity, (b) pigmentation, (c) hemolysis of rabbit blood agar, (d) gelatinase activity, (e) mannitol fermentation, and (f) growth on 25 per cent normal human serum agar.

Seventy-two per cent were coagulase positive with titers ranging from 1:1 to 1:2560. Eighty-five per cent were *S. aureus*, 80 per cent showed hemolytic activity on rabbit blood agar, 42 per cent liquefied gelatin, and 30 per cent fermented mannitol. All of the coagulase positive strains grew confluently (4+) on 25 per cent human serum agar, whereas 6 of the coagulase negative strains showed 2+ growth and 22 gave 1+ growth under the same conditions.

Metabolic studies. An attempt was made to demonstrate the antistaphylococcal activity of human serum for coagulase negative strains by

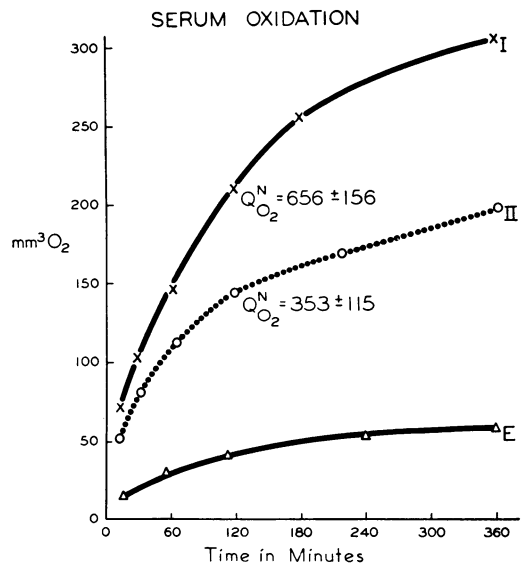


Figure 1. Oxidation of normal human serum by coagulase positive and coagulase negative strains of *Staphylococcus aureus*. Flask contents: 1.0 ml adjusted cell suspension, 0.5 ml normal human serum, 0.5 ml phosphate buffer pH 7.0, and 0.2 ml 40 per cent KOH in center well.

Curve I, coagulase positive strains; curve II, coagulase negative strains; and curve E, endogenous (mean of both groups—see text).

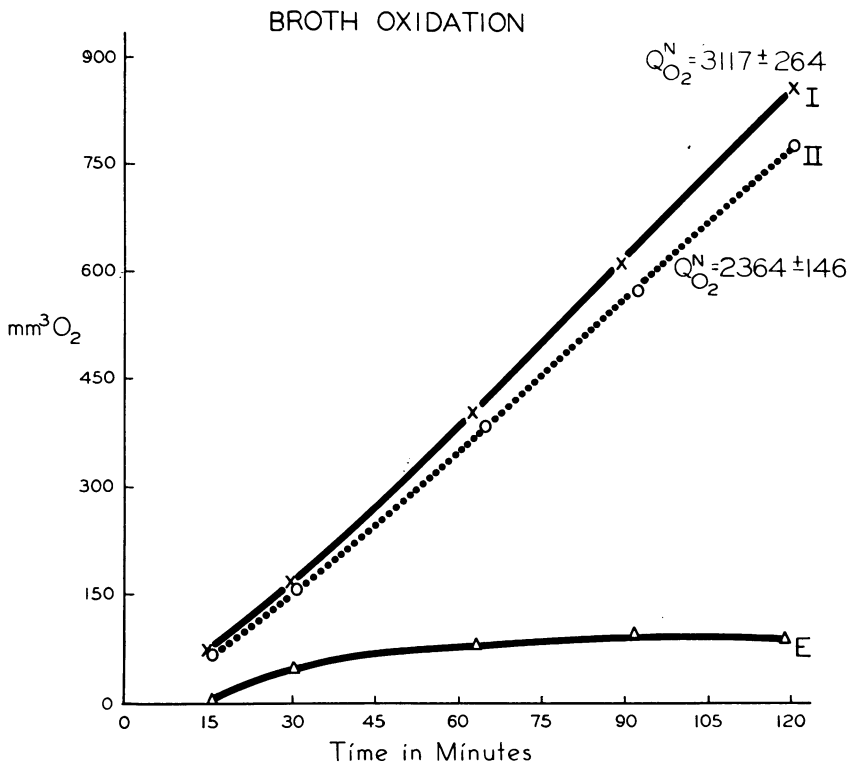


Figure 2. Oxidation of brain heart infusion broth by coagulase positive and coagulase negative strains of *Staphylococcus aureus*. Flask contents: 1.0 ml adjusted cell suspension, 0.5 ml brain heart infusion broth, 0.5 ml phosphate buffer pH 7.0, and 0.2 ml 40 per cent KOH in center well.

Curve I, coagulase positive, strains; curve II, coagulase negative strains; and, curve E, endogenous (see figure 1).

manometric techniques. A random selection of 12 coagulase positive and 12 coagulase negative strains was made from our collection and the oxygen uptake in air with normal human serum as substrate was determined. Figure 1 shows a plot of the mean values calculated from these experiments. The endogenous respiration rates for both groups of organisms were so similar they were averaged and plotted as one curve. In every case the coagulase negative strains showed a distinctly lower rate of respiration than the coagulase positive strains. This contrasted sharply with the respiration of these strains in brain heart infusion broth. Figure 2 shows a composite plot of the same strains with broth as substrate. It should be mentioned that all the strains used in these studies were *S. aureus* and hemolytic on rabbit blood agar. Similar results were obtained when the coagulase positive and coagulase negative strains were grown in undiluted normal human serum and brain heart infusion broth.

Figures 3 and 4 show the growth measured turbidimetrically. Because of changes in the optical properties of serum during growth of the organisms, identical tubes were removed at the indicated intervals, the bacteria separated by centrifugation, washed once with distilled water, and resuspended in a constant volume of distilled water for turbidity measurements. The readings were made in a Coleman spectrophotometer at 660 $m\mu$ wave length.

Attempts to detect differences in the rate of oxidation of glucose and pyruvate by both groups of organisms were without success. Both coagulase positive and coagulase negative strains respired equally well with these defined substrates.

The possibility that normal human serum did not contain sufficient nutrient for growth of coagulase negative strains, or was deficient in an essential growth factor for these strains, was next considered. Serum diluted 1:2 with distilled

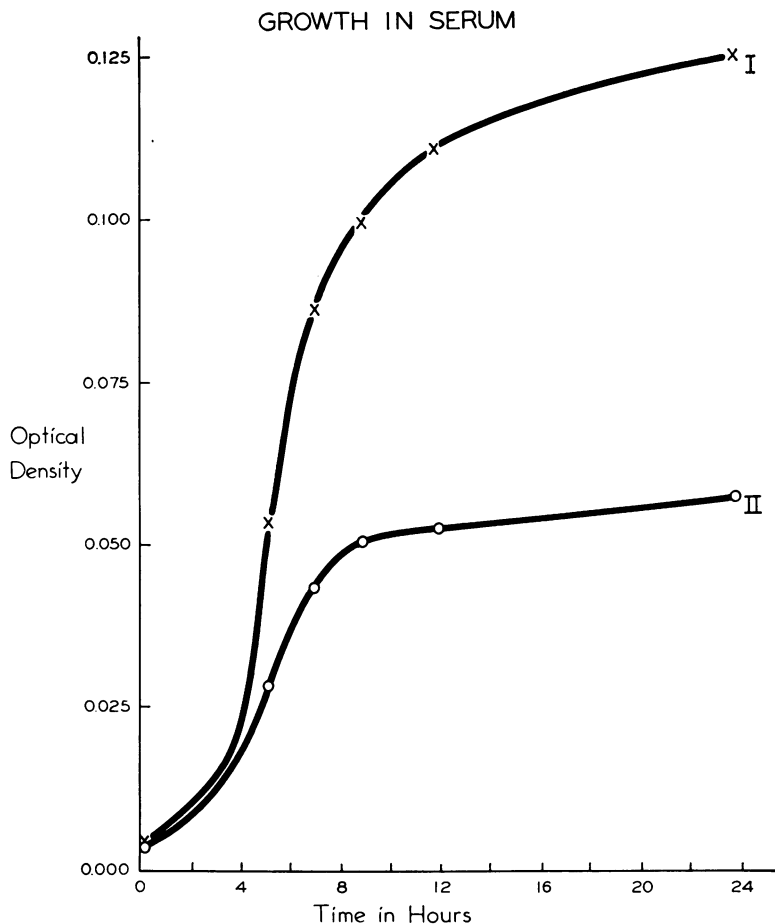


Figure 3. Growth of coagulase positive and coagulase negative strains of *Staphylococcus aureus* in normal human serum.

Curve I, coagulase positive strains; and curve II, coagulase negative strains.

water was heated in a boiling water bath for 10 min, the heat coagulable material removed by centrifugation, and the supernatant used as substrate. Figure 5 shows the results of these experiments. It is clear that serum devoid of heat coagulable material contains sufficient nutrients to support the respiration of both coagulase positive and coagulase negative strains to a comparable degree, and would tend to rule out lack of appropriate or sufficient nutrients as an explanation of the metabolic differences seen in whole serum. Serum dialyzate was also shown to support active respiration of both groups of organisms equally well.

The permeability of the coagulase positive and coagulase negative strains was studied by comparing the oxygen uptake of sonically disinte-

grated cells with serum as substrate. Ten ml of the cell suspensions were disintegrated in a Raytheon 10 kc, 250 w oscillator for 1.5 hr, and used directly. Microscopic observation of the broken cell suspensions showed essentially complete disruption of the cells. An occasional single unbroken cell could be found in 20 to 30 oil immersion fields. Centrifugation of the broken cell suspensions to remove particulate matter depressed the oxygen uptake to such low levels that the whole suspensions were used without centrifugation. The disintegrated cells of both coagulase positive and coagulase negative strains behaved in a manner similar to the unbroken cells. The respiration of the broken coagulase positive strains was considerably greater ($Q(O_2)N = 343$) than that of the coagulase negative strains ($Q(O_2)N = 143$).

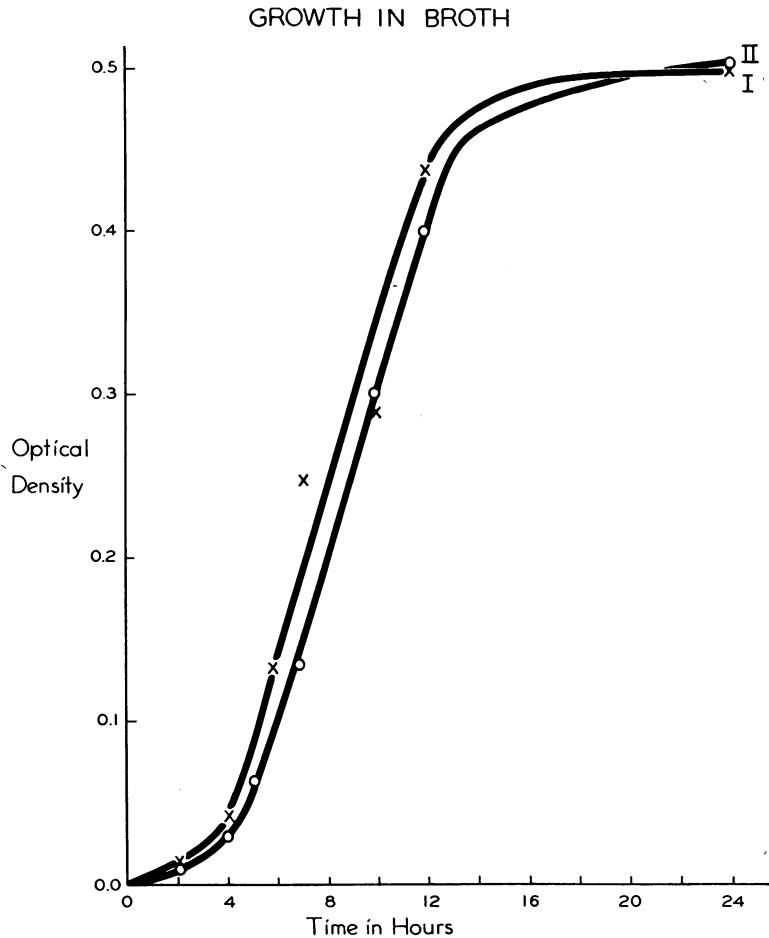


Figure 4. Growth of coagulase positive and coagulase negative strains of *Staphylococcus aureus* in brain heart infusion broth.

Curve I, coagulase positive strains; and curve II, coagulase negative strains.

These results would seem to indicate that the differences in respiratory activity of coagulase positive and coagulase negative strains with serum as substrate are not due to differences in the permeability of the strains to substrate.

It was shown in previous studies (Ekstedt and Nungester, 1955; Ekstedt, 1956a, b) that coagulase negative strains of staphylococci could be induced to grow luxuriantly in normal human serum by adding partially purified cell-free coagulase to the system. It was of interest to ascertain whether a similar response could be demonstrated manometrically. The oxygen uptake of coagulase positive and coagulase negative strains in serum was compared in the presence and absence of 1 mg additional purified

coagulase. Figure 6 shows the results of these experiments. It is evident that exogenous coagulase stimulates the respiration of both coagulase positive and coagulase negative staphylococci. The rate of respiration of coagulase negative organisms in the presence of coagulase was comparable to that of the coagulase positive strains in the absence of additional coagulase. The stimulation in oxygen uptake at the end of 6 hr with the coagulase negative strains was approximately 2 times greater than the uptake of the coagulase positive strains under the same conditions. No increase in oxygen uptake above the endogenous was seen in flasks containing only coagulase, indicating that the coagulase itself was not being utilized as substrate by the organisms.

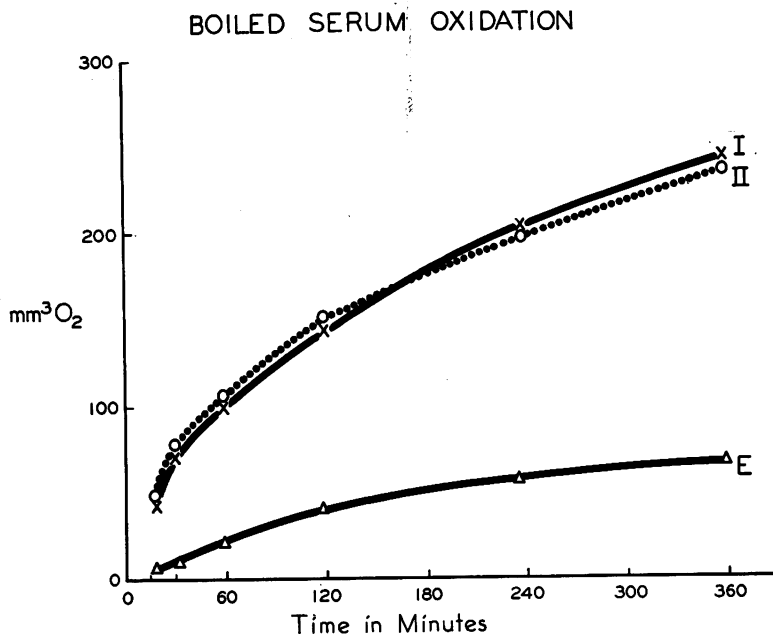


Figure 5. Oxidation of boiled serum supernatant by coagulase positive and coagulase negative strains of *Staphylococcus aureus*. Flask contents: 1.0 ml adjusted cell suspension, 1.0 ml 1:2 buffer diluted boiled serum, and 0.2 ml 40 per cent KOH in center well.

Curve I, coagulase positive strains; curve II, coagulase negative strains; and curve E, endogenous (see figure 1).

DISCUSSION

The human serum antistaphylococcal factor is a heat stable component of normal serum which is more active against coagulase negative strains. The factor has been shown to require calcium ions as a cofactor, is inactivated by digestion with pepsin, and prolonged heating at temperatures above 60 C destroys its activity (Ekstedt and Nungester, 1955; Ekstedt, 1956a, b). The system is probably identical to the serum β -lysins. Recently Myrvik *et al.* (1958), working with the rabbit and human serum bacteriocidin system for *Bacillus subtilis*, have shown that in addition to calcium, bicarbonate ions are required as cofactor in the human system, whereas with rabbit serum only bicarbonate ions are required. A recent review (Skarnes and Watson, 1957) indicated that Myrvik was unable to confirm our previous results, but neglected to mention that he was working at that time solely with the rabbit system.

The inhibition of coagulase negative staphylo-

cocci by the human serum factor has been demonstrated by manometric techniques and the reversal of the inhibition by the addition of purified coagulase has been confirmed. That nutritional differences in coagulase positive and coagulase negative strains are responsible for the differences seen in the growth and respiration of these two groups in serum is not likely, since serum devoid of heat coagulable material contains sufficient nutrients to support active respiration and growth of both to a comparable degree.

Permeability of the coagulase positive and coagulase negative strains to substrate also did not seem to play a role, since sonically disintegrated cells behaved in the same manner as whole cells.

It is interesting to note that the addition of exogenous purified coagulase also stimulates the respiration of coagulase positive organisms to some extent. Preliminary evidence also seems to indicate that in the presence of increased amounts of the γ -globulin fraction of serum, coagulase positive strains can also be inhibited. It appears

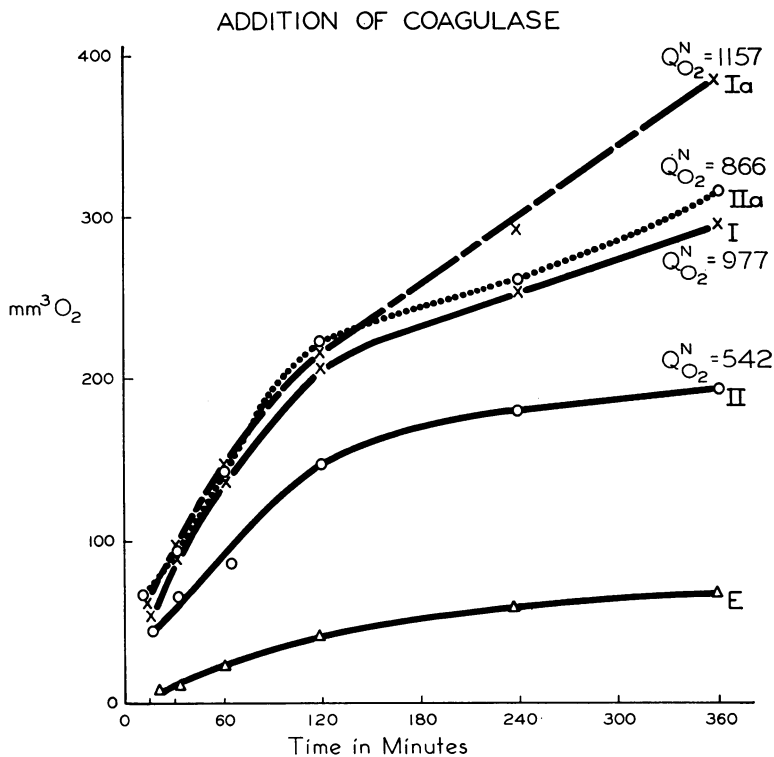


Figure 6. Effect of coagulase on the oxidation of normal human serum by coagulase positive and coagulase negative strains of *Staphylococcus aureus*. Flask contents: 1.0 ml adjusted cell suspension, 0.5 ml normal human serum, 0.5 ml phosphate buffer pH 7.0 (I and II), 0.5 ml buffer containing 1.0 mg coagulase (Ia and IIa), and 0.2 ml 40 per cent KOH in center well.

Curve I, coagulase positive strains; curve II, coagulase negative strains; curve Ia, coagulase positive strains plus coagulase; curve IIa, coagulase negative strains plus coagulase; and curve E, endogenous (see figure 1).

that there is a direct antagonism between the antibacterial serum factor and coagulase. Whether this is of a competitive or noncompetitive nature remains to be determined.

Although our purified coagulase preparation was shown to have esterase activity, using tributyrin as substrate, it should be pointed out that Drummond and Tager (1958) have also shown this activity in their most highly purified coagulase preparations. Recently Haughton and Duthie (1959) have presented evidence to show that electrophoretically homogenous coagulase hydrolyzes *N* α -toluene-*p*-sulfonyl-L-arginine-methyl ester. Whether the esterase activity of their preparations is distinct from the coagulase activity is not clear. That our results could be ascribed to the contamination of our coagulase with esterase is not likely since we have demon-

strated that many coagulase negative strains also possess strong tributyrinase activity, and recently Drummond and Tager (1959) have shown a dissociation of the clotting activity and tributyrinase activity.

SUMMARY

Coagulase positive strains of *Staphylococcus aureus* respired in human serum much more actively than did coagulase negative strains. Both coagulase positive and coagulase negative strains oxidized glucose and pyruvate equally well. Permeability could not be shown to be responsible for the differential rate of utilization of serum by coagulase positive and coagulase negative staphylococci. Differences in nutritional requirements were not a factor since boiled serum was shown to support active growth and respira-

tion of both coagulase positive and coagulase negative strains.

Purified coagulase added to the system stimulated the respiration of both coagulase positive and coagulase negative strains. The negative strains were stimulated about twice as much as the positive strains.

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