STUDIES ON STAPHYLOCOCCI

III. Further Studies on Purification and Mechanism of Action of an Antibacterial Human Serum Factor[1]

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A previous report (Yotis and Ekstedt, 1959) showed that normal human serum contains a substance which inhibits the growth and respiration of coagulase negative strains of staphylococci, but coagulase positive strains resist its action and grow or respire actively under the same conditions. When antistaphylococcal serum is treated with partially purified coagulase, the inhibitory activity is neutralized and coagulase negative strains grow or respire as well as coagulase positive staphylococci.

Further study of this phenomenon has shown that the antistaphylococcal serum factor is localized in a water soluble globulin fraction of human, rabbit, and horse serum. Its activity can be demonstrated manometrically with glucose as a substrate, and the factor has antibacterial action for *Bacillus subtilis* and *Micrococcus lysodeikticus*.

MATERIALS AND METHODS

The source, maintenance, and cultivation of the cultures used, the serum source, the preparation of purified coagulase, and the technique of the manometric studies have been described (Yotis and Ekstedt, 1959).

Serum fractionation. Fresh human serum pooled from at least three donors was diluted with an equal volume of distilled water, chilled to 0 C and sufficient solid ammonium sulfate added slowly to raise the concentration to 25 per cent saturation. The amount of ammonium sulfate added was calculated with the assumption that at 0 C, 70 g per 100 ml represented 100 per cent saturation. The precipitate formed was separated by centrifugation at 0 C, dissolved in distilled water, dialyzed against running tap water for 24 hr, and lyophilized.

The supernatant from the 25 per cent saturation fraction was raised successively to 50 per cent and 75 per cent saturation with ammonium sulfate to give three fractions, and the final supernatant. These were also dialyzed and lyophilized. The fractions were stored in closed containers in a deep freeze.

The 25 per cent ammonium sulfate fraction upon dialysis separated into water soluble and water insoluble fractions, which were separated and lyophilized.

Boiled serum supernatant fluid. In some preliminary experiments boiled serum supernatant was used as a substrate in the metabolic studies with the thought of providing possible cofactors for the antibacterial activity. Preparation of the boiled serum supernatant has been described (Yotis and Ekstedt, 1959).

RESULTS

Fractionation studies. The precipitates resulting from 25 per cent, 50 per cent, and 75 per cent saturation of human serum with ammonium sulfate, as well as the remaining supernatant, were tested separately for their ability to inhibit the respiration of coagulase positive and coagulase negative staphylococcal strains. Boiled serum supernatant was used as a substrate. The results shown in figure 1 are those obtained with the 25 per cent fraction. This fraction at a concentration of 5 mg per flask inhibited the oxidation of substrate by coagulase negative strains and had no detectable effect on coagulase positive strains. The remaining fractions showed no effect

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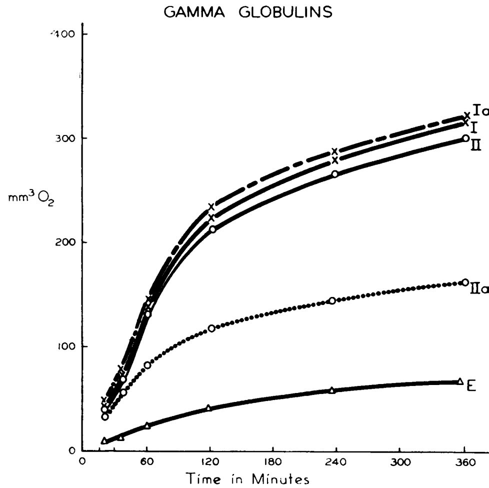

Figure 1. Effect of gamma globulin on the oxidation of boiled serum supernatant by staphylococci. Flask contents: 1 ml cell suspension (*I* and *II*), 1 ml cell suspension containing 5 mg gamma globulin (*Ia* and *IIa*), 0.5 ml boiled serum supernatant, 0.5 ml phosphate buffer, pH 7.0, 0.2 ml 40 per cent KOH in the center well. *Curve I*, coagulase positive strains; *curve Ia*, coagulase positive strains plus gamma globulin; *curve II*, coagulase negative strains; *curve IIa*, coagulase negative strains plus gamma globulin; and *curve E*, endogenous.

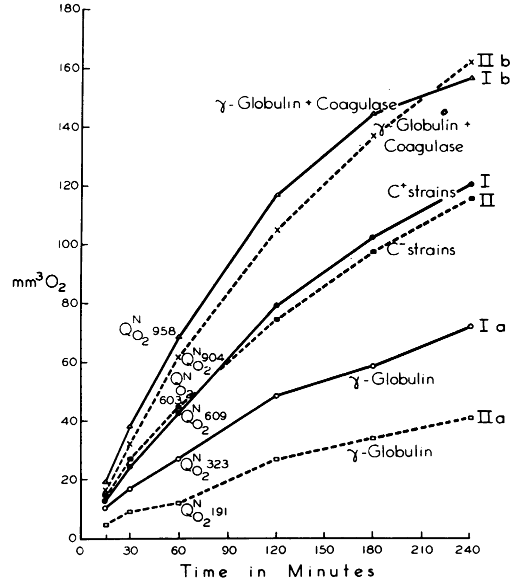

Figure 2. Effect of human gamma globulin and coagulase on the oxidation of glucose by staphylococci. Flask contents: 0.5 ml cell suspension, 0.5 ml 5.5 × 10^{-3} M glucose containing 3 μM ATP, 1.0 ml phosphate buffer, pH 7.0, and 0.2 ml 40 per cent KOH in center well.

on either coagulase positive or coagulase negative staphylococci.

Inhibition of glucose oxidation. To learn more about the mechanism of action of the serum factor, and to determine what cofactors if any were required for its action, experiments with a defined substrate were designed. Accordingly the activity of the 25 per cent globulin fraction from serum was determined on resting cells with glucose as substrate. These experiments were carried out as follows. To a series of screw-capped tubes containing 60 mg of the 25 per cent fraction were added 1.5 ml of a standardized cell suspension of either coagulase positive or coagulase negative strains. The mixtures were placed at 4 C for 1 hr and were shaken manually every 12 min. After this treatment the mixtures were dispensed into duplicate Warburg flasks and their oxygen consumption determined with glucose as a substrate. In several experiments the cell suspensions were treated with 1 mg per ml of partially purified coagulase at room temperature for 5 min before they were exposed to the globulin fraction. The results of these experiments are summarized in figure 2. When both coagulase positive and coagulase negative strains of staphylococci were exposed to 40 mg per ml of the globulin fraction their ability to oxidize glucose was impaired. Prior treatment of the cells with coagulase protected them from the antistaphylococcal serum factor.

Further purification of the serum factor. During dialysis of the 25 per cent ammonium sulfate fraction from human serum against water, a precipitate of water insoluble globulins formed. The precipitate and supernatant were tested individually and in combination for their ability to inhibit oxidation of glucose by coagulase positive and coagulase negative strains. The results of these experiments indicated that the antistaphylococcal activity resided primarily in the water soluble fraction of the 25 per cent ammonium sulfate precipitate of human serum. The water soluble fraction comprised 55 per cent of the whole 25 per cent ammonium sulfate fraction, the insoluble fraction approximately 45 per cent.

Distribution in other mammalian serums. Experiments were done to determine whether a similar antistaphylococcal activity was in the

serum from other species. Globulin fractions were prepared from rabbit, horse, and bovine serum in a manner identical to that used in the preparation of the active human serum fraction. These fractions were tested as described above, by manometric assay of the cells. It was found that both rabbit and horse serum globulin were active in inhibiting the oxidation of glucose by staphylococci, but preparations from bovine serum were without effect.

Action of the antistaphylococcal factor on other microorganisms. To determine the specificity of action of the human globulin antistaphylococcal factor, the effect of this substance on the oxidation of glucose by a variety of species of microorganisms was determined. Standardized cell suspensions of *Escherichia coli*, *Proteus vulgaris*, *Neisseria perflava*, *Lactobacillus casei*, *Bacillus megaterium*, *Bacillus subtilis*, *Micrococcus lysodeikticus*, *Diplococcus pneumoniae*, *Streptococcus pyogenes*, and *Streptococcus faecalis* were prepared and exposed to the human globulin fraction as previously described. The respiration of resting cell suspensions of *B. subtilis* and *M. lysodeikticus* on glucose was markedly inhibited by exposure of the cells to the globulin antibacterial factor. No such effect was observed with the other organisms studied.

Experiments were done to determine whether coagulase could protect these organisms from the serum factor, as had been shown with the staphylococci. Washed suspensions of *B. subtilis* and *M. lysodeikticus* were treated with 1 mg partially purified coagulase per ml of cell suspension for 5 min at room temperature. The cells were then exposed to 40 mg per ml of the whole 25 per cent globulin fraction for 1 hr at 4 C and tested manometrically as before. Separate samples of the cell suspensions were exposed to the serum factor without prior treatment with coagulase. Washed unexposed cells served as a control. Prior treatment with coagulase protected *B. subtilis*, but not *M. lysodeikticus*, from the antibacterial serum factor.

The observation that *M. lysodeikticus* was susceptible to the action of the 25 per cent globulin fraction led to a search for lysozyme activity in this fraction. These assays were done with Difco lysozyme substrate, lysozyme, and lysozyme buffer, using the procedure recommended by the manufacturers. No reduction in the turbidity of a standard suspension of killed *M. lysodeikticus* (lysozyme substrate) could be detected when equal volumes of the suspension were mixed with a 40 mg per ml solution of the globulin fraction.

Effect of varying times of exposure. In early experiments the cell suspensions were exposed arbitrarily to the serum factor for 1 hr at 4 C. To determine the velocity of the reaction and the optimal exposure time, 10 coagulase negative strains of *Staphylococcus aureus* were selected. These were prepared and exposed to 40 mg of the globulin fraction per ml of cell suspension for 0, 5, 15, 30, 45, and 60 min at 4 C. After this exposure their ability to oxidize glucose was determined manometrically. Exposure to the antibacterial globulin fraction for 60 min resulted in the greatest loss of cellular activity. Exposure up to 3 hr produced no further decrease in the oxidative activities of the organisms.

Effect of concentration. To determine the specific activity of the water soluble globulin fraction, washed cell suspensions were exposed to varying concentrations of the fraction for 60 min. Exposure to 10 mg per ml resulted in approximately a 25 per cent reduction of oxidation of glucose by the cells, whereas 40 mg per ml reduced the activity almost 60 per cent. Higher concentration produced no greater reduction.

Effect of ionic strength. In previous experiments the organisms after growth in brain heart infusion broth (Difco) were harvested, washed, and standardized in water. In attempting to study the pH optimum of the antibacterial serum globulin, 0.15 M buffers at a wide range of pH were prepared and the cells exposed to the serum factor in these buffers. Little or no activity could be demonstrated at any pH. These results led to a consideration of the effect of ionic strength on the reaction. The cells were grown, harvested, and washed three times with distilled water. Standardized suspensions were prepared in pH 7.0 phosphate buffer at molarities ranging from 10^{-1} to 10^{-5}. Each suspension was treated with 40 mg of the water soluble globulin fraction per ml for 1 hr at 4 C. The suspensions were tested manometrically for their ability to oxidize glucose. Figure 3 shows the results of these experiments. Only when the cells were exposed to the globulin fraction in water or buffers of 10^{-4} M or less was there a significant reduction of glucose oxidation.

Effect of pH. When an understanding of the

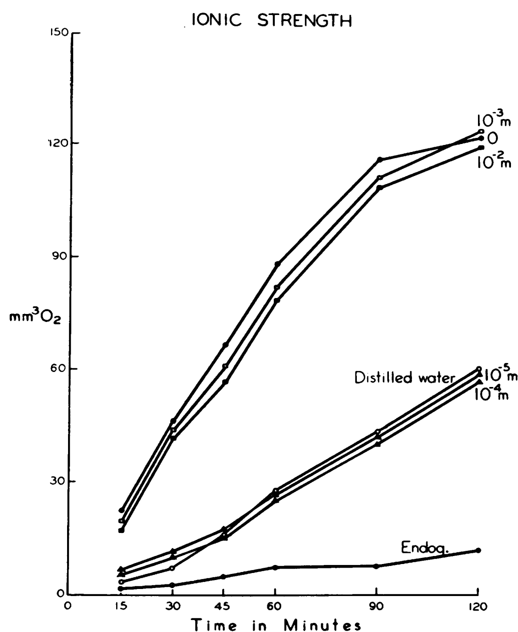

Figure 3. Effect of ionic strength on the action of the water soluble globulin portion. Flask contents: 0.5 ml cell suspension, 0.5 ml 5.5×10^{-3} M glucose containing 3 μM ATP, 1.0 ml phosphate buffer, pH 7.0, and 0.2 ml 40 per cent KOH in the center well.

ionic strength requirements of the reaction had been reached, experiments were performed to determine the optimal pH for the antibacterial activity of the water soluble globulin fraction. Phosphate buffers at 10^{-4} M ranging from pH 6 to 9, were prepared, and washed standardized cell suspensions of staphylococci were exposed to 40 mg of the fraction per ml at each pH. After exposure the cells were tested manometrically. It was found that the antibacterial serum factor reacted equally well at all pH levels tested.

Heat stability of the antibacterial factor. Ekstedt (1956) showed that the antistaphylococcal activity of undiluted human serum was stable to heating at 60 C for 30 min. To test the effect of temperature on the partially purified water soluble globulin fraction, 12 per cent aqueous solutions (60 mg per 0.5 ml) were prepared and samples held for 30 min at 4, 25, 37, 56, and 70 C. Standardized cell suspensions were added to the tubes to bring the concentration of globulin to 40 mg per ml of cell suspension. These mixtures were allowed to react for 1 hr at 4 C and the manometric assay performed. No significant difference in the activity of the globulin fraction could be detected, even after heating at 70 C for 30 min.

Antagonism of the water soluble globulin fraction by coagulase. It was shown previously that partially purified coagulase, when used to treat washed cell suspensions of staphylococci, protected them against the antibacterial action of human serum and the purified active fractions. The experiments described below were undertaken to obtain quantitative data on the antagonism of the water soluble globulin fraction by coagulase. To tubes containing 2 mg partially purified coagulase, 4, 8, 16, and 50 ml of the usual standardized cell suspensions of 10 strains of coagulase negative staphylococci were added. This resulted in cells treated with 0.5, 0.25, 0.125, and 0.05 mg coagulase per ml of suspension.

The coagulase was allowed to react with the cells for 20 min at 37 C. At the end of this time 1.5 ml samples of each suspension were added to tubes containing 60 mg of the water soluble globulin fraction and the mixtures allowed to react for 1 hr at 4 C. After this treatment manometric studies were performed. The results showed that 0.25 mg of partially purified coagulase per ml of cell suspension completely protected the cells against the action of 40 mg per ml of the antibacterial globulin fraction. The antibacterial effect of the globulin fraction was decreased about 50 per cent by 0.125 mg of coagulase, while 0.05 mg was below the level required to demonstrate any protective effect.

Assay of the water soluble globulin fraction for anticoagulase. In view of the ability of coagulase to neutralize the antibacterial activity of the globulin fraction, titration of the serum factor for anticoagulase activity was carried out.

A solution of partially purified coagulase was prepared using 2 per cent peptone-saline containing 1:5000 merthiolate as a diluent. This solution contained four minimal clotting doses per ml, one minimal clotting dose being defined as the least amount capable of producing a solid clot in undiluted noninhibitory human plasma in 4 hr at 37 C, and 20 hr at room temperature. The water soluble globulin fraction was dissolved in the above diluent at a concentration of 40 mg per ml and serial 2-fold dilutions prepared in 0.25 ml volume. To each dilution of the serum factor 0.25 ml of the coagulase solution concontaining 1 minimal clotting dose was added

TABLE 1

Effect of human globulin and coagulase on the viability of strains of Staphylococcus aureus

	Mean Number of Viable Organisms*					
	Coagulase positive			Coagulase negative		
	Cell treatment					
	None	Globulin	Coagulase and globulin	None	Globulin	Coagulase and globulin
No oscillation	2.4×10^9	2.5×10^8	1.2×10^9	6.0×10^8	5.2×10^7	4.4×10^8
SD†	±1.9	±2.2	±4.3	±4.6	±7.0	±5.2
Oscillation	2.1×10^9	2.8×10^8	1.3×10^9	7.8×10^8	6.7×10^7	2.1×10^8
SD	±3.8	±2.9	±1.2	±5.6	±10.1	±2.7

* The values represent the mean and standard deviations calculated from five separate experiments, each with coagulase positive and coagulase negative strains.

† SD = standard deviation.

and the tubes incubated at 37 C for 1 hr. After this preliminary incubation, 0.5 ml of undiluted human plasma was added to each tube and incubation continued for 4 hr at 37 C, and 20 hr at room temperature. The last tube showing no clot formation was taken as the end point. In each of five experiments, the anticoagulase titer of the globulin fraction was 1:8.

Effect of the globulin fraction on the viability. A number of the observations reported here (ionic strength, time of reaction, pH experiments, heat stability) made it doubtful that the antibacterial serum factor, at the concentrations used, functioned as a metabolic inhibitor, but suggested rather a direct lethal and possibly lytic action on the cells. The decrease in glucose oxidation might have been a reflection of direct bactericidal action. On a number of occasions the cells, after exposure to the serum factor, were observed by phase contrast microscopy. In addition to a certain amount of clumping of the cells, there appeared to be a change in their optical opacity. In order to determine the effect of the water soluble globulin fraction on the viability of staphylococci, the usual bacterial suspensions were prepared and divided into three samples of 1.5 ml in duplicate. The first samples served as controls, the second were exposed to 40 mg per ml of the globulin fraction as previously described, and the third were treated with 1 mg coagulase per ml for 5 min and then exposed to 40 mg of the globulin fraction. After 1 hr exposure at 4 C one of the samples from each treatment was shaken in a Mickle disintegrator for 90 sec to break up the aggregates, and pour plates were made after appropriate dilution. Preliminary observations showed that this brief agitation in the Mickle dispersed the agglutinated cells without affecting their viability. The duplicate samples from each treatment were diluted and plated without agitation in the Mickle disintegrator. The results of these experiments are shown in table 1 and represent the mean counts obtained in five separate experiments. As can be seen, the globulin fraction at a concentration of 40 mg per ml of cell suspension has a lethal activity against both coagulase positive and coagulase negative strains, producing a 90 per cent reduction in viability in both cases. Furthermore, treating the cell suspensions with coagulase protected them against the lethal action of the antibacterial factor.

Electron microscopic observations. To study the morphological changes in the cells when exposed to the water soluble globulin fraction, electron microscopic observations of various preparations were made. Figure 4 shows the control cells which had been washed, suspended in water, allowed to stand for 1 hr at 4 C, and dried on the electron microscope grids. Figure 5 shows the cells exposed to 40 mg of the water soluble globulin fraction per ml of suspension for 1 hr at 4 C. The cells appear clumped and many appear to be undergoing lysis. Cells still intact appear less dense and particulate structures were apparent in the cytoplasm. Cells treated with

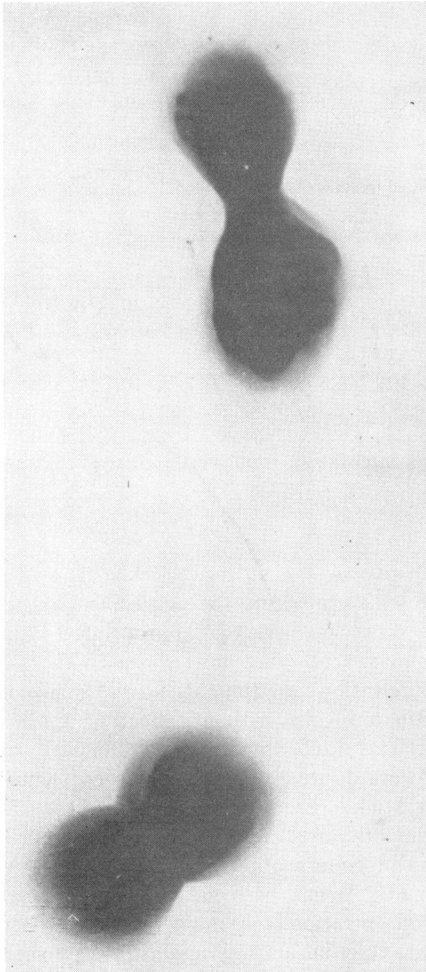

Figure 4. Staphylococcus aureus, no treatment, ×14,750.

coagulase prior to exposure to the globulin fraction, although clumped, showed no evidence of lysis.

Properties of the water soluble globulin factor. The antibacterial water soluble serum globulin fraction was insoluble in absolute ethanol, ether, and acetone. It gave positive reactions in the Millon, glyoxylic, xanthoproteic, biuret, and cystine-cysteine sulfur tests. It gave precipitates with concentrated mineral acids, heavy metals, ethanol, heat, and alkaloidal reagents which could not be dissolved. With alkali there was no precipitate but hydrolysis occurred. The Molisch test was negative both before and after acid hydrolysis.

DISCUSSION

An antibacterial factor is present in human, rabbit, and horse serum, but not detected in bovine serum. It is localized in the water soluble fraction of the proteins precipitated by 25 per cent saturation by ammonium sulfate. When coagulase negative staphylococci were treated with this fraction at a concentration of 5 mg per ml of cell suspension, a marked loss in their ability to oxidize glucose occurred. This concentration is close to the concentration of gamma globulin in human serum. Coagulase positive staphylococci were unaffected at this concentration of the serum factor, but when treated with 40 mg per ml of standard cell suspension were also markedly inhibited in their oxidation of glucose.

The action of the inhibitory serum factor is not specific for staphylococci, since under appro-

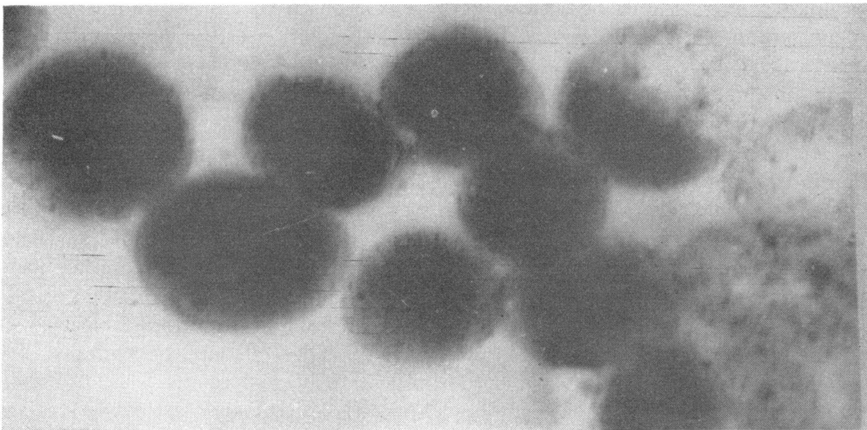

Figure 5. Staphylococcus aureus treated with 40 mg per ml of the water soluble globulin portion for 1 hr at 4 C, ×14,750.

priate conditions, other gram-positive organisms also may be adversely affected. It will be of interest in the future to study the susceptible microorganisms for similarities in structure or function which may serve as substrate for the action of the antibacterial factor.

The results show that the antibacterial water soluble globulin fraction from human serum has a direct lethal and partially lytic effect on staphylococci. These results could explain the marked inhibition of glucose oxidation when susceptible species of microorganisms are exposed to this substance, and make metabolic inhibition as the mechanism of action of the serum factor less probable.

The inability to detect antibacterial action of the partially purified serum factor when the microorganisms were exposed in buffers from 10^{-1} to 10^{-3} M suggests that under conditions approaching isotonicity, lysis of the exposed cells would not occur. It is clear, however, from earlier studies (Yotis and Ekstedt, 1959) that whole human serum under physiological conditions does alter the cells of coagulase negative staphylococci in some manner which can be detected by a decrease in their ability to oxidize substrate. An alteration in their ability to concentrate certain substrates or a change in permeability upon exposure to antibacterial human serum would be worth studying.

The function of coagulase in reversing the antibacterial activity of the partially purified serum factor remains to be satisfactorily explained. It has been shown (Ekstedt and Yotis, 1960) that coagulase negative staphylococci can adsorb partially purified coagulase from solution. This adsorbed coagulase remains rather firmly attached and can be detected on the cells after five washings with distilled water. We postulate that the function of coagulase is to protect susceptible cells from the antibacterial factor by physically preventing the reaction of the serum factor with its substrate in the cells.

Three activities have been demonstrated in the water soluble globulin fraction from human serum. This fraction has some agglutinating action in high concentration, possesses a low titer of anticoagulase, and exhibits antibacterial activity against three species of microorganisms. It is difficult to state whether these activities are different manifestations of the same substance, or distinct entities. It is unlikely that the agglutinating activity and the antibacterial activity are identical since agglutination can be observed in those cells exposed first to coagulase and than to the serum factor, while antibacterial activity is not demonstrable under these conditions. In general, agglutinating antibody does not affect the metabolic activities of microorganisms (Oldfelt, 1942). It is also difficult to conceive in what way anticoagulase might act detrimentally against coagulase negative staphylococci or *B. subtilis* and *M. lysodeikticus*.

SUMMARY

A water soluble globulin fraction from normal human serum was shown to have a direct lethal and lytic action on *Staphylococcus aureus* when used in sufficient concentration. The activity of the fraction could be demonstrated only when the cells were exposed in aqueous suspensions or in buffers below 10^{-4} M. The antibacterial factor was stable to heating at 70 C for 30 min, and its activity was the same over a pH range from 6 to 9.

A substance of similar activity was demonstrated in rabbit and horse serum, but not in bovine serum.

The serum globulin fraction was shown to inhibit also the oxidation of glucose by *Bacillus subtilis* and *Micrococcus lysodeikticus*.

The antibacterial activity of the fraction could be reversed by treating staphylococci or *B. subtilis* with coagulase. *M. lysodeikticus* could not be thus protected.

The mechanism of action of the serum factor in relation to its antibacterial, agglutinating, and anticoagulase activity is discussed.

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