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The Effect of Rat and Rabbit Sera on the Metabolism of Coagulase Positive and Coagulase Negative Staphylococci

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THE EFFECT OF RAT AND RABBIT SERA ON THE METABOLISM OF COAGULASE POSITIVE AND COAGULASE NEGATIVE STAPHYLOCOCCI

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LIFE

Jesse S. Ortiz was born on October 18, 1933 in Barranquitas, Puerto Rico.

He was graduated from Central High School in San Juan, Puerto Rico in 1952, and received a Certificate in Medical Technology from the Eastern School for Physicians' Aides in New York City in 1954. He worked as a laboratory technician at Irvington House, Irvington, N.Y. from 1954-1955.

In 1955, he entered Northwestern University on a part-time basis while working at Northwestern University Medical School as a medical laboratory supervisor, and was graduated with a Bachelors of Philosophy in 1961. He began his graduate studies at Loyola University in 1962.

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STATEMENT OF THE PROBLEM

Many investigators have demonstrated the existence of antimicrobial substances in blood serum, leukocytes, platelets, and other tissue fluids. The majority of these substances, dependent on complement and properdin for their action, have been well defined for the gram negative organisms.

Studies on antibacterial factors against gram positive organisms have been conducted with only a few species of this group. The sera of humans, rabbits, and rats have been studied extensively for their activity against Bacillus subtilis. Some studies with the same organism have been done with platelets or platelet extracts.

Human and rabbit sera have also been extensively studied for their activity against Staphylococcus aureus. A great deal of confusion has arisen regarding the identities of the anti-staphylococcal factors and their role in natural resistance to the infectious processes.

The aim of the present study is a systematic comparison of the anti-staphylococcal activity of rabbit and rat serum with respect to anti-respiratory and bactericidal action against both coagulase positive and coagulase negative staphylococci.

HISTORY

1. Antibacterial Factors in Serum and Tissue.

Factors present in normal serum against bacteria have been described as bactericidal, bacteriostatic and antirespiratory. The bactericidal effect of serum for gram positive organisms was independent of complement, and attributed to the B-lysins (Pettersson, A., 1926). He found that the beta-lysin was unlike alexin in that it acted principally against gram positive bacteria. It required temperatures of 64° to 75°C. for one half hour to become inactivated, withstood dialysis and extraction with fat solvents, and was somewhat active at low temperatures.

Mackie and Finkelstein (1932) reported a thermostable substance in animal sera that withstood heating at 55°C. for one half hour and was active only against gram positive bacteria. They obtained most frequent and most pronounced effects with an attenuated strain of Bacillus anthracis, avirulent pneumococci and Micrococcus lysodeikticus. This thermostable substance appeared to be unrelated to "plakins", "leukins", or "lysozyme". They arranged various animal species in descending order of the activity of their "thermostable" bactericidins: Rabbit (most active), rat, horse, human, pig, sheep, ox, guinea pig and pigeon (rarely active). They also concluded that individual animals of the same species vary considerably in the bactericidal activity of their sera.

Tillet (1937) reported a bactericidal substance against hemolytic streptococci in the sera of patients with acute infections. This substance was present in high concentration during the active phase of the disease,

but was absent or greatly diminished in potency after recovery. The anti-streptococcal substance was absent, in all instances, in normal individuals.

In a series of papers, Myrvik and his associates (1955, 1956) investigated a serum bactericidin active against Bacillus subtilis and Micrococcus pyogenes var. albus, and quantitated this bactericidin in the sera of rat, rabbit, human, guinea pig, cow and dog. High levels of bactericidin were found only in the rat and rabbit sera. Sera of healthy humans contain only small amounts of antibacterial substance, but the levels were increased several fold during the acute phase of various illnesses. They were not successful in their attempts to extract bactericidin substances from liver, spleen, polymorphonuclear leucocytes and monocytes of rabbits.

Myrvik et al. (1958) found that the antibacterial substance in human serum required both calcium ions and bicarbonate ions for its activity, though only bicarbonate ions were required in the rabbit system. Bactericidal activity of rabbit serum was lost upon dialysis in saline, but was restored upon the addition of bovine serum dialysates. The antibacterial activity showed a wide range of pH (from 6-9), but was lost at pH ranges from 3-5.

Myrvik (1960) was able to demonstrate that the serum bactericidal activity was due to two non-dialyzable components; complement and lysozyme were excluded as participants. Component I from rabbits was able to form a complete bactericidal system with component II from human serum. Naff et al (1958) showed that the serum level of bactericidin activity against B. subtilis was greatly elevated in patients with myocardial infarction. This finding confirmed Tillet's work.

Jacox (1950) reported on a bactericidal factor against B. subtilis found in patients with acute infections, carcinomas and other diseases. This bactericidal substance was not related to complement, C-reactive protein, or to specific antibodies. Jacox's bactericidal factor was inactivated by decalcification of the serum, but regained activity when an optimal concentration of calcium was added. The addition of magnesium did not cause reactivation of this substance.

Hirsch (1960) studied platelets as a source of bactericidal factors against B. subtilis, Bacillus megaterium and S. aureus. Serum derived from plasma which had been centrifuged in siliconized glassware to decrease the number of platelet showed a much reduced bactericidal activity against these gram positive organisms when compared with serum obtained from platelet rich plasma. Platelet rich plasma was as active as serum obtained from blood allowed to clot normally. The substance released by platelets during the clotting process was heat-stable, and was not found in heparinized or citrated plasma. Human platelet-rich plasma was less bactericidal than rabbit platelet rich-plasma.

Further studies on platelet action against B. subtilis by Jago and Jacox (1961) indicated that rabbit and rat platelets contained two non-dialyzable components, which, together with bicarbonate ions, produced potent bactericidin for B. subtilis. The authors claimed that the platelet factors appear to be identical to the serum bactericidal substances, and suggested that disruption of platelets during blood clotting releases the platelet factors into the serum.

Both components are present in rabbit and rat platelets and are absent in normal human platelets. Component I is present in high concentration in rabbit and rat sera, but is either absent or present only in low concentrations in sheep, bovine, horse and human sera. Component II is found in sheep, horse, bovine, rabbit, rat and normal human serum.

2. Antistaphylococcal Factors

"The close correlation between the ability of staphylococci to clot blood plasma and their capacity to produce disease, and the corresponding absence of this property in non-pathogenic strains, have led to the assumption that the enzyme, coagulase, plays some role in the pathogenicity of disease." (Blair, 1962)

Hale and Smith (1945) reported on the influence of coagulase on the phagocytosis of staphylococci. They observed that coagulase negative strains were readily phagocytized in citrated blood, while coagulase positive strains were not. Furthermore, with certain species of animals, inhibition of phagocytosis was demonstrable with plasma not normally coagulable by coagulase, provided that coagulase activator was added to the mixture.

Further studies on the role of coagulase in staphylococcal infections by Smith, Hale and Smith (1947) suggest that coagulase is a major factor in the initiation and development of staphylococcal lesions, but that once the organisms have gained a foothold, other factors come into play.

Rogers and Tompsett (1952) observed that coagulase positive staphylococci ultimately were ingested by leukocytes and were capable of sur-

vival within leukocytes for a longer period of time than coagulase negative organisms.

Spink and Vivino (1942) reported that coagulase positive strains of staphylococci resisted the bactericidal action of human defibrinated blood, much more so than coagulase negative strains.

Studies by Ekstedt and Nungester (1955) and Ekstedt (1955) revealed that normal human serum contains an antistaphylococcal factor and that its activity can be inhibited by the addition of exogenous coagulase. Myrvik (1956) was unable to confirm the role of coagulase in the neutralization of this serum factor. Unlike Ekstedt, he found that the addition of crude coagulase to rabbit sera did not impair bactericidal action toward susceptible bacterial strains. It should be pointed out, however, that Myrvik worked with rabbit sera, and he used crude coagulase and visual methods to determine bactericidal activity, whereas Ekstedt worked with human serum and partially purified coagulase, and used plate counts to determine the action of the antistaphylococcal factor.

It has been shown that human sera has less bactericidal activity than rabbit sera under the same conditions (Myrvik, 1956).

Fletcher (1962) showed that the antistaphylococcal factor is demonstrable for coagulase negative strains, in serum diluted to a concentration of 17.5 per cent and buffered pH values of 8.2 - 8.8. The staphylococcus inhibitor was stable to boiling for ten minutes and was not destroyed by the addition of heat killed organisms or by the addition of concentrated preparations of coagulase. However, many proteinaceous materials, including

peptone, destroyed the inhibitor when added to the serum in fairly high (5%)concentrations.

Borowski and Tybusz (1963) established a correlation between the quantitatively expressed ability of staphylococci to produce coagulase and their resistance to the bactericidal action of human sera. They concluded that human sera contain different quantities of antistaphylococcal factor.

Yotis and Ekstedt (1960) have shown that the antistaphylococcal serum factor is localized in a water soluble globulin fraction of human, rabbit and horse sera and has a direct lethal, and partially lytic, action on coagulase negative staphylococci.

Yotis (1962) reported further purification of the human serum antistaphylococcal factor. He found that this partially purified serum factor was able to protect mice when virulent staphylococci were subjected to its action for one hour before injection.

Recently, Donalson et al. (1964) described a method for the purification of beta lysin from normal rabbit serum. This method, which involves filtration and elution of the absorbed beta-lysin from Seitz filters, resulted in a 1000 - 5000 fold concentration of the active substance.

3. Respiratory Action

Martin (1932) suggested the oxygen consumption of growing cultures of bacteria as a criterion of growth.

Grieg and Hoogerheide (1941) showed that oxygen uptake of growing cultures of bacteria was directly proportional to bacterial

content. This relationship was valid for Proteus vulgaris, S. aureus, Pseudomonas fluorescence and a yeast. Under favorable conditions, measurement of the rate of oxygen uptake constitutes a convenient method for the measurement of the rate of growth.

Wood et al. (1958) described a method for measuring the antibacterial activity of sera from different animal species against B.subtilis. They used the Warburg apparatus and did concomitant studies with the micro Kjeldahl to confirm their results. Yotis and Ekstedt (1959) showed that coagulase positive strains of staphylococci respired in human serum much more actively than did coagulase negative strains; although coagulase positive and coagulase negative strain oxidized glucose and pyruvate equally well. The addition of purified coagulase 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.

Bornside et al. (1963) showed that serum of patients with neoplastic diseases have an elevated antirespiratory activity against a strain of B. subtilis. They did not observe this antirespiratory activity with sera from normal individuals. Further work by Bornside et al. (1964) indicated that the antirespiratory activity of serum was diminished by being heated at 56°C. for thirty minutes. The antirespiratory activity was decreased by the addition of excess ferric ions to the system, though reversal was not, however, obtained by the addition of transferrin. Addition of aluminum, magnesium, manganese, copper, cobolt or zinc ions had no such effect. The effect of ferric ions was on the serum and not on the bacilli.

A two-fold dilution of rabbit serum eliminated antirespiratory activity, but showed less effect on bactericidal activity. The bactericidal activity of rabbit serum was detected quickly upon mixture of serum and bacilli, but the antirespiratory effect was usually discernible after two hours. By these methods of assay, there was no statistically significant association between bactericidal and antirespiratory activity. Alcoholic fractionation of serum indicated that the antirespiratory agent was a globulin, but study of globulins from man and animals failed to identify any single globulin as the consistent antirespiratory agent.

4. Absorption Studies

Absorption methods have been applied in the removal of related or closely related substances from a system. Jacox (1950) used C-carbohydrate and pneumococcus XXVII in an attempt to remove bactericidal activity against B. subtilis from human sera. His experiments reveal that bactericidal activity was still present in the absorbed sera.

Myrvik and Wieser (1955) claimed that the bactericidin in rabbit serum against B. subtilis was of cationic nature readily absorbable by bentonite.

Yotis (1963) showed the antistaphylococcal factor to be absorbed by both coagulase positive and coagulase negative staphylococci and by B. subtilis. Escherichia coli, Neisseria catarrhalis, Bacillus megaterium, Proteus vulgaris, Mycobacterium phlei and Saccharomyces cerevisiae failed to absorb the serum factor.

Fletcher (1962) claimed that the inhibitor in human serum for

coagulase negative staphylococci was not abolished by live or heat-killed suspensions of S. aureus or by coagulase. It seems that Fletcher worked with a system somewhat different from those of Jacox, Myrvik and Yotis.

MATERIALS AND METHODS

Cultures: Forty-four coagulase positive and coagulase negative strains of Staphylococcus aureus were tested for glucose and D-mannitol utilization, gelatin liquification, pigmentation and coagulase production. Fermentation of carbohydrates was done both anaerobically and aerobically according to the method of Hugh and Leifson (O-F medium, 1953). The basic medium consisted of 0.1% casitone, 0.1% yeast extract, 0.05% Tris (Hydroxymethyl) aminomethane buffer, 0.3% agar, 0.5% NaCL and 0.001% bromethymol blue as indicator. The pH was adjusted to 7.5 with 0.1N NaOH. Dextrose and D-mannitol were prepared in 10% concentration and sterilized separately. Ten ml of the respective carbohydrates were added aseptically to 90 ml of the above medium, giving a final concentration of 1%.

Two tubes of each sugar broth were inoculated per culture. To one tube 0.5 ml sterile petrolatum was added to establish anaerobic conditions. Cultures were incubated at 37°C. for 48 hours after which they were read. The nutrient gelatin cultures were incubated at 37°C. for 7 days, then placed in cold water until the control was solid. Trypticase soy broth and Trypticase soy agar (BBL) were inoculated and incubated at room temperature for 48 hours and then checked for pigment production and grum stain reactions.

Five strains of each, coagulase positive and coagulase negative staphylococci, were selected for studies of serum factor activity. These strains were kept in stock on Trypticase soy agar slants in screw capped tubes. Transfer to new slants was done every two weeks. Each transfer

was followed by coagulase titration and sugar utilization to make sure no changes had taken place on storage.

Coagulase titration: Coagulase titrations were done on the supernatant of cultures grown for 24 hours in Brain Heart Infusion broth (Difco). One half ml of fresh rabbit citrated plasma (1% citrate), diluted 1:4 in sterile 1% Bacto peptone saline was added to each 0.5 ml of supernatant. When dilutions of supernatants were required, these were done in sterile 1% peptone saline. The supernatant plasma mixture was incubated at 37°C. for 4 hours and then checked for clot formation. The tubes were then left at room temperature for 24 hours. If no clot was apparent at this time, the culture was described as "coagulase negative".

Sera: Serum was prepared from blood collected from the central ear artery of adult unanesthetized New Zealand rabbits, and from the dorsal artery of adult male rats anesthetized with ether. Blood was allowed to clot for one hour. The clot was rimmed with sterile applicator sticks and then centrifuged for 15 minutes at 2,500 rpm. The clear serum was separated from the clot and re-centrifuged for ten minutes at 2,500 rpm. Unless specified, the serum was used in all instances within two hours after bleeding.

Serum Antirespiratory Activity: Several staphylococcal strains were used in this study. Cells were harvested routinely from an 18 hour culture of heavily seeded Trypticase soy agar (BBL) plates incubated at 37°C. The cells were washed two times in sterile distilled water and then resuspended in 5 ml sterile distilled water with a few glass beads. A

homogeneous suspension was made. The cells were adjusted to an optical density of 1 (500 Klett units) using a 420 millimicron wave-length blue filter. Serum antirespiratory activity was measured manometrically according to the Warburg method of Wood et al. (1958). The total Warburg flask volume of 2.2 ml consisted of 1 ml serum, 0.5 ml cells (500 Klett units), 0.5 ml of 0.1M potassium phosphate buffer at pH 7.2, and 0.2 ml of 40% KOH in the center well. Two flasks were used for each strain and 2 flasks containing buffer instead of serum served as the endogenous respiration control. The flasks were equillibrated for 15 min at 37°C. Subsequent readings were taken every 15 minutes for the first hour and at 30 minute intervals thereafter. The mean volume of oxygen consumed by the endogenous control was subtracted from the mean volume of oxygen consumed by each strain. The difference was used to calculate microliters of oxygen uptake per mg. dry weight of cells.

Dry weights were determined by drying at 95°C to constant weight 10 ml portions of washed bacterial suspensions adjusted to a Klett reading of 500. Five samples were used for each strain and the mean dry weight was recorded. Oxygen consumption was calculated per mg. dry weight of each strain.

Serum Bactericidal Activity: Cells for serum bactericidal activity were grown in 10 ml of Trypticase soy broth for 18 hours. Cultures were gram stained prior to their use. Cells were harvested by centrifugation and washed 2 times in sterile distilled water. The packed cells were resuspended in 5 ml of sterile distilled water with a few

glass beads. A homogeneous suspension was prepared by manual shaking. The culture was then adjusted to an optical density of 0.1 (50 Klett units) by the use of a 420 millimicron wave-length blue filter. This suspension was found to contain from 10 to 5 x 10^7 cells per 0.1 ml.

One-tenth ml (0.1 ml) of cell suspension was inoculated into 2 ml of serum in a heavy wall conical centrifuge tube and incubated at 37°C. for 18 to 24 hours. Then a 0.1 ml sample was removed and dilutions made for plate counts.

Plate Counts: Dilutions were made in sterile distilled water. One tenth (0.1 ml) ml of the proper dilution (previously determined) was inoculated into 15 ml of Trypticase soy agar and a plate poured. Two different dilutions were plated per strain. The plates were incubated at 37°C. overnight. The colonies were counted in each plate and multiplied by the dilution factor to obtain the number of organisms per ml. The remaining portion of the serum culture was centrifuged at 5,000 rpm for 15 minutes. The packed cells were washed two times and then they were resuspended in 5 ml distilled water with glass beads. After shaking it to obtain a homogeneous suspension, the optical density was taken using a 420 millimicrons wave-length blue filter.

Preliminary studies indicated that bactericidal activity could be demonstrated within 2 hours. However, concomitant studies of both bactericidal activity and optical density were desired for the present study. Therefore 18-24 hours were allowed to elapse before plate counts and O.D. measurements were made.

Salts: 0.4M stock solutions of sodium oxalate, sodium citrate, magnesium chloride, and calcium chloride were prepared. Solutions of ferric chloride ($FeCl_3$), manganese sulfate ($MnSO_4$-H_2O), magnesium chloride ($MgCl_2$-$6H_2O$) and zinc sulfate ($ZnSO_4$-$7H_2O$) were also prepared at a concentration of 30 mg per ml.

Absorption Studies:

1. Bentonite. Two grams of bentonite (200 mesh), were suspended in 100 ml saline and boiled for 30 minutes. The boiled suspension was centrifuged at 3,000 rpm for 15 minutes. The supernatant was discarded and the sedimented bentonite was washed 2 times in sterile saline. The washed bentonite was resuspended in 200 ml sterile saline, a homogeneous suspension prepared and the solution was stored in the icebox.

For absorption experiments, the stock solution was shaken vigorously by hand and the appropriate amount (10 ml = 100 mg) pipetted into a centrifuge tube; this was then centrifuged at 5,000 rpm and the supernatant discarded. The sedimented bentonite was used to absorb serum.

2. Cells. Cells for absorption were grown in 200 ml Trypticase soy broth for 18 hours at 37°C. Then the cells were heat-killed at 60°C. for 1 hour and centrifuged at 3,000 rpm for 15 minutes. The supernatant broth was discarded, the sedimented cells were washed 2 times with sterile distilled water and the packed cells used to absorb 2 ml of serum.

Absorption Procedures: Homogeneous suspensions of cells or bentonite and serum were incubated at 37°C. for one-half hour, followed by 1½ hours at 4°C. The absorbed mixture was then centrifuged at 4,000 rpm

for 15 minutes. The clear serum was transferred to a heavy wall conical centrifuge tube and centrifuged once more at 5,000 rpm for 15 minutes. The clear serum was collected aseptically, placed into screw capped tubes, and used for bactericidal tests. Pour plates were made with samples of the serum to check for sterility.

RESULTS

Survey of Strains: Forty-four strains of staphylococci recently isolated from clinical cases were studied for a)coagulase production, b)glucose and d-mannitol fermentation, c)gelatinase activity and d)pigmentation. Table I shows the results of the study. Seventy-seven per cent were classified as Staphylococcus aureus. The other 23% were considered to be Staphylococcus epidermiditis. Ninety-six per cent fermented glucose, and 77% fermented d-mannitol. Seven per cent oxidized d-mannitol and 16% did not. Ninety-one per cent liquified gelatin and 68% produced yellow pigment. Two strains which did not utilize carbohydrates at all were discarded. Those that oxidized d-mannitol were also thought unsuitable for the present study.

The coagulase positive strains selected for further study clotted citrated plasma within 4 hours. The coagulase negative strains selected failed to clot citrated plasma within 24 hours, did not utilize d-mannitol or liquify gelatin.

Metabolic Studies: Five coagulase positive and 5 coagulase negative strains were selected for metabolic studies. The oxygen uptake in air was determined manometrically with Trypticase soy broth (TSB), Rabbit and Rat serum as substrates. Figure I shows the respiration of both coagulase positive and coagulase negative staphylococci with TSB as substrate. It can be seen that both coagulase positive and coagulase negative strains respire equally well in broth. Although the respiration rate of coagulase negative strains is a little less than that of coagulase positive

strains, the differences are small. The endogenous respiration for the two groups was so similar that the data were averaged and plotted as one curve.

Figure II shows the respiration of coagulase positive and coagulase negative strains with rabbit serum as substrate. The data revealed that coagulase positive strains respire very well in rabbit serum, while coagulase negative strains respire very poorly. This difference was attributed to the presence in rabbit serum of an antirespiratory factor against coagulase negative staphylococci. This factor is not apparent with coagulase positive strains. This may be due to the ability of coagulase to neutralize the serum antirespiratory activity. (Yotis, 1963)

Figure III shows the respiration of coagulase positive and coagulase negative staphylococci with rat serum as substrate. This figure shows that coagulase positive strains respire much better than do coagulase negative strains.

A comparison of the oxidation rate of the coagulase positive and coagulase negative strains in rabbit and rat sera (Figure IV) indicates that coagulase positive strains respire much better in rabbit serum than in rat serum. Coagulase negative strains respire in rabbit serum almost as well as coagulase positive strains do in rat serum. These differences might be due to the presence in rat serum of more antibacterial factor against staphylococci than in rabbit serum. The differences in oxidation rate of coagulase positive and coagulase negative strains in rat serum are not as marked as the differences in the oxidation rate of these strains in rabbit serum.

Effect on Antirespiratory Activity by Ferric Ion: Bornside et al. (1964) showed that the antirespiratory activity of various sera against B. subtilis was reversed by the addition of ferric ion to the serum. Experiments were performed to determine the concentration of iron needed to reverse the antirespiratory activity of rabbit serum. Table II shows the oxidation of rabbit serum with both coagulase positive and coagulase negative staphylococci with various concentrations of ferric ion. In the presence of increasing amounts of ferric ions, the inhibitory effect of rabbit serum on oxygen consumption by coagulase negative staphylococci was decreased. The oxygen uptake approached that of the coagulase positive strain controls and in some cases it exceeded the controls. It was noticed that the decreased effect on the system was most pronounced with 1.5 mg of iron per ml of serum. Subsequent experiments were thus set up using 1.5 mg of iron for the reversal of antirespiratory activity of both rabbit and rat sera, Figures V, VI, and VII. Studies using glucose and ATP as substrate indicated that the reversal of antirespiratory activity by ferric ions was due to some direct action of iron on the serum and not on the cells. Figure VIII shows that ferric ions has no effect on the respiration of coagulase negative staphylococci when glucose is used as a substrate.

Ferric ions was the only metal effective in decreasing respiratory inhibition. When the same concentration of other metal ions was employed, the following were without effect: magnesium chloride, zinc sulfate and manganese sulfate (Table III).

Bactericidal Activity: The kinetics of bactericidal activity

were investigated using rabbit and rat serum. One tenth (0.1) ml of culture with optical density of 50 Klett units was inoculated in 2 ml of rat and rabbit serum and in TSB. Samples were incubated at 37°C. for 18 to 24 hours. One tenth ml portions were removed, serial dilutions were made in sterile distilled water and Trypticase soy agar plates were poured. The rest of the sample was centrifuged, washed 2 times in distilled water, resuspended in 5 ml distilled water and the optical density was determined.

Figure IX shows the growth of coagulase positive and coagulase negative staphylococci in TSB, rat and rabbit sera measured turbidimetrically. Apparently the coagulase positive strains multiply in rat and rabbit sera at about the same rate as in broth. Even though there is some difference, this difference is not pronounced. On the other hand, coagulase negative strains failed to multiply in rat and rabbit sera, but grew just as well as the coagulase positive strains in the Trypticase soy broth.

Effect of Inoculum: Different concentrations of the bacteria were inoculated into rat and rabbit sera to determine if there were a difference in the concentration of antistaphylococcal factor in the two species. Table IV shows the growth of coagulase negative staphylococci with increasing concentrations of inoculum measured turbidimetrically. With rabbit serum the coagulase negative staphylococci grow well when the inoculum is increased to an optical density of 0.2. However, the same was not true for rat serum, which required an inoculum of an optical density of 1.4 before growth could be detected. This indicates that the bactericidal activity of rat serum against coagulase negative staphylococci

is much greater than that of rabbit serum for the same organisms. With coagulase positive organisms there was growth in both rat and rabbit sera. However, the growth of these strains in rat serum was consistently less than that in the rabbit serum.

Effect of Dilution on the Bactericidal Activity of Rat and Rabbit Serum: Rat and rabbit sera were diluted in Trypticase soy broth. Table V shows a typical dilution experiment for coagulase negative staphylococci. A dilution of 20% (1 part broth plus 4 parts serum) of rabbit serum showed no bactericidal activity against coagulase negative staphylococci. On the contrary, it took a 50% dilution (1 part broth plus 1 part serum) of rat serum to decrease bactericidal activity by 100%. Again these differences showed that rat serum is much more potent in bactericidal activity against these strains than is rabbit serum.

Effect of Heat on the Bactericidal Activity of Rat and Rabbit Sera: Rat and rabbit sera were heated at 56°C. for 30 minutes, 56°C. for 1 hour, 60°C. for 1 hour, and 60°C. for 2 hours. Bactericidal tests were performed with the heated and the unheated sera using 0.1 ml culture with an optical density of 50 Klett units as inoculum. Table VI shows a typical experiment on the effect of heat on the two sera. Although optical density measurements were not reliable as a measure of antibacterial activity in this test, poured plates showed the antibacterial activity of both rat and rabbit serum is not destroyed by heating each serum at 56°C. for 1 hour. Therefore the antibacterial activity of rat and rabbit sera is not dependent on complement and properdin since these two substances are

heat labile and destroyed at 56°C. for 30 minutes. Heating at 60°C. for 2 hours destroyed the bactericidal activity of both rat and rabbit sera.

Differences between Serum and Plasma on the Bactericidal Activity Against Coagulase Negative Staphylococci: Fresh rat and rabbit sera and fresh citrated plasmas were used in an experiment with coagulase negative staphylococci. Table VII shows that the sera had a potent bactericidal effect on coagulase negative staphylococci, while the citrated plasma did not show this effect. The addition of calcium ions sufficient to clot the plasma did not restore the bactericidal activity.

Effect of Calcium ions on the Bactericidal Activity of Rat and Rabbit Sera Against Coagulase Negative Staphylococci: In view of the divalent cation requirement for the antibacterial substance in human and rabbit sera (Jacox, 1950; and Myrvik et al. 1958), it was anticipated that sodium citrate and sodium oxalate would inactivate the bactericidal activity of rat and rabbit sera and that this activity should be restored to its original activity by the addition of divalent cations such as calcium and magnesium. Experiments were performed by adding 0.3 ml of 0.4 M solutions of sodium citrate and of sodium oxalate to 2 ml of rat and rabbit sera. After fifteen minutes equimolar concentrations of calcium chloride, magnesium chloride, and sodium chloride were added. A one-tenth ml inoculum of an optical density of 50 Klett units was inoculated into each sample. These were incubated at 37°C. Poured plates were made at 2 hours and at 20 hours.

Table VIII shows the results of such experiments. The results indicate that calcium ions are not capable of reactivating the system

once it has been destroyed by sodium citrate and sodium oxalate. Although certain bactericidal activity was observed in oxalated serum, no increase in this activity could be demonstrated after the addition of calcium ions to the system. Magnesium ions were also without effect.

Absorption Experiments: Myrvik and Weiser (1955) were able to absorb bactericidal activity against B. subtilis from rabbit serum by the use of bentonite particles. Yotis (1963) showed that the anti-staphylococcal factor of human serum was absorbed by coagulase positive and coagulase negative staphylococci and by B. subtilis, but not by some other organisms.

Experiments were set up for absorption employing 100 mgs of bentonite per ml of serum and the heat killed washed bacterial cells of 200 ml overnight cultures of Escherichia coli, Streptococcus pyogenes, S. aureus, and S. epidermiditis. Absorption was done by incubating serum and absorbing particles at 37°C. for one-half hour followed by one and one-half hour incubation at 4°C. The cells were collected by centrifugation and the clear supernatants were used for bactericidal tests. Table IX shows the mean values of five experiments. The experimental data showed that the bactericidal activity of rat and rabbit sera is absorbed by both S. aureus and S. epidermiditis. The activity is somewhat reduced by E. coli and S. pyogenes with rabbit serum and is absorbed by bentonite in rabbit serum. The bactericidal activity of rat serum is somewhat decreased by bentonite but not absorbed by E. coli or S. pyogenes. These experiments show that there is a difference between the bactericidal activity of

rat and rabbit sera. Whether or not this difference is due to a difference in concentration of antibacterial factor of the two species cannot be decided from the above experiments.

Effect on Bactericidal Activity by Ferric ions: Studies by Bornside et al. (1964) showed that serum antirespiratory activity against B. subtilis was decreased by the addition of ferric ions to the serum. Bactericidal tests were done employing 1.5 mg ferric chloride per ml of rat and of rabbit serum. Table X shows the results of a typical experiment. The addition of 1.5 mg of ferric chloride to the serum completely inhibited the bactericidal activity of both rat and rabbit sera, and thus Bornside's results were confirmed.

DISCUSSION

The antibacterial activity of rat and rabbit sera is similar in many respects: a) heat stability, b) inhibition by ferric ions, c)absorption by staphylococci, d) and once inactivated by sodium citrate and sodium oxalate not being restored to its original activity.

It is clear that both rat and rabbit sera have a remarkable capacity to inhibit multiplication of coagulase negative staphylococci. This inhibiting effect was not, however, found against coagulase positive strains of staphylococci.

Although coagulase negative strains of staphylococci respire poorly in rat and rabbit sera as compared with coagulase positive strains, both coagulase negative and coagulase positive strains respire better in rabbit serum than they do in rat serum. A 20% dilution of rabbit serum abolishes its bactericidal activity, while it takes a 50% dilution of rat serum to abolish its bactericidal activity. Furthermore, when the inoculum was increased, it was evident that rabbit serum did not destroy bacterial concentrations that were readily destroyed by rat serum. This indicates that the antibacterial activity of rat serum is much greater than that of rabbit serum.

Certain characteristics of the serum antirespiratory and bactericidal substances are of significance in differentiating them from specific antibody protein. The one of greater importance is that staphylococcal antirespiratory activity and bactericidal action can be decreased by the addition of ferric ion to the system.

Since no difference could be found between the oxygen uptake of cells in glucose alone, and in glucose plus ferric ions, it would seem that ferric ions acted directly on the serum and not on the cells.

A great difference was found in the concentration of ferric ions required to decrease the antirespiratory activity of staphylococci and that of B. subtilis. While Bornside (1964) used 30 micrograms of ferric ions for the inhibition of antirespiratory activity against B. subtilis, the same system for staphylococci required 1500 micrograms of ferric ions for complete inhibition. It should be pointed out that normal animal and human sera contain 94 to 143 micrograms of iron per ml (Ramsey, 1953) and thus the need for 1500 micrograms of this metal for the inhibition of serum antistaphylococcal activity represents an entirely artificial situation for the establishment of staphylococci in the host.

The reported antibacterial activity against B. subtilis may be due to a number of serum proteins. This possibility is indicated by the reported variations of heat inactivation characteristics of antibacterial factors. While the serum bactericidal substance was markedly inactivated after heating at 56°C. for 60 minutes (Jacox, 1950), and completely destroyed after heating at 60°C. for 2 hours (Myrvik et al., 1955), the bacteriostatic substance withstood 60 minutes of heating at 56°C. (Martin, 1962). The antirespiratory agent is described as withstanding heating for 30 minutes at 56°C. (Wood et al, 1958), and only slightly diminished after heating at 56°C. for 30 minutes (Bornside et al., 1964).

The bactericidal activity of rat and of rabbit serum in this

report was not diminished by heating at 56°C. for 2 hours or by heating at 60°C. for 1 hour but was completely destroyed after heating at 60°C for 2 hours. The results of these experiments are in accord with those of Myrvik and Weiser, since they showed that serum bactericidal activity against B. subtilis was destroyed by heating at 60°C. for 2 hours.

Unlike any of the cases cited above, Pettersson (1926), described a thermostable bactericidal substance in serum which he named "beta-lysin", this acted mainly against gram positive bacteria and required temperatures of 64°C. to 75°C. for 30 minutes for its inactivation.

It is of interest to note that unlike published reports (Jacox, 1950; and Myrvik, 1958), on the ability of calcium ions to restore from human and rabbit sera, the bactericidal activity for B. subtilis, there are indications in the present report that this may not be the case for the restoration from rat and rabbit sera of bactericidal activity for staphylococci. However inoculum concentrations did vary. While the above authors used an inoculum of 10^3 to 10^4 cells in the B. subtilis system, 10^7 staphylococcal cells were employed in the present study. This may explain the discrepancy.

Absorption studies confirmed published reports by Yotis (1963), in that this substance is absorbed by both coagulase positive and coagulase negative staphylococci. Bentonite, however, absorbed the antibacterial substance from rabbit serum (as found by Myrvik, 1955), but did not do so from rat serum. There is, therefore, an obvious difference between the bactericidal activity of rat and rabbit sera. Equal volumes of both

sera and the same concentration of bentonite was used for absorption. However, since rat serum contains approximately 4 times more antibacterial substance than the rabbit serum, the inability of bentonite to absorb the rat serum antibacterial agent may be due to the high levels of this agent in the rat serum.

SUMMARY

The mechanisms of body defense have not been clearly elucidated. There are obvious differences in these mechanisms between species. Rats are clearly resistant to staphylococcal infections while rabbits are more susceptible (Hale and Smith, 1945). The present study indicates that:

1. Coagulase positive staphylococci respire much better in both rat and rabbit sera than do coagulase negative staphylococci.

2. Antirespiratory activity of rat and rabbit sera against staphylococci was decreased by excessive amounts of ferric ions.

3. Ferric ion inhibition of serum bactericidal activity was due to direct action on the serum and not on the bacterial cells.

4. Coagulase positive organisms grow very well in both rat and rabbit serum while coagulase negative organisms are readily killed by these sera.

5. Rat serum is more inhibiting for staphylococci than rabbit serum.

6. Inhibition of bactericidal activity by sodium citrate and sodium oxalate was not reversed by calcium ions in either rat or rabbit serum.

7. Absorption of the antibacterial substance from both rat and rabbit serum was accomplished by heat killed staphylococci.

8. Bentonite absorbed the antibacterial substance from rabbit serum, but failed to do so from rat serum.

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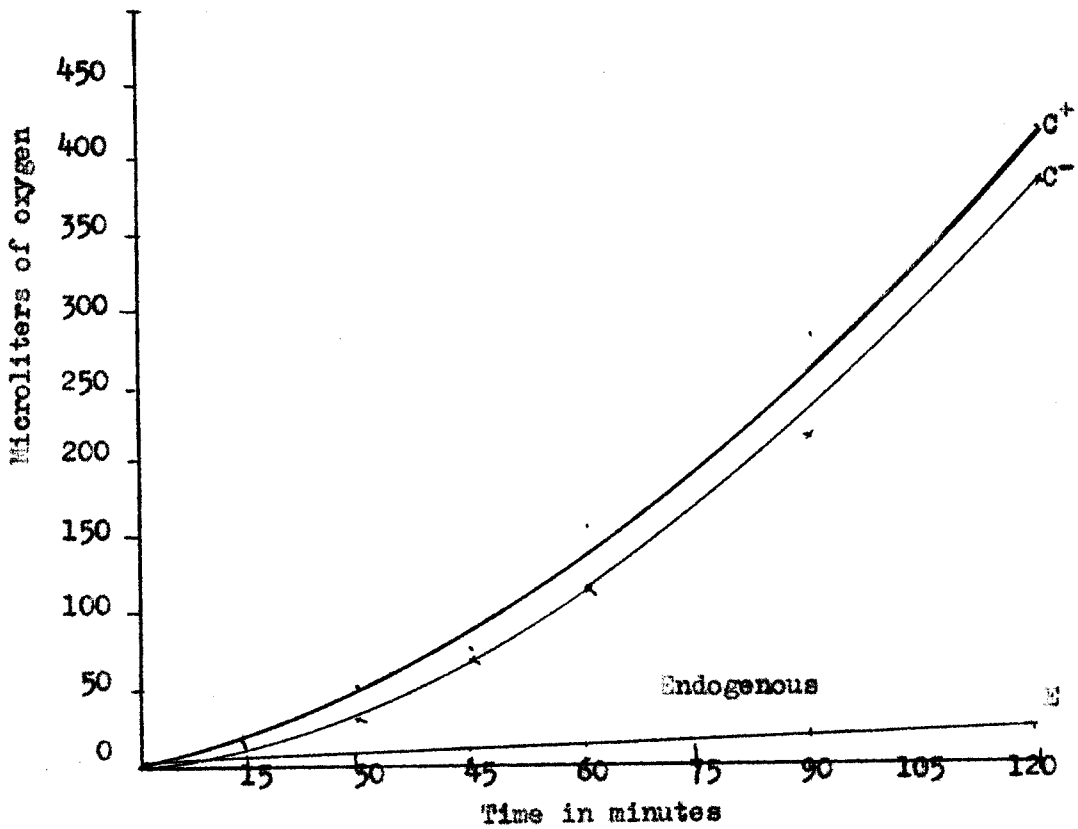

Fig. I. Oxidation of trypticase soy broth by coagulase positive (C^+) and coagulase negative (C^-) staphylococci.

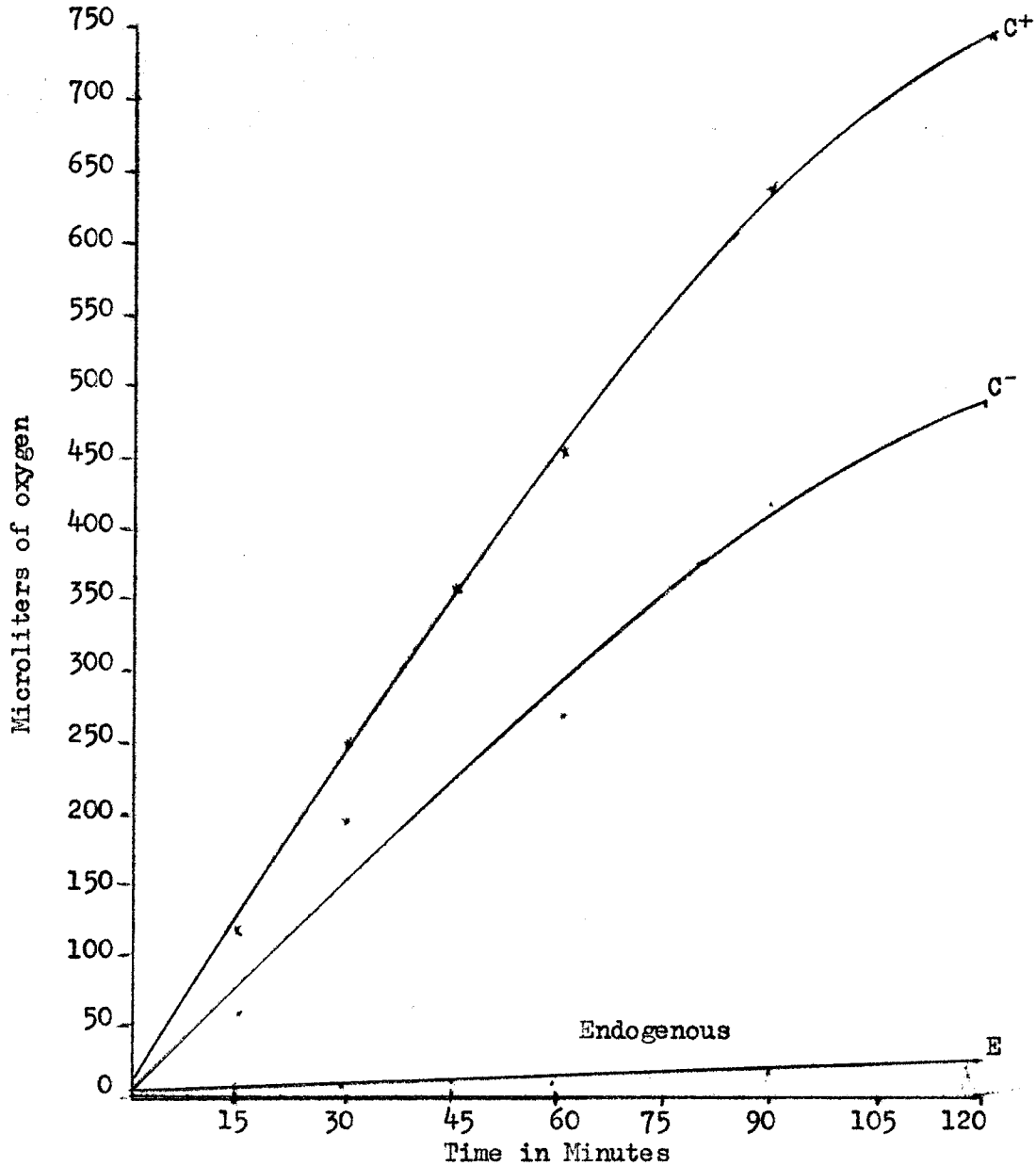

Fig. II. Oxidation of normal rabbit serum by coagulase positive (C^+) and coagulase negative (C^-) staphylococci.

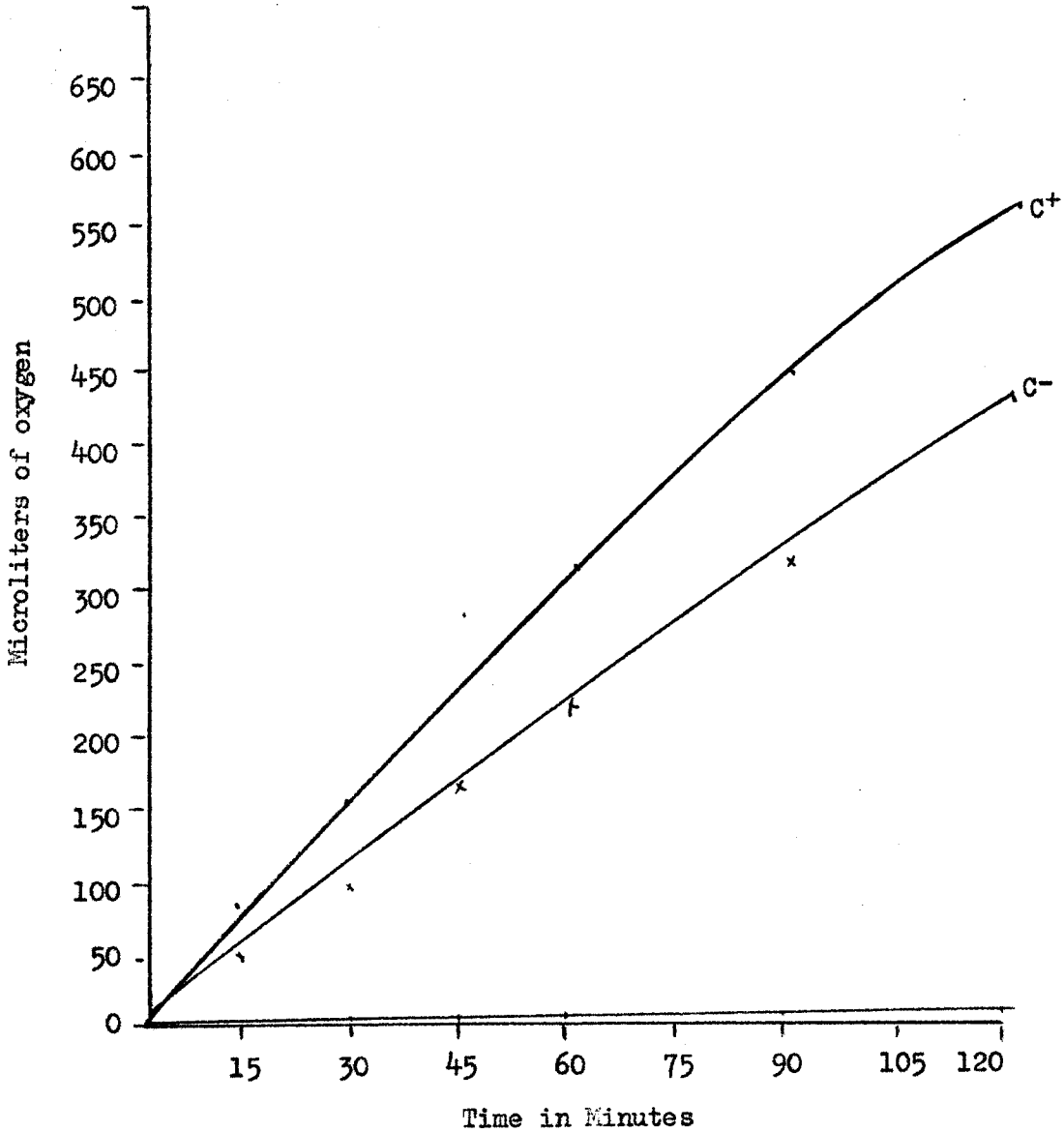

Fig. III. Oxidation of normal rat serum by coagulase positive (C^+) and coagulase negative (C^-) staphylococci.

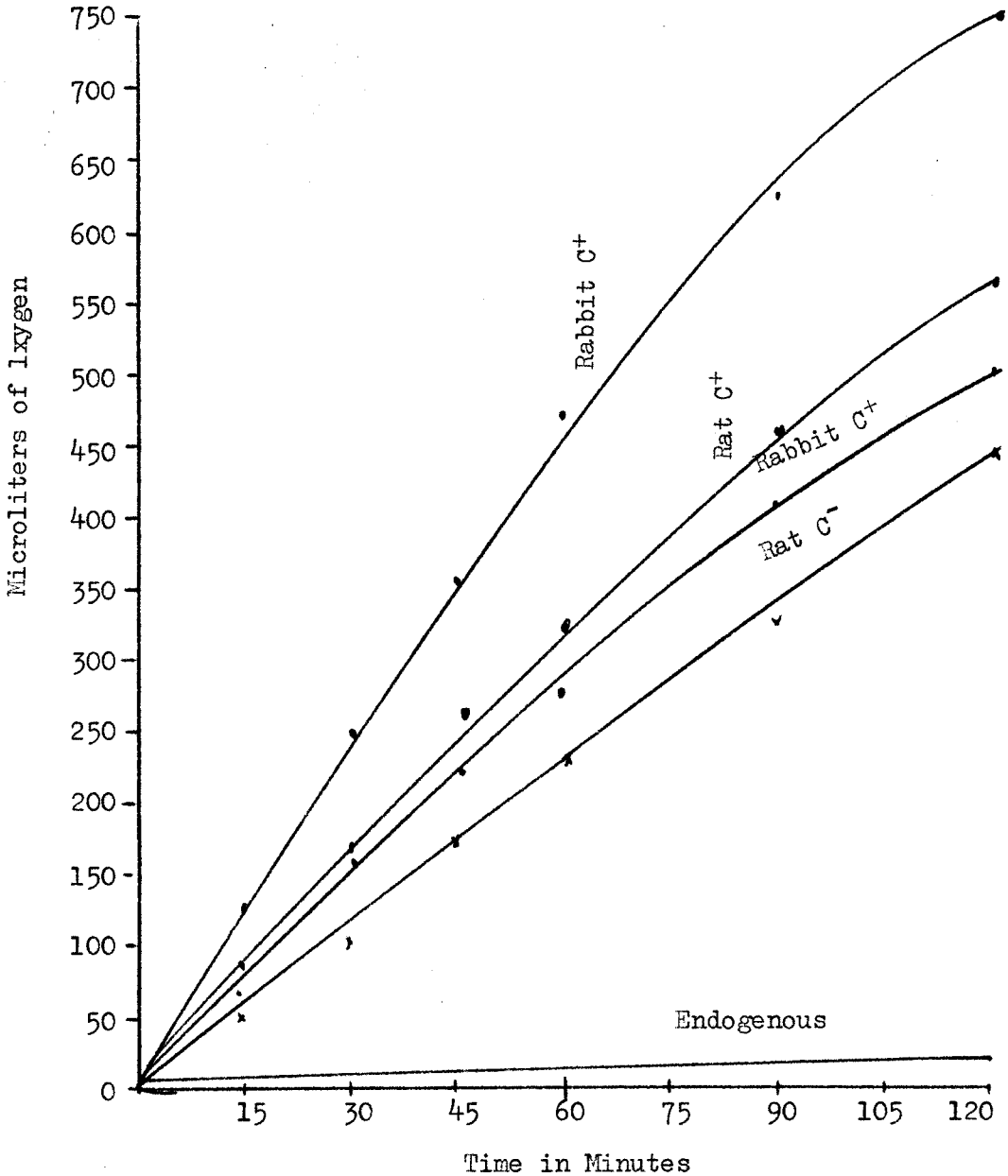

Fig. IV. A comparison in the oxidation of rat and rabbit serum by coagulase positive (C^+) and coagulase negative (C^-) staphylococci.

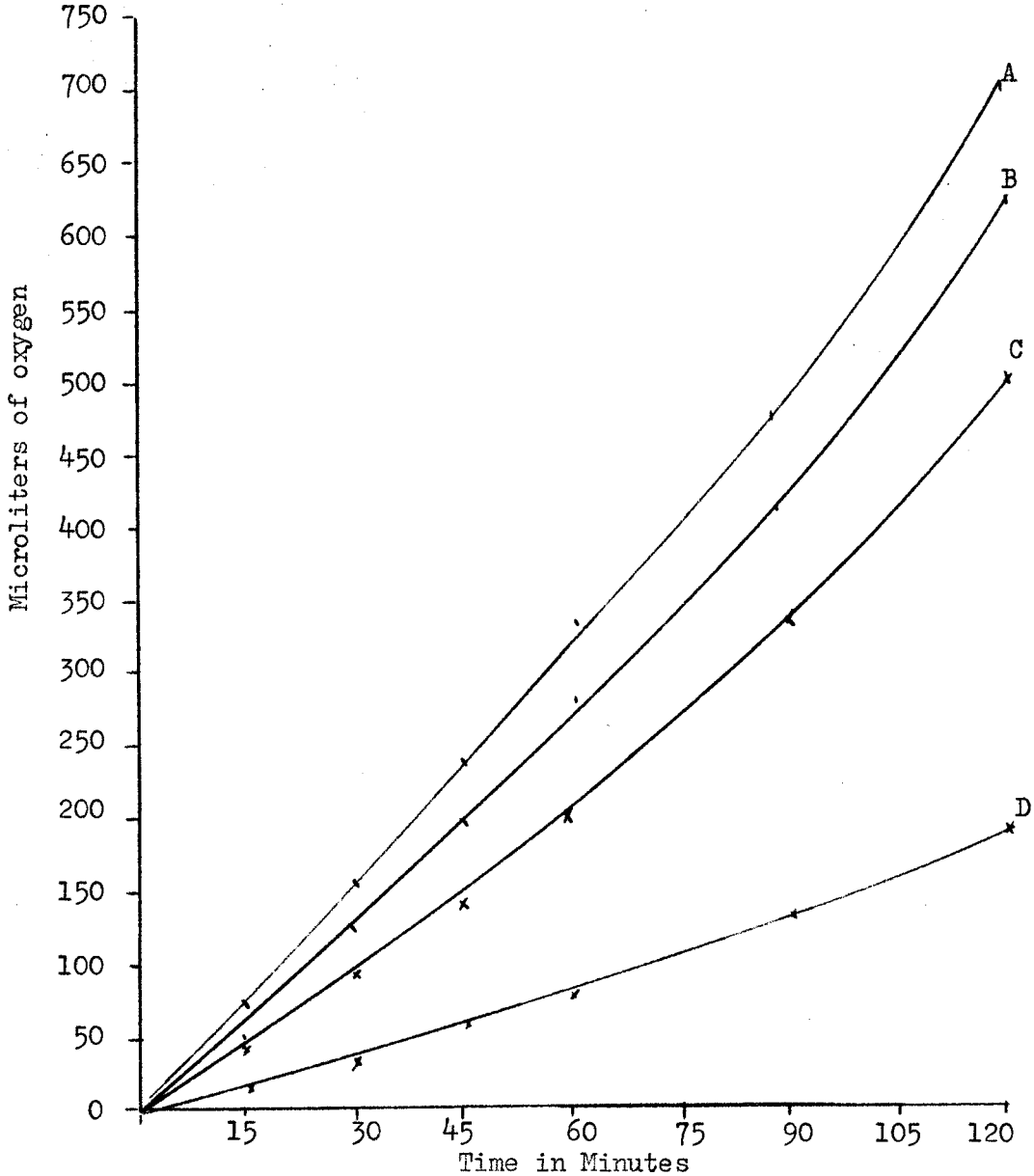

Fig. V. Effect on the antirespiratory activity in normal rabbit serum by ferric ions.

Curve: A = Coag.pos. plus 1.5 mg Fe^{+++}
B = " " Control
C = Coag.neg. plus 1.5 mg Fe^{+++}
D = " " Control

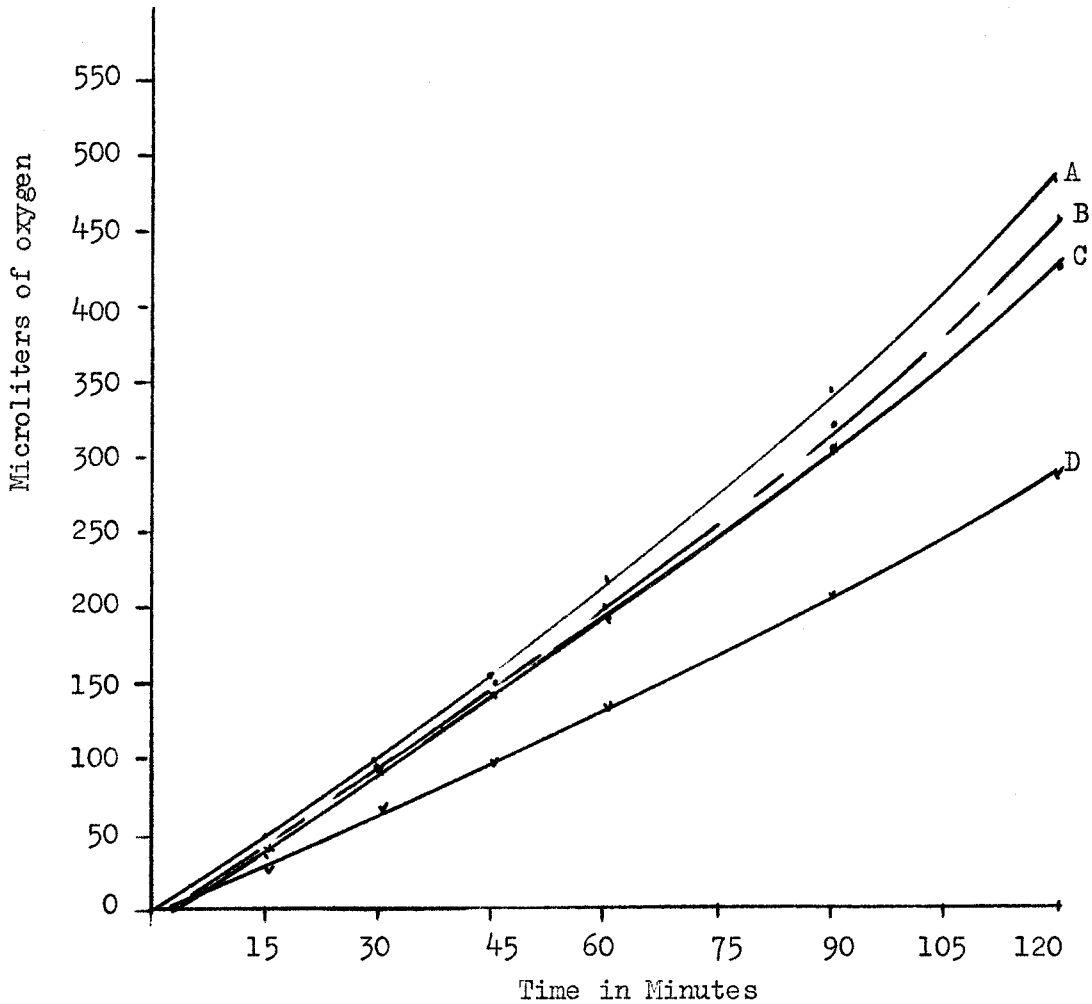

Fig. VI. Effect on the antirespiratory activity of normal rat serum by ferric ions.

Curve: A = Coag. pos. plus 1.5 mg Fe^{+++}
B = " neg. " " " "
C = " pos. control
D = " neg. control

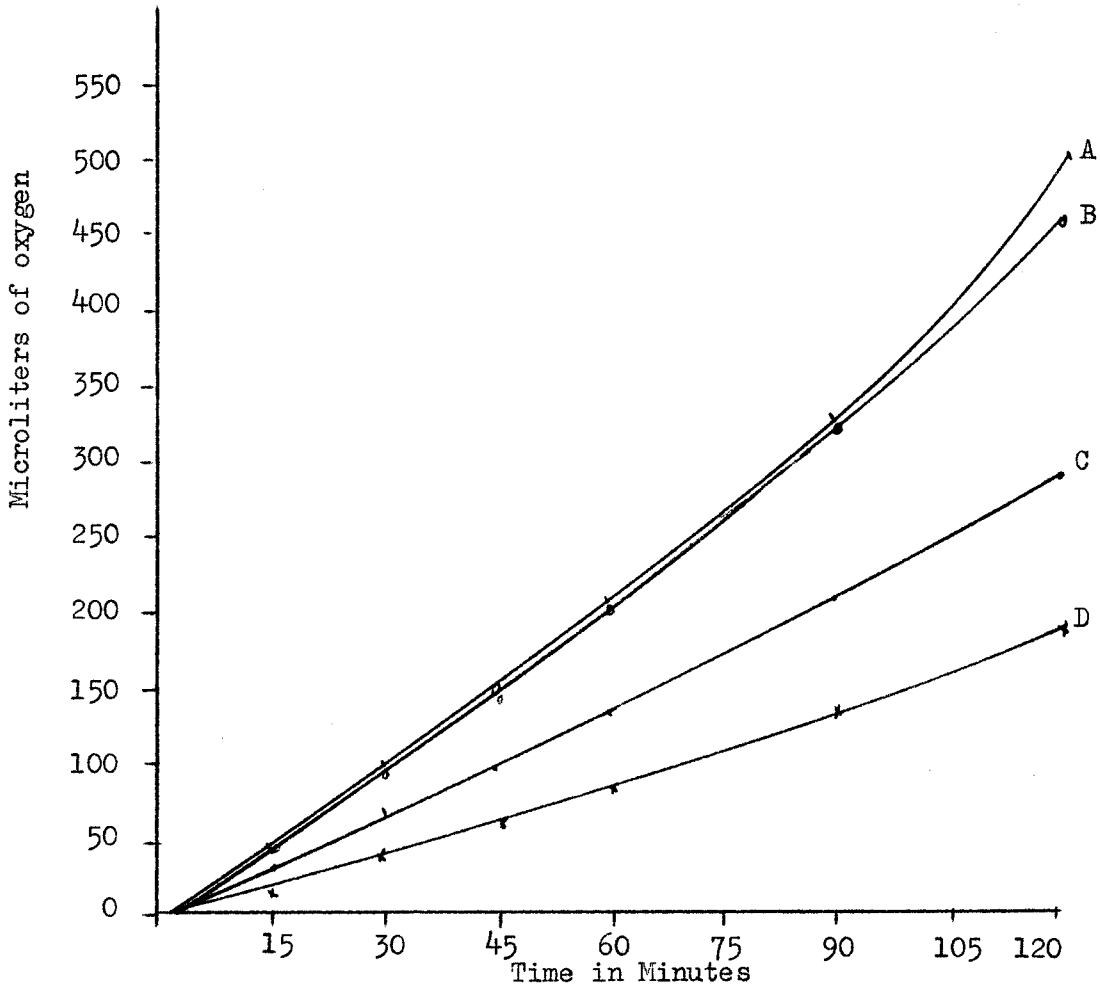

Fig. VII. Comparison of the decrease of antirespiratory activity of rat and rabbit serum by ferric ions.

Curve: A = Coag. neg. plus 1.5 mg Fe^{+++} in rabbit serum
B = Coag. neg. plus 1.5 mg Fe^{+++} in rat serum
C = Coag. neg. control in rat serum
D = Coag. neg. control in rabbit serum

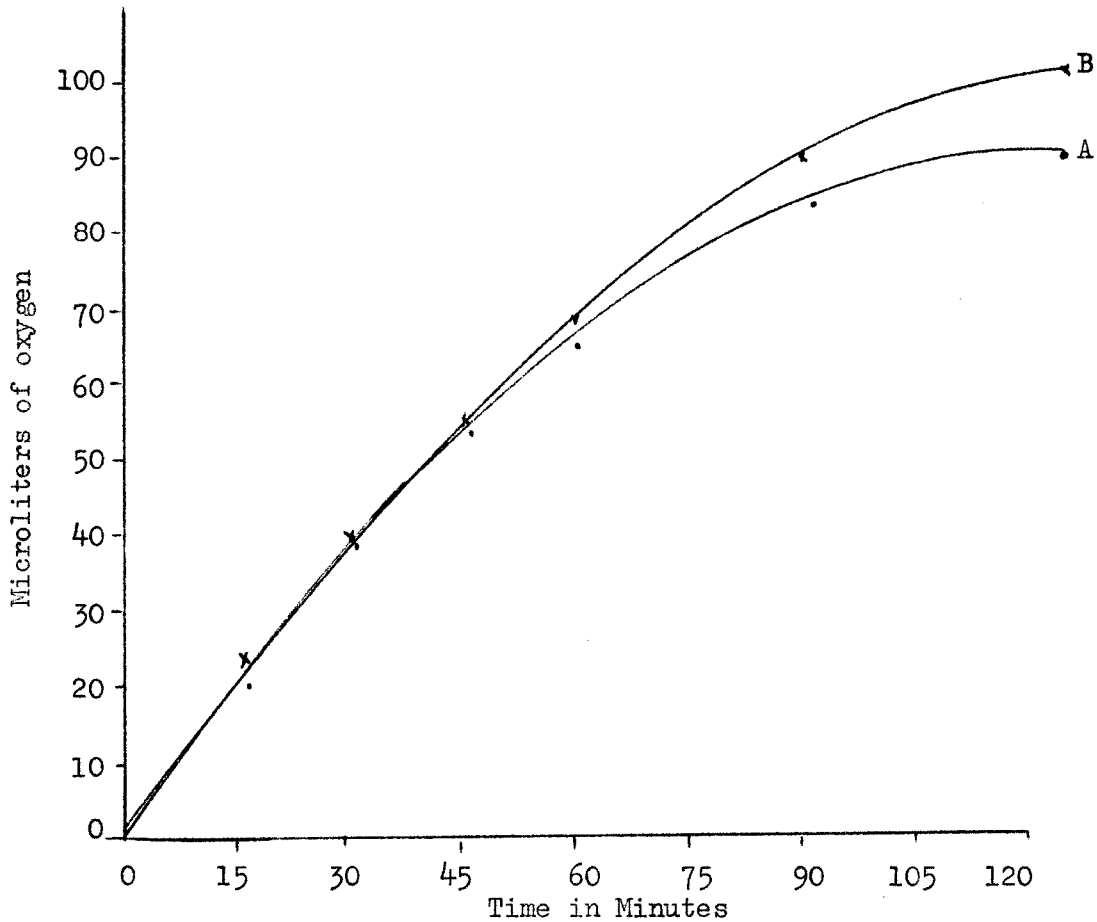

Fig. VIII. Effect of ferric ions on the oxidation of glucose by coagulase negative staphylococci:

Curve: A = Glucose plus 1.5 mg Fe^{+++}
B = Glucose control

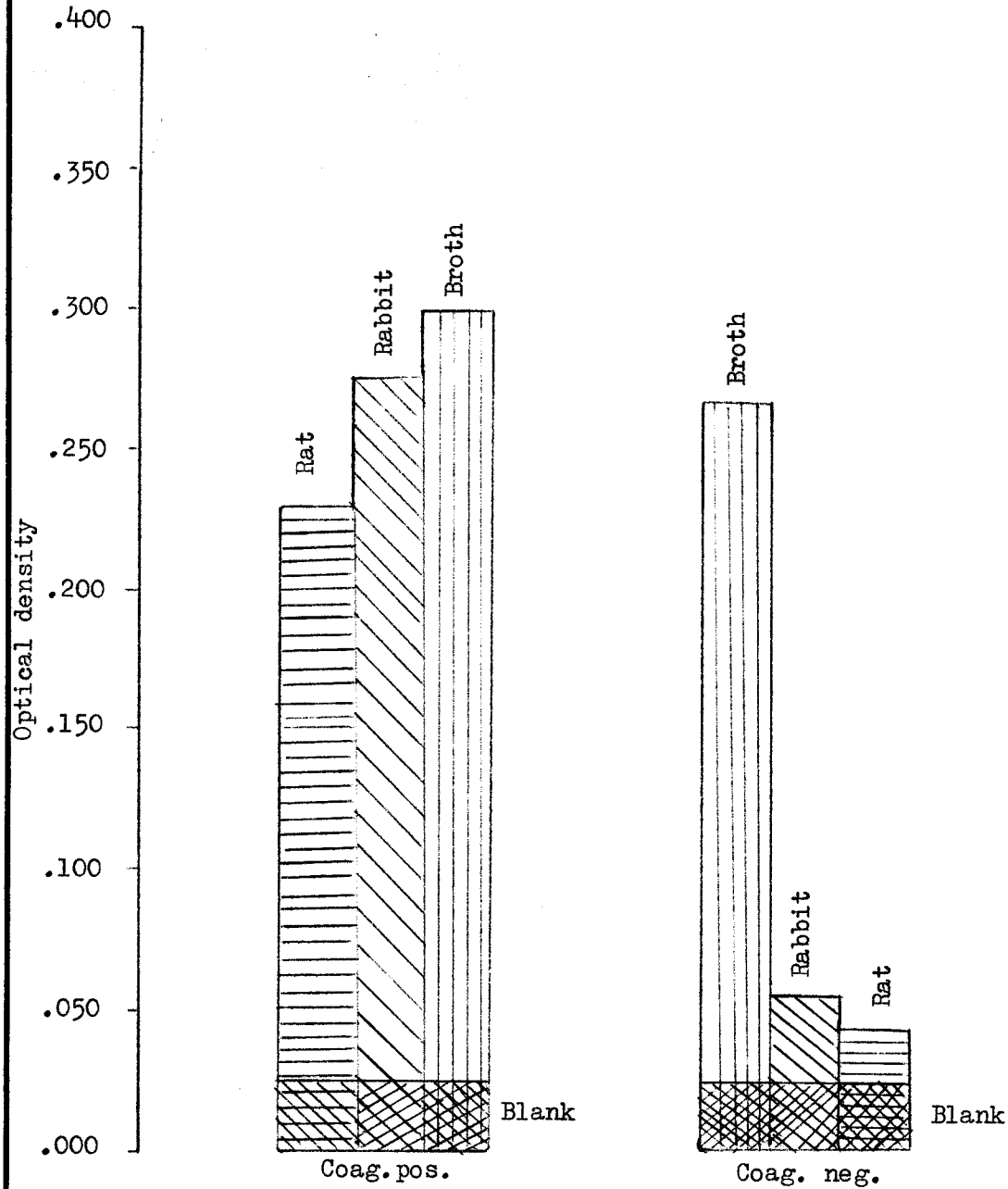

Fig. IX. Growth of coagulase positive and coagulase negative staphylococci in broth, rat and rabbit serum.

TABLE I.

PHYSIOLOGICAL CHARACTERISTICS OF THE TEST MICROORGANISMS

Strain	Phage Type	Gluc.	D-mann.	Gel.	Coag.	Pigment
52277	29/52/52A/79/80	F	F	+	+	G.Y.
52294	187	F	F	+	+	G.Y.
52298	52/52A/80	F	F	+	+	G.Y.
52311	80/81	F	F	+	+	G.Y.
52314	7/47/53/54/75/77/83a	F	F	+	+	G.Y.
52366	33/3c/55/71	F	F	+	+	G.Y.
220H	———	F	O	-	-	W.
246H	———	F	-	-	-	W.
296	29/52/52A	F	F	+	+	G.Y.
299	6/7/42E/47/53/54/75/77	F	F	+	+	G.Y.
303	52/79	F	F	+	+	G.Y.
309	55/71	F	F	+	+	G.Y.
313	80/81	F	F	+	+	G.Y.
316	52/52A/80/81	F	F	+	+	G.Y.
322	7	F	F	+	+	G.Y.
323	3A/71	F	F	+	+	Y
328	6/42E/47/75	F	F	+	+	Y
330	3B/71	F	F	+	+	G.Y.
334	29/52	F	F	+	+	G.Y.
335	52A/79	F	F	+	+	G.Y.
344	3A	F	F	+	+	G.Y.
368	6/53/83A	F	F	+	+	G.Y.

TABLE I. cont.

Strain	Phage Type	Gluc.	D-mann.	Gel.	Coag.	Pigment
Gel28	_____	F	-	+	-	W
11	_____	F	O	-	-	W
12	_____	F	-	+	-	W
13	_____	F	-	+	-	W
14	_____	F	O	-	-	W
15	_____	F	-	+	-	W
21	not typed	F	F	+	+	G.Y.
22	" "	F	F	+	+	G.Y.
357H	80/3A/79	F	F	+	+	W
362H	80/3A/79/7	F	F	+	+	W
363H	80/3A/79	F	F	+	+	W
365H	80/81/79	F	F	+	+	G.Y.
372H	3B/71/3C/79/3A	F	F	+	+	G.Y.
390H	79/3A/80	F	F	+	+	G.Y.
391H	79/80	F	F	+	+	G.Y.
392H	79/80	F	F	+	+	W
393H	79/42E/80/81/7	F	F	+	+	G.Y.
394H	79/3A	F	F	+	+	G.Y.
395H	79/3A/80	F	F	+	+	G.Y.
Bromb	79/53/80	F	F	+	+	G.Y.
354H	_____	-	-	+	-	W
Pearl	_____	-	-	+	-	W

F = Fermenter
O = Oxidizer
+ = Positive
- = Negative

G.Y. = Golden Yellow
Y = Yellow
W = White

TABLE II

EFFECT OF FERRIC ION CONCENTRATION ON THE ANTIRESPIRATORY ACTIVITY OF RABBIT AND RAT SERUM AGAINST COAGULASE POSITIVE AND COAGULASE NEGATIVE STAPHYLOCOCCI

	Coag. Neg. Strains						Coag. Pos. Strains	
Serum	Ferric ion, mg/ml						Ferric ion, mg/ml	
	0.0	0.15	0.30	1.20	1.50	1.70	0.0	1.50
	ul O_2						ul O_2	
Rabbit	12	30	15	14	40	16	48	62
	30	32	32	46	86	52	122	152
	56	36	48	88	140	94	200	240
	80	46	62	140	196	148	292	346
	136	70	96	262	332	334	502	568
	190	88	120	408	500	416	624	702
Rat	24	--	--	--	32	--	32	32
	66	--	--	--	98	--	96	96
	96	--	--	--	148	--	140	160
	132	--	--	--	200	--	190	220
	208	--	--	--	330	--	304	348
	288	--	--	--	454	--	426	482
Glucose + ATP	26	--	--	--	12	--		
	40	--	--	--	16	--		
	56	--	--	--	52	--		
	60	--	--	--	64	--		
	82	--	--	--	80	--		
	102	--	--	--	90	--		

TABLE III

EFFECT OF IRON, MAGNESIUM, ZINC AND MANGANESE IONS ON THE ANTIRESPIRATORY ACTIVITY OF RAT AND RABBIT SERUM AGAINST COAGULASE NEGATIVE STAPHYLOCOCCI

Control	Iron	Magnesium	Zinc	Manganese
		(ul O_2)		
Rabbit Serum				
12	40	16	25	15
30	86	36	45	32
56	140	58	66	49
80	196	80	86	69
136	332	131	129	103
190	500	193	176	147
Rat Serum				
14	32	18	24	20
38	98	44	47	44
64	148	80	77	74
98	200	117	110	108
167	330	199	184	180
239	454	248	264	242
Glucose				
26	12	8	--	6
40	16	10	--	26
56	52	24	--	42
60	64	36	--	62
80	80	64	--	94
102	90	76	--	106

TABLE IV

EFFECT OF INOCULUM CONCENTRATION ON THE BACTERICIDAL ACTIVITY OF RAT AND RABBIT SERUM AGAINST STAPHYLOCOCCI

1. Turbidimetric Determinations

		Inoculum Concentration in O.D.						
Strain	Serum	0.1	0.2	0.4	0.6	0.8	1.0	1.4
Coag.Pos.	Rat	.123	.204	.188	.178	.153	.182	.210
	Rabbit	.285	.319	.316	.318			
Coag.Neg.	Rat	.034	.023	.027	.031	.054	.057	.165
	Rabbit	.046	.189	.200	.215			

2. Plate Counts

		Inoculum Concentration, Cells		
Strain	Serum	4.3×10^7	3.2×10^8	3.9×10^9
Coag.Pos.	Rat	6.3×10^7	6.3×10^8	4.3×10^8
	Rabbit	9.5×10^7	1.2×10^8	7.1×10^9
Coag.Neg.	Rat	2.2×10^5	2.5×10^6	3.6×10^6
	Rabbit	4.1×10^5	9.3×10^7	5.0×10^7

TABLE V

EFFECT OF DILUTION ON THE BACTERICIDAL ACTIVITY OF RAT AND RABBIT SERUM AGAINST COAGULASE NEGATIVE STAPHYLOCOCCI

Serum (ml)	Broth (ml)	Dil. (%)	Rabbit Serum		Rat Serum	
			O.D.	Plate Count	O.D.	Plate Count
2.0	0.0	0	.023	4.4×10^5	.013	2.2×10^5
1.8	0.2	10	.059	4.4×10^6	.018	3.3×10^5
1.6	0.4	20	.116	4.0×10^7	.028	2.5×10^5
1.4	0.6	30	.129	3.8×10^7	.080	3.5×10^5
1.2	0.8	40	.166	6.0×10^8	.122	5.6×10^6
1.0	1.0	50	.173	2.4×10^9	.204	3.7×10^7
0.8	1.2	60	.173	2.5×10^9	.279	7.2×10^8
0.6	1.4	70	.213	8.2×10^9	.311	2.0×10^9
0.4	1.6	80	.272	5.7×10^9	.323	4.0×10^9
0.2	1.8	90	.263	6.2×10^9	.311	3.2×10^9
0.0	2.0	100	.268	5.5×10^9	.260	5.5×10^9

Inoculum = 2.5×10^7

TABLE VI

THE EFFECT OF HEAT ON THE BACTERICIDAL ACTIVITY OF RAT AND RABBIT SERUM AGAINST COAGULASE POSITIVE AND COAGULASE NEGATIVE STAPHYLOCOCCI

1. Optiocal Density Determinations

Strain	Serum	Undiluted Serum	Serum Heated at: 56 C/30 min.	56 C/1 hr.	60/1 hr.	60 C/2 hr.
Coag. Neg.	Rat	.010	.012	.014	.031	.043
	Rabbit	.005	.021	.040	.020	.040
Coag. Pos.	Rat	.178	.174	--	--	--
	Rabbit	.265	.244	--	--	--

2. Plate Counts

Coag. Neg.	Rat	2.2×10^5	2.1×10^5	2.1×10^5	2.0×10^5	4.2×10^7
	Rabbit	6.0×10^4	4.1×10^5	4.1×10^5	4.7×10^5	8.0×10^7
Coag. Pos.	Rat	3.1×10^8	1.8×10^8	---	---	---
	Rabbit	8.3×10^8	5.1×10^8	---	---	---

Inoculum:

Coag. Pos.	Coag. Neg.
4.3×10^7	3.1×10^7

TABLE VII

GROWTH OF COAGULASE NEGATIVE STAPHYLOCOCCI IN SERUM AND CITRATED PLASMA

	Rabbit		Rat	
Culture Medium	O.D.	Plate Count	O.D.	Plate Count
Plasma	0.115	2.3×10^8	0.127	1.9×10^7
Serum	0.009	7.7×10^4	0.009	2.3×10^4
Broth	0.300	8.8×10^8	0.300	8.8×10^8

Inoculum = 1×10^7

TABLE VIII

THE EFFECT OF CALCIUM ON THE BACTERICIDAL ACTIVITY OF RAT AND RABBIT SERUM AGAINST COAGULASE NEGATIVE STAPHYLOCOCCI

	0.4M Solutions of:					Plate Count	
Serum	$Na_2C_2O_4$	$Na_2C_6H_5O_7$	NaCl	$CaCl_2$	$MgCl_2$	Rat	Rabbit
(ml)	(ml)	(ml)	(ml)	(ml)	(ml)		
2.0	---	0.3	0.3	---	---	5.2×10^7	2.8×10^7
2.0	---	0.3	---	0.3	---	6.1×10^7	5.6×10^7
2.0	---	0.3	---	---	0.3	3.4×10^7	6.2×10^7
2.0	0.3	---	0.3	---	---	3.0×10^6	1.3×10^6
2.0	0.3	---	---	0.3	---	2.2×10^6	5.5×10^6
2.0	0.3	---	---	---	0.3	5.6×10^6	1.1×10^7
2.0	---	---	0.6	---	---	8.5×10^5	5.4×10^6
2.0 (broth)	---	---	0.6	---	---	3.5×10^8	3.5×10^8

Inoculum = 3.6×10^7

TABLE IX

ABSORPTION OF BACTERICIDAL ACTIVITY FROM RAT AND RABBIT SERUM

Absorbing Agent	Rat Serum		Rabbit Serum	
	O.D.	Plate Count	O.D.	Viable Count
E. coli	.016	1.0×10^5	.036	3.1×10^6
S. pyogenes	.017	2.4×10^5	.036	3.5×10^6
S. epidermiditis	.126	1.5×10^7	.070	2.33×10^7
S. aureus	.153	1.9×10^7	.089	6.65×10^7
Bentonite	.027	1.0×10^6	.090	4.5×10^7
Serum control	.030	1.5×10^5	.020	2.6×10^5
Broth control	.280	3.2×10^8	.280	3.2×10^8

Inoculum = 2.0×10^7

TABLE X

EFFECT ON BACTERICIDAL ACTIVITY OF RAT AND RABBIT SERUM BY FERRIC IONS

Strain	Serum	1.5 mg. Fe^{+++}	Control
Coag. Positive	Rat	4.4×10^7	5.7×10^7
	Rabbit	1.4×10^8	1.2×10^8
Coag. Negative	Rat	5.7×10^7	5.3×10^4
	Rabbit	1.0×10^8	1.0×10^4

Inoculum = 1×10^7

STATISTICAL DATA

Statistical analysis of the data reveals a "P" value of less than 0.001 for both oxygen uptake and bactericidal action for both coagulase negative and coagulase positive staphylococci in rat and rabbit sera. No significant difference was found between rat and rabbit sera.

	C^+	C^-	C^+	C^-
Oxygen uptake = ul O_2/mg/hr	206 ± 30.3 (N=10) P=(0.001)	122 ± 26.6 (N=10) P=(0.001)	330 ± 66 (N=10) P=(0.001)	171 ± 58.8 (N=10) P=(0.001)
Bactericidal action				
Optical Density = units/2 ml Serum	.217 ± .042 (N=6) P=(0.001)	.018 ± .012 (N=5) P=(0.001)	.272 ± .099 (N=9) P=(0.001)	.0468 ± .030 (N=5) P=(0.001)

APPROVAL SHEET

The thesis submitted by Jesse S. Ortiz has been read and approved by three members of the faculty of the Stritch School of Medicine, Loyola University.

The final copies have been examined by the director of the thesis and the signature which appears below verifies the fact that any necessary changes have been incorporated, and that the thesis is now given final approval with reference to content, form, and mechanical accuracy.

The thesis is therefore accepted in partial fulfillment of the requirements for the Degree of Master of Science.

January 18, 1965
Date

William W. Yotis
Signature of Adviser