THE USE OF STILBESTROL, LYSOZYME AND POLYVALENT VACCINES IN THE CONTROL OF EXPERIMENTAL STAPHYLOCOCCAL INFECTIONS IN LABORATORY ANIMALS

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This is to certify that the

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presented by

CHESTER A. HORNBECK

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of the requirements for

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O. L. Jacklin
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ABSTRACT

THE USE OF STILBESTROL, LYSOZYME, AND POLYVALENT VACCINES IN THE CONTROL OF EXPERIMENTAL STAPHYLOCOCCAL INFECTIONS IN LABORATORY ANIMALS

by Chester A. Hornbeck

The major goal of this study was the investigation of substances not biologically related to antibiotics which would be potentially therapeutic as well as prophylactic for experimental staphylococcal infections in animals. The agents considered were diethylstilbestrol (stilbestrol), lysozyme, and polyvalent vaccines.

Stilbestrol was administered to mice by three routes: (1) subcutaneously after mixing in corn oil; (2) intravenously in 50% (v/v) alcohol-water solution; and (3) orally in food for 12 days preceding infection and 14 days afterwards. The challenge dose of Staphylococcus aureus was given 5 days after the subcutaneous treatment and within minutes after intravenous administration of the stilbestrol solution. Based on percent survival there appeared to be no suppression of the infection. No marked difference in decrease of staphylococci was noted in the blood or liver of treated mice compared with untreated controls. The evidence

suggested that stilbestrol was not of therapeutic or prophylactic value in the management of experimental staphylococcal infection in mice.

In vitro use of lysozyme had no effect upon coagulase and the latter had no effect on lysozyme. Using 100 μg/ml of lysozyme, no inhibition of growth of staphylococci was noted; however, adsorption of lysozyme to the bacterial cell was shown by the agglutination of such lysozyme-combined cells by both antilysozyme serum and the globulin fraction prepared from the antiserum.

When treatment was started within several hours of the experimental staphylococcal infection, 60 to 75% of lysozyme-treated mice survived infections which killed all the untreated controls. Some 60% of the mice treated with antilysozyme globulin died after challenge with a normally non-lethal dose of staphylococci.

The use of semi-purified coagulase alone or in conjunction with a whole cell vaccine prepared from several phage types did not protect against an intracutaneous challenge dose of staphylococci.

Polyvalent vaccines inactivated with alkyldimethylbenzyl ammonium chloride (Zephiran) evoked greater protective

response than those inactivated with either formalin or heat. Polyvalent vaccines of recent isolates from human lesions caused more protective response in rabbits than those from laboratory strains.

If carminic acid, an agent known to saturate the reticulo-endothelial system, was administered together with intracutaneous injection of polyvalent vaccine, an increased resistance to subsequent challenge was found. No similar effect was observed when immunization was performed via the intramuscular route.

The amount of total rabbit serum protein was not grossly changed either after immunization or after intracutaneous infection. There appeared to be a slight decrease in the albumin fraction and a concomitant appearance of an α_3-globulin. Only γ-globulin fell slightly below normal levels after challenge.

Of the three enzymes studied, serum glutamic oxalacetic transaminase, serum glutamic pyruvic transaminase, and ornithine carbamyl transferase, only the latter was found to show increased activity. Slight increases were observed after immunization, but abnormal values were seen only after

the infective dose. The highest activity level was associated with glycogen infiltration of the liver, suggesting hepatic damage.

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INTRODUCTION

When Ogston (1881) presented his report to the British Medical Association on the investigation of some 82 abcesses, he distinguished between what he called "chain-micrococci" and "grouped-micrococci" or staphylococci. Since that time, a host of investigators have amassed a voluminous literature in their ceaseless probing of this ubiquitous species.

This common pathogen has been the subject of continuing chemotherapeutic efforts. In the early years, Fleming (1924) pointed out that many antiseptics in common use were quite toxic to the tissue cells. With this fact in mind, his discovery of penicillin in 1928 was truly epoch-making. The impact upon the management of staphylococcal infections by penicillin and the wholly new group of antibiotics that rapidly followed can hardly be overestimated.

The widespread occurrence of resistant staphylococcal infections, especially in hospitals, has caused almost universal interest. Finland et al. (1959) demonstrated that over a 23 year period from 1935 to 1957, staphylococcal bacteremia increased almost fourfold at Boston City Hospital with over a twofold increase since 1947.

On the other hand, Rogers (1959) in studies based on two hundred consecutive medical service patients subjected to post-mortem examinations between 1938 and 1940, and an equal number in 1957 and 1958, found no such increase in staphylococcal related deaths. However, in an earlier report (1956) he pointed out that although the incidence of staphylococcal pneumonia had doubled during the antimicrobial era, in general, the population acquiring the disease was composed primarily of elderly or chronically ill patients.

The conflicting reports illustrate the difficulty in interpreting studies from various areas which may have different environmental conditions, but more importantly they underscore the effects of the emergence of antibiotic resistant strains of staphylococci.

In the early 1940's it became apparent that staphylococci from patients treated with penicillin could show greatly increased resistance to penicillin. As each new antibiotic made its appearance, resistant strains of staphylococci followed in its wake.

Anderson (1954) isolated from a brain abscess a staphylococcus which was resistant to five antibiotics. Bondi et

al. (1954) in a survey using seven antibiotics (penicillin, streptomycin, chloramphenicol, chlorotetracycline, oxytetracycline, erythromycin, and carbomycin) found only 22% of their strains sensitive to all seven. Forty percent were resistant to penicillin, streptomycin, chlorotetracycline, and oxytetracycline, while 4% were resistant to all of these and chloramphenicol as well. Various combinations of antibiotics have given good response in therapy; yet the problem of resistance to these agents remains unsolved.

In this study, it was thought that the potentially therapeutic as well as prophylactic value of agents not related to the antibiotics could profitably be considered. Stilbestrol, lysozyme, and various vaccines were investigated.

HISTORICAL REVIEW

Stilbestrol

Diethylstilbesterol (stilbestrol) was introduced by Dodds, Goldberg et al. (1938) during their studies of stilbene compounds. Allen (1941) found that stilbestrol differed from the natural estrogen in that it was active both parenterally and by mouth. Faulkner (1943) in assaying the bacterial action of the estrogens found that stilbestrol had some bactericidal action on all Gram-positive organisms tested. The action was not rapid in low concentration since it required two hours to kill Staphylococcus aureus at a 1:100 dilution and eighteen to twenty-four hours in 1:50,000 parts.

Bocovo et al. (1952) found that stilbestrol was an effective fungistatic agent against such organisms as Blastomyces dermatitidis, Blastomyces braziliensis, Histoplasma capsulatum, and Coccidioides immitis. In Chile, Urba and her coworkers (1953) reported that the naturally occurring steroid hormones had no effect upon the growth of Escherichia coli but that stilbestrol stopped growth after an initial stimulation.

San Clemente and MacKenzie (1957) in determining the effect of stilbestrol upon the growth of some common micro-organisms found that a wide spectrum of bacteria were inhibited; in general, the Gram-positive organisms were the most susceptible. Of the Gram-negative organisms, Alcaligenes and Proteus were the most resistant; Klebsiella the least.

Frank and Pounden (1961) have studied the effect of stilbestrol and progesterone in the growth of mastitis-producing bacteria. They found that the strain of staphylococcus used was inhibited by concentrations ranging between 0.1 μg and 5.0 μg per ml.

Little work has been reported using stilbestrol for treatment or prophylaxis in vivo in any infective process. Nicol and coworkers (1961) have reported beneficial therapeutic effects. Gorman (1961) found that saturated aqueous solution of stilbestrol used to produce vaginal epithilial cornification in a human female "did not exacerbate an existing trichomonas infection in one patient."

Quite apart from the investigations concerning the bactericidal effect of stilbestrol are those involving the response of the reticulo-endothelial system (RES) to the

various stilbene derivatives. Nicol et al. (1958) reported on the effect of various stilbene compounds in the phagocytic activity of the RES. The activity was measured by determining the rate of disappearance of a known amount of carbon particles (approximately 250 Å) from the circulating blood, samples being taken at short intervals following intravenous injection in mice. Log values of the absorptiometric readings lay along a straight line when plotted against time, and the slope of the line was taken as the phagocytic index denoted by the symbol "K." They found the strongest stimulants (producing increased K values) possessed the following features: (1) two p-hydroxy phenol groups attached to adjacent carbon atoms, (2) unsaturation, and (3) absence of H atoms at the α and β carbons.

Bilby and Nicol (1958) investigated the effect of several natural steroids on the phagocytic activity of the RES. They found estrogenic steroids caused rapid removal of the carbon particles from the blood with K values from 47 to 81 compared to the normal K value of 13. Progesterone caused a definite depression to a value of 7. Testosterone and cholesterol and other steroids had little or no effect.

Nicol and Ware (1960a) reported that daily doses of

100 mg of stilbestrol (delivered subcutaneously in 0.05 ml of Arachis oil) for six days caused definite RES stimulation. At dosage levels of 0.2 mg through 0.6 mg (highest level used) there was more than four to six-fold increase in phagocytic activity. Concomitantly there was a weight reduction of 8 to 18% in the mice. Extending their work, these authors (1960b) found the duration of stimulation remained high for approximately one week and by the eighth or ninth day returned to normal limits. More recently, Nicol et al. (1961) have related the K value to total body weight as well as the combined weight of liver and spleen from which they derived a phagocytic index. This index was considered a corrective factor which takes into account the enlargement of these organs.

Heller et al. (1957) considering the response of RES function to natural and synthetic estrogens, found that both substances have a powerful stimulating effect on phagocytic velocity. They found statistically significant splenomegaly and hepatomegaly; however, based on radiocolloid studies, activity per gram was reduced. They reported no significant change in blood and bone marrow smears between experimental and control groups. It was suggested that the

increased activity was in all probability due to surface phenomena. Since there was no increase in RES elements, the net effect was due to increased efficiency of the RES cells.

Cuppage and Blockworth (1960) have demonstrated that stilbestrol induced intrahepatic bile duct hyperplasia in rabbits. During long term administration, they reported considerable liver damage in some cases.

Johnson et al. (1961) reported on the enzymic hydrolysis of stilbestrol diphosphate both in vivo and in vitro. They found this compound to be rapidly hydrolyzed by the phosphatase present in homogenates of rat liver, spleen, prostate, and kidney. Following intravenous administration of the compound, free stilbestrol was detected as early as five minutes after injection, reached a maximum in fifteen minutes, and was nearly gone in one hundred twenty minutes.

Thus there appeared to be two attributes of stilbestrol which could potentially influence the course of infection in animals: (1) a bactericidal effect, and (2) a stimulating effect on the RES.

Lysozyme

In 1922 Fleming described a powerful bacteriolytic substance capable of lysing thick suspensions of certain bacteria. This agent, to which he gave the name lysozyme, was found widely distributed in nature, i.e., in tears, nasal mucus, saliva, blood serum, many tissues, and in certain secretions; but not in urine, sweat or cerebrospinal fluid. Lysozyme was present also in plant substances. Of the materials examined, egg white was found to be the richest source. Fleming evidently attached general significance to the finding of this antibiotic substance so widely distributed in the animal kingdom, and even suggested that natural resistance of some animals to certain pathogens may have this underlying protective mechanism. This view was not shared by Goldsworthy and Florey (1930) who investigated the lysozyme concentration of various secretions in different species and pointed out that few pathogens were affected by it.

A large number of bacteria, both Gram-positive and Gram-negative, have been subjected to the activity of lysozyme over the past years. The sensitivity of Bacillus, Micrococcus, Staphylococcus, Proteus, and Brucella to

lysozyme was confirmed in early investigations. The Gram-negative bacteria, when tested under conditions giving rapid lysis of indicator organisms, generally have been more resistant; however, the distinction is not sharp since the sensitivity of certain Gram-negative bacteria is comparable to that of many Gram-positive organisms. Early observations by various workers of the microscopic sequence of changes affected by lysozyme have been in complete agreement in reporting a marked swelling of the cells which occurred before lysis and that this swelling was probably due to an alteration of the cell wall. Meyer et al. (1936) believed that the lysis resulted from hydrolysis of a mucoid substance in the bacterial membrane. Boasson (1938) concluded that the action of lysozyme on the cell wall of Micrococcus lysodeikticus was to make it more permeable to the cellular contents. The substrate acted upon was identified by Epstein and Chain (1940) as a high molecular weight polysaccharide found in the insoluble fraction of mechanically disrupted M. lysodeikticus.

Lysozyme is an enzyme belonging to the carbohydrases and brings about hydrolysis of a polysaccharide fraction which is not water-soluble. This polysaccharide substrate

has been found in all lysozyme-sensitive species tested. In some cases there is no lysis but death of the cell still results from its action. The hydrolysis yields reducing sugars of which half can be detected as acetyl hexosamine, and the depolymerization of the polysaccharide is associated with lowered viscosity (Meyer and Hahnel, 1946).

Babudieri and Biette (1945) and Kern et al. (1951) using electron microscopy, reported the following sequence of events with the sensitive M. lysodeikticus: swelling of cells, cell wall rupture and dispersal of contents and membrane leaving only two small electron dense bodies from each lysed cell. Ultracentrifugal analysis of lysozyme digested bacteria has shown that the major components have a molecular weight of approximately 10,000 to 20,000. Some of the products are sufficiently small to pass through dialysis tubing and it was found by Salton (1956) that 50% of the original weight of the cell was dialysable after complete digestion of M. lysodeikticus walls by lysozyme.

The observations of Fleming and Allison (1927) that an active form of lysozyme is present in leucocytes in sufficient concentration to accomplish intracellular digestion of certain human pathogens provided the early impetus for succeeding studies.

Recently Weiser et al. (1958) reported that no convincing proof was obtained by them to indicate that crystalline egg white can exert salutary effects on the course of experimental tuberculosis of mice.

On the other hand, Oshima and coworkers (1961) have shown that lysozyme appears to be the main tuberculostatic factor present in extracts of granulomatous lungs. Melsom and Weiser (1958) found lysozyme to have a therapeutic action on pneumococcosis of mice as indicated by a marked prolongation of survival time.

This author is not aware of investigations on the use of lysozyme as a possible therapeutic agent on staphylococcal infection. The paucity of work in this area may be due to conflicting reports on the in vitro susceptibility of staphylococci.

Kern et al. (1951) found that coagulase positive strains of Staphylococcus aureus are not lysed by lysozyme; although electron microscopy showed dissolution of the outer viscous portion of the cell wall, and very high concentrations of lysozyme produced a slow drop in the viable cell count. Mitchell and Moyle (1956) reported that the treatment of S. aureus with high concentrations of lysozyme in buffer

with sucrose as an osmotic stabilizer results in loss of cell wall and the liberation of protoplasts.

Brumfitt (1959) showed that deacetylation of acetyl-glucosamine of the cell wall of a certain strain of S. aureus failed to render it sensitive to lysozyme in contrast to other organisms listed. One reason for this finding would be that the lysozyme-sensitive bonds are absent from the cell wall. Mitchell and Moyle (1957) suggest that this is not true for all strains of staphylococci. As Weiser et al. (1958) have pointed out, in vitro insusceptibility per se does not indicate an equal in vivo insensitivity since possible synergistic effects may occur, and that in some tissues very high concentrations may be found. Moreover, Savini and Mercurelli (1947) have suggested on the basis of their experiments that lysozyme has an opsonic action on staphylococci rendering them more prone to phagocytosis. Melsom and Weiser (1958) found a greater tendency for pneumococci to adhere to phagocytes in mice treated with lysozyme. These various reports have stimulated this investigation of lysozyme in experimental staphylococcosis of mice.

Immunological Considerations

In the decades preceding the introduction of antibiotics, Forssman (1935) was almost alone in stressing the importance of antibacterial immunity in staphylococcal infection. Panton and Valentine (1929) injected washed suspensions intradermally in rabbits and by taking pus production as their indicator effect were able to show that repeated cutaneous infections gave rabbits partial immunity to a large dose. Forssman (1935) observed that this type of immunity could be passively transferred to normal animals. He found also that formalinized staphylococci as well as living ones can set up an immunity which may be sufficient to protect rabbits against a lethal dose of staphylococci. Heat-killed staphylococcal vaccines proved disappointing therapeutically (Rigdon, 1937). Washed, formalinized, or heat-killed suspensions of staphylococci, unlike the living organisms, failed to cause increased antitoxin titers (Kitching and Farrell, 1936; Downie, 1937) and animals so-treated reacted like controls to challenge with toxin. Farrell and Kitching (1940) found some protective power in immune horse sera against the homologous but not against the heterologous strains when tested in mice. The

protection did not appear to depend on alpha toxin or agglutinin content. Staphylococci isolated from lesions are by no means always agglutinated by the patient's serum and the demonstration of agglutinins seems to be of little clinical importance (Lichty et al., 1943).

The advent of the antibiotic era caused diminished interest in staphylococcal vaccines whether of whole organisms, antigen fractions, or toxoids. The continued, though limited, use of these preparations has been an indication of the failure of other therapeutic measures. In recent years, noticeable increase in demands for autogenous vaccines are concurrent with the increasing frequency of antibiotic resistant staphylococcal strains.

McCoy and Kennedy (1960), reporting on autogenous vaccines in sixty patients with various staphylococcal infections, indicated excellent response in 73%, improvement in 18%, and failure in 9%. Ten intracutaneous injections were used. The initial amount given was 0.01 ml, and the largest amount was 0.08 ml. The authors concluded that the mild action of Zephiran was one of the principal reasons for the vaccine's beneficial effect.

Greenberg and Cooper (1960) prepared a polyvalent

somatic antigen by combining the enzyme-lysed (Dornase) fractions of a number of vaccines prepared from selected phage types of S. aureus. The strains of S. aureus were chosen so as to give as broad a coverage as possible of the phage type being currently isolated from infections. They found good protection against both lethal and skin infecting doses. Protection against intradermal infection was obtained only when the rabbits were immunized intradermally or intramuscularly along with subcutaneous injection.

Greenberg et al. (1961) found that the vaccine described previously (1960) produced undesirable cutaneous reactions in humans. Since they considered the growth medium used to be a possible cause of toxicity, a synthetic fluid medium based on Medium No. 589 of Healy et al. (1955) was devised. The vaccine produced in this chemically defined medium was relatively free of reaction. There appeared to be little difference in the immunizing capacity.

MATERIALS AND METHODS

Organisms and Culture Media

A set of twenty-four international phage-propagating strains of staphylococci (Blair and Carr, 1953) was obtained from Dr. John E. Blair, Hospital for Joint Diseases, New York, New York; and the six Seto-Wilson phage-propagating strains were received from Dr. J. B. Wilson, University of Wisconsin, Madison, Wisconsin. The Smith strain of staphylococcus was provided by Dr. Myron Fisher, Parke, Davis & Co., Detroit, Michigan. Nine strains of staphylococci, recent isolates from carriers or lesions, were supplied by the Michigan Department of Health Laboratories, Lansing, Michigan. In this study, all strains were grown on brain heart infusion agar (Difco) at 37 C for 18 to 22 hours. The stock cultures were maintained on brain heart infusion agar at 5 C and were subcultured at monthly intervals.

Laboratory Animals

The rabbits used were virgin female New Zeeland albinos, ranging in weight from 2.0 to 2.5 kilograms. They were maintained on Rockland rabbit pellets.

The experimental mice were White Swiss (Webster strain) of both sexes and weighed 20 to 25 grams. They were maintained on Rockland Mouse Diet pellets.

Administration of Organisms and Their Estimation

In mice three routes of challenge were used: intravenous, intracerebral, and intraperitoneal. For intracerebral administration, the organisms were suspended in physiological saline to control 100,000 cells per 0.03 ml (Figure 1). This volume was delivered with a 0.1 ml syringe through a 27 gauge (3/8") needle. The intravenous challenge dose consisted of 0.1 ml of suspension whose density ranged from 1×10^7 to 1×10^8 cells per ml using a 1.0 ml syringe and 3/4", 27 gauge needle. Intraperitoneal injections were delivered with 2 ml syringes and 1/2", 24 gauge needles. The Smith strain of S. aureus was used in all routes of inoculation.

Tissue assay was carried out by killing the mice, removing the tissues aseptically, then homogenizing the tissue with sufficient diluent in sterile grinding mortars.[1]

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[1] E. H. Sargent & Co., Chicago, Illinois.

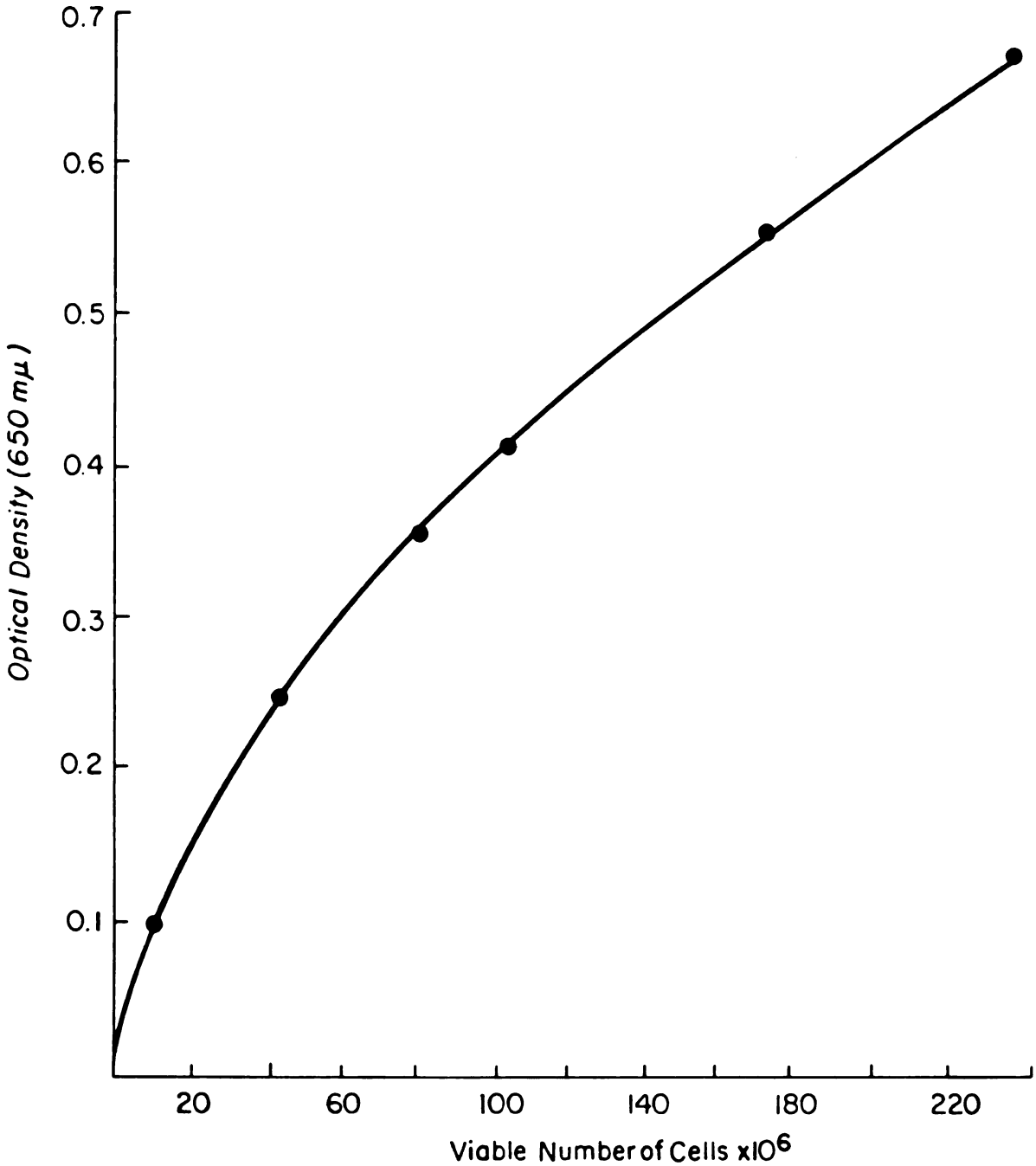

Figure 1. Calibration curve showing relationship between optical density (650 mμ) and the numbers of viable cells of Staphylococcus aureus, Smith strain, suspended in physiological saline.

Suitable dilutions were plated in duplicate and viable cell counts determined per whole tissue or in the case of blood, per ml.

Blood was obtained from the tail vein, drawn up into heparinized 20 λ pipettes, and discharged into suitable diluent. Further dilutions were made, plated in duplicate, and staphylococcal colonies counted after incubation at 37 C for one day.

The intracutaneous route of injection was used in rabbits to allow for simultaneous comparison of several strains of staphylococci in the dermal layer of the same animal. The infecting dose was contained in 0.1 ml buffered saline solution delivered by 1.0 ml syringe through 5/8", 25 gauge needle.

The effect of the challenge dose was measured by noting the diameter of both the zone of erythema and pyogenesis. In some instances differential and leucocyte counts were made on blood taken by cardiac puncture.

Blood for serum analysis was allowed to clot at room temperature for several hours, then held at 5 C until separation of serum from the clot was complete. Sera were held at -20 C if not immediately used.

Preparation and Administration of Stilbestrol Solutions

Crystalline stilbestrol was prepared for oral, subcutaneous, and intravenous use. For oral administration, a slurry was prepared of ground Rockland Mouse Diet pellets and stilbestrol solution to give a concentration of 1 mg per gram of dry food. Corn oil containing 500 μg per 0.1 ml of dissolved stilbestrol was used for subcutaneous injection (0.1 ml). Intravenous dosage was 0.05 ml of a 50% (v/v) alcoholic solution of stilbestrol containing 250 μg per 0.05 ml.

Lysozyme

In vitro studies

Purified, crystalline lysozyme[2] prepared from egg white was used. Stock solutions were prepared in phosphate buffer, sterilized by filtration, and stored at 5 C. Lysozyme was prepared for immunization by alum precipitation by the method of Kabat and Meyer (1961).

Potency of lysozyme was determined using a modification of the method of Hartsell and Smolelis (1949). The modifications included incubation at 37 C for ten minutes, instead

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[2]Difco Laboratories, Inc., Detroit, Michigan.

of twenty minutes at room temperature; and substrate concentration was decreased to give an optical density of 0.78 instead of 1.0 to compensate for the change in incubation. Activity of this enzyme was measured in terms of μg of purified lysozyme, shown by the change in optical density of the substrate (inactivated, dried M. lysodeikticus, Difco) versus the quantity of crystalline lysozyme (Figure 2).

Inhibition of lysozyme both by antilysozyme serum and its globulin fraction was accomplished by the reaction of suitable dilutions of antiserum or reconstituted globulin with known concentrations of lysozyme in equal volume. Approximately eighteen hours at 5 C gave maximum inhibition. Lysozyme inhibition was expressed in terms of an inhibition index. This index was calculated by dividing the optical density of the control by the optical density of the sample containing antibody, then obtaining the reciprocal of quotient.

The intereffects of lysozyme and coagulase were determined by adding variable quantities of lysozyme to brain heart infusion broth (BHI) prior to seeding with one of several strains of S. aureus. After a growth period of one day the organisms were sedimented and the supernatant fluid

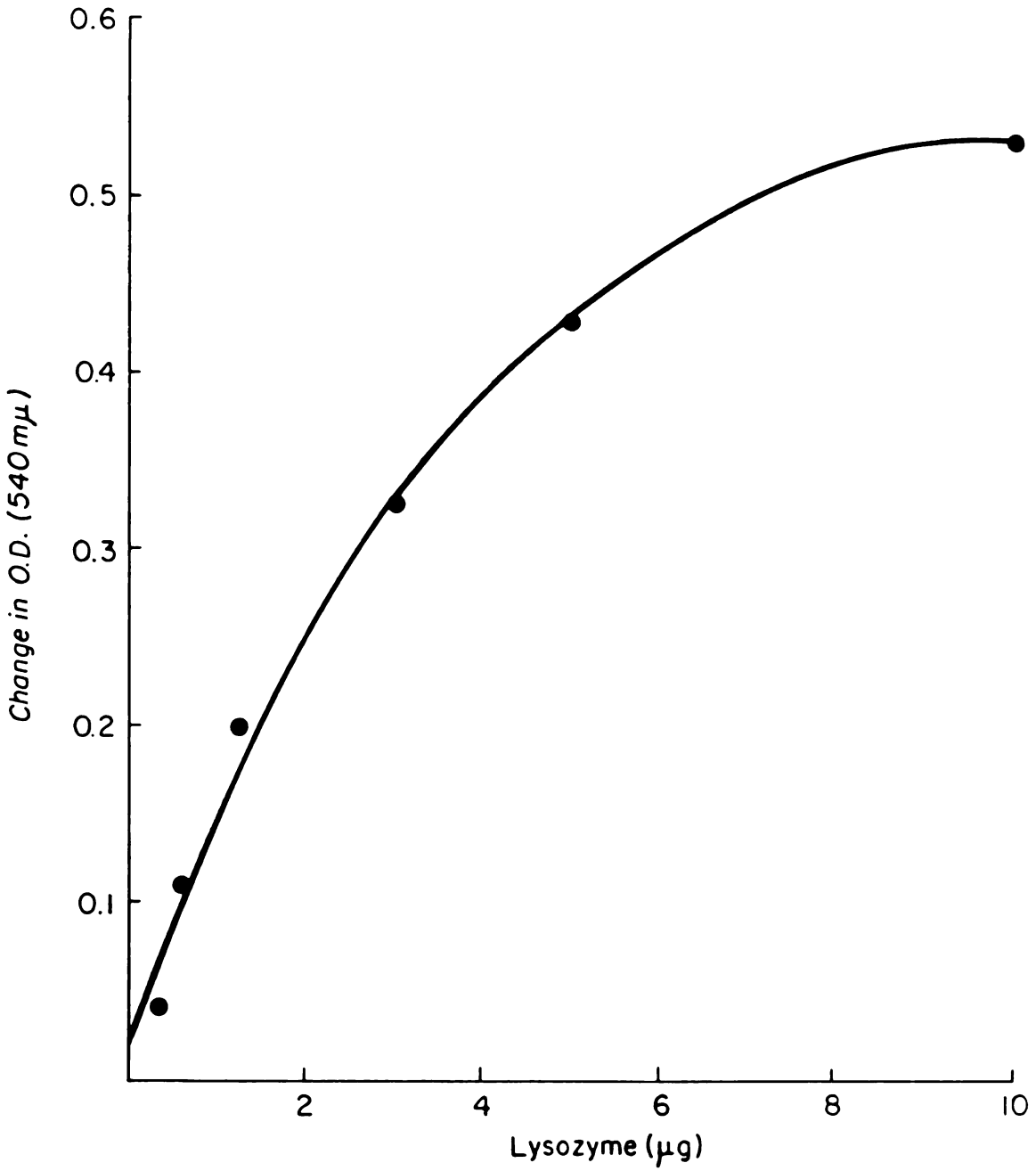

Figure 2. Changes in optical density (540 mμ) resulting from the lytic action of crystalline lysozyme upon a constant suspension (OD of 0.77) of substrate prepared from dried cells of Micrococcus lysodeikticus.

tested for both enzyme activities. The effect of lysozyme on growth was also determined by resuspending the packed cells in fresh BHI containing no lysozyme and comparing the density of growth to that of the control.

Stability of the stock lysozyme and substrate was maintained for more than one month at 5 C.

In vivo studies

In vivo activity of lysozyme was determined by drawing blood from the tail vein into 20 λ pipettes. The blood was delivered into buffered physiological saline rather than phosphate buffer to avoid hemolysis. The erythrocytes were sedimented and the supernatant fluid tested for lysozyme activity. When lysozyme was studied for its therapeutic effects upon an experimental staphylococcal infection in mice, the dose was 1000 μg, given either subcutaneously or intravenously.

Antilysozyme Globulin

Alum precipitated lysozyme was prepared and injected in rabbits at bi-weekly intervals, 5 ml in each buttock. Rabbits were bled by cardiac puncture, serum collected, assayed,

then stored at -20 C. When immunogenic response was poor, lysozyme emulsified in Freund's complete adjuvant[3] (Freund and McDermott, 1951) was used. Pooled sera of sufficient potency were used for fractionation to obtain globulin (Kendall, 1937). After completion of the fractionation, thimerosal was added to the fluid to a final concentration of 1:10,000; the fluid was filtered and stored in aliquots at -20 C. For in vivo administration, the dose consisted of 0.2 ml of the globulin reconstituted to half its original serum volume. Only the subcutaneous route of inoculation was employed. The test animals received the challenge dose of staphylococci just before the injection of antilysozyme globulin was given.

Coagulase(s)

The coagulase used was purified principally by the methods developed by Tager (1948) and more recently by Blobel et al. (1960). Additional modifications are described here. The supernatant culture fluid was adjusted to pH 3.8 with 4N hydrochloric acid. The resulting precipitate was collected by continuous flow centrifugation,

[3]Difco Laboratories, Inc., Detroit, Michigan.

dissolved in 50 ml distilled water, and adjusted to pH 7.2 with 0.66 M disodium phosphate buffer. After mixing in the cold, the insolubles were removed by centrifugation at 12,100 x G for twenty minutes. The supernatant fluid was precipitated by adding 95% ethanol kept at -20 C to a final concentration of 70% (v/v). Precipitation was continued for twenty hours; then the precipitate was collected by centrifugation at 1,950 x G. The precipitate was resuspended in distilled water, adjusted to pH 7.2, and diluted to 50 ml.

The insoluble material was removed by centrifugation and the cycle of ethanol precipitation repeated. The final clear solution was concentrated approximately four-fold by dialysis against polyvinyl pyrrolidone[4] for twenty-four hours at 5 C. The resulting coagulase preparation was then lyophilized and stored at -20 C.

Vaccines

Staphylococcal vaccines were prepared by harvesting the organisms grown from eighteen to twenty hours on BHI agar slants using 2 ml of buffered physiological saline. The organisms were sedimented, resuspended in fresh saline

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[4]Oxford Laboratories, Redwood City, California.

and resedimented. Density was adjusted to 6 x 10^8 organisms per ml and inactivated as follows: Fifteen ml of bacterial suspension were mixed with 2.5 ml Zephiran solution (1:1000) and incubated thirty minutes at 37 C with occasional gentle mixing. Heat inactivation was carried out by incubation at 56 C for ninety minutes. Formalin inactivation was accomplished by addition of 0.1 ml of 40% formaldehyde per 10 ml of suspension giving a final concentration of about 0.4%. Sterility tests were made in both BHI broth and fluid thioglycolate medium.

In certain experiments, Carmine Red (as carminic acid) was concomitantly used with the vaccine in a sterile 1% solution (Murray, 1961).

Immunization Protocol

Paired rabbits were used in all immunization procedures. Coagulase emulsified in Freund's complete adjuvant (1-2 mg per ml) was given weekly for 3 weeks subcutaneously in the nape of the neck. Vaccination with bacterial vaccine was by: (1) multiple intracutaneous injections, or (2) three intramuscular injections each followed immediately by an intracutaneous injection. The multiple intracutaneous

injections were given every other day starting with 0.01 ml and increasing by 0.01 ml for each successive injection to 0.04 ml. Increases thereafter were 0.02 ml to 0.08 ml, and four more injections of 0.08 ml for a total of 10 completed the immunization schedule.

Intramuscular immunizations consisted of the injection of 1.0 ml of vaccine into the buttock followed by an intracutaneous inoculation (0.04 ml). This regimen was carried out weekly for three weeks.

When Carmine Red was used, it was given subcutaneously in the nape of the neck in 2.5 ml quantities. Rabbits immunized by multiple intracutaneous injections received Carmine Red every third immunization dose, while the rabbits immunized intramuscularly were given Carmine Red at each immunization period.

Agglutination Tests

The test organisms were suspended in 1.8% sodium chloride solution so as to contain 1×10^9 organisms per ml (Nunnery, 1959). One volume of this suspension was added to a series of test tubes containing serially diluted serum in like volume. The tubes were incubated

at 37 C for sixty minutes and then held at 5 C overnight. Agglutination was read with a 3.5 power Bausch and Lomb hand lens. Agglutination of lysozyme-coated staphylococci was done by reconstituting the globulin to volume, and therein suspending the coated cells to a density of approximately 1×10^9. The agglutinations were read as 4, 3, 2, 1, $\pm$, or negative. The titer was defined as the reciprocal of the highest dilution giving a 2 reading.

Serum Determinations

Total serum proteins

Total rabbit serum protein was determined using the Biuret method, Gornall et al. (1944). Bovine serum albumin of known concentration was used to prepare a standard curve (Figure 3). Rabbit total serum protein was determined for normal serum, serum after immunization, and serum following intracutaneous infection.

Serum electrophoretic patterns

Electrophoretic fractionation was carried out using cellulose acetate paper strips[5] (Kohn, 1957) 2.5 cm x 12 cm,

[5]Consolidated Laboratories, Inc., Chicago, Illinois.

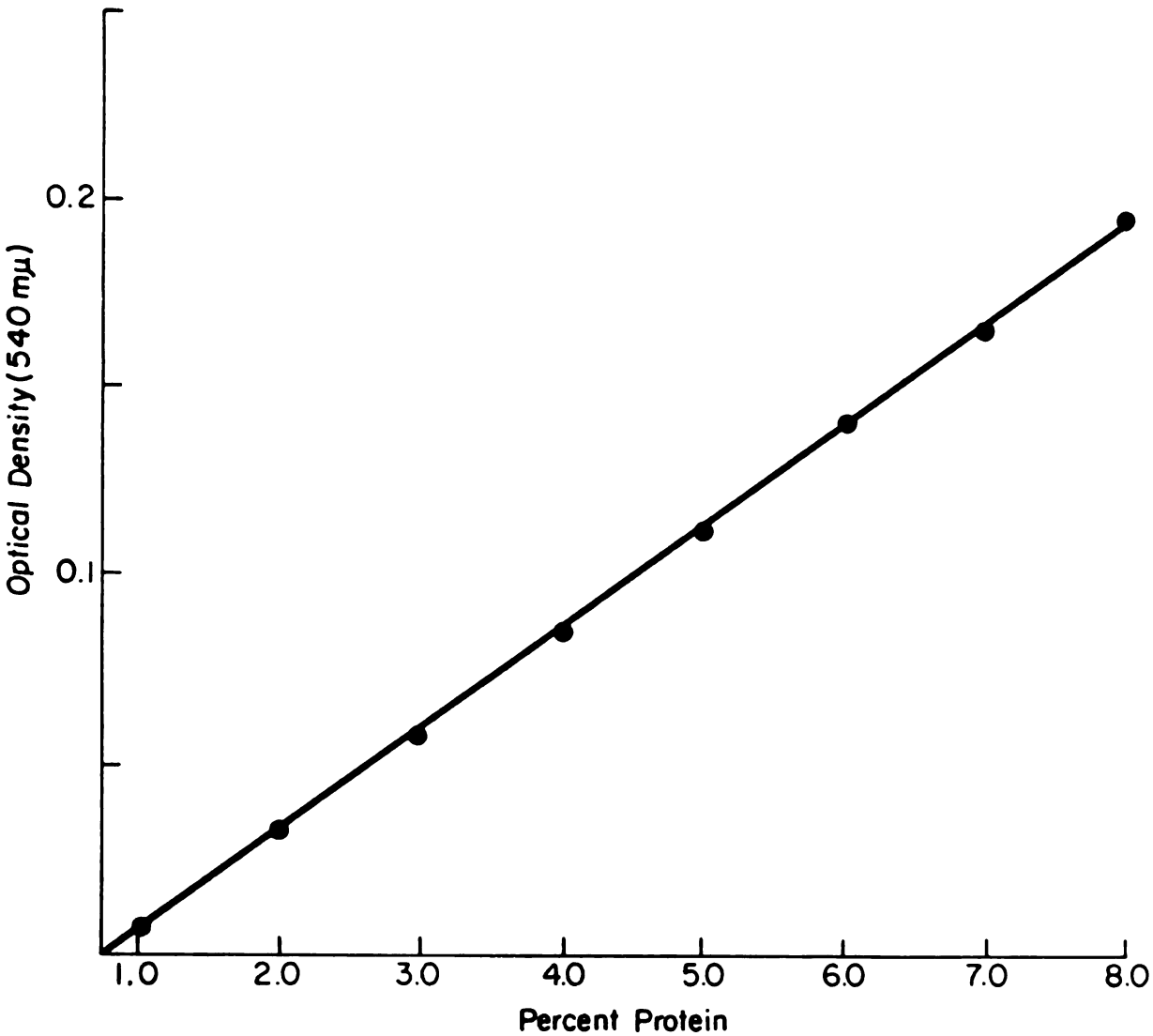

Figure 3. Reference calibration curve measuring percent total protein against optical density (540 mμ) using standardized bovine serum albumin prepared in physiological saline.

and Shandon Universal Electrophoresis Apparatus.[6]

The electrode buffer was composed of veronal 13.8 gm, sodium veronal 87.6 gm, calcium lactate 3.84 gm, and distilled water to 10 liters. The strip buffer was composed of veronal 16.6 gm, sodium veronal 105.1 gm, calcium lactate 15.36 gm, and distilled water to 10 liters. The strip buffer was diluted 1:3 before using. For each serum analysis, 10 λ of specimen was used. A constant current of 275 volts with approximately 0.45 mamp per cm width of strip produced separations of 6 to 8 cm after eighty minutes. After the separation was finished, the strips were immersed in fresh 3% aqueous trichloroacetic acid (TCA) for 5 minutes, then stained with Ponceau S stain (2% in 3% TCA) for 5 minutes. Clearing of the strip background was accomplished by washing the strips in three successive trays of 5% acetic acid (Korotozer et al., 1961). The strips were dried under pressure to prevent curling. The stained fractions were excised and eluted with 0.1 N sodium hydroxide. The optical densities at 560 mμ were found, and subsequently the percent protein determined from the curve (Figure 3).

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[6]Consolidated Laboratories, Inc., Chicago, Illinois.

Serum transferases

Three enzymes, serum glutamic oxalacetic transaminase (SGOT), serum glutamic pyruvic transaminase (SGPT), and ornithine carbamyl transferase (OCT) were included in this study (Molander et al., 1953; and Wroblewski and La Duce, 1956).

The enzyme SGOT catalyzes the following general reaction:

$$\alpha \text{ Ketoglutarate} + \text{L-Aspartate} \longrightarrow \text{Glutamate} + \text{Oxalacetate}$$

The enzyme SGPT catalyzes the following general reaction:

$$\alpha \text{ Ketoglutarate} + \text{L-Alanine} \longrightarrow \text{Glutamate} + \text{Pyruvate}$$

In the method used for this study (Reitman and Frankel, 1956, modifying the Karmen, 1955 procedure) the amount of oxalacetate or pyruvate which forms a highly colored hydrazone with 2,4-dinitrophenyl hydrazine hydrochloride in sixty minutes is determined colorimetrically. The calibration curve, Figure 4, for this analytical procedure plots optical density (505 mμ) against arbitrary Sigma-Frankel units per ml as well as certain ratios of pyruvate to α-ketoglutarate.

The enzyme OCT catalyzes the following special reaction:

$$\text{Citrulline} \xrightarrow{\text{Arsenate}} \text{Ornithine} + \text{Carbon Dioxide} + \text{Ammonia}$$

This reaction forms the basis for determining OCT activity by measuring the rate of ammonia formation when

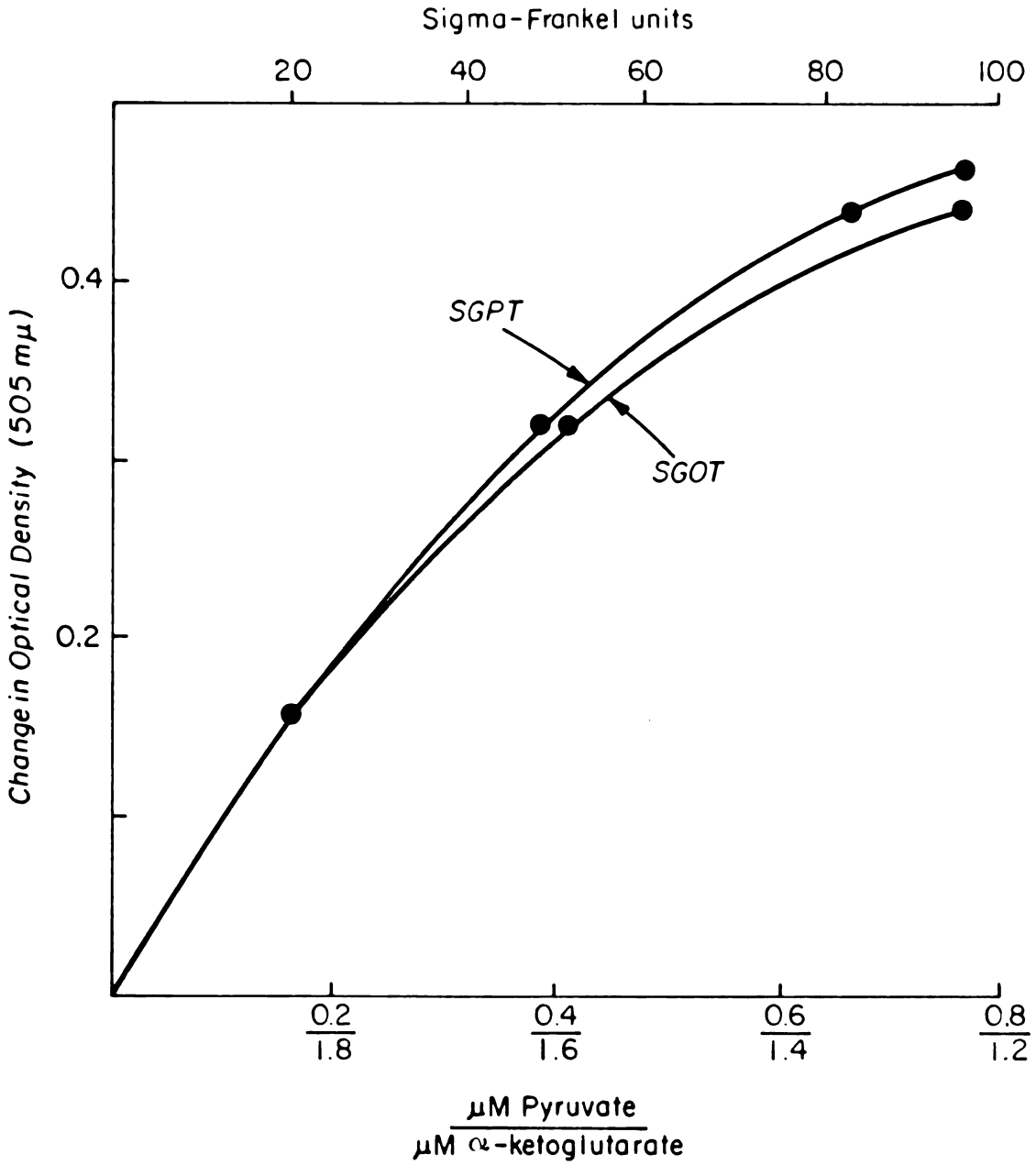

Figure 4. Relation between the activity of transaminase and change in optical density, expressed both in Sigma-Frankel units and as concentration of pyruvate and corresponding decrease in α-ketoglutarate (total 2 uM), as measured by their 2,4-dinitrophenyl hydrazones.

citrulline is incubated in the presence of an arsenate. This method (Reichard, 1957) determines the amount of ammonia absorbed by 0.01 N hydrochloric acid in a Conway microdiffusion unit by nesslerization. Optical density at 410 mμwas compared to the standard curve (Figure 5), with results stated in μM ammonia per 0.5 ml serum. Serum samples from each animal were measured prior to treatment, post-immunization, and post-challenge.

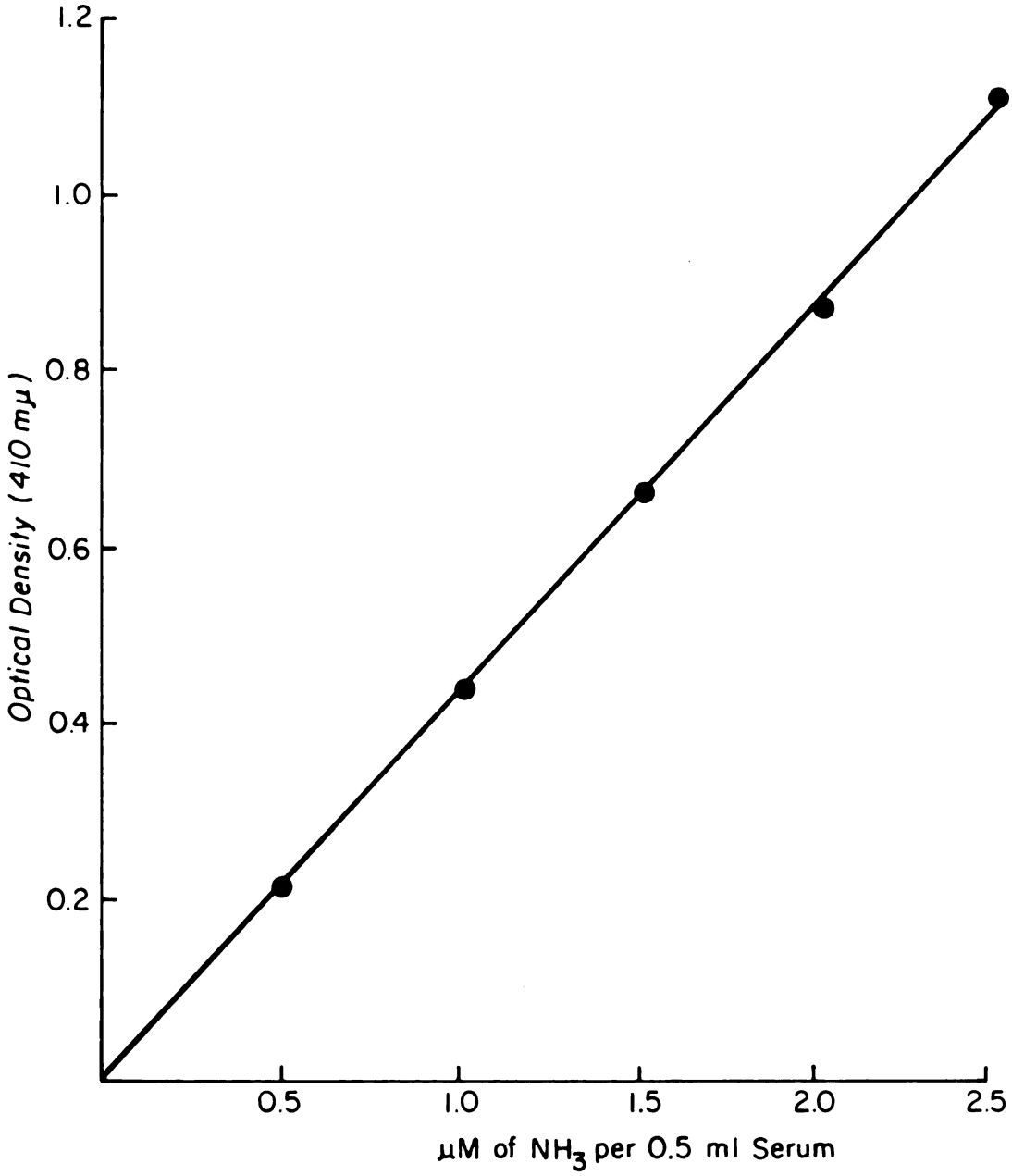

Figure 5. Reference calibration curve measuring ornithine carbamyl transferase expressed as μM of NH_3 per 0.5 ml of serum (determined by nesslerization) plotted against optical density (410 mμ).

RESULTS

Untreated and Experimentally Infected Mice

The data in Figure 6 show the disappearance of staphylococci from the blood as well as the uptake by certain tissues at several intervals following a caudal intravenous challenge dose of 1×10^7 organisms. The intravenous route of inoculation was selected as the most desirable since preliminary experiments showed survival time was the longest when compared to either the intracerebral or intraperitoneal route. It was assumed also that this route of injection produced an infection which most closely resembled the staphylococcal septicemia occurring in clinical cases.

The greatest number of viable organisms was noted in the blood sample where there was a thousand-fold reduction in viable organisms in fifteen minutes; and after thirty minutes there remained only 0.1% of the original number injected. The liver retained 90% of the infecting dose at thirty minutes while the lungs accounted for 1%. After two hours, the kidneys showed an increase over the initial number of staphylococci injected which indicated an early establishment

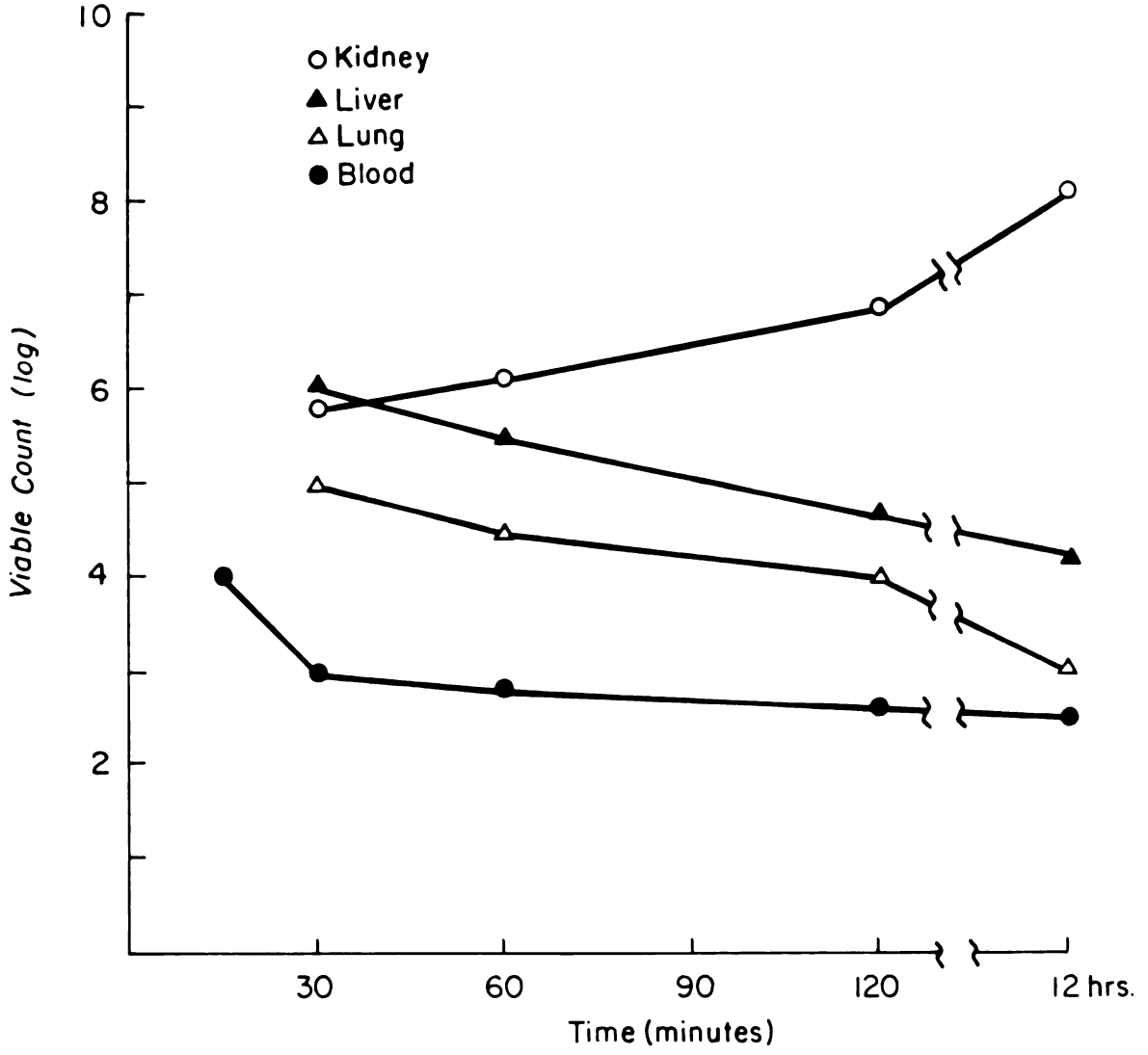

Figure 6. Viable counts per whole tissue taken from mice after intravenous injection with the Smith strain of Staphylococcus aureus.

and reproduction in this tissue. Assays beyond twelve hours showed incomplete removal of bacteria from blood, liver, and lungs; although levels were very low in these tissues, the kidneys consistently harbored a much higher population.

Effect of Stilbestrol upon Experimental Infection

Table 1 lists the viable counts of staphylococci per liver at two intervals of time after challenge of the mice undergoing treatment with stilbestrol via various routes of administration. In all groups of animals with and without treatment and regardless of route of administration, the liver captured from 7 to 20% of the challenge. However, the number of viable organisms measured at the second interval was considerably smaller than the first.

The result of the intravenous injection of the challenge dose of staphylococci as measured in the bloodstream is shown in Table 2. In both the control group and those receiving the oral and subcutaneous stilbestrol, there was found a marked reduction in numbers of bacteria. However, the group receiving stilbestrol intravenously showed a bacterial reduction of only approximately one-quarter as much as the others.

TABLE 1. Viable cell counts per liver of mice treated with stilbestrol via various routes after challenge with Staphylococcus aureus.

Route	Dosage Period	Liver Counts After Challenge[5]	
		20 min	70 min
		X 10^5	X 10^5
Oral[1]	6 days	7.5	3.2
	12 days	15.0	11.0
Subcutaneous[2]	Daily for 5 days	20.0	5.2
Intravenous[3]	Single injection	13.0	9.0
Control[4]	----------	11.0	5.0

[1]Mice were fed orally ad libitum with prepared Rockland mouse diet containing 100 µg of stilbestrol per gram of feed.

[2]Mice were given a daily injection of 500 µg of stilbestrol in 0.1 ml of corn oil.

[3]Mice were injected with 250 µg of stilbestrol contained in 0.05 ml of 50% ethanol.

[4]Control mice were fed plain Rockland mouse diet during entire experimental period.

[5]Challenge dose of 10 million organisms was administered IV.

TABLE 2. Viable cell counts per ml of blood of mice treated with stilbestrol via various routes* after challenge with Staphylococcus aureus.

Route	Dosage Period	Liver Counts After Challenge	
		20 min	70 min
		X 10^3	X 10^3
Oral	6 days	12.1	5.3
	12 days	9.3	4.3
Subcutaneous	Daily for 5 days	12.6	4.7
Intravenous	Single injection	42.0	18.2
Control	----------	10.0	2.6

*Experimental conditions same as in Table 1.

Table 3 shows the survival time after experimental infection of all groups of mice regardless of treatment. Of the untreated controls only 5% failed to survive at least thirteen days. With the exception of 20% of the group which were treated with stilbestrol subcutaneously, all other stilbestrol treated animals died after the experimental infection. Under the conditions of this experiment stilbestrol exerted no prophylactic action against the staphylococcal infection. In comparison with the oral control group which received no medication, the control groups receiving plain corn oil and plain 50% ethanol showed some decreased resistance to the challenge dose.

Effects of Lysozyme upon *Staphylococcus aureus*

In vitro studies

Typical results of the effects of lysozyme and coagulase upon each other are shown in Table 4. In concentrations of 100 µg per ml, lysozyme exerted no demonstrable antagonistic or synergistic effect upon coagulase. None of the coagulases from the seven strains had any effect upon the lysozyme. It was observed also that incubation for eighteen to twenty hours at 37 C had little effect upon lysozyme activity.

TABLE 3. Survival of mice challenged with Staphylococcus aureus after treatment with stilbestrol via various routes.*

Administration Route	Dosage Period	Survivors / Total	Mortality %	Maximum Days of Survival
Oral	6 days	0/15	100	12
	12 days	0/15	100	4
	None (control)	19/20	5	13
Subcutaneous	Daily (5 days)	4/20	80	6
	None (control)	17/20	15	5
Intravenous	One injection	0/15	100	2
	None (control)	8/10	20	5

*Experimental conditions as in Table 1.

TABLE 4. The inactivity of lysozyme and coagulase upon each other during active growth of Staphylococcus aureus.

Strain	Actual Tests		Activity Index**	
	Supernatant Fluid	Supernatant Fluid Plus Lysozyme*	Supernatant Fluid	Supernatant Fluid Plus Lysozyme
	Reciprocal Coagulase Titer			
3C	Neg[1]	Neg	0.93	4.70
6	Neg	Neg	1.00	4.30
70	1280	1280	0.98	5.05
44A	640	640	1.00	4.90
Smith	640	320	0.98	4.00
7	320	320	0.95	5.20
80/81	160	320	0.95	4.80
Test Controls				
Medium (uninoculated)			1.00	
Supernatant fluid after growth			0.98	
Supernatant fluid and lysozyme after growth				5.20
Fresh medium and lysozyme				5.15

*100 μg per ml, diluted 1:10 for testing.

**OD_{540} $\frac{\text{substrate control}}{\text{test}}$

[1]Negative at 1:20 dilution.

Table 5 summarizes the effect of lysozyme on total bacterial growth. In the case of three strains there was a very slight reduction in optical density, while there was an opposite effect shown by the other four. Apparently lysozyme had no appreciable effect at the concentration of 100 μg per ml of medium.

Results of lysozyme inhibition experiments are summarized in Table 6. Dilutions of 1:5 and 1:10 both serum and globulin, showed complete inhibition of lysozyme with respect to the controls containing substrate only. There seemed to be little if any difference between the antiserum or globulin in potency.

Since the data shown in Table 4 gave little indication of lysozyme inactivation by growing cells, it was decided to expose lower concentrations of lysozyme to increasing numbers of various strains of staphylococci. The organisms were harvested from BHI broth, and washed three times prior to mixing with the lysozyme solution. After the mixture was allowed to stand approximately eighteen hours at 37 C, it was centrifuged, and the supernatant examined for enzyme activity. The sedimented organisms were washed four times with phosphate buffer, and resuspended in buffered

TABLE 5. The relative inactivity of lysozyme upon growth of various strains of Staphylococcus aureus* in vitro.

Strains	Medium Only	Medium Containing Lysozyme
	X 10^8	X 10^8
3C	9.5	8.5
6	12.1	14.5
70	20.0	21.0
44A	15.5	14.0
Smith	11.1	13.2
7	34.7	31.2
80/81	23.5	24.5

*Number of organisms after 18 hours incubation.

TABLE 6. Lysozyme inhibition by antilysozyme serum and antilysozyme globulin.

Test Material	Dilution of Antibody Material	Inhibition Index*
Pooled rabbit sera	1:5	1.00
	1:10	0.98
	1:20	0.77
	1:40	0.71
	1:80	0.34
Control**		1.00
Globulin	1:5	1.00
	1:10	1.00
	1:20	0.83
	1:40	0.67
	1:80	0.33
Control**		1.00
Lysozyme control[1]		5.90

*Reciprocal of OD_{540} $\frac{\text{Control}}{\text{Test}}$

**Serum (or globulin) and substrate only.

[1]10 μg per ml.

physiological saline for agglutination tests. Figure 7 reflects the adsorptive capacity of four strains, 6, 80/81, 7, and Smith, previously mentioned in Table 5. It can be seen that all strains caused decrease in the lytic activity of the lysozyme solution. No one strain appeared to display superior adsorptive ability. Further, the most adsorptive strain, PS 6, removed less than 50% of the initial lysozyme activity present before absorption with the cells.

In vivo studies

The effectiveness of lysozyme as a therapeutic agent in experimental infections of mice was examined. The presumed prophylactic dose of 1000 μg in 0.1 ml was given simultaneously or just prior to the challenge infection. The observed averaged results are shown in Table 7. Regardless of treatment route, groups receiving lysozyme all showed increased survival rates over the untreated controls. The highest survival rate was found among those receiving lysozyme immediately after challenge. The control animals receiving lysozyme only showed no untoward effects.

The effect of in vivo administration of antilysozyme globulin upon subsequent sublethal challenge infection in

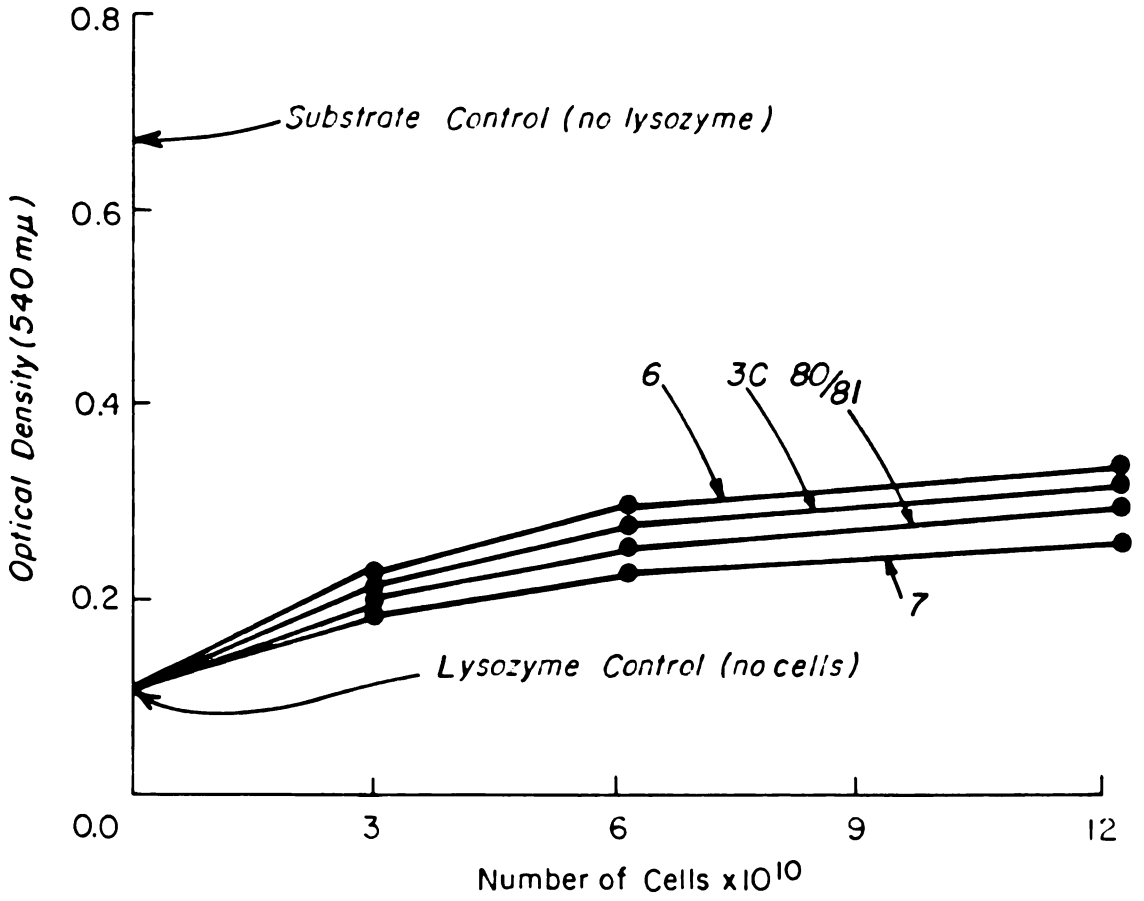

Figure 7. Decrease in lytic activity of lysozyme solution (10 ug per ml) after absorption by each of four strains of washed staphylococcal cells.

TABLE 7. Effect of lysozyme upon experimental staphylococcal infection in mice.

Treatment with Lysozyme	Challenge Dose	Survivors / Total	Survivors
			%
Intravenous	Simultaneous	18/30	60
Intracutaneous	Preceding treatment	21/32	66
Intracutaneous	Following treatment	23/31	74
None	Yes	0/15	0
Intracutaneous	None	15/15	100
Intravenous	None	15/15	100

mice was investigated. Typical results are given in Table 8. There appears to be some interference with the defense mechanism of the host by the antilysozyme globulin as indicated by a decrease in survival percent. Additional experiments where treatment was given several hours before challenge showed survival rates of more than 80%. Treatment several hours after challenge gave results slightly more comparable to the control group which all survived.

Effect of Various Vaccines

A series of rabbits immunized with partially purified coagulase was challenged by intracutaneous injection using thirteen strains of staphylococci. The degree of resulting erythema and pyogenesis was measured. The results are presented in Table 9. It is noted that the coagulase-treated animals showed an exaggerated response to the challenge dose over the controls. With respect to degree of erythema strains 77 and 83 produced the greatest contrast over the controls.

Polyvalent vaccine, inactivated by one of three methods, was administered to another series of rabbits. The response to intracutaneous infection is summarized in Table 10.

TABLE 8. Effect of antilysozyme globulin upon sublethal experimental staphylococcal infection of mice.

Route of Treatment	Treatment	Challenge Dose	Survivors / Total	Survivors %
Intracutaneous	Yes	Preceding treatment	4/10	40
Intracutaneous	Yes	None	10/10	100
	None	Yes	10/10	100*

*Two mice ill, but survived experimental period.

TABLE 9. Responses to intracutaneous infection of rabbits immunized with partially purified coagulase.

Phage Strains	Immunized Rabbits		Controls	
	Erythema	Pyogenesis	Erythema	Pyogenesis
	Avg cm		Avg cm	
6, 55, 73	0.9 - 1.0	0.1	0.8	0.1
187, 3A, 42D*, 53, 70, 81	1.1 - 1.2	0.3	1.0	0.1
29, 80*	1.3 - 1.4	0.4	1.1	0.2
77, 83*	1.5 - 1.6	0.5	1.0	0.3

*Homologous strains.

TABLE 10. Responses to intracutaneous infection of rabbits immunized with polyvalent vaccine.

Vaccine and Administration	Immunized Rabbits		Controls	
	Erythema	Pyogenesis	Erythema	Pyogenesis
	Avg cm		Avg cm	
Multiple I.C.[1]				
A-H *	0.8	0.05		
A-F **	0.4	< 0.05	0.9	0.1
A-Z***	0.1	< 0.05		
I.M. and I.C.[2]				
A-H	0.6	0.05		
A-F	0.5	0.05	1.0	0.1
A-Z	0.2	< 0.05		

*A-H is heat inactivated
**A-F is formalin inactivated
***A-Z is Zephiran inactivated

[1]Multiple intracutaneous injections (8 - 10)

[2]Intramuscular injection (1), followed immediately by intracutaneous injection (1). Repeated twice.

The data presented in this table indicate that the heat-inactivated vaccine was the least effective of those tested. Little protection was evident when the intracutaneous route of immunization was used. Formalin inactivated vaccine in both cases was of intermediate effectiveness. Again, in both methods of immunization, Zephiran seemed to be the most efficacious inactivating agent when administered by multiple intracutaneous injection.

Rabbits, previously immunized with coagulases from S. aureus strains of phage groups I and II, were injected with the Zephiran-inactivated polyvalent vaccine intradermally in order to determine if any increased protective effect could be demonstrated upon challenge. It can be seen from Table 11 that the control animals responded to the challenge dose with a smaller degree of erythema and pyogenesis than those injected previously with coagulase(s) and polyvalent vaccine. Pyogenesis in all cases was not critically different.

Using the data accumulated during these studies, the relative infective index was calculated from degree of erythema and pyogenesis of the immunized groups over the control groups. In Table 12 an infective index of less

TABLE 11. Responses to intracutaneous infection of rabbits immunized with various coagulases and subsequently with polyvalent vaccine.

Strains	Coagulases					
	Group I		Group II		Control Animals	
	Erythema	Pyogenesis	Erythema	Pyogenesis	Erythema	Pyogenesis
	Avg cm		Avg cm		Avg cm	
3A	1.6	0.2	1.2	0.3	1.2	0.3
55	1.1	0.1	1.1	0.1	0.8	0.1
29	1.2	0.1	1.3	0.1	1.1	0.1
80	1.5	0.2	1.1	0.2	1.0	0.1
81	1.3	0.0	1.1	0.1	0.5	0.0
83	1.5	0.2	1.2	0.1	0.6	0.1
42D	2.0	0.1	1.3	0.2	1.0	0.1

TABLE 12. Infective index of immunized rabbits.

Strain	Vaccine	Vaccine and Coagulase	Coagulase Only
6, 55, 73	0.5*	1.28	1.25
187, 3A, 42D, 53, 70, 81	0.47	1.51	1.30
29, 80	0.39	1.15	1.35
77, 83	0.41	2.15	1.57
Controls	1.00	1.00	1.00

*Calculation of infective index: Ratio of average diameter of erythema plus average diameter of pyogenesis of the immunized animal to the average diameter of erythema plus average diameter of pyogenesis of the control animal.

$$\text{Infective index} = \frac{0.5 + 0.1}{1.0 + 0.2} = 0.5$$

than unity signifies protection against the infective dose while a value greater than unity signifies decreased protection when compared to the untreated controls. From the results it is seen that coagulase alone or in combination with polyvalent vaccine exacerbated the challenge infection whereas the vaccine alone reduced the response to infection by 50% or more.

It is common knowledge that continued cultivation on the usual laboratory media tends to lower virulence of microorganisms. Data showing the protection offered by a polyvalent vaccine prepared from the stock phage propagating strains against a challenge dose of freshly isolated staphylococci are found in Table 13. In comparison with results from control rabbits observed in preceding tables, the erythematous response to the recent isolates was, in most cases, more than two-fold greater while the pyogenesis was three to ten times greater. Protection against the challenge dose of recently isolated strains was not as marked as that against old stock strains.

Another series of paired rabbits was immunized with a polyvalent vaccine prepared from six recent isolates. In this series, Carmine Red was administered subcutaneously

TABLE 13. Responses to intracutaneous infection of rabbits immunized with recent isolates from human lesions.

Vaccine and Administration	Immunized Rabbits		Controls	
	Ery-thema	Pyogen-esis	Ery-thema	Pyogen-esis
	Avg cm		Avg cm	
A-Z[1] Multiple I.C.	1.1	0.5		
B-Z[2] Multiple I.C.	1.8	0.5		
			2.0	1.2
A-Z I.M. and I.C.	1.5	0.7		
A-Z I.M. and I.C.	1.3	0.5		

[1]Vaccine A-Z: 25% propagating strain (PS) 42D, 20% PS6, 25% PS 3A, 25% PS 29, and 5% PS 187.

[2]Vaccine A-Z: 25% PS 42D, 20% PS 77, 25% PS 55, 25% PS 80, and 5% PS 187.

Both vaccines were Zephiran inactivated.

TABLE 14. Responses to intracutaneous infection of rabbits immunized with recent isolates from human lesions.

Administration Adjunct[1]	Immunized* Rabbits		Controls	
	Ery-thema	Pyogen-esis	Ery-thema	Pyogen-esis
	Avg cm		Avg cm	
Multiple I.C.	0.9	< 0.05		
Multiple I.C. Carmine Red	0.1	0.00		
			1.4	0.7
I.M. and I.C.	0.3	< 0.05		
I.M. and I.C. Carmine Red	0.4	< 0.05		

[1]Carmine Red (carminic acid) was administered subcutaneously just prior to the vaccine.

*A polyvalent vaccine made from 6 strains of the recent isolates, inactivated with Zephiran.

as a saturant of the RES just before vaccine injection. The average figures for erythema and pyogenesis are given in Table 14. These data indicate a good degree of protection; the infective index ranged from less than 0.1 to a maximum of 0.43. Carmine Red seemed to cause an increase in protective effect when used in conjunction with the intracutaneous route of vaccination. There was no similar effect when the intramuscular-intracutaneous route was utilized. It is of interest to note that the virulence as indicated in the controls decreased from that shown in Table 13, but it was still greater than that of the old stock strains.

Serum protein studies

In addition to dermal response, a series of hematological parameters were selected for quantitative measurement which included electrophoretic analysis of the serum proteins and determination of a group of serum transferases.

Table 15 gives the results obtained from determination of total serum protein of rabbits immunized with polyvalent vaccine. There was no particular change resulting from either immunization or subsequent infection.

Table 16 shows the results of the electrophoretic analysis of various protein components averaged from the sera

TABLE 15. The effect upon total serum protein of rabbits by polyvalent vaccine and subsequent challenge dose of Staphylococcus aureus.

Rabbit	Percent Total Protein		
	Normal	Post-Immunization	Post-Challenge (48 hr)
1	5.6	6.1	6.5
2	6.35	6.35	6.1
3	5.9	5.5	5.7
4	5.5	5.0	4.85
5	5.5	6.1	6.1
6	5.9	6.35	6.35
7	6.1	6.1	5.7
8	6.1	6.2	5.9
9*	5.9	6.0	6.3
10*	6.0	6.5	6.4
Avg	5.88	6.02	5.99

*Non-immunized controls.

TABLE 16. The effect upon the electrophoretic pattern of rabbit serum proteins by polyvalent vaccine and subsequent challenge by Staphylococcus aureus.

Fraction	Average* Percent of Fraction Found		
	Normal	Post-Immunization	Post-Challenge (48 hr)
Albumin	61.3	59.6	55.8
Globulins			
α_1	7.5	8.1	8.8
α_2	7.8	6.9	8.6
α_3	-	-	4.9
β_1	9.1	8.3	9.1
β_2	6.4	8.5	6.4
λ	7.9	8.6	7.4

*Average of 10 rabbits.

of ten rabbits. Two days after the challenge dose the α-globulins showed a small increase while the β-globulins remained relatively unchanged. After an initial rise of about 9% following immunization, the γ-globulin decreased to a value slightly below normal in 48 hours.

Serum transferases

Serum glutamic oxalacetic transaminase values are shown in Table 17. The only rabbit (#10) showing an elevated SGOT value had a focal subacute granulomatous myocarditis which was probably caused by several cardiac punctures. The other rabbits gave values within normal limits.

Serum glutamic pyruvic transaminase activities are given in Table 18. All values were normal, falling mostly in the lower half of the range.

Abnormally elevated ornithine carbamyl transferase values, Table 19, were found in all post-challenge serum samples. In the majority of cases little difference between immunized rabbits and controls was noted. Pathological examination of several livers indicated glycogen infiltration. In all cases, there was a slight increase of OCT activity in all sera taken six days after immunization.

TABLE 17. The effect upon rabbit serum glutamic oxalacetic transaminase values by polyvalent vaccine and subsequent challenge dose of Staphylococcus aureus.

Rabbit	Normal	Post-Immunized	Post-Challenge (48 hr)
		Sigma-Frankel Units*	
1	16	19	16
2	16	38	27
3	14	22	21
4	11	17	22
5	22	53	27
6	30	19	38
7	22	30	30
8	21	45	32
9**	22	22	26
10**	21	97	53

*Value in Sigma-Frankel (S-F) units; 1 unit will form 4.82×10^{-4} μM of glutamate per minute (pH 7.5 @ 25 C). Normal values 8 - 40, post infarction 40 - 200, and liver necrosis to 2000.

**Non-immunized rabbits.

TABLE 18. The effect upon serum glutamic pyruvic transaminase values[1] of rabbit serum by polyvalent vaccine and subsequent challenge with Staphylococcus aureus.

Rabbit	Normal	Post-Immunization	Post-Challenge (48 hr)
1	14	16	11
2	24	11	14
3	11	14	16
4	10	21	22
5	18	18	16
6	19	11	16
7	16	12	14
8	20	27	28
9*	19	19	21
10*	16	19	24

[1]S-F Units; tentative normal values, 5 - 35, post infarction 40 - 100, liver necrosis over 100.

*Non-immunized controls.

TABLE 19. The effect upon rabbit ornithine carbamyl transferase values[1] by polyvalent vaccine and subsequent challenge dose of Staphylococcus aureus.

Rabbit	Sera Examined		
	Normal	Post-Immunization	Post-Challenge (48 hr)
1	0.07	0.12	0.53
2	0.12	0.14	0.62
3	0.15	0.21	0.31
4	0.15	0.33	0.67
5	0.13	0.23	0.27
6	0.15	-	0.42
7	0.02	-	0.53
9*	0.30	-	0.53
10*	0.30	-	0.57

[1]Values of μM NH_3 per 0.5 ml of serum, normal range 0 - 0.25, cirrhosis of liver 0.5 - 1.2, acute cholecystitis 1.0 - 8.0, and infectious hepatitis 0.5 - 25.

*Non-immunized controls.

DISCUSSION

Stilbestrol

From results in Tables 1 and 2, it is clear that administration of stilbestrol, regardless of route, failed to increase materially the disappearance of viable organisms from the blood and tissues. From smears of peripheral blood, the number of macrophages was shown to remain relatively constant. Heller et al. (1957) believed that in the absence of increased numbers of cells the explanation must lie in the probability that increased disappearance of carbon was caused by an enhancement of the activity of each cell. While this explanation may well be correct, one must consider the great difference between an inert carbon particle and the dynamic surface of a bacterial cell. Evidence from the present studies did not indicate increased phagocytic activity under the conditions used. Had there been increased phagocytic activity, a longer survival period and a lower mortality rate would have been expected. Neither alternative was seen.

The groups receiving the stilbestrol over the longest period of time showed the lowest capacity to remove the

microorganisms from the bloodstream. Cuppage and Blockworth (1960) reported that administration of stilbestrol for 50 to 100 days caused distinctly abnormal bile duct proliferation in rabbits. In fact 80% of the liver was replaced by bile duct cells in one rabbit. The injection intravenously of mice with ethanol-stilbestrol solution caused a reduction of about 75% in the ability of the liver to remove staphylococci. Since the administered amount of this solution was approximately 2% of the total blood volume, this treatment was probably the most physiologically disturbing since death occurred rapidly within 48 hours. Perhaps the longest dosage period used in this study may also have caused subclinical liver damage which reduced the trapping of bacteria. Frank and Pounden (1961) found that mastitis-producing staphylococci were inhibited by 3.5 μg per ml of stilbestrol in vitro. On the basis of the regimen in this study blood levels of more than 3.5 μg per ml were possibly obtained; yet, compared to the controls, no marked reduction of viable staphylococci occurred. Again the difficulty of comparison between the test tube and the animal system is underscored.

Heller et al. (1957) found significant hepatomegaly

following stilbestrol administration in mice, and further, that based on radiocolloid studies the activity per gram of liver was decreased. It is suggested therefore that stilbestrol administration may adversely effect the liver causing interference with normal organism trapping efficiency. This in turn could hamper the normal defense response resulting in a subsequent fatal outcome.

It was concluded that stilbestrol administered under the conditions given here was not valuable as a therapeutic agent in experimental staphylococcal infection of mice.

Lysozyme

According to Elek (1959) the known production of proteinases by staphylococci may lead to auto-destruction of coagulase (Birch-Herschfield, 1940). Since egg white inhibits the proteinase action against coagulase (Lominske, Morrison, and Smith, 1955), it was desirable to study possible interaction between lysozyme and coagulase. Moreover, because Ekstedt and Nungester (1955) and Yotis (1962) have shown that coagulase exerts an inhibiting effect on an active protective factor in normal serum, possible

interaction of these two enzymes could create a unique effect upon the host-staphylococcus relationship. As previously indicated, data presented in Table 4 reveal no antagonism between coagulase and lysozyme. The combination gave no evidence of any synergistic relationship. Although there appeared to be no definite effect upon total bacterial population as measured by optical density, slight decreases in lysozyme activity suggested the possibility of bacterial adsorption of lysozyme. This supposition was subsequently borne out when after repeated washing, the cells were consistently agglutinated by both antilysozyme serum and its globulin fraction. Since the titer never exceeded 1:20, perhaps a limited number of active sites were available for lattice formation. According to Raffel's discussion of the lattice hypothesis (1961), it is probable that the lysozyme-antiserum specific aggregate has the maximum spatial freedom for lattice formation for saturating the available active sites. The agglutination reaction, on the other hand, suffers first from the fact that the basic properties of lysozyme would be attracted by negatively charged groups on the bacterial surface. Secondly, as a result of the adsorption of the lysozyme, the residual

sites available on the exposed facets may be inadequate to provide the minimum necessary lattice for strong agglutination.

The results of tests in mice with lysozyme (Table 7) indicated a moderate degree of protection. Both the intravenous and subcutaneous routes gave similar results. Time of administration of lysozyme and the challenge dose was immaterial provided the interval between the two was short. If the interval extended beyond two hours, the detectable life of the enzyme, protection dropped off sharply. This protective action seemed to be related to the degree and recency of bacterial adsorption of additional lysozyme. Savini and Mercurelli (1947) reported that the adsorption of lysozyme did render the staphylococci more susceptible to phagocytosis. Also Melsom and Weisser (1958) reported that in lysozyme-treated mice there was a greater tendency for pneumococci to adhere to phagocytes.

The position that the lysozyme was a factor in the protective action was strengthened by the observation that the administration of antilysozyme globulin decreased by 60% the survival of mice challenged with a normally sublethal number of staphylococci (Table 8).

Recently, Flannagan and Lionette (1955) showed the lysozyme content of blood was due to its liberation from damaged polymorphonuclear cells since plasma cells, erythrocytes, lymphocytes, and platelets contain none. This concentration suggests a relatively constant source of lysozyme to participate in phagocytosis. Thus, the injection of antilysozyme globulin could appreciably reduce phagocytic action by neutralizing naturally occurring lysozyme. Even though it is probable that the passive specific antibody would be quickly destroyed, the amount of globulin solution was the equivalent of approximately 16% of the total blood volume of a normal adult mouse. It seems quite reasonable to assume that this rather massive dose of antilysozyme globulin could effect, at least temporarily, neutralization of normally present lysozyme. This action could, in turn, allow the infecting organisms to overwhelm the host as a result of the altered defense mechanism.

It has been reported by Lepow et al. (1959) that partially purified properdin contains a small amount of lysozyme. It is interesting to contemplate the possible role of lysozyme in the known inhibitory effect of properdin on some bacteria and viruses.

Vaccines

The polyvalent vaccines used in this study excluded treatment with Dornase. However, the results of Greenberg and Cooper (1960) with polyvalent vaccine subjected to Dornase indicated that in every instance their preparation protected against a greater variety of strains than one without the Dornase treatment.

McCoy and Kennedy (1960) found good therapeutic results using an autogenous whole cell vaccine in antibiotic-resistant staphylococcal infections. In agreement with our findings (Table 10) these authors also preferred the use of a minimum quantity of a mild antiseptic over the drastic denaturation caused by heat. They also believed that only essential subculturing be done to lessen changes in bacterial cells due to artificial culture medium.

None of the above workers considered the possible use of coagulase as potentially valuable in either a vaccine system or alone. As previously mentioned, the fact that coagulase appeared to promote virulence in coagulase-negative staphylococci suggested several experiments to determine whether immunization with this substance would reduce the virulence of staphylococci injected into rabbits.

The experimental results (Tables 9 and 11) using semi-purified coagulase were not encouraging when used alone or combined with a polyvalent vaccine. The heightened erythematous and pyogenic response when coagulase had been used suggested at first a hypersensitivity phenomenon. If this were the case, one would expect the greatest reaction with the most active coagulase producing strains; however, this was not borne out. The strain (PS 70) producing the greatest amount of coagulase ranked in the middle so far as infective response is concerned (Table 9).

In January, 1962, Yotis presented evidence that coagulase appears to neutralize some serum factor with antibacterial activity located in the supernatant portion obtained following 65% $(NH_4)_2SO_4$ saturation of the water-soluble globulin portion and precipitation by ethanol.

Since the rabbits were injected with coagulase and Freund's adjuvant, the protracted release of coagulase from the sterile abscess formed from inoculation may well have served to keep the antibacterial activity in the rabbit serum at a constant and prolonged low level, allowing a given infective dose to produce a greater reaction than in the control. It seems, also, that this effect could perhaps

be interfering with antibody production after vaccination.

Derbyshire and Helliwell (1962) reported that their α-lysin-coagulase-leucocidin vaccine enhanced resistance to a challenge of staphylococci via the mammary gland. The severe mastitis found in one-third of the experimental group may have been due to continued release of coagulase from the $AlPO_4$ used as the adsorbant for the vaccine.

Also in the matter of inactivation, Zephiran was found to be the least disruptive of the cationic agents tested against staphylococci as demonstrated by a lower percent of phosphorus and nitrogen released into the medium (Hotchkiss, 1946). This leakage has been employed as a measure of permeability of the bacterial cell.

The study of total serum proteins (Table 15) was not rewarding. The experimental infection had no measurable effect upon the total protein. The variation for each period assayed was approximately 3%.

When the various serum fractions were quantitated after elution from the acetate paper strips (Table 16), there appeared to be a slight decrease in the albumin fraction, the most marked reduction occurring 48 hours after intravenous staphylococcal injection. The γ-globulin fraction

was the only one to show a decrease below normal values 48 hours after challenge. Presumably this would be expected since antibody is concentrated in this fraction. Caution needs to be exercised in assessing these changes in the separate reactions because failure to properly relate the changes to the total protein may give a false impression of the degree of change.

The enzymic response in serum of rabbits to both immunization and experimental infection was considered potentially valuable in understanding systemic and tissue effects. White (1960) suggests that enzymes showing activity in serum are normally there as the natural products of cellular wear and tear; therefore, increased metabolism of any major tissue resulting in greater cell replacement should be reflected in increased levels of its constituent enzymes in the blood at a given time. Three enzymes were selected which are involved in hepatic disorders, serum glutamic oxalacetic transaminase (SGOT), serum glutamic pyruvic transaminase (SGPT), and ornithine carbamyl transferase (OCT). The first two are not solely restricted to liver, but are fairly widely distributed in mammalian tissue. OCT is highly localized in liver to the extent that abnormal

activity is found only in patients with hepatitis (Reichard, 1957).

The major detoxicating organ is the liver; thus, if immunization provokes a systemic response by the introduction of foreign material requiring inactivation, an increase in liver activity should produce an increase in the enzymes listed above. No general increase in either SGOT or SGPT was found either after immunization or after the challenge dose. A subacute granulomatous myocarditis determined after necropsy of a rabbit with an abnormally high SGOT may have been caused by cardiac punctures (Table 17).

The OCT values were found to be increased moderately after immunization, but the high values attained after challenge indicate liver disturbance. Rabbits displaying the highest values were necropsied and glycogen infiltration of the liver was found by pathological examination. These findings intimate that rabbits undergoing immunization may show detectable liver involvement in terms of elevated OCT levels.

SUMMARY

The effect of stilbestrol, lysozyme, and various vaccines upon the experimental infection of laboratory animals with Staphylococcus aureus has been studied.

Tissue localization and blood clearance of staphylococci were determined in mice experimentally infected by intravenous inoculation.

Mice were variously treated with stilbestrol in food, or dissolved in corn oil and injected intracutaneously, or dissolved in 50% (v/v) alcohol-water solution and administered intravenously. The results suggested that in vivo use of stilbestrol exacerbated a subsequent infection with staphylococci.

Concentrations of 100 μg per ml of lysozyme had no apparent effect upon growth of staphylococci in vitro. Lysozyme activity was completely inhibited by both antilysozyme serum and its globulin fraction in vitro. No apparent interaction occurred between lysozyme and coagulase in the in vitro studies.

Washed staphylococci were found to adsorb lysozyme from solution and were agglutinated by both antilysozyme serum and its globulin fraction.

In vivo tests in mice indicated that lysozyme exerted a therapeutic effect based upon increased rate of survival against staphylococcal infection. On the other hand, anti-lysozyme globulin administered by subcutaneous injection increased the mortality rate with normally sublethal infecting doses.

Semi-purified coagulase used as an immunizing agent alone or followed with polyvalent bacterial vaccines failed to promote resistance in rabbits after intracutaneous infection. The superiority of the methods for inactivating the vaccines was in the order of Zephiran, formalin, and heat. Polyvalent vaccines made from recent isolates of human lesions showed a better immunizing ability and a greater virulence than the laboratory strains. In the use of Carmine Red as an adjuvant during the immunization schedule with polyvalent vaccine, results found after the challenge dose indicated that there was some beneficial action if multiple intracutaneous administration had been used. The results were less favorable if immunization had been accomplished by the intramuscular route.

Rabbit total serum protein showed no essential change after either immunization or intracutaneous infection.

Electrophoretic separation of rabbit serum using cellulose acetate strips indicated a slight decrease in albumin after infection, and the appearance of an α_3-globulin. After infection, only the γ-globulin decreased slightly below the normal value.

Enzyme studies revealed that neither serum glutamic oxalacetic transaminase or serum glutamic pyruvic transaminase showed abnormal activity after immunization or infection. Ornithine carbamyl transferase values were slightly increased after immunization. After intracutaneous infection, values exceeded normal limits but were below clinical levels. The livers from several rabbits in which the OCT values were higher were examined histologically. The observed glycogen infiltration indicated hepatic damage.

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