STAPHYLOCOCCAL ACID PHOSPHATASE: ANTIGENICITY, ASSAY OF ITS ANTIBODY, AND PROTECTION STUDIES

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ABSTRACT

STAPHYLOCOCCAL ACID PHOSPHATASE:
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By

Barbara K. Bodner

Purified staphylococcal acid phosphatase was prepared from Staphylococcus aureus (PS 55) according to Malveaux and San Clemente (1969). In a series of ten injections given every other day, three New Zealand White rabbits were inoculated with the purified enzyme, mixed 1:1 with Freund's complete adjuvant. The first five injections were given in the foot pad and the last five subcutaneously in the cervical region. One rabbit received a total of 25 units phosphatase, the second, 50 units, and the third, 100 units. One unit of acid phosphatase is the amount necessary to liberate from p-nitrophenylphosphate, disodium salt, one μM of p-nitrophenol per minute at 37 C. Antibody was first detected on day 12 following the first inoculation in the two rabbits given the largest amount of phosphatase. Eventually all three rabbits produced an

antibody titer of 1:16. Titers were determined by an immunodiffusion test in which serial dilutions of antisera were permitted to diffuse against purified staphylococcal acid phosphatase. Development of precipitin lines and identification of the antibody was made with a specific stain. A colorimetric procedure for antiphosphatase quantitation was also developed; however, it is not as sensitive as the immunodiffusion method.

As determined by mercaptoethanol treatment of an antiphosphatase serum sample, antibodies to staphylococcal acid phosphatase were apparently localized in the IgG serum fraction.

Serum samples from normal and staphylococcus infected individuals were tested for staphylococcal antiphosphatase. The antibody was not found in sera from normal individuals or those with acute staphylococcal disease. It was detected in most serum samples from individuals with chronic staphylococcal skin disease and those in which deep tissues were infected. Its presence, therefore, seems to indicate a chronic or severe staphylococcal infection.

Three rabbits previously immunized with the purified staphylococcal acid phosphatase, and one nonimmunized

control rabbit, were challenged with subcutaneous injections of Staphylococcus aureus (PS 55) and talc. The talc served as a foreign body irritant. The degree of erythema and necrosis, as well as the total and differential leukocyte counts were determined at 24, 48, 72, 96 hr and then at frequent intervals until healing occurred. Data obtained the first week following inoculation suggested that the antiphosphatase was providing some protection. Later the immunized rabbits' lesions increased in severity and took longer to heal than the control rabbit, probably due to the organisms' ability eventually to overcome the antiphosphatase or to delayed hypersensitivity.

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INTRODUCTION

Most studies concerned with the nature of staphylococcal disease and its prevention have focused on certain bacterial products toxic to the infected individual. While it is now obvious that no one factor is responsible for virulence, it is also apparent that products other than toxic ones should be investigated.

Staphylococcal acid phosphatase, which produces no known toxic effect, has been considered a reliable indicator of pathogenicity in staphylococci (Barber, Brooksbank and Kuper, 1951; White and Pickett, 1953; Gupta and Charavarti, 1954). The enzyme has been purified, characterized and localized (Malveaux and San Clemente, 1969), and there is some evidence that it is antigenic in rabbits and cows (Arai and San Clemente, 1971).

This investigation was undertaken to confirm the antigenicity of staphylococcal acid phosphatase. If there should be an antibody response we would proceed to develop a rapid, easy method of antibody assay, and subsequently use this assay to screen sera of staphylococcal infected

and noninfected individuals for antiphosphatase. Furthermore, we would determine the possible protective effect of an acid phosphatase immunization program to intracutaneously injected, virulent Staphylococcus aureus.

REVIEW OF LITERATURE

Acid Phosphatase as an Indicator of Pathogenicity

The majority of the publications on staphylococcal acid phosphatase involve its correlation with pathogenicity. Since coagulase production is the classic indicator of pathogenicity, many workers have correlated the occurrence of phosphatase with coagulase. Barber, Brooksbank and Kuper (1951) tested strains of staphylococci for phosphatase, glucuronidase and sulfatase but only phosphatase correlated with coagulase production. All 160 coagulase-positive organisms tested were phosphatase-positive, and of 75 coagulase-negative strains only one was phosphatase-positive. Similar results were reported by White and Pickett (1953), despite their use of a different phosphatase test system. In another study 400 strains of *Staphylococcus aureus* were screened for phosphatase activity and approximately 90% were both coagulase- and phosphatase-positive (Gupta and Charavarti, 1954). Correlation between these two enzymes was further

substantiated by Adolfo and Gallardo (1959) who reported 98.7% agreement. After consideration of mannitol fermentation, hyaluronidase, coagulase and phosphatase production, Angyal (1960) concluded that coagulase was the most reliable indicator of potential pathogenicity. However, he recommended that a coagulase-negative strain be regarded as pathogenic if it was positive for phosphatase or hyaluronidase.

Several investigators have tried to relate the enzyme production of staphylococci to phage pattern. When comparing phage type, coagulase, hyaluronidase and phosphatase activity Fodor, Rizgonyi and Csepke (1962) noted that Staphylococcus aureus, phage type 80/81, had low coagulase and hyaluronidase and high phosphatase activity. They explained, however, that phosphatase could not be regarded as the sole indicator of virulence since virulent strains from other phage types had low phosphatase activity. Cannon and Hawn (1963) reported that the relationship between phosphatase production and penicillin resistance was inconsistent. However they observed a close relationship between phosphatase production and phage type. They also found the greatest percentage of high phosphatase producing staphylococci in the 80/81 and Group I strains, regardless

of source. Other reports substantiating these results were published by Solomon and San Clemente (1963) and Pan and Blumenthal (1961). In the strains tested by Solomon and San Clemente coagulase and phosphatase activity and mannitol fermentation all occurred together or not at all. The quantitative results of Pan and Blumenthal (1961) supported previous qualitative evidence that acid phosphatase activity was higher in coagulase-positive strains than in coagulase-negative ones; coagulase-positive strains of S. aureus produced an average of five times more acid phosphatase than coagulase-negative strains. But individual coagulase-negative and -positive cultures could not be separated on the basis of their acid phosphatase content since some coagulase-negative strains produced more phosphatase than coagulase-positive strains. In a later study Kedzia et al. (1966) found a quantitative difference in the phosphatase production of organisms isolated from various types of infection. They demonstrated that strains isolated from healthy carriers produced considerably less phosphatase than those from cases of septicemia and pyemia as well as furunculosis. Moreover, the activity of strains from septicemia and pyemia was generally greater than that of the strains from

furuncles. These authors suggested that organisms isolated from patients had a higher acid phosphatase activity because of adaptation to the acid pH within the lesion. There has been some disagreement with the observation that phage Group I contained the majority of high phosphatase producers. Choudhuri and Chakrabarty (1970) reported that phage Group II had maximal correlation with α and β hemolysin, lipase and phosphatase production. Earlier work by Thatcher and Simon (1956) showed isolates from phage Group IV to be more consistent in the production of coagulase, phosphatase and of potent amounts of α and β hemolysins and enterotoxins. Since some of their strains were isolated from dairy foods, they were interested in finding a good correlation between coagulase or phosphatase and toxin production. However, neither of these enzymes consistently indicated toxigenic strains.

Acid phosphatase has been demonstrated in a variety of microorganisms and yet little is known about the physiological role of this enzyme and how it contributes to pathogenicity (Tirunarayanan and Lundbeck, 1968). Based on work done with Escherichia coli, Torriani (1960) suggested that phosphate, a product of phosphatase action, controlled the level of enzyme synthesis via a negative

feed back system. Such a phosphate-phosphatase regulatory system could play an important part in the phosphorous economy of the cell. However Kuo and Blumenthal (1961) reported that such a phosphate repressible system could not be found in Staphylococcus aureus. Ivler (1965) theorized that some staphylococcal enzymes might contribute to virulence by maintaining the cell at a high rate of metabolic activity. Kedzia et al. (1966) presented extensive evidence that phosphatase activity is closely related to some mechanism for regulating the concentration of the inorganic phosphate pool and consequent metabolic processes within the bacterial cell. They cited the study by Volk (1954) who found that bacterial cells grown in the presence of sodium fluoride were unable to ferment phosphorylated compounds. It was theorized that phosphorylated compounds did not penetrate the membrane freely, and previous growth in fluoride must have prevented formation of the phosphatase necessary for dephosphorylation and subsequent penetration of these compounds. Moreover they suggested that the high phosphatase activity in clinical strains might play an important role in the intensity of penetration of some compounds into the cell and thus in the regulation of the bacteria's phosphate pool. A clue

to the action of phosphatase in the infected host was reported by Mukherjee et al. (1965) who found an increase in host inorganic phosphate associated with staphylococcal infection. Staphylococcal acid phosphatase could contribute to the fall of organic phosphate and the rise of inorganic phosphate in the host, although the substrate involved in vivo is not known.

Although the concept of phosphatase as an indicator of virulence is well supported, several conflicting studies have been published. Yoshida and Takeuchi (1970) found that diffuse variants exhibited lower coagulase, deoxyribonuclease, phosphatase and hemolysin activity than the compact strain they were derived from. The diffuse strain was encapsulated and these authors suggested that its virulence in mice was not related to any extracellular products but rather to the capsule and its size. In a study of over 500 clinical isolates (Morton and Cohn, 1972) 200 of 224 coagulase-positive organisms produced phosphatase as also did 19 of 96 coagulase-negative organisms. The authors concluded that phosphatase production did not correlate well with coagulase production. Strains of staphylococci from a great variety of clinical sources that did not produce either coagulase or

deoxyribonuclease were isolated as frequently as strains which produced these substances. Therefore they concluded that the production of coagulase and deoxyribonuclease were not necessarily indicative of potential pathogenicity of these organisms for man.

Acid Phosphatase Preparation

A number of methods, yielding products of different degrees of purity, have been used to extract acid phosphatase from microorganisms. Sevag et al. (1954) used water extraction to remove acid phosphatase from Brewers Yeast. A partially purified phosphatase preparation was obtained when extraction was followed by ammonium sulfate fractionation and dialysis against water. An "osmotic shock procedure" has been used to release a group of enzymes from E. coli (Dvorak, Brockman and Heppel, 1967). This technique was used as the first step in preparation of acid phosphatase followed by chromatography on Sephadex ion exchangers, zone electrophoresis and gel filtration (Hofsten and Porath, 1962). A highly purified acid phosphatase was obtained from Neurospora crassa by using

ethanol fractionation, precipitation with cetyltrimethylammonium bromide (CTAB) and ammonium sulfate and finally fractionation on a diethylaminoethyl (DEAE)-cellulose column (Kuo and Blumenthal, 1961). Precipitation with ammonium sulfate has been the standard procedure for preparation of various staphylococcal enzymes and toxins. Using this method, deoxyribonuclease has been precipitated by Anfinsen, Rumley and Taniuchi (1963), leukocidin by Woodin (1959), hemolysin by Bernheimer and Schwartz (1963) and staphylokinase by Glanville (1963). Vesterberg et al. (1967) using isoelectric focusing were able to separate all these proteins with less loss of biological activity than occurred with ammonium sulfate precipitation. Malveaux and San Clemente (1969) developed a method of extraction and purification of staphylococcal acid phosphatase by eluting it from the surface of S. aureus cells with 1.0 M KCl (pH 8.5) by gentle agitation and then purifying it 44-fold by two cycles of dialysis and gel filtration.

Some investigators have attempted to increase acid phosphatase production by varying culture conditions. Torriani (1960) reported that E. coli alkaline phosphatase was only formed when inorganic phosphorus became limiting

in the medium. In contrast Hofsten (1961) had shown that the nature and concentration of the phosphate source for growth had no marked influence on the formation of E. coli acid phosphatase. He also reported that acid phosphatase activity varied considerably with the composition of the growth medium. For example, succinate or glycerol as the carbon source supported synthesis of high levels of acid phosphatase, whereas carbohydrates, such as glucose, had a repressive effect. A biphasic growth medium was used by Pan and Blumenthal (1961) for production of staphylococcal acid phosphatase. The highest yields were obtained when Brain-Heart-Infusion medium was used and the flasks were shaken. Arvidson, Holme and Wadstrom (1971) compared staphylococcal enzyme and toxin production using membrane covered solid medium and liquid media in shake flasks and stirred, aerated fermentors. For all enzymes, cultivation in liquid media was superior to membrane plates, and yeast extract was required as a medium component for high yield of cells and extracellular proteins. Maximal bacterial yields were obtained in aerated cultures, but it was necessary to control foaming to avoid inactivation of enzymes. Another paper by these authors (Arvidson, Holme and Wadstrom, 1971) described the importance of optimal

pH of the culture medium. The maximal yield of acid phosphatase was obtained at pH 6.5-8.0.

Determination of Staphylococcal Acid Phosphatase Activity

A method for the detection of staphylococcal acid phosphatase was first reported by Barber and Kuper (1951). They incorporated the substrate, phenolphthalein diphosphate, into either nutrient broth or agar. If the substrate was cleaved by growing colonies the surrounding medium turned pink when exposed to ammonia fumes. A more rapid phosphatase test was described by White and Pickett (1953). By using phenyl disodium phosphate as the substrate results were available in four hours instead of the twelve to eighteen hours required for the first procedure. An indicator, 2,6, dibromo-N-chloro-p-quinoneimine was used to detect substrate cleavage. Others (Lovell, 1958) have incorporated this same substrate into nutrient agar and thereby lengthened the procedure to overnight incubation. Phosphatase liberates free p-nitrophenol, a dye whose yellow color is intensified at an alkaline pH. A

recent report applied the indigogenic principal used to demonstrate tissue enzymes to detection of staphylococcal acid phosphatase (Von der Muehll, Ludwick and Wolf, 1972). The substrate, 5-bromo-4-chloro-3-indolyl phosphate, offered the advantage of precise enzyme location with very little or no diffusion. They stated that only pathogenic staphylococci produced a blue green precipitate after thirty minutes incubation with the substrate. In addition they listed a number of the Enterobacteriaceae in which acid phosphatase was detected by this method. Barnes and Morris (1957) published a quantitative method using the phenyl disodium phosphate substrate used by White and Pickett (1953) in their screening test. The resulting colored product is quantitated by spectrophotometry after thirty minutes incubation. Cannon and Hawn (1963) modified this procedure by substituting phenolphthalein diphosphate as the substrate which required eighteen hours incubation.

Immunological Aspects

Schlamowitz (1953) reported production of antibodies in rabbits against dog intestine alkaline

phosphatase by injecting an alum suspension of the antigen intravenously. Antibodies to mouse duodenal and jejunal phosphatase have also been described by Moog and Angeletti (1962). They indicated that there was an immunological difference between the phosphatases from these different parts of the mouse intestine. Sevag et al. (1954) reported production of antiphosphatase by subcutaneous and intravenous immunization of rabbits with dried and live yeasts and with partially purified yeast phosphatase. Their work dealt with the nature of the linkage between phosphatase and antiphosphatase causing, under optimal conditions, the complete inhibition of the enzyme. Antibodies to human prostatic acid phosphatase have been described by Shulman et al. (1964), who used them to quantitate prostatic acid phosphatase by gel diffusion. Antiserum was prepared by inoculating rabbits intraperitoneally with normal human serum or human prostatic fluid. Some evidence for the antigenicity of staphylococcal acid phosphatase in cows and rabbits had been published by Arai and San Clemente (1971). They injected equal parts of purified staphylococcal acid phosphatase and Freund's complete adjuvant intramuscularly into a cow and into rabbit foot

pads. Antibodies were detected in the cow and rabbits by precipitin and hemagglutination tests.

There were several reports in the literature of assay methods for antibodies to staphylococcal components that might be used as diagnostic tools. Perfiliev (1971) described the presence of antihyaluronidase in acute purulent staphylococcal disease of the abdominal cavity and the apparent correlation between the antibody level and the severity and duration of the disease. Bergquist (1952) noted the occurrence of staphylococcal antihyaluronidase in tuberculosis patients with multiple infections. He suggested that the presence of antihyaluronidase indicated that tuberculous tissue may be subjected to constant or intermittent action of bacterial hyaluronidase which may promote the spreading of tubercle bacilli and lead to progression of the disease. Daugharty, Martin and White (1966) found antibodies against teichoic acids and a large number of type specific antigens in pooled concentrated human globulin using Ouchterlony double diffusion. Antibodies to these same components were detectable in unconcentrated sera from subjects with active staphylococcal disease. Based on these findings Martin, Crowder and White (1972) reported the development of an agar gel

diffusion test to differentiate between staphylococcal and streptococcal endocarditis. Teichoic acid antibodies were present in the sera of patients with known staphylococcal endocarditis but not in patients without endocarditis or other staphylococcal infections, or in patients with streptococcal endocarditis. Arai and San Clemente (1971) were able to detect antistaphylococcal acid phosphatase in both serum and milk of mastitic cows by a passive hemagglutination technique. The procedure was proposed as a diagnostic test for staphylococcal mastitis. Morris and Hobbs (1971) reported an immunodiffusion test for diagnosis of bovine mastitis; however, the test utilized a nonspecific antigen thought to be an immunoglobulin produced by lymphocytes in the udder.

Early in the immunological investigation of staphylococcal infection it became evident that vaccination with the organism failed to provide protection in the same manner as was possible with other bacterial diseases. As a result, attention was then focused on the question of antitoxic immunity and α hemolysin was one of the first toxins to be investigated. Most early work failed to provide convincing evidence of immunity due to antihemolysin (Forssman, 1938; Flaum, 1938; Downey, 1937). The few

favorable results indicated antitoxin immunity under the conditions of the experiment, but not any significant protection against natural infection. Parish and Cannon (1960) were able to produce high levels of antitoxin to hemolysin in rabbits and horses. When immunized rabbits were challenged by intradermal or subcutaneous injection of virulent staphylococci, the resulting lesions showed a minimal amount of necrosis, were less extensive, and healed more rapidly than those produced in controls. However, immunity was antitoxic and not antibacterial. Leukocidin toxoid has been used for therapeutic immunization in cases of chronic osteomyelitis (Mudd, Gladstone and Lenhart, 1965). The toxoid was efficient in eliciting antitoxin in human subjects; either healthy persons or those suffering from chronic staphylococcal infections. Clinical impressions of the cases indicated that patients did appreciably better than would have been expected without immune therapy; however, it was not feasible to observe matched control cases without such therapy. Joy and Harrison (1964) investigated the protection of rabbits against staphylococcal infection following immunization with staphylocoagulase toxin or toxoid. The antibody appeared to give some protection against homologous

organisms and an occasional heterologous strain. The number of survivors in the immunized group was significant at 4 days but not at 21 days which suggested that anti-coagulase interfered with the pathological processes that lead to death shortly after injection of staphylococci and, secondly, that it failed to prevent the chronic type of disease that eventually leads to death. Hornbeck and San Clemente (1962) challenged coagulase-immunized rabbits with intradermal inoculation of staphylococci and noted no diminution in erythema and pyogenesis compared to control animals. In fact, previous sensitization with coagulase appeared to exacerbate dermal response to experimental infection. Stockland reported production of antistaphylococcal LDH in rabbits; however, he observed little, if any, protective effect to intracutaneous challenge. Antibodies to staphylococcal lipase (Lundbeck and Tirunarayanan, 1967) and hyaluronidase (Bergquist, 1952) have been detected in human sera, but little work has been done to determine whether or not they were protective. In an effort to produce antibacterial immunity, other investigators have used a variety of staphylococcal products. A distinctive, highly potent, immunizing polysaccharide, called specific polysaccharide antigen (SPA), has been

prepared from Smith type staphylococci (Fisher, Devlin and Erlandson, 1963). Specific polysaccharide antigen will stimulate the appearance of protective serum antibody in mice, dogs, cows and humans but not in guinea pigs, rabbits and monkeys. The immunized animals resisted challenge by various strains of S. aureus as well as the Smith strain. San Clemente, Renshaw and Fisher (1965) injected various combinations of SPA, coagulase and lipase into cows and rabbits. The resulting antisera were tested for mouse protective capacity against the Smith strain. The rabbit sera did not provide any protective antibodies; however, cow sera showed that SPA and either lipase or coagulase produced higher titers of protective antibody than SPA alone. Others have tried using combination vaccines (Blobel and Berman, 1964). Vaccination of dairy cattle with a staphylococcal bacterin-toxoid to which coagulase and egg yolk factor were added stimulated the production of α and β antihemolysins, "agglutinin," antileukocidin and antibodies inhibiting coagulase and egg-yolk factor. Vaccinated cattle had increased resistance to mammary infections with the homologous, but not heterologous strains. The incidence and severity of clinical cases of mastitis, including those caused by

heterologous strains of staphylococci, were markedly reduced in vaccinated cows. In an attempt to develop a vaccine that would give protection against a large number of strains, Greenberg and Cooper (1960) prepared a polyvalent somatic antigen vaccine by combining enzyme lysed fractions of a number of different phage types of S. aureus. Immunized rabbits were protected from challenge by subcutaneous injection of a variety of S. aureus strains. They found that greater protection was given by lysed polyvalent somatic antigens than nonlysed, indicating that antibodies to intracellular antigens play a greater part in immunity to staphylococcal infections than antibodies to surface or extracellular antigens. These vaccines have been used in humans and proved to be helpful (Greenberg, 1963). Yoshida and Ekstedt (1968) found antiteichoic acid to be the antibody in hyperimmune rabbit antisera against heat-killed S. aureus (Smith strain) that protected mice against challenge with homologous organisms. These same workers (Ekstedt and Yoshida, 1969) noted that mice immunized by sublethal doses of living Staphylococcus aureus strains other than the Smith strain were significantly more resistant to challenge with the Smith strain than animals immunized with heat killed organisms.

The possible role of hypersensitivity in staphylococcal infection was suggested by Panton and Valentine (1929) who studied the effect of repeated skin infections in rabbits. More recently, Johanovsky (1958) reported producing a state of hypersensitivity in rabbits by repeated intradermal and intravenous infections with small doses of staphylococci. Spleen cells or peritoneal exudate cells of these rabbits were transferred intraperitoneally to normal rabbits. Transfer of the cells resulted in delayed hypersensitivity and increased susceptibility to staphylococcal infection in the recipients. In a study by Johnson, Cluff and Goshi (1961) the effect of previous experience with staphylococcal infection upon response of the host to subsequent challenge by the organism was investigated. They found that repeated staphylococcal infection was associated with the development of delayed hypersensitivity unaccompanied by the appearance of demonstrable serum antibody. The delayed hypersensitivity produced increased infectivity of the organism in the skin of the sensitized animal characterized by intensification of the lesions seen with large bacterial inocula and induction of abcesses with inocula incapable of producing any lesion in normal rabbit skin. These same

authors (Goshi et al. 1961) theorized that the inflammation associated with hypersensitivity reactions may be partially responsible for the infectivity of staphylococci. Their results showed that areas of nonspecific inflammation were more susceptible to infection although the effect was never as impressive as that observed with delayed hypersensitivity. Furthermore, they concluded that inflammation could be expected to influence infectivity only when factors of nonspecific resistance play an important role in conditioning the virulence of the organism. In another study (Goshi, Cluff and Johnson, 1961) nonspecific resistance so restricted the infection that the effect of acquired immunity could not be demonstrated. By comparison it was found that high levels of serum antibodies to α hemolysin or dermonecrotoxin were associated with a striking increase in resistance to infection in necrotic tissue. Taubler (1968) reported delayed hypersensitivity to staphylococci induced by eight weekly subcutaneous injections in mice. The hypersensitivity could be transferred to normal mice via lymphoid cells but could not be transferred with plasma, nonviable cells, or cell-free extracts. In a study by Martin, Crowder and White (1967) skin hypersensitivity to

staphylococcal antigen A and teichoic acids was demonstrated in adult subjects. They found that incubation of leukocytes with high titer teichoic acid antisera resulted in the release of lysozyme from the leukocytes, which suggested a role for the enzyme in the skin reactions observed.

MATERIALS AND METHODS

Organism Cultivation and Maintenance

Staphylococcus aureus PS 55 of the International Blair series (Blair and Carr, 1960) of phage propagating strains was the source of enzyme in this study. A stock culture was maintained at 4 C on Trypticase Soy Agar (Baltimore Biological Laboratories, Baltimore, Md.) and was transferred every six weeks.

Production of Acid Phosphatase

Media

In a pilot study to determine the medium for optimal acid phosphatase production, cells were cultivated in Trypticase Soy Broth (Baltimore Biological Laboratories, Baltimore, Md.), casein hydrolysate medium (Stutzenberger, San Clemente and Vedehra, 1966), a modified casein hydrolysate medium with succinate substituted for glucose, CCY medium (Arvidson, Holme and Wadstrom,

1971) and a biphasic growth medium (Pan and Blumenthal, 1961). Flasks containing 500 ml of each medium and a biphasic growth medium containing 50 ml of a 2% agar base and 3% Trypticase Soy Broth and layered with 12.5 ml of 3% Trypticase Soy broth as the liquid phase, were inoculated with 1 ml overnight Trypticase Soy Broth culture (PS 55). They were incubated at 37 C while being agitated on a reciprocal shaker. At intervals over a 24 hr period, and after recording the optical density (O.D.) at 625 nm, whole culture acid phosphatase was determined according to the method of Barnes and Morris (1957). The experiment was repeated using only Trypticase Soy Broth and CCY medium. At this time culture pH was adjusted to 7.5 and Dow B Antifoam (Dow Chemicals, Midland, Mich.) was added at a concentration of 0.05 ml per liter of media. Again O.D. at 625 nm and whole culture acid phosphatase were determined. Trypticase Soy Broth was the medium of choice and was used for the remaining acid phosphatase production.

Cultural Conditions

Cultivation was performed in a MF 214 Microferm unit (New Brunswick Scientific Co., New Brunswick, N.J.)

in 10 liters of Trypticase Soy Broth at 37 C. A propeller speed of 200 rpm was maintained. The air flow rate was 1.5-2.0 liters per min at 1 atmosphere pressure. The pH of the medium was adjusted to 7.5 before sterilization and did not decrease more than one pH unit during the 24 hr cultivation period. To control foaming Dow B Antifoam was added before sterilization to a concentration of 0.05 ml per liter of media and additional aliquots were added as needed during cultivation. Overnight shake flask cultures in 100 ml Trypticase Soy Broth served as inocula for the 10 liter cultures. Cells were collected by continuous-flow centrifugation using a Serval Model SS-1 centrifuge equipped with the Szent-Gyorgi and Blum continuous-flow system (Ivan Sorval, Inc., Norwalk, Conn.).

Purification of Acid Phosphatase

Purified staphylococcal acid phosphatase was obtained from cells (PS 55) using a slightly modified procedure of Malveaux and San Clemente (1969). The whole cells were washed once with 500 ml of a 0.1 M KCl/0.05 M Tris-chloride solution (pH 8.5), centrifuged, and

resuspended in the same volume of 1.0 M KCl/0.5 M Tris-chloride (pH 8.5). The wash solution was discarded. Elution was effected by gentle agitation of the washed cells on a reciprocal shaker at 4 C for 60 minutes. The cells were separated from the eluted material by centrifugation and the resulting supernatant fluid was dialyzed against 10 volumes of 0.01 M Tris-chloride (pH 8.5) for 12 hours at 4 C. The precipitate formed during dialysis was sedimented in a Serval Model RC-2 refrigerated centrifuge (Ivan Sorval, Inc., Norwalk, Conn.) at 4 C, was redissolved in 1.0 M KCl/0.5 M Tris-chloride (pH 8.5), and was redialyzed as described above. The precipitate formed after the second dialysis was dissolved in several milliliters of 1.0 M KCl/0.5 M Tris-chloride. A Sephadex (Pharmacia Fine Chemicals, Piscataway, N.J.) G-100 column (2.5 by 38 cm) was equilibrated with this same solution and the void volume determined with Blue Dextran 2000 indicator (Pharmacia Fine Chemicals, Piscataway, N.J.) at 4 C. The sample (2 ml), containing sucrose at a concentration of 10%, was layered on the column and was eluted with the same solution while 4 ml fractions were collected on a Fractomat fraction collector (Buchler Instruments, Fort Lee, N.J.). Each fraction was scanned at 260 nm and

280 nm with a Beckman DU spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif.). Those fractions with the highest protein content were assayed for acid phosphatase activity and those with the maximum were combined. This purified product was used in the remainder of the study.

Quantitative and Qualitative Methods

Assays on the Purified Enzyme

Acid phosphatase activity was measured by a method modified from Barnes and Morris (1957) using 0.08% p-nitrophenylphosphate, disodium salt (Calbiochem, Los Angeles, Calif.) as the substrate buffered at pH 5.2 with sodium acetate. When a blank was included, acidification with HCl was not necessary to eliminate color due to free p-nitrophenol. Crystalline p-nitrophenol (Matheson, Coleman and Bell, Norwood, Ohio.) was used to prepare a standard curve.

Protein determinations were made according to the method of Lowry *et al.* (1951), using crystallized bovine

Fraction V albumin (Sigma Chemical Co., St. Louis, Mo.) for protein standards.

A number of tests were made on the purified acid phosphatase to determine the presence or absence of associated staphylococcal products. Coagulase was measured by the tube method (Bailey and Scott, 1970) at 37 C using rabbit plasma (Difco Laboratories, Detroit, Mich.). Deoxyribonuclease activity was analyzed by placing 0.2 ml of the purified fraction into wells cut in Deoxyribonuclease Test Agar (Baltimore Biological Laboratories, Baltimore, Md.). The plates were incubated overnight at 37 C and flooded with 1 N HCl. A clear zone around the well indicated deoxyribonuclease activity. Lipase activity was determined according to the method of Nachlas and Seligman (1949), using p-nitrophenylpalmitate (California Corporation for Biochemical Research, Los Angeles, Calif.) as the substrate. The reaction occurred in veronal buffer (pH 7.4) at 40 C. The presence of a yellow color following incubation for one hour indicated lipase activity. Fibrinolysin activity was analyzed by streaking the sample on fibrin plates made by adding citrate bovine fibrinogen (Sigma Chemical Co., St. Louis, Mo.) to warm

nutrient agar. The plates were read after incubation overnight at 37 C.

Antiphosphatase Detection and Semiquantitative Assay

Qualitatively, antibodies were detected by immunodiffusion using Ion Agar No. 2 (Colab Laboratories, Glenwood, Ill.), 0.85% in 5% NaCl at pH 8.1. Two ml of agar were layered on a microscope slide and a symmetrical five well pattern was cut. Each well had a diameter of 1.5 mm and the distance between the central and outside wells was 4.5 mm. Antigen (48.6 units/ml) was added to the center well. One unit of acid phosphatase is the amount necessary to liberate one μM of p-nitrophenol per minute at 37 C. Development was allowed to proceed overnight at room temperature. Quantitatively, antiphosphatase titers were determined on agar slides with a 14 well pattern in 3 rows. Antiserum was diluted with a commercial microtiter loop and plastic plates (Cooke Engineering Co., Alexandria, Va.). Antigen was placed in the middle row of four wells.

Concentration of sera was sometimes necessary to detect antibody and was accomplished using two different

procedures. Sera were first concentrated by addition of approximately 0.2 gm Lyphogel (Gelman Instrument Co., Ann Arbor, Mich.) to 1 ml serum. The mixture was allowed to stand at room temperature for five hours for maximal concentration. Sera negative for antiphosphatase following this procedure were treated with ammonium sulfate to precipitate gamma globulin. One-third saturation was obtained by dropwise addition of the proper volume of saturated ammonium sulfate. The mixture was adjusted to pH 7.8 with 0.5 N NaOH and continually stirred for 2 hr. The resulting precipitate was removed by centrifugation and resuspended in saline to the original volume. This procedure was repeated two additional times and the final precipitate was resuspended in borate buffered saline (pH 8.2) to one half its original volume.

Staining of the precipitin bands was carried out by adapting the Gomori histochemical stain for acid phosphatase as described by Shulman et al. (1964). This utilized the following reagents: a) Buffer: 0.05 M acetic acid-sodium acetate; pH 5.0. Lead nitrate was incorporated into the buffer at a concentration of 1.33 g/liter. b) Substrate: 3% sodium β glycerophosphate

prepared in water. This substrate was newly prepared prior to use. c) Rinse: 1% acetic acid. d) Stain: 2% yellow ammonium sulfide. Slides were incubated in the buffer-substrate mixture (10:1) at 37 C for 15 min, rinsed in distilled water, then 1% acetic acid, and then immersed in 2% ammonium sulfide for 1 to 2 min. Precipitin lines containing acid phosphatase stained a deep brown.

Colorimetric Antiphosphatase Assay

Antiphosphatase was also quantitated by a colorimetric procedure based on the acid phosphatase determination of Barnes and Morris (1957). Optimal proportion of antigen and antibody to be used in the colorimetric procedure was initially determined by a simplified quantitative precipitin test using agar gel diffusion (Nowotny, 1969). Serial dilutions of antigen were made using a microtiter plate and loop. A constant amount of antibody was added to each well and the mixture was incubated for 1 hr at 37 C. A microscope slide previously covered with 2 ml Ion Agar No. 2 (0.85% in 5% NaCl) was prepared by cutting a row of seven wells (1.5 mm diameter) in the center of the slide and a trough above and below the row

of wells. Following incubation, each well on the slide was filled with some of the mixture from the corresponding microtiter well. The top trough was filled with antiserum and the bottom well with antigen. The slides were read after overnight incubation at 37 C.

Prior to performing the colorimetric assay the sera were inactivated at 56 C for 30 min and diluted 1:10 if they were known to contain high levels of the antibody such as were found in rabbits immunized with large amounts of purified acid phosphatase. Equal amounts (0.2 ml) of serum and purified acid phosphatase (2 units) were mixed and incubated for 1 hr at 25 C. Following incubation the mixture was centrifuged to sediment the fine precipitate present. Four tubes were labeled: test, control, total acid phosphatase, and blank. The following additions were made: 0.1 ml supernatant from the reaction mixture to the tube labeled test; 0.1 ml serum diluted 1:1 with saline to the control tube; 0.1 ml acid phosphatase diluted 1:1 with saline to the total acid phosphatase tube; and 0.1 ml saline to the blank. Next 0.3 ml of 0.08% p-nitrophenyl-phosphate, disodium was added to each tube followed by 2.6 ml sodium acetate buffer (pH 5.2). The tubes were incubated at 37 C for 30 min after which the reaction was

stopped by addition of 1.0 ml 1 M NaOH. The O.D. of each solution was read at 400 nm against the blank set at zero O.D. in a Bausch and Lomb Spectronic 20 spectrophotometer (Bausch and Lomb, Rochester, N.Y.). The O.D. of the control, which indicated the amount of serum acid phosphatase, was subtracted from the O.D. of the test. The ratio of the O.D. of the corrected test to the O.D. of the total acid phosphatase gave the percentage of acid phosphatase that remained in solution. We preferred to take the difference between this value and 100% and expressed our answer as percentage of acid phosphatase bound.

Total White Blood Count and Differential

Blood was withdrawn from the central ear artery using a 22 gauge needle (Becton, Dickinson and Co., Columbus, Neb.) and 5 ml glass syringe. It was immediately transferred to a Vacutainer tube (Becton, Dickinson and Co., Columbus, Neb.) containing ethylenediaminetetraacetic acid (EDTA) anticoagulant. The whole blood was diluted 1:500 in Isoton (Coulter Electronics, Hialeah, Fla.) using a Dade automatic dilutor model 2D (Dade Reagents, Inc., Miami, Fla.). Duplicate diluted samples were each counted

twice on a Coulter Model B counter (Coulter Electronics, Hialeah, Fla.) and an average value was calculated. A thin smear of blood was made on a microscope slide and stained using Wright-Giemsa stain (Harleco, Philadelphia, Pa.). A differential count of 100 white blood cells (WBC) was made twice and an average taken. Normal values were determined by performing total and differential WBC counts on four rabbits on the four days prior to their inoculation.

Immunology

Experimental Animals

Adult male New Zealand White rabbits weighing approximately 10 kg were caged individually and supplied with a diet of Triumph Rabbit Pellets (John K. Van Den Bosch Co., Zeeland, Mich.).

Immunization Schedule

In a series of 10 injections given every other day, three rabbits were inoculated with purified

staphylococcal acid phosphatase, mixed 1:1 with Freund's complete adjuvant (Difco, Detroit, Mich.). The first five injections, using 25-gauge needles, were given in the foot pad and the last five subcutaneously in the cervical region. One rabbit received a total of 25 units phosphatase, the second 50 units, and the third 100 units. One unit of acid phosphatase is the amount necessary to liberate from p-nitrophenyl disodium salt, one μM of p-nitrophenol per minute at 37 C. The control rabbit was given only Freund's complete adjuvant.

Serum Collection and Preparation

After rubbing the ear with xylene, serum samples (about 0.5 to 1.0 ml) were collected using a 22 gauge needle and 5 ml glass syringe from the central ear artery. The blood was allowed to coagulate at room temperature and the serum was then separated by centrifugation. The serum was transferred to a tube by Pasteur pipette and stored at -20 C.

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Pilot Protection Studies
on Immunized Rabbits

Since rabbits are known to be relatively resistant to skin infection with Staphylococcus aureus, a preliminary experiment was conducted to determine the number of organisms necessary to establish infection in a normal rabbit; also the feasibility of using a foreign body irritant to enhance infection was explored. Two types of irritants were investigated. Following a method described by Elek (1956) sterile cotton sutures of uniform size were soaked in dilutions of Staphylococcus aureus (PS 55) prepared from an overnight Trypticase Soy Broth culture. Each dilution differed logarithmically from 10^8 organisms/ml to 10^3 organisms/ml. The concentrations were determined by plate counts done in triplicate (Manual of Microbiological Methods, 1957). The second method (Dajani and Wannamaker, 1970) involved injecting inocula containing talcum powder (Mallinkrodt Chemical Works, St. Louis, Mo.) at a concentration of 1 mg/ml. Again concentrations of organisms ranged from 10^8 organisms/ml to 10^3 organisms/ml.

The backs of two nonimmunized rabbits were shaved and depilated. Along one side of each rabbit sutures

treated as described above were inserted using one stitch which was secured with a double knot leaving some slack to accommodate swelling. A suture soaked in sterile saline was the control. Along the other side 0.1 ml subcutaneous injections of the organism and talc suspensions described above were given by means of a 25 gauge needle and tuberculin syringe. A suspension of sterile saline and talc was injected for the control.

Based on the results of this experiment the talcum powder irritant was chosen in the remaining protection studies. The plan was slightly modified by increasing the number of organisms injected. The dilutions ranged from 4.4×10^{10} organisms/ml to 4.4×10^{5} organisms/ml. In all other aspects the procedure described above was followed. The degree of erythema and necrosis, as well as the total and differential leukocyte counts were determined at 24, 48, 72, 96 hr and then at frequent intervals until healing occurred.

Antisera Absorption

The following organisms were used in the absorption experiment: Klebsiella pneumoniae; Enterobacter

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aerogenes; Escherichia coli; Streptococcus faecalis; Proteus vulgaris; Serratia marcescens; Staphylococcus aureus (PS 55). In addition purchased alkaline phosphatase (hog and calf intestinal mucosa) and acid phosphatase (potato and wheat germ) were used (Sigma Chemical Co., St. Louis, Mo.).

The organisms were inoculated into a tube of Trypticase Soy Broth and grown overnight at 37 C. They were separated from the culture medium by centrifugation and resuspended in 0.05 M Tris-chloride buffer (pH 8.2) at a concentration of 0.5 g (wet wt) per ml. To release the firmly and loosely bound acid phosphatase each cell suspension was subjected to sonic oscillation (sonic treatment for 15 sec was alternated with cooling for 30 sec) in a MSE 100 watt ultrasonic disintegrator (Measuring and Scientific Equipment Ltd., London, England). Rod-shaped organisms were sonicated for 2 min while coccal organisms were sonicated 4 min. Equal parts (0.2 ml) of the cell lysate and serum containing antiphosphatase and equal parts of the purchased enzyme (30 units/ml) and immune serum were mixed and incubated for 1 hr at 25 C and overnight at 4 C. A negative control consisted of untreated sera incubated in the same manner; for a

positive control, purified staphylococcal acid phosphatase (30 units/ml) was added to the serum. Following incubation the suspensions were centrifuged and the anti-phosphatase in the supernatant fluid titered by the immunodiffusion method previously described. In addition the suspensions were tested for acid phosphatase using the quantitative procedure of Barnes and Morris (1957).

Results indicated that at least 30 units/ml of acid phosphatase were necessary before absorption of antibody took place. Therefore the procedure was repeated using 24 hr shake flask (100 ml) cultures of Staphylococcus aureus (PS 55) and Klebsiella pneumoniae. The cells were centrifuged and treated as described above.

Mercaptoethanol Treatment of Antisera

Serum with a high titer of staphylococcal anti-phosphatase was treated with mercaptoethanol according to Yoshida and Ekstedt (1968). Serum (1 ml) was mixed with an equal volume of 0.2 M 2-mercaptoethanol (Eastman Organic Chemicals, Rochester, N.Y.) and allowed to react at 4 C for 18 hr. The mixture was then dialyzed against 0.02 M iodoacetic acid (Matheson, Coleman and Bell,

Norwood, Ohio.) in 0.02 M phosphate-buffered saline for 2 hr and in fresh phosphate-buffered saline (0.02 M) for 24 hr at 4 C. The control serum was treated in the same manner, except phosphate-buffered saline (0.02 M) was used instead of mercaptoethanol.

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RESULTS

Preparation of Staphylococcal Acid Phosphatase

Cultural Modifications

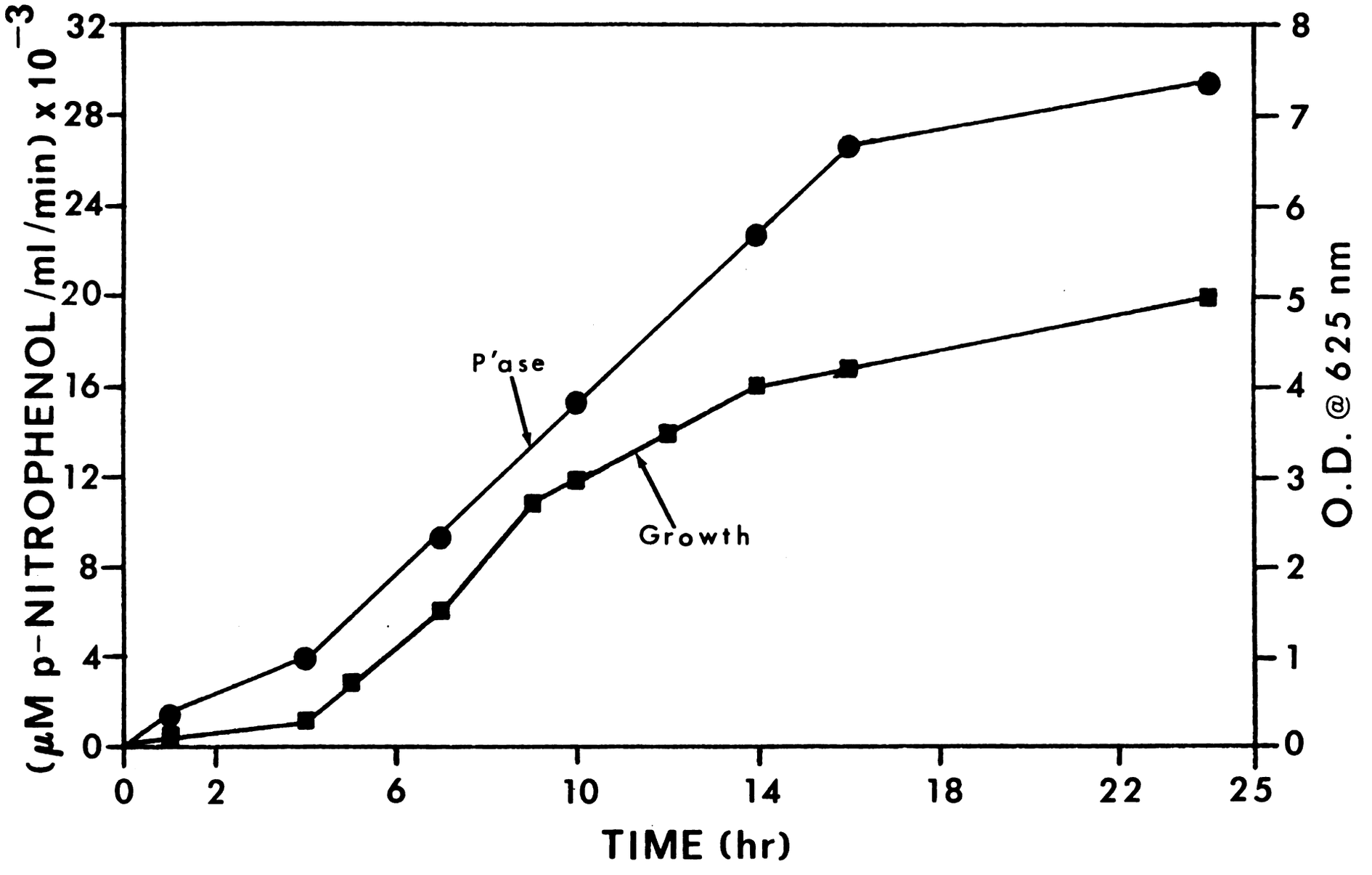
To determine the medium for maximal acid phosphatase production Staphylococcus aureus (PS 55) was cultivated in Trypticase Soy Broth, casein hydrolysate, a modified casein hydrolysate with succinate substituted for glucose, CCY, and a biphasic growth medium. In all five media growth increased most rapidly between 8 and 20 hr and slowly thereafter. The peak acid phosphatase concentration was reached in approximately 22 hr and subsequently decreased sharply, presumably due to denaturation. The original casein hydrolysate medium and the succinate substituted medium were eliminated because both growth and acid phosphatase production were far below that observed in the other media. The biphasic growth medium was also excluded because it was confined to the use of relatively small volumes of media. The experiment was repeated using both Trypticase Soy Broth and CCY medium

in which the pH and foaming were controlled to reduce enzyme denaturation. The rate of acid phosphatase production in whole culture paralleled cell density in Trypticase Soy Broth shake culture (Fig. 1). In the CCY medium the rate of whole culture acid phosphatase production also increased with cell number (Fig. 2), but the amount of growth was about one half of that obtained in Trypticase Soy Broth and consequently the total amount of acid phosphatase elaborated was much less. In both cases the control of pH and foaming seemed to inhibit the enzyme denaturation that otherwise occurred by 22 hr (Fig. 3). Also, slightly higher levels of acid phosphatase were reached prior to 22 hr in the controlled media. Based on these results, Trypticase Soy Broth (pH 7.5, antifoam concentration 0.05 ml/liter) was chosen for acid phosphatase production.

Acid Phosphatase Purification

Loosely bound acid phosphatase was extracted and purified (Malveaux and San Clemente, 1969) from *Staphylococcus aureus* cells grown in 20 liters of culture. The final product contained 0.63 mg of protein/ml and an acid

Fig. 1.--Rate of whole culture acid phosphatase production and cell growth (O.D. at 625 nm) measured in shake cultures (Tryp-ticase Soy Broth) of S. aureus (PS 55) at 37 C, pH 7.5, Dow B antifoam 0.05 ml/liter.


(μM p-NITROPHENOL/ml/min) x 10⁻³
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TIME (hr)

Fig. 2.--Rate of whole culture acid phosphatase production and cell growth (O.D. at 625 nm) measured in shake cultures (CCY medium) of S. aureus PS 55 at 37 C, pH 7.5, Dow B antifoam 0.05 ml/liter.

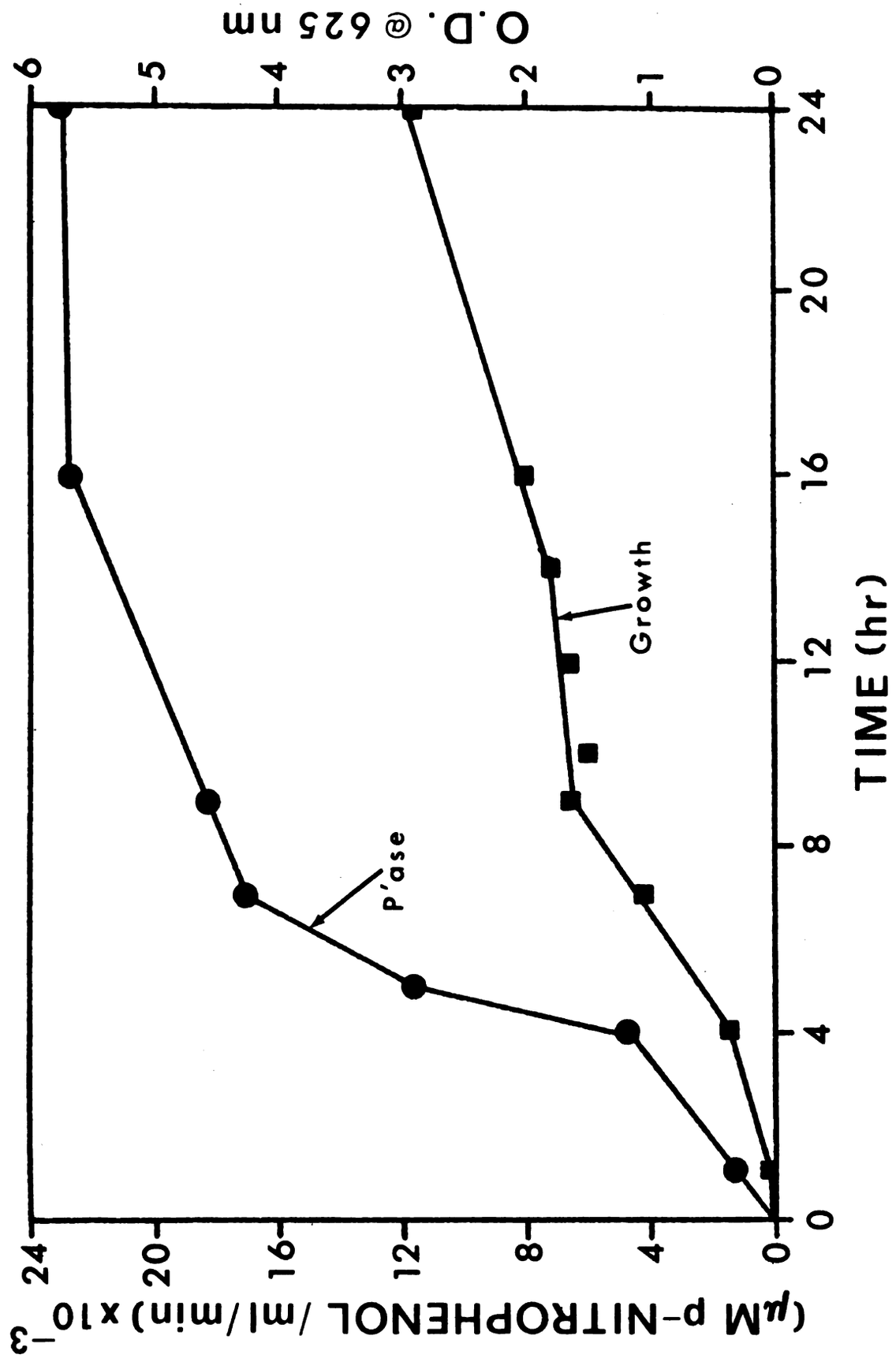
(μM p-NITROPHENOL /ml/min) x 10⁻³
O.D. @ 625 nm
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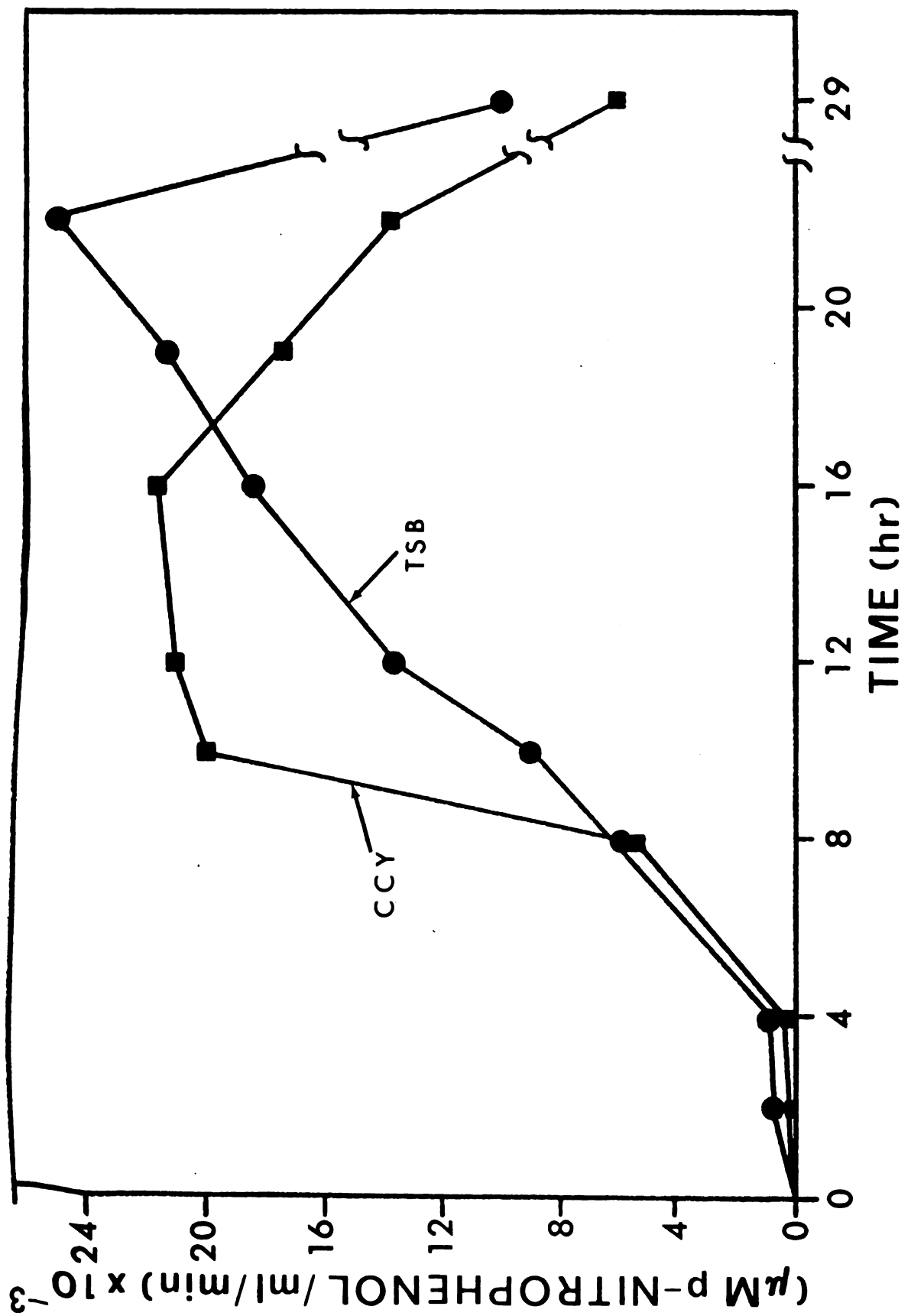
Fig. 3.--Rate of whole culture (S. aureus PS 55) acid phosphatase production measured in shake cultures of Trypticase Soy Broth and CCY medium at 37 C, pH 7.5, and without anti-foam.


(μM p-NITROPHENOL /ml/min) x 10⁻³
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TIME (hr)
TSB
CCY

phosphatase activity of 48.6 units. One unit of acid phosphatase is the amount necessary to liberate 1 μM of p-nitrophenol/min at 37 C. The 280/260 ratio of the product was 1.4 or approximately 0.75% nucleic acid (Manual of Microbiological Methods, 1957). Tests for coagulase, fibrinolysin and deoxyribonuclease were negative; however, a qualitative test indicated that a small amount of lipase was present.

Colorimetric Antiphosphatase Assay

Quantitative precipitin tests were done to determine the optimal antigen-antibody ratio for the test. Precipitin lines between the center wells and the upper trough indicated that an antigen excess was present in the antigen-antibody mixture. Precipitin lines between the center wells and the lower trough indicated antibody excess. At the equivalence point there were no precipitin lines. The results of these tests indicated that 2 units of antigen provided a margin of slight excess when incubated with the antiphosphatase levels present in most sera. However, when high antibody levels were present, as in the

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rabbits immunized with large amounts of pure acid phosphatase, it was necessary to dilute the sera 1:10.

Table 1 lists the amount of antiphosphatase in rabbit sera determined by the colorimetric test compared with the immunodiffusion titer. Sera negative for antiphosphatase by the immunodiffusion method were also negative using the colorimetric procedure as shown in Table 2. Very low levels of the antibody, detectable in horse serum by immunodiffusion, were not detected by the less sensitive colorimetric procedure.

Immunodiffusion Antiphosphatase Assay

Parameters of the Assay

Initially, immunodiffusion of immunized rabbit serum versus purified acid phosphatase was attempted using 0.85% Ion Agar No. 2 in 0.85% saline (pH 8.1). No precipitin lines formed, but a broad, diffuse ring around the antigen well indicated impeded mobility of the antigen. When the salt (NaCl) concentration was increased, the enhanced solubility of the antigen produced sharp precipitin lines. No further enhancement of the pattern was

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TABLE 1.--Assay of staphylococcal antiphosphatase in sera of immunized and control rabbits using immunodiffusion and colorimetric methods.

Animals	Immunodiffusion titer	Colorimetric test- % of acid phosphatase bound
Rabbit 1[a]	1:4	49.6
Rabbit 2[b]	1:4	49.0
Rabbit 3[c]	1:8	74.5
Control Rabbit[d]	0	0

[a]Received 25 units staphylococcal acid phosphatase 90 days prior to these determinations.

[b]Received 50 units staphylococcal acid phosphatase 90 days prior to these determinations.

[c]Received 100 units staphylococcal acid phosphatase 90 days prior to these determinations.

[d]Did not receive any staphylococcal acid phosphatase.

TABLE 2.--Staphylococcal antiphosphatase titer in concentrated and unconcentrated sera of normal and infected subjects using immunodiffusion and colorimetric methods.

Source of sera	Number of samples	Immunodiffusion unconc.	Immunodiffusion conc.	Colorimetric
Human	29 normal	-	-	
	infected: Patient D[a]	-	+	
	Patient S[b]	-	-	-
	Patient F[b]	-	-	-
	Patient 1[c]	+	+	
	Patient 2[c]	-	+	
	Patient 3[c]	-	-	
	Patient 4[c]	-	-	
Cow	5 normal	-	-	
	1 infected[d]	-	-	
Horse	1 infected[e]	-	+	-
	post vaccine[f]	+	+	-
Dog	1 infected	-	-	

[a]Chronic staphylococcal skin infection; received autogenous vaccine.

[b]Acute staphylococcal skin infection.

[c]Staphylococcal endocarditis.

[d]Streptococcal mastitis.

[e]Chronic cellulitis from which a Staphylococcus aureus and a Streptococcus were cultured.

[f]Sera of same horse following administration of an autogenous vaccine containing both organisms.

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observed when the saline concentration exceeded 5%. Substitution of the sodium salt with 1.0 M KCl/0.5 M Tris-chloride did not increase the sensitivity of the test. Precipitin bands generally occurred after 4 to 6 hr incubation at 25 C. Incubation at 37 C did not increase the rate of diffusion or appear to have any effect on the pattern. The concentration of agar (0.85%) or the pH (8.1) were not critical but were within the parameters generally suggested for immunodiffusion.

Staining Immunodiffusion Assays

Application of a staining procedure by Shulman et al. (1954) specifically stained phosphatase-antiphosphatase precipitin bands dark brown. The phosphate was split from the β-glycerophosphate substrate by acid phosphatase at pH 5.0. Lead in the substrate solution was precipitated as lead phosphate and was visualized as brown lead sulfide when incubated with ammonium sulfide. Due to this specificity, bands formed by other antigen-antibody complexes remained unstained. When β-glycerophosphate was absent, no staining occurred. Although enzymes are usually completely inhibited by their

antibodies, the action of acid phosphatase during the staining procedure indicated that it was active despite coprecipitation with its antibody. This had previously been observed only in systems where the substrate had a low molecular weight (Cinander, 1963). However, when p-nitrophenolphosphate (M.W. 263) or phenolphthalein diphosphate (M.W. 562) were substituted for β-glycerophosphate (M.W. 216) staining was not inhibited.

Figure 4 shows a stained antiphosphatase assay on two rabbit sera; one in the top row of wells, the second in the bottom row. Both sera formed precipitin lines as far as the third well which was a 1:8 dilution.

Antiphosphatase Titers

Immunization of Rabbits

Three rabbits were immunized over a 20-day period with increasing amounts of acid phosphatase. Their sera were tested for antibody at frequent intervals (Table 3). On Day 12, following the first inoculation, antibodies were detected in Rabbits two and three by immunodiffusion.

Fig. 4.--Stained precipitin lines obtained during the titration of antiphosphatase by immunodiffusion of two different rabbit serum samples. Each has a titer of 1:8.

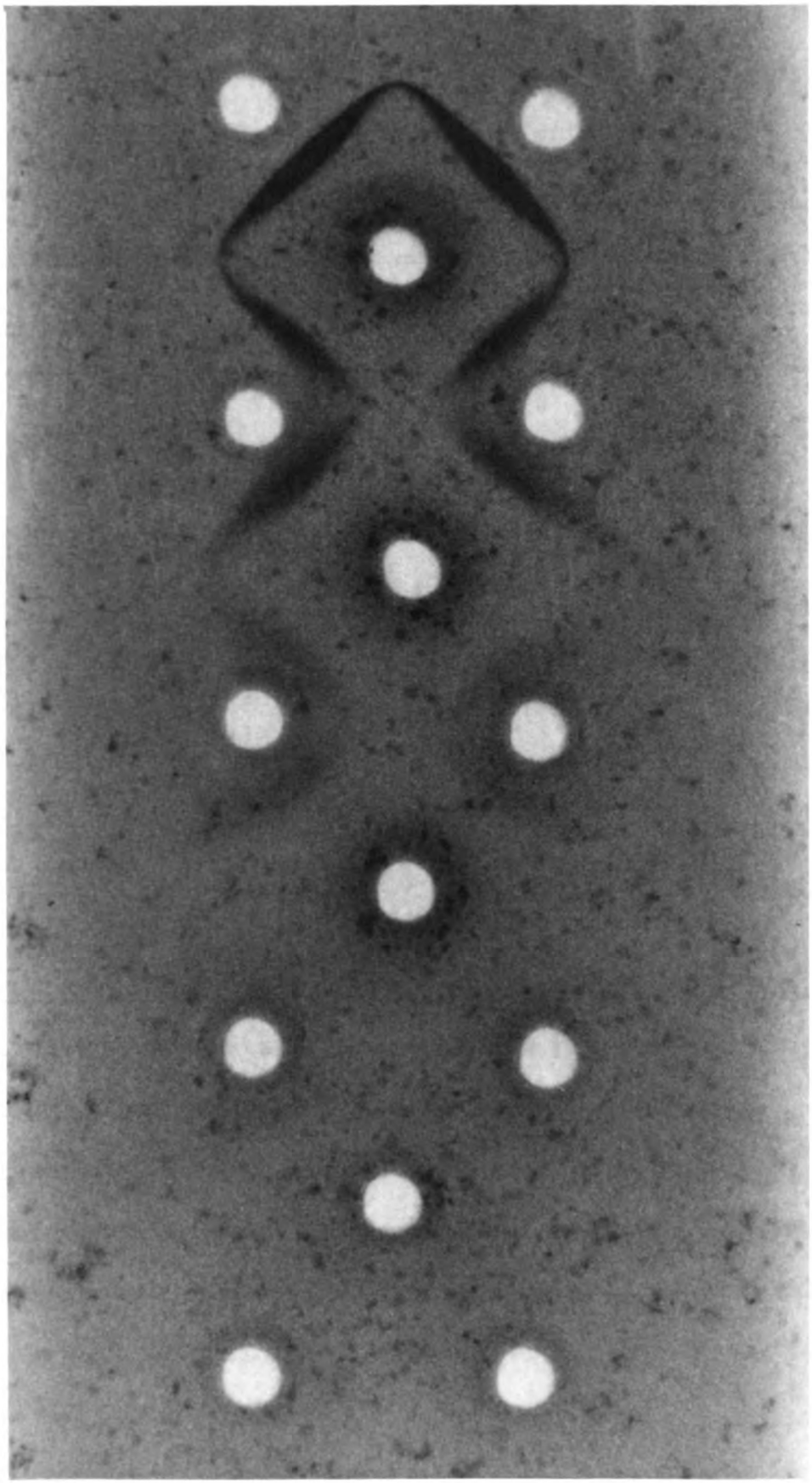

TABLE 3.--Course of staphylococcal antiphosphatase titers in rabbits immunized with purified staphylococcal acid phosphatase.

Days postimmunization	Rabbit 1[a]	Rabbit 2[b]	Rabbit 3[c]
0	0	0	0
12	0	+	+
20[d]	1:2	1:8	1:4
26	1:2	1:8	1:8
33	1:8	1:16	1:8
41	1:16	1:16	1:16
55	1:16	1:16	1:16
65[e]	1:16	1:16	1:16
70	1:16	1:16	1:16
90	1:4	1:4	1:8

[a]Received 25 units staphylococcal acid phosphatase.

[b]Received 50 units staphylococcal acid phosphatase.

[c]Received 100 units staphylococcal acid phosphatase.

[d]Date of tenth and final injection of acid phosphatase.

[e]Staphylococcal skin infection established in all rabbits.

Approximately one week later antibodies were detected in Rabbit one and titers in the other two rabbits had further increased. By Day 41 all rabbits had reached a maximal titer of 1:16 which remained unchanged despite the establishment of a staphylococcal (Staphylococcus aureus PS 55) skin infection on Day 65. Titers determined on Day 90 showed a significant decrease.

Several of these serum samples were tested by the colorimetric technique and Table 2 shows these values compared with immunodiffusion titers on the same sera.

Antiphosphatase Titers in Normal and Infected Subjects

Human, cow, horse, and dog sera from normal and infected subjects were tested for antiphosphatase (Table 2). Unconcentrated and concentrated human sera from 29 normal individuals were tested for antiphosphatase by immunodiffusion and found to be negative. Several patients with a history of staphylococcal infection were also tested. Human patient D had a history of chronic staphylococcal skin infections that did not respond to antibiotic treatment. He had been injected with one series of an autogeneous vaccine that produced little, if any, relief.

Antibodies were detected in his serum only after concentration with Lyphogel or in the gamma globulin fraction after ammonium sulfate precipitation. Human patients S and F had acute staphylococcal skin infections, and antibodies could not be detected in their serum using either immunodiffusion or colorimetric procedures. Human patients 1, 2, 3, and 4 had staphylococcal endocarditis. Antibody was found in one patient's unconcentrated serum and in a second following concentration. In three of the sera an antibody other than antiphosphatase was detected but could not be identified.

Sera from five normal cows and one with streptococcal mastitis were tested and found to be negative for antiphosphatase.

A horse with a chronic staphylococcal-streptococcal cellulitis that involved a large part of one leg was found to have antiphosphatase detectable only by immunodiffusion using concentrated serum. Following administration of an autogeneous vaccine containing both organisms the antibody was detected in unconcentrated serum.

Serum from a dog with a staphylococcal skin infection of short duration was negative for antiphosphatase.

Cross Reaction of Staphylococcal Antiphosphatase with Nonhomologous Phosphatase

Table 4 shows the results of absorption of antiphosphatase serum with lysates of organisms containing various amounts of acid phosphatase and with four commercially prepared enzymes. Results of the positive control and the Staphylococcus aureus lysate indicated that a minimum of 30 units of acid phosphatase were necessary for absorption to occur. It was possible to obtain this concentration with only one other organism, Klebsiella pneumoniae, and with the four purchased phosphatases. There was no absorption of antibody by any organism or enzyme other than the homologous one.

Antiphosphatase Immunoglobulin

To determine which immunoglobulin fraction contained antiphosphatase, serum containing antibody was treated with 2-mercaptoethanol. If IgM were responsible for antiphosphatase activity, incubation of the antiserum with mercaptoethanol would result in dissociation of the

TABLE 4.--Absorption of rabbit staphylococcal antiphosphatase serum.

Source	Acid phosphatase (units)	Titer of absorbed sera
Staphylococcus aureus (PS 55)	17.6	1:16
	37.5[b]	1:8
Klebsiella pneumoniae	15.6[a]	1:16
	43.8[b]	1:16
Escherichia coli	0.7[a]	1:16
Enterobacter aerogenes	0.3[a]	1:16
Serratia marcescens	1.4[a]	1:16
Proteus vulgaris	0.04[a]	1:16
Streptococcus faecalis	0.2[a]	1:16
Acid phosphatase (potato)	30.0[b]	1:16
Acid phosphatase (Wheat germ)	30.0[b]	1:16
Alkaline phosphatase (hog mucosa)	30.0[b]	1:16
Alkaline phosphatase (calf intestinal mucosa)	30.0[b]	1:16
Positive control (purified staphylococcal acid phosphatase)	30.0	1:4
Negative control (unabsorbed serum)	-	1:16

[a]All of these sources were tested prior to realizing that 30 or more units of acid phosphatase were required to obtain optimal proportion of antigen and antibody.

[b]The fact that 30 or more units of nonhomologous acid phosphatase did not absorb antibody indicates the specificity of the phosphatase-antiphosphatase system.

pentameric IgM fraction into ineffective subunits accompanied by a decrease in antibody titer. The antiphosphatase titer of serum treated with mercaptoethanol and an untreated control serum were determined by immunodiffusion (Table 5). Both had a titer of 1:8 indicating that the mercaptoethanol had no effect on the antibody.

TABLE 5.--Effect of 2-mercaptoethanol on the titer of rabbit antiphosphatase.

Serum samples	Antiphosphatase titer	
	Mercaptoethanol	No Mercaptoethanol
Normal[a]	none	none
Antiphosphatase	1:8	1:8

[a]No antiphosphatase activity.

Pilot Protection Studies on Rabbits Immunized with Acid Phosphatase

Staphylococcal skin infections were established, by subcutaneous injection of organisms and talc, in three rabbits during the period of maximal antiphosphatase

titer (1:16) and in one nonimmunized control rabbit. Total and differential WBC count and lesion severity were determined at 24, 48, 72, 96 hr and at frequent intervals until healing occurred. Table 6 shows normal WBC values for all the rabbits and the WBC values post inoculation. Table 7 lists the differential for the granulocytic and lymphocytic series so that relative shifts may be observed.

At 24 hr post inoculation, lesions formed at the sites of the three most concentrated injections (4.4×10^9, 4.4×10^8 and 4.4×10^7 organisms/0.1 ml) were red and slightly swollen in the immunized rabbits. In the control rabbit these lesions were also red and swollen and in addition contained some pus. There was no reaction at any control site where suspensions of sterile saline and talc had been injected. After 48 hr the lesions had reached their maximal size and there occurred no further increase in intensity. The lesion produced by the highest number of organisms (4.4×10^9 organisms/0.1 ml) was approximately 2 cm in diameter, the second lesion was 1.0-1.5 cm in diameter and the third 0.6-1.0 cm in diameter. By 96 hr the lesions of the immunized rabbits appeared to have diminished in intensity while those of the control

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TABLE 6.--Normal WBC of experimental rabbits prior to immunization with different amounts of acid phosphatase and counts taken approximately 2 months later following establishment, by subcutaneous inoculation, of a staphylococcal skin infection.

Animal	Average normal WBC	Average post inoculation WBC				
		24 hr	48 hr	72 hr	96 hr	288 hr
Rabbit 1[a]	15,500	16,500	17,800	13,000	14,000	13,100
Rabbit 2[b]	11,450	13,900	18,000	17,500	14,600	15,200
Rabbit 3[c]	10,500	11,750	13,500	13,400	11,400	11,800
Control[d]	12,650	9,450	9,750	13,000	12,400	10,900

[a]Received 25 units staphylococcal acid phosphatase 65 days prior to inoculation.

[b]Received 50 units staphylococcal acid phosphatase 65 days prior to inoculation.

[c]Received 100 units staphylococcal acid phosphatase 65 days prior to inoculation.

[d]Did not receive any staphylococcal acid phosphatase.

TABLE 7.--Normal differential counts of experimental rabbits prior to immunization with different amounts of acid phosphatase and counts taken approximately 2 months later following establishment, by subcutaneous inoculation, of a staphylococcal skin infection.

Animal	White cell type	Differential Count					
		Normal	Hours post inoculation				
			24 hr	48 hr	72 hr	96 hr	288 hr
Rabbit 1	neut.[a]	24	85	33	29	40	34
	stabs[b]	0	0	0	0	0	0
	lymph.[c]	73	15	66	67	57	60
Rabbit 2	neut.	38	84	52	55	61	55
	stabs	0	4	1	6	0	0
	lymph.	55	10	44	35	27	42
Rabbit 3	neut.	37	77	39	40	52	37
	stabs	0	1	0	0	0	0
	lymph.	59	22	57	58	46	58
Control	neut.	30	65	13	22	32	35
	stabs	0	1	0	0	0	0
	lymph.	75	33	86	75	67	63

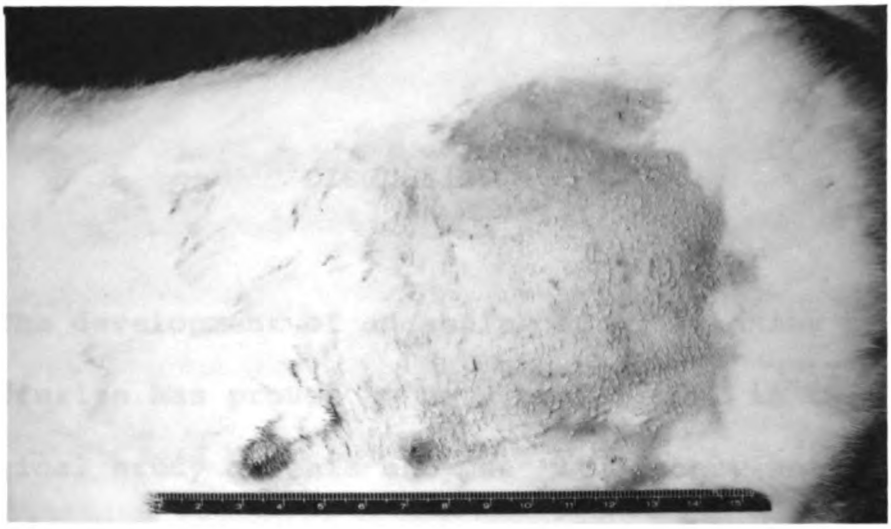
[a] neut. = neutrophils

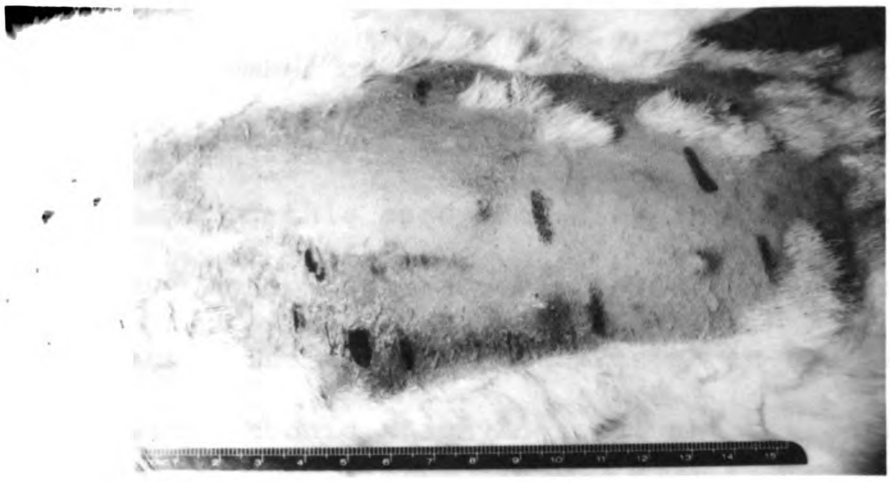
[b] stabs = immature neutrophils

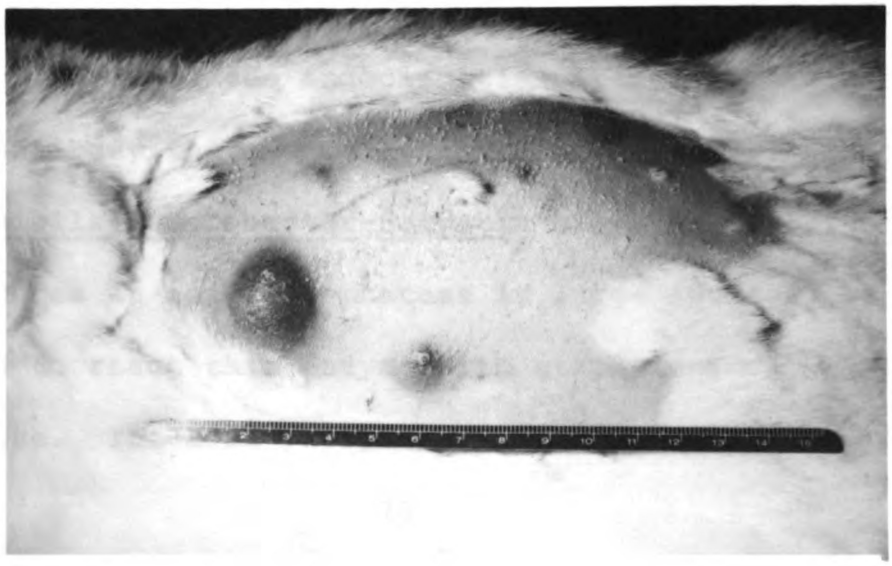
[c] lymph. = lymphocytes

rabbit were redder and contained a large amount of pus. At approximately 1 week post inoculation the immunized rabbit lesions, which had appeared to be healing, became extremely red, raised and fluctuating (Fig. 5). Within the next 5 days many of the lesions developed pus and became necrotic. At first necrosis occurred in lesions produced by the highest number of organisms (4.4×10^9 organisms/0.1 ml) and eventually it occurred in the other two lesions. Following suppuration healing became evident and was complete by 1 month post inoculation. The control rabbit's lesions which had become necrotic much earlier (96 hr), also healed sooner than the immunized rabbits (approximately 17 days post inoculation).

Fig. 5.--Lesions in rabbit 1, rabbit 2 and rabbit 3 occurring at 1 week post inoculation with viable Staphylococcus aureus.







DISCUSSION

The development of an antiphosphatase assay by immunodiffusion has proved to be a useful tool in the immunological study of this enzyme. It is easy and rapid to perform and is more sensitive than the colorimetric test. Concentrated sera are easily tested with this method. Since the number of serum samples available for testing was small, our data are only an indication of the possible usefulness of this assay for diagnosis of staphylococcal disease. Antiphosphatase was not detectable in normal human serum. The antibody was present in the sera of individuals with chronic staphylococcal skin infection and with infections of the deep tissues such as staphylococcal cellulitis and endocarditis. In addition serum samples of three of the endocarditis patients contained another antibody that we were unable to identify. Since the Klebsiella-Enterobacter-Serratia group of organisms also produces an acid phosphatase in large amounts, we attempted to react this enzyme with staphylococcal antiphosphatase. The acid phosphatase of these organisms did

not cross react with antibody to staphylococcal acid phosphatase; nor was there any cross reaction between this antibody and acid phosphatase preparations of plant origin. Evidently staphylococcal antiphosphatase was a specific indicator of a chronic or severe staphylococcal infection.

There are several implications that might be drawn from the staining reaction used in the immunodiffusion test. Since the enzyme had to react with the substrate to produce the staining effect, it was evident that specific aggregation with its antibody does not inhibit reactivity. It is theorized that the enzyme acted on the substrate through catalytic sites not blocked by the antibody. This usually occurs with substrates having a molecular weight less than 200 (Cinander, 1963). The staphylococcal antiphosphatase system did not comply with this observation since the substrate, β-glycerophosphate, had a molecular weight of 216. Substitution with substrates of higher molecular weight (p-nitrophenylphosphate, M.W. 263; and phenolphthalein diphosphate, M.W. 562) did not inhibit staining. Therefore one can conclude that factors other than the molecular weight of the substrate were involved. Cinander (1959) indicated the complexity of this problem by citing other variables in the inhibition of enzyme by

its antibody. The degree of inhibition appears to depend on the quality of the antibody as determined by the conditions of immunization, the relative quantity of antibody, the period of incubation and in some instances, on the concentration of substrates. Sevag, Newcomb and Miller (1954) reported that dissociation occurred in mixtures of α-glycerophosphate and yeast antiphosphatase in both antigen and antibody excess. There is some evidence for similar dissociation of staphylococcal phosphatase and its antibody. In the colorimetric antiphosphatase determination antigen was still detected in the supernate when antibody was in excess. It was not within the scope of this project to determine which of these factors was responsible for continued reactivity of the precipitated phosphatase; however, this reactivity was notable because of the effect it might have on the protective role of this antibody in vivo.

Some investigators (Rogers and Melly, 1965) have concluded that, due to repeated subclinical infections with staphylococci, most adults had developed a maximal titer of antibodies which could not be further increased. Our finding, that rabbits immunized with different doses of staphylococcal acid phosphatase had all achieved the

same titer which was not enhanced by subsequent imposition of a staphylococcal skin infection, seemed to support this thesis. However, while it appears that a peak titer had been reached, it should be noted that it was much higher than was found in individuals with chronic staphylococcal disease. These same authors claimed that patients with active staphylococcal infection did not demonstrate higher titers of antistaphylococcal antibodies than did normal adults. We found the opposite to be true; i.e., anti-phosphatase antibodies were not detectable unless chronic or severe staphylococcal disease was present. Therefore from our experience with this particular antibody we concluded that increased immunization was worth pursuing.

Ekstedt and Yoshida (1968) observed that the antibody in hyperimmune rabbit antisera specific for Staphylococcus aureus teichoic acid was associated with the IgM fraction. The titer persisted for only a short time (2 to 3 weeks) leading them to suspect that there may be a lack of immunological memory in animals undergoing IgM response exclusively. This nonrecognition was suggested as a reason for repeated reinfection of man by staphylococci. For this reason we considered determination of the immunoglobulin class of antiphosphatase important to

our study. Incubation of the rabbit antiphosphatase serum with mercaptoethanol did not reduce the titer of the serum indicating that the antibody was in the IgG fraction.

The high phosphatase activity of pathogenic staphylococci as compared to saprophytic nonpathogenic strains (Barber, Brooksbank and Kuper, 1951) prompted us to examine the protective capacity of phosphatase immunized rabbits to subcutaneous injection of Staphylococcus aureus (PS 55). It has been suggested (Kedzia et al., 1966) that phosphatase was involved in regulating the concentration of the inorganic phosphate pool of the organism. Presumably neutralization of acid phosphatase activity would therefore seriously impair metabolic functioning of the staphylococcal organisms. Following challenge of acid phosphatase immunized animals with subcutaneous inoculation of Staphylococcus aureus (PS 55), total and differential WBC counts were determined in anticipation of obtaining quantitative evidence of protection in the immunized animals. Dajani and Wannamaker (1972) reported hamster total and differential WBC count values observed during experimental staphylococcal skin infection. Of 12 infected animals the WBC counts were normal in all but one that exhibited leukocytosis, and neutrophilia was marked

in all instances. In our experiment a moderate increase in the WBC within 48 hr was observed in the immunized rabbits, but no response in the control rabbit. A neutrophilia and lymphopenia was observed in the differential of all four rabbits at 24 hr which returned to normal by 48 hr. The rise in the WBC seen in the immunized rabbits might be interpreted as a superior ability to respond to infection; however, for the most part, the results appeared inconclusive. The degree of inflammation and necrosis was also used as a criterion. At the onset lesions in the control rabbit were more severe than in the immunized rabbit. There was pus formation by 24 hr in the control rabbit and necrosis by 96 hr. By comparison the immunized rabbits' lesions were red and swollen with no evidence of pus formation or necrosis. One might conclude at this point that the organisms were able to multiply rapidly and produce enough toxins to cause necrosis in the control rabbit, while in the immunized rabbits multiplication of the organisms was inhibited. This course changed abruptly at 1 week post inoculation when the lesions in the immunized rabbits became intensely red, raised and fluctuating. Eventually these lesions became necrotic and healed two weeks later than the control rabbit lesions.

There are several possible explanations for this phenomenon. Antiphosphatase might have succeeded in inhibiting multiplication of organisms early in infection but this defense was eventually overcome. Similar evidence was noted in protective studies following immunization with coagulase (Harrison, 1964). In this study, the number of survivors in the immunized group was significant at 4 days but not at 21 days. The author suggested that anticoagulase interfered with the pathological processes that lead to death shortly after injection of large numbers of staphylococci, but that it failed to prevent the chronic type of disease that led to death later on. If this were the case, a combination of selected purified staphylococcal products, including acid phosphatase, might elicit a more protective antibody combination. If this sudden lesion intensification was due to the ability of the organism to finally reach a high titer, one would expect a concomitant rise in the WBC. Another explanation for this reaction might be that it was the result of delayed hypersensitivity. Johnson, Cluff and Goshi (1961) found that repeated staphylococcal infection of rabbit skin was associated with the development of delayed hypersensitivity unaccompanied by the appearance of demonstrable

serum antibody. Furthermore, they noted that the delayed hypersensitivity resulted in an increased infectivity of the organism in the skin of the sensitized animal, characterized by intensification of the lesions. It would be interesting to test this hypothesis by attempting to transfer this hypersensitivity by means of lymphoid cells. If this reaction were proved to be a result of delayed hypersensitivity, perhaps the protective effect of anti-phosphatase could be better assessed using lethality studies in small animals such as mice.

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