IMMUNE AND ANTIBODY RESPONSE IN RABBITS AND DAIRY CATTLE AGAINST STAPHYLOCOCCAL VACCINES

BRAHMA SINGH MALIK

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IMMUNE AND ANTIBODY RESPONSE IN 
RABBITS AND DAIRY CATTLE AGAINST 
STAPHYLOCOCCAL VACCINES

BY
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This dissertation has been examined and approved.

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May 10, 1964 Date
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INTRODUCTION

Bovine mastitis continues to rank as one of the most costly diseases of dairy cattle in many parts of the world. It is estimated that it causes an annual loss of about 250 million dollars in the United States and about 5 to 6 million pounds in Great Britain. It is reported from various parts of the world that the rate of bovine mastitis due to staphylococci is on the increase. The incidence in some of the badly neglected herds in the United States is as high as 60%. On the basis of a ten-year survey the incidence reported in Great Britain was 13.5%. During the same period, from more than 1000 abnormal udder secretions, 41.8% isolates were staphylococci.

No effective methods have as yet been developed for the control of staphylococcal bovine mastitis. A number of research workers have attempted to establish the value of staphylococcal toxoids, bacterins, or bacterin-toxoids for increasing the resistance of animals or man to Staphylococcus aureus infections. It is recognized that antitoxin and antibacterial immunity in S. aureus infections are different. Vaccination with toxoids stimulates antitoxin response which protects tissue damages due to bacterial toxins but may not prevent or eliminate the infection. On the other hand, bacterial vaccines produce antibodies only against cells and will not prevent tissue damages due to various toxins.
elaborated by the cells during the process of infection. A combination of both may be the best compromise. Some workers prefer the use of lysed cell vaccines rather than the ones having whole cells. However, it has recently been pointed out that for successful phagocytosis of certain strains, the presence of antibodies against bacterial cell surface antigens are obligatory; antibodies against lysed cell antigen in such cases are not enough. It has also been demonstrated that certain strains of \textit{S. aureus} may contain a variety of surface and protoplasmonic antigens. Some may be strain specific and others are shared by different strains. Further studies are needed to determine the value of polyvalent vaccines for immunizing dairy cattle against \textit{S. aureus} mastitis and to determine what antigenic substances produced by this organism are of particular value in stimulating an immune response to this disease.

In this study, an effort was made to determine the influence of certain factors on the production of various diffusible products by strains of \textit{S. aureus} isolated from bovine mastitis. Various chemical and physical means of inactivation of the bacterial cells and the toxins for vaccine preparations were studied. A comparison of the immunizing properties of the whole and disrupted cell vaccines was made. The effects of vaccines with and without adjuvants were investigated in relation to antibody production. An attempt was also made to correlate various types of antibodies with resistance to \textit{S. aureus} infection.
A. FACTORS AFFECTING THE PRODUCTION OF ALPHA 
AND BETA TOXINS AND COAGULASE BY S. AUREUS 

1. Temperature. Very little work has been done on 
the optimum temperature requirements for the production of 
various types of S. aureus toxins. Chapman (1947) claimed 
that certain coagulase positive strains of S. aureus have 
an optimum growth temperature of 30 C. Davies (1951) exam-
ined S. aureus strains for coagulase production at dif-f 
erent temperatures. He found some production between 
the temperatures of 20 C and 45 C, but the maximum yield 
was obtained at 35 C. Recently, Bungay (1961) examined 
three strains for the production of staphylokinase, hyal-
uronidase, the Panton-Valentine leucocidin (P.V. leucocidin), 
and alpha hemolysin at the temperatures of 35 C to 43 C. 
Staphylokinase yield was maximum at 35 C and lowest at 43 C. 
There was no difference in the amount of cell growth at 
these temperatures. Hyaluronidase, leucocidin, and alpha 
hemolysin production were tested in cultures grown at 37 C 
and 41 C temperatures. Although there was no difference in 
the yield of leucocidin and alpha hemolysin, the production 
of hyaluronidase was 5 to 6 times higher in the cultures 
grown at 37 C than at 41 C. 

2. Aeration and carbon dioxide. Various means 
have been employed to increase the yield of S. aureus toxins 
in liquid media. Walbum (1922) pointed out that a slightly
acid reaction of the medium was optimal for toxin production. Parker et al (1925-26) increased toxin production by growing the cultures in an atmosphere of 10% CO$_2$. The increased yield was attributed to the buffering action of this gas. Burnet (1930) added small amounts of agar in the liquid medium to achieve the same purpose. Later when devices for shaking the cultures mechanically were available for increased aeration, the use of agar was discontinued. Casman (1938, 1940) was able to increase the titer by bubbling a mixture of 20% CO$_2$ and 80% O$_2$ into the medium. The author compared different methods of mechanical agitation; shallow liquid cultures aerated by continuous gentle rocking method gave best results which were comparable to the yield obtained by semi solid agar method. Favorite and Hammon (1941) reported no alpha toxin production on the shaking machine; horizontal rotation of the culture flasks, however, gave good yield. They also found that semi solid agar cultures without shaking gave highest toxin titers. A gas mixture of either 50% CO$_2$ and 50% O$_2$ or 25% CO$_2$ and 75% air was found superior to other mixtures. Daily replacement of the gaseous mixture did not increase the yield of toxins. Duthie and Wylie (1945) obtained very high yield of toxins by continuous gentle rocking of shallow liquid culture. Christie and North (1941) observed that, unlike alpha lysin, beta lysin production is independent of the increased concentration of CO$_2$. It is also not inhibited by anaerobiosis. Davies (1951) produced higher coagulase
titers by stirring the surface of the medium gently with a stream of air. Fahlberg and Marston (1960) found that concentrations of 5%, 20%, 30%, and 40% CO$_2$ were not stimulatory and in some cases rather inhibited coagulase production. Shaking of the culture medium reduced the yield of coagulase of one strain and had no effect on two other strains.

3. **Medium.** Good titers of *S. aureus* toxins were obtained in peptic digest media by Ramon (1936), in pepsin digested horse meat by Ramon et al (1941), in acid hydrolysate of meat by Mercier and Pillet (1946), and in casein hydrolysate supplemented with thiamine, nicotinic acid and glucose by Favorite and Hammon (1941). Bramann and Norlin (1951) reported excellent toxin production in casein hydrolysate to which yeast extract had been added. Slanetz et al (1959) and Bass and Higginbotham (1960) obtained high yields in veal infusion broth.

Some success has been achieved in producing toxins in chemically defined media. Gladstone (1938) obtained good yields of alpha toxin in a medium containing arginine, glycine, alanine, proline, valine, phenylalanine, cystine, tryptophan, and methionine. The same amino acids were required for hemolysin production as were needed for growth but the concentrations of arginine and glycine necessary for toxin production were higher.

For coagulase production either brain heart infusion dialysate (BHID) broth (Fahlberg and Marston, 1960) or BHID with some additional trace elements (Borchardt et al, 1963)
have been successfully used. Other media used for coagula-
lose production are heart infusion broth (Blobel et al., 1960) and digest broth (Lominski et al., 1962).

For large scale production of leucocidin Woodin
(1961) used a medium which contained 20 g casamino acids,
20 g Na glycerophosphate, 14.4 ml Na lactate 70% syrup,
0.25 ml MgSO$_4$.7H$_2$O 16% solution, 0.125 ml MnSO$_4$.4H$_2$O 6.4% solution, 2 ml FeSO$_4$.7H$_2$O 0.32% solution, 0.41 g KH$_2$PO$_4$,
3.1 g Na$_2$HPO$_4$.2H$_2$O, and 20 g oxid yeast diffusate. Dist-
tilled water was added to make the final volume to 1000 ml,
the pH adjusted to 7.4 and the medium autoclaved at 15 lbs
for 20 minutes. This medium was also used by Bungay (1961)
for the production of staphylokinase, P.V. leucocidin, and
hyaluronidase.

4. Incubation time. Parker et al. (1925-26) ob-
tained good yield of toxin using an atmosphere of 10% CO$_2$
and an incubation period of 48 hours. Gladstone (1938)
found that the rate of alpha lysin production in a synthe-
tic medium was high after the first two days of incubation
and reaching maximum on the fourth or fifth day. Favorite
and Hammon (1941) reported that in casin hydrolysate me-
dium, hemolysin production increased up to the second day,
then fluctuated slightly during the next five days of in-
cubation. Mangalo et al. (1954) found a time lag between
the beginning of the exponential phase of growth and the
appearance of toxin in the culture medium. Davies (1951)
under certain defined cultural conditions reported a large
amount of coagulase within a few minutes in the later part of the lag phase of bacterial growth. Fahlberg and Marston (1960) using brain heart infusion broth were able to obtain good titer of coagulase within six hours of incubation. They thought that there was no necessity of longer incubation for higher production. Gale (1943) reported that hyaluronidase and coagulase stop increasing along with the stoppage of bacterial growth. Rogers (1954, 1955) concluded that coagulase production has a lag phase similar to the lag phase of bacterial growth. Production then starts slowly and stops before growth. The lag phase of hyaluronidase production is greater than the lag in growth. Its production then occurs with a greater speed and stops increasing abruptly sooner than the growth stops. Valentine (1936) found that P.V. leucocidin appears within the first 24 hours of incubation and, being oxygen labile, thereafter decreases in amount. Woodin (1959) obtained maximum yield of P.V. leucocidin in eight to ten hours in casamino acid yeast casein medium. Bungay (1961) reported that the production of staphylokinase at first lagged behind the growth and then reached maximum in about six and one half hours.

B. INACTIVATING AGENTS

Since living staphylococci and active toxins cannot be used for immunization purposes, a constant search has been made to find suitable inactivating agents for use in the preparation of staphylococcal vaccines. Most earlier
workers used heat killed preparations. Downie (1937) reported that rabbits immunized with formalinized vaccines developed smaller lesions than those immunized with heat treated vaccines following challenge with *S. aureus* cultures. Forssman (1938) maintained that both live and formalin killed vaccines protected rabbits against a lethal challenge dose. Heat killed vaccines were useless. Rigdon (1937) reported that the heat killed vaccines were disappointing for therapeutic purposes. These and other results disfavoring heat inactivated vaccines are supported by recent serological studies of Oeding (1953) and Haukenes and Oeding (1960). Their studies indicate that *S. aureus* possesses both heat stable and heat labile antigens. During the process of heat treatment, heat labile antigens may become denatured and lose their antigenic properties.

Some workers have used disrupted live cell vaccines. Cohen et al. (1958) and Stamp (1961) treated live cells of *S. aureus* ultra-sonically for use in immunization studies in rabbits. They also disrupted cells in a ball mill or by a simple shaking device. Gengou (1932), Holm et al. (1936a, b), and Boger et al. (1960) used filtered phage lysed vaccines. Greenberg and co-workers (1960, 1961) tested phenolysed heat killed cells after lysing with deoxyribonuclease enzyme. Rabbits vaccinated with these preparations were found to have an increased resistance to challenge doses of *S. aureus*.

Burnet (1929) reported the inactivation of staphylo-
coccal toxins with formaldehyde. Burnet and Freeman (1932) found that the rate of detoxification, within wide limits, was proportional to the amount of formaldehyde used. Kitching and Farrell (1938) used 0.5 to 1% formalin and found complete detoxification in 24 hours at 37 C. They claimed that rapid detoxification led to less drop in antigenic potency. Wilson (1939) reported that at a given temperature there was an optimum incubation period to detoxify the toxin completely with a minimal loss of antigenic properties.

Apart from formaldehyde, other means of detoxification have occasionally been employed. Li (1936) discovered that photodynamic inactivation by methylene blue produced a toxoid which was as effective as formalized toxoid. Parfentjev et al. (1941) advocated the use of toxoids prepared by means of peptic digestion. This was reported to be less irritating than the formol toxoid. Ramon and Richou (1963) treated toxin with 10% papain solution for six hours at 45 C and concluded that papain treated toxoid had produced nearly as strong immunity in rabbits as untreated toxoids. Orlans and Jones (1958) reported that the level of beta antitoxin in the blood serum of rabbits immunized with beta-propiolactone (BPL) treated toxoid was much higher than the levels obtained with formalinized toxoid. It was also noted that the precipitation lines using gel diffusion technique were more clear and appeared sooner with BPL treated toxoid. Flaming (1960, 1962) compared the toxoiding action of formalin and BPL on the basis of antitoxin response in
immunized cows and rabbits and concluded that the BPL treated toxoid was more active in stimulating both alpha and beta antitoxin production. He thought that formalin, particularly in higher concentrations, damages the antigenicity of the toxin.

Much less attention has been paid to the detoxification of toxins other than alpha and beta hemolysins. Gengou (1933) found 0.3% formaldehyde at 37°C led to complete inactivation of coagulase enzyme within 48 hours. Walker et al. (1947) used trypsin and pepsin enzymes for coagulase inactivation. Harrison (1963) inactivated coagulase with iodine or BPL. The reagents needed were in proportion to the protein content of the sample rather than its specific activity.

C. IMMUNE AND ANTIBODY RESPONSE TO S. AUREUS ANTIGENS

1. Antibodies in non-immunized animals. For controlled immunological studies of various staphylococcal vaccines, the level of normal antibodies among the experimental animals should be established. Numerous reports indicate that S. aureus antibodies may be present in the blood of animals due to natural exposure to S. aureus infections. The agglutinin and the alpha antitoxins are the two types of antibodies which have been investigated to the greatest extent. Agglutinating antibodies against coagulase positive staphylococci have been reported in the sera
of non-immunized rabbits (Ramon, Richou, and Djourichitch, 1936b; Van Loghem, 1946; Pillet and Orta, 1952; Cohen et al., 1961; Oeding, 1960; and Boger et al., 1960), mice (Cohen et al., 1963), guinea pigs (Ramon, Richou, and Djourichitch, 1936a), and human beings (Boger et al., 1960; Jensen, 1958 and others). Boger et al., (1960) employed a modified agglutinating technique in the fluid medium and demonstrated agglutinating antibodies in the blood sera of normal animals belonging to a number of species including the cow, sheep, goat, and horse. Rountree and Barbour (1952) found hemagglutinating antibodies in the sera of non-immunized human beings to the erythrocyte coating substance (probably a polysaccharide) present in the pathogenic staphylococci. Recently, other types of antibodies viz. antibodies detected by fluorescent technique and precipitating antibodies in rabbits (Cohen et al., 1961) and precipitating (Jensen, 1958 and Finkelstein and Sulkin, 1958), protecting (Fisher, 1959) and incomplete antibodies (Neter and Gorzynski, 1959) in human beings have been reported.

The presence of different antitoxins and in particular the alpha antitoxin in the blood serum of human beings has drawn much attention and has extensively been dealt with by Elek (1959). An international standard for alpha antitoxin was established by Smith and Ipsin in 1938. This reduced the discrepancies that had been reported in alpha antitoxin levels in the blood of humans and animals. It is estimated that about 75% of all adult humans have alpha
antitoxin titers between 0.5 and 2.0 international units in their blood serum (Elek, 1959).

There has been a great interest among various workers in studying the normal alpha antitoxin in the blood sera of different species of animals including dairy cows. Antitoxin in the blood of non-immunized pigeons (Richou, 1936), rats (Bryce and Burnet, 1932; Richou, 1936), guinea pigs and rabbits (Ramon et al., 1935), sheep (Ramon et al., 1935; Minnet, 1937, 1939; and Foggie, 1948), and goats (Minnet, 1937 and Derbyshire, 1960a) has been demonstrated. The level of normal antitoxin in horses and pigs is reported up to 15 I.U., in guinea pigs up to 10 I.U., in sheep up to 5 I.U., and in rabbits up to 2 I.U. per ml of blood sera.

The normal values of alpha antitoxin in the blood serum of dairy cattle has been investigated by Minnet (1936, 1937), Gwatkin (1937), Ramon et al. (1935, 1951, 1952a, b, c and 1953), Richou and Holstein (1941a), Richou et al. (1941, 1952a, b), Miller and Heishman (1943), Lasmanis and Spencer (1954), Slanetz et al. (1959), Derbyshire (1960a, b), Brown (1962a, b, 1963), Blobel and Berman (1962), and Spencer et al. (1963). It is estimated that three out of every four cows show antitoxin in their blood serum. Brown (1960), while reviewing the work of other workers, reported that about 63% of the cattle above the age of two years had at least one unit of antitoxin per ml. An equivalent value on the other hand is found only in about 20% of the cattle.
belonging to the age group of two years and under. Roughly, about 25% of the cattle belonging to the category contain more than four international units of antitoxin, while only about 2% of the younger age group carry four units or more.

Cows with *S. aureus* infection in their udders have usually shown higher level of antitoxin in their blood and milk whey than non-infected cows. Brown (1960) estimated that about 90% of the cows having staphylococcal udder infections had at least one international unit of antitoxin as opposed to only 57% of the cows without such infection. Minnet (1937) and Miller and Heishman (1943) apparently noted no absolute correlation of the age of cows and the udder infections to the blood antitoxin. An increase of blood serum antitoxin has been reported after the parturition of the cows (Minnet, 1937; Edwards and Smith, 1959). Such an increase has been generally attributed to be due to the clinical infections and an increased multiplication of the *S. aureus* organisms within the udder tissues. Brown (1960) found some correlation between the number of quarters infected and the level of antitoxin in the blood.

The presence of alpha antitoxin has also been investigated in the milk whey of cows (Minnet, 1937; Miller and Heishman, 1943; Lasmanis and Spencer, 1954; Thorne, 1958; Edwards and Smith, 1959; Derbyshire, 1960b; and Blöbel and Berman, 1962). The quantity of antitoxin in whey was almost always much less than in blood serum of such animals, although it was usually higher in colostrum
(Minnet, 1937; Miller and Heishman, 1943; Edwards and Smith, 1959; and Blobel and Berman, 1962). The antitoxin titers of colostrum were found to drop to a considerable extent within three to four days after parturition. Minnet (1937), Edwards and Smith (1959), and Blobel and Berman (1962) have found some degree of relationship between the antitoxin titers of blood serum and the milk whey; the latter was found to increase with the increase of serum titers.

The presence of alpha antitoxin in the blood of newborn calves has also been investigated by some workers. Ramon et al. (1951) reported it to be lower than in the blood serum of their mothers. Edwards and Smith (1959) and Blobel and Berman (1962) found no antitoxin in the blood of newborn calves before they were allowed to nurse; within two days of nursing, all calves developed some antitoxin in their blood which was still lower than the titers of their mothers. The antibodies persisted in the sera of the calves for two to five weeks. The level of alpha antitoxin in calves was found to have some relation with the titers of the blood serum and the colostrum of their mothers. Ramon et al. (1951) reported that calves are unable to synthesize antibodies in their systems, until they are about five months old. Recently, Thorne and Wallmark (1961) observed much lower antitoxin titers in the blood of young animals upon immunization than in older animals.

Beta antitoxin in the blood, milk, and colostrum of dairy cows has been demonstrated by Minnet (1936, 1937),
Ramon et al. (1952b), and Richou et al. (1952a, b). The occurrence of beta antitoxin in dairy cows is reported to be about 10 to 20% less than alpha antitoxin (Richou et al., 1952a and Edwards and Smith, 1959). Beta antitoxin has also found to increase with the age of the animal and the infection in the udder. The level of beta antitoxin is found higher in colostrum than in the blood which then drops gradually after parturition.

The occurrence of antibodies against other diffusible products of S. aureus has rarely been investigated in the non-immunized animals. Gross (1933) observed that substances, presumably antibodies in nature, inhibiting coagulase were demonstrable in a proportion of human sera, especially from patients suffering from chronic staphylococcal infections. Lominski and Roberts (1946) found that antibodies to coagulase were more common in the serum of healthy subjects than in the patients with major staphylococcal infections. Ramelkemp et al. (1950) demonstrated antibodies against three different types of coagulase antigens in the normal human beings. Howell (1954) reported the presence of antihyaluronidase in the serum of dairy cows.

2. Immune and antibody response to the vaccines.

The antibody titers against cellular and diffusible antigens of S. aureus frequently increase considerably in response to artificial immunization. Such actively produced antibodies usually increase resistance against natural and experimental infections. Unlike most other microorganisms,
**S. aureus** possesses both invasive as well as toxigenic properties to spread and establish its infection in the host tissues.

For prophylactic and therapeutic purposes various **S. aureus** vaccines having cellular and/or diffusible antigens have been tried in different species of animals with variable results. Although from an immunological point of view any vaccine consisting of living unmodified cells would be ideal, such a vaccine cannot be used for its obvious disadvantages. Derbyshire (1961b) used live **S. aureus** vaccine to immunize goats with some success. Brodie et al. (1958) used living attenuated cell vaccine in rabbits and claimed to have protected them against a lethal dose of virulent **S. aureus** culture. For producing hypersensitivity in rabbits, Panton and Valentine (1929) and Johanovsky (1958b) used small doses of live organisms.

Normally, microorganisms inactivated by some physical or chemical means are used for immunization. The vaccine may contain intact whole cells or disrupted cells, thereby liberating the somatic antigens into the fluid medium. So far, little is known about the immunizing properties of the vaccines prepared from disrupted cells, although Greenberg and Cooper (1960) and Stamp (1961) claimed to have obtained a high immune response with such vaccines.

a. **Immune and antibody response in rabbits and mice.** There has been a great difference of opinions about the efficacy of the bacterial and toxoid vaccines. Forssman (1938) was able to protect rabbits against a lethal
dose of virulent culture after immunizing them with a formalinized whole cell vaccine. He found that such animals did not have hemolysins in their blood serum and the protection was afforded by antibacterial substances. Greenberg and Cooper (1960) and Stamp (1961) recently reported further evidence in support of the antibacterial immunity on the basis of their work in rabbits. Koenig et al. (1962) clearly differentiated the antitoxic and antibacterial immunity. In their experiments, mice immunized with toxoid were protected to a lethal dose of alpha hemolysin but succumbed to such a dose of living cocci. On the other hand, immunization with a killed cell vaccine could not protect the mice against a lethal dose of alpha hemolysin; a lethal dose of living staphylococci had no ill effect on such mice.

Most workers during the 1930's stressed the therapeutic and prophylactic values of toxoids. Burnet (1929), Kitching and Farrell (1936), Franco (1936), and Dolman (1937) reported that antitoxic immunity developed actively or transferred passively into the rabbits showed considerable resistance or increased survival time against a lethal dose of staphylococci. The resistance was found largely on the provocation of an adequate titer of specific circulating antitoxins. Others (Conner and McKie, 1933) have found no relation between the antihemolytic titers and the prolonged survival time against a challenge dose. Animals showing circulating antitoxins were said to have developed fewer
lesions and showed greater resistance to bacteremia (Gengou, 1932; Franco, 1936; and Downie, 1937) than control animals. Rabbits immunized with formalinized culture filtrates showed more active phagocytosis than control rabbits (Downie, 1937). According to Franco (1936), in addition to the development of antitoxin in the blood serum, a lesser amount of agglutinins was also synthesized on toxoid vaccination. The protecting value of agglutinins and other antibacterial substances was considered doubtful by Kitching and Farrell (1936). Parish and Cannon (1960) considered that antitoxic immunity was only one aspect of the control of bacterial infections. Antitoxin itself was unable to dispose of the invading organisms; it simply afforded a temporary recovery by abscess formation in the various organs. Parenteral injections of antitoxin into non-immunized mice have also conferred immunity against a lethal dose of homologous (Farrell and Kitching, 1940) or heterologous strains (Frappier and Sonia, 1956).

b. Immune and antibody response in sheep and goats.
Altara (1933) was probably the first to attempt to cure sheep suffering from gangrenous mastitis and pustular dermatitis of the mammary glands. The author reported a definite improvement of the cases after the second injection of a polyvalent vaccine. Improvement occurred within seven or eight days after the first injection. For therapeutic purposes in sheep mastitis, Minnet (1939) recommended two toxoid injections of 5 ml each at an interval of about three
weeks. Local effects could be prevented by intramammary injections of toxoids; subcutaneous injections were, however, more beneficial for systemic reactions. Alum precipitated toxoids were found superior to plain toxoids and protected sheep for at least three months. An average level of 8 to 24 units of antitoxin adequately protected sheep from any ill effect. McLay et al. (1946), on the other hand, reported that in sheep vaccinated with toxoid, there was no improvement in experimentally produced mastitis. The treatment with toxoid, however, was started after the clinical symptoms were visible.

Attempts have been made to protect sheep and goats against staphylococcal infections prophylactically by means of staphylococcal vaccines. Foggie (1943) used toxoid which reduced the incidence of tick pyemia in sheep but was not satisfactory for the treatment of affected lambs. Foggie (1948) in his subsequent studies immunized ewes with toxoid prior to lambing. This produced a high degree of antitoxins in the vaccinated animals. The passive immunity conferred upon the lambs from their mothers declined steadily from the first week of birth and was unable to protect them from natural or artificial infections. The production of antitoxin in response to toxoid did not occur before the age of 25 days. Spencer et al. (1956) reported that six sheep vaccinated with a dried formalinized culture suspended in 10% aqueous aluminum hydroxide could not be protected against an intramammary challenge dose of organisms. There
was not high antitoxin production in any of these sheep.

Pilet et al. (1959a, b) immunized sheep with a bacterin-toxoid via the teat canal which showed a considerable resistance to a challenge dose two to three weeks later. The protection significantly decreased in about a month's time and disappeared in 48 days. For a good protection at least three to four injections are needed; the last one or two could be given intramuscularly. Plommet and Gall (1963a) immunized different groups of sheep with various types of vaccines employing one or more successive injections by local or parenteral routes. Parenteral injections stimulated higher antitoxin than the local injections both in the blood and the whey of the milk. All animals became infected when challenged with 8 to 60 virulent organisms through the teat canal. It was reported that immunity is related to antitoxin content which reduced the severity of infection. There was no difference in the degree of resistance shown by parenteral or local routes of injections. In another experiment Plommet and Gall (1963b) vaccinated two groups of 24 sheep with four local and one parenteral booster injection. Each group received injections of bacterin-toxoid prepared from a different strain. One strain was alpha beta producer and the other alpha beta delta toxin producer. None of the sheep showed a difference in resistance against the challenge organism. There was also no difference in the immune response of the two groups proving that delta toxin does not play any important role in the
virulence of staphylococci in ewes.

Derbyshire (1960a) compared different types of vaccines and concluded that formalinized cell toxoid with aluminum hydroxide gel stimulated highest antibody production which was also maintained for a longer duration than was the case after injection of vaccines without adjuvants. Adjuvant cell toxoid conferred a high degree of resistance against a massive challenge dose of the homologous strain by intramammary route. Derbyshire (1961a) observed that such goats also showed protection against the challenge dose of one heterologous strain but not against three other heterologous strains. The reaction against these three heterologous strains was far less severe than in non-immunized goats, which suffered from a severe gangrenous mastitis. Goats vaccinated with the adjuvant cell toxoid of strain BB showed no protection when challenged with the homologous strain. The blood serum from goats vaccinated with strain BB, when transferred parenterally to a group of mice, could not protect such mice against the challenge dose of this strain; however, they showed resistance against the challenge dose of one heterologous strain. Derbyshire (1961b) in subsequent work found that when subcutaneous abscesses on the skin and udders of goats were produced by living cocci of strain BB, they showed better protection against a massive intramammary challenge dose of this strain. Derbyshire and Helliwell (1962) vaccinated goats with a vaccine containing alpha hemolysin, coagulase, and
leucocidin. These goats showed a greater degree of resistance against strain BB than those immunized with the adjuvant cell toxoid of strain BB. When such goats were challenged with strain 201, the immunity was comparable to the adjuvant cell toxoid of this same strain.

c. Immune and antibody response in the dairy cows.
The literature dealing with vaccination in bovine staphylococcal mastitis is fairly recent. Gwatkin (1937) reported that toxoid, when used therapeutically was unable to eliminate the infection from cows udders. Minnet (1937), while admitting the shortcomings of toxoid therapy, recommended its use over other forms of treatment. Wilson (1942), Gould (1942), and Collinson (1949) found favorable results by the toxoid treatment in staphylococcal mastitis. Richou and Holstein (1941a, b, and 1944) reported that vaccination with toxoids gave complete recovery in about 75% of the cows suffering from staphylococcal mastitis. The cases which did not respond to the treatment usually suffered from mixed infections. There was also a great improvement in the milk quality of the cows suffering from chronic infections. The injections of toxoids were repeated every five days. Richou and Thieulin (1955) later on recommended the vaccine treatment supplemented with antibiotics. Ramon et al. (1951, 1952a, b, c, and 1953) advocated the use of alpha and beta toxoids for the treatment of staphylococcal mastitis. These authors recommended the incorporation of certain types of killed bacterial cells (viz. staphylococci,
Streptococcus agalactiae or Escherichia coli) either alone or in combination with potassium alum in the toxoid; the cells enhanced the level of immunity. The treatment with the help of staphylococcal toxoids was preferred over the antibiotics. Plommet (1960) treated 10 out of 19 staphylococcal infected quarters by injecting bacterin-toxoids in 2.5 ml doses through the teat canals. Most of the recovered quarters responded to treatment after the first injection; for others three to four injections had to be repeated at five to eight day intervals. Thorne and Wallmark (1961) treated bovine staphylococcal mastitis with toxoids and obtained favorable results. The authors observed that in the young animals the rise of the antistaphylolysin titers proved to be less marked than in the older cows.

Few vaccine trials have been made in the field bovine herds and the infection rates compared with the non-vaccinated cows. Kearney and Maloney (1943) carried a 16 month survey in a herd of 80 cows following the use of an autogenous staphylococcal toxoid and streptococcal anaculture. Thirty-eight cows were clinically infected with mastitis before vaccination. It was claimed that three courses of injections spread during a period of nine months cleared the infections in the majority of the cases. Any new clinical case was less severe.

Pearson (1959) used staphylococcal autogenous toxoid vaccine for the prevention of mastitis infection in a dairy herd. In 25 randomly selected young cows, four doses
of 3 to 5 ml each at a weekly interval were given intramuscularly. Every six months a booster dose of 5 ml vaccine was given to each cow, and results on the basis of cultural examinations were compared with 18 control cows. The authors reported significant differences in the number of staphylococcal infections and clinical flare ups between the two groups. During the first 18 months, only 1.9% of the milk samples from the vaccinated cows showed major staphylococcal infections, as compared to 8.1% in the control cows. Only one case of clinical mastitis was observed in the vaccinated group against nine such cases in the non-vaccinated cows. During the following 12 months, there was no noticeable difference in the clinical mastitis cases between the vaccinated and non-vaccinated groups.

Slanetz et al. (1959) carried out vaccination studies in several field herds spread over a period of about five years. In their first experiment, 31 cows belonging to a large dairy herd were vaccinated with bacterin-toxoid, while 39 cows served as controls. During the following period of 18 months, there was a slight reduction (from 16% to 14.1%) in the infection rate of the vaccinated cows. In the control group, however, the number of infected quarters increased three times in the cows of one barn and two times in the cows of the second barn. Only 17 clinically infected quarters were reported among the 31 vaccinated cows. On the other hand, the number of clinical infections among the 39 control cows was 79. The level of alpha antitoxin in the
vaccinated group was significantly higher than in the control cows.

In their experiments No. 2 and No. 3, the authors vaccinated 100 dairy cows and employed another 77 cows as non-vaccinated controls. The vaccine used was either toxoid (in experiment No. 2) or bacterin-toxoid (in experiment No. 3) prepared in their own laboratory or obtained from a commercial company.* The vaccinated group received two to three injections of 10 ml each at an interval of four weeks. The booster dose, if given, was injected after another six months. In these experiments, immunization stimulated the production of higher antibodies and increased the resistance to staphylococcal mastitis. The spread of infection was prevented and there was a marked reduction in the number of acute flare ups.

Slanetz et al. (1963) further continued their staphylococcic vaccine studies on four herds. In herds one, two, and three there was a considerable reduction in the incidence of infected quarters in the vaccinated cows as compared with the control groups. In the fourth herd all of the cows were vaccinated and their results could not be compared with non-vaccinated animals, although the incidence of infection was much less after vaccination. There were no newly infected quarters during the test period of two years after immunization.

* American Cyanamid Co., Princeton, New Jersey.
A few staphylococcal vaccine trials have been carried out in experimental cows under more controlled conditions. Spencer et al. (1956), after obtaining certain preliminary immunization experience in rabbits, vaccinated intramuscularly nine cows with a dried formalinized culture (bacterin-toxoid) suspended in 10% aqueous aluminum hydroxide. All but one cow had a good response of antihemolysin production which varied from 51 to 102 units after two weeks of vaccination. Intramammary challenge with the homologous strain produced more severe reactions in all the five quarters of the non-vaccinated than in the 16 quarters of the vaccinated cows. Nine of the 16 quarters of the vaccinated cows developed chronic infection following the challenge dose.

Slanetz et al. (1959) vaccinated four cows with two injections of bacterin-toxoid at four week intervals. After one to two months, all of the vaccinated and an equal number of control cows were challenged by the intramammary route with one to five billion organisms of the homologous strain. All of the experimental cows resisted the challenge dose much better than the control cows.

Slanetz, Bartley, and Allen (1963) subsequently carried out other staphylococcal immunity trials in cows. In their first experiment four cows were injected intramuscularly with two 5 ml doses of a commercially available bacterin-toxoid.* One month after the second injection,
all the quarters of the four experimental and four control
cows were challenged via the teat canal with a massive dose
of the homologous organism. At the end of the second month,
it was observed that the number of infected quarters in the
experimental and control cows was 9 and 14 respectively.
The infected quarters of all the animals were treated with
chlortetracycline dihydrostreptomycin and neomycin, and the
experimental cows received another two injections of 10 ml
bacterin-toxoid at a monthly interval. On challenge, the
rate of infection in the vaccinated cows was shown to be
about half (6 out of 13 quarters) of the control cows (12
out of 14 quarters).

In the second experiment, the authors vaccinated
another eight cows. The details and procedures of immuni-
zation pertaining to the doses of vaccine, intervals of
injections and the type of vaccine were more or less the
same as are mentioned in their first experiment of this
series. Two quarters of each cow were challenged with the
homologous and the other two with a heterologous strain.
At the end of the observation period, about 75% of the
quarters belonging to the experimental cows were found to
be infected. The infection in the quarters of the control
cows was nearly 100%. The reaction in the quarters of the
non-vaccinated cows was more severe than in the vaccinated
cows. All the infected quarters were treated and the experi-
mental group received one booster injection of 10 ml bacterin-
toxoid. On rechallenge, the percentage of infected quarters in the control group was almost twice that of the experimental cows.

In the last experiment of this series, Slanetz et al. (1963) immunized eight more cows and an equal number served as controls. The vaccine used was a polyvalent bacterin-toxoid inactivated by 1% beta-propiolactone. One homologous and three heterologous strains were used to challenge all the four quarters of all of the cows. The homologous strain (strain 570) infected 14 out of 16 quarters. The infected quarters developed acute mastitis and all these cows also suffered from systemic disturbances. The two cows which resisted the infection against this strain belonged to the group of experimental cows and had a high level of alpha antitoxin in their blood serum. One heterologous strain infected six out of eight quarters of the control cows. The clinical response with strain 570 was so rapid and severe that other heterologous strains did not get sufficient time to set up the infection. Quarters other than the ones challenged with strain 570 got infected with this strain.

Derbyshire (1960b) vaccinated four cows with a monovalent adjuvant cell toxoid in the first month of their first lactation. The doses used during this course of immunization were 5 ml, 10 ml, and 10 ml during the first week spaced at two day intervals and a dose of 10 ml after a period of another three weeks. One week after the
secondary injection, one quarter of all the vaccinated and
two non-vaccinated cows were challenged through the teat
canal with a 10 ml whole broth culture of the homologous
strain. The challenged quarters of the control cows de-
veloped a severe purulent mastitis. In the vaccinated cows,
however, the reaction was very transient and milder. All
cows continued to be infected during the entire period of
three weeks under observation.

Blobel and Berman (1962) immunized 18 cows with a
formalinized 72 hour whole broth culture of a strain iso-
lated from a case of bovine mastitis. The vaccine contained
measured amounts of coagulase and egg yolk factor. All cows
received seven intramuscular injections, three at two week
intervals and the remaining four booster injections three
to six months apart. After the four weeks of the first in-
jec tion series, the average alpha and beta hemolysins in
the vaccinated cows was 38.2 and 48.4 I.U. respectively.
The titers of antibodies against coagulase and egg yolk
factors were low. Six weeks after the first injection,
all the vaccinated and an equal number of non-vaccinated
cows were exposed to infection through the media of two
infected cows, each placed at the head of one of the two
milking lines. The two cows were infected via the teat
canals with four different strains (one homologous and
three heterologous). It was noted that the infection with
the vaccine strain occurred most frequently in the non-
vaccinated cows. One of the three heterologous strains
disappeared quickly from the herd and later on, other new strains were isolated from the herd. An increased resistance among the vaccinated cows was observed against the vaccine strain. Although there was no appreciable difference in the number of infected quarters with the heterologous strain, the reactions were more severe and persisted longer in the non-vaccinated than in the vaccinated cows.

**Monovalent versus polyvalent vaccines.** *S. aureus* possesses a number of surface and cytoplasmic antigens. Different strains possess both group and type specific antigens (Oeding, 1952; Malik and Singh, 1960; and Slanetz and Bartley, 1962). The strains isolated from a single and self contained source usually possess similar antigenic structures. This consideration has led some workers to use autogenous vaccines for better results (Altara, 1933; Kearney and Maloney, 1943; and Pearson, 1959). When it is not possible to use autogenous vaccines, it is considered desirable to include more than one antigenic type in the vaccine. Considering the drawbacks of a monovalent vaccine, Slanetz et al. (1959) advocated the incorporation of more than one type of strain in the vaccine. There are published reports where the immunized animals could not be protected against the challenge dose of heterologous strains. Derbyshire (1961a) reported that goats vaccinated with the vaccine prepared from a strain conferred a high degree of immunity against the challenge dose of the homologous and one heterologous strain but not against three other heter-
ologous strains. Similar reports are available from immunological studies in cattle (Blobel and Berman, 1962 and Slanetz et al., 1963). It has also been observed in goats (Derbyshire, 1961a) and cattle (Slanetz et al., 1963) that at times, vaccines prepared from some strains do not afford protection even against the challenge dose of such particular homologous strains.

**Immunological significance of individual diffusible antigens.** The merits of alpha toxoid have earlier been discussed in some detail. So far, little information is available on the immune and antibody responses of other *S. aureus* soluble antigens.

The level of beta antitoxin in the blood serum of vaccinated animals has usually been investigated with alpha antitoxin. Most of the *S. aureus* strains from bovine sources are alpha-beta hemolytic and the toxoids prepared from such strains usually stimulated both types of antihemolysins. Brown (1960) and Blobel and Berman (1962) have reported that the titers of beta antitoxin usually correlate with the titers of alpha antitoxin in the blood serum of cows. Derbyshire and Helliwell (1962) on the basis of their studies in goats reported that beta hemolysin does not appear to play any significant role in the virulence of or in the immunity against staphylococci. Virtually nothing is known about the immunological significance of delta lysin. It has been suggested that delta lysin and leucolysin i.e., the third type of leucocidin, are identical
(Gladstone and van Heyningen, 1957). The hemolytic activity of delta lysin is reported to be non-specifically neutralized by the normal serum (Marks, 1951). Recently Plommet and Gall (1963b) reported that delta toxin does not play any important role in the virulence of staphylococcal mastitis in ewes.

The antigenicity of coagulase has been known for the last 30 years (Gross, 1933). Ramelkemp et al. (1950) described three and Duthie and Lorenz (1952) described four different antigenic types of staphylocoagulase. Lominski (1949) found that coagulase inhibitory human serum conferred immunity in rabbits. Boake (1956) demonstrated that anti-coagulase increased the resistance of experimental rabbits against the intravenous challenge with coagulase positive strains. Blobel and Berman (1961) observed a rapid depletion of blood fibrinogen in rabbits following the intravenous injection of a purified coagulase. Hyperimmunized rabbits, however, were fully protected against fibrinogen depletion and pulmonary vascular blockage to coagulase doses lethal for normal animals. Lominski et al. (1962) immunized rabbits with toxic filtrates free of hemolysins and obtained good coagulase antibody response. The rabbits immunized with coagulase preparation from a phage group III strain were protected against the challenge dose of a phage group III strain but not against phage group I strain. On the other hand, rabbits immunized with coagulase preparation from a phage group I staphylococcus showed no resistance to
either of the two phage group staphylococci. Derbyshire (1960a) was unable to detect any anticoagulase titer in the sera of the goats immunized with adjuvant cell toxoid vaccine. Blobel and Berman (1962) demonstrated an increased anticoagulase titer in the serum of vaccinated cattle after each booster injection. The authors concluded that to obtain a higher antibody titer, a large amount of coagulase has to be incorporated into the vaccine. In cattle not much is known about the role of coagulase antibodies in staphylococcal immunity. Derbyshire and Helliwell (1962), from their work in goats, concluded that coagulase plays an insignificant role in the mechanism of virulence and contributes nothing towards immunity.

During recent years, there has been a revival of interest in studies of the P.V. leucocidins and the role of their antibodies in staphylococcal infections. Panton and Valentine (1932) and Valentine (1936) drew attention to the high antigenicity of the P.V. leucocidin. Woodin (1959) described a fast and a slow antigenic fraction (F and S) of the P.V. leucocidin. On the basis of clinical observations and the antileucocidin titers in the human patients, Butler and Valentine (1943) suggested the immunological importance of leucocidin. Johanosky (1958a), Towers and Gladstone (1958) and Gladstone et al. (1962) observed a direct relationship between the resistance to staphylococcal infections and the antileucocidin titers among the human patients. They found no such correlation
with anti alpha hemolysin. Sebek et al. (1959) noted a seven fold reduction in the incidence of mastitis in human mothers after immunizing them during pregnancy with a toxoid containing high leucocidin. Derbyshire and Helliwell (1962) immunized goats with toxoids containing alpha hemolysin, coagulase, and leucocidin. The goats showed considerable resistance to a massive challenge dose of organisms.

The immunizing role of the antibodies against other S. aureus diffusible products is not known. Derbyshire (1961a) was unable to detect antibodies against hyaluronidase, lipase, and protease enzymes in the sera of immunized goats. Blobel and Berman (1962) reported that in cattle the antibodies inhibiting the egg yolk factor almost remained at the pre-vaccination level until more partially purified egg yolk factor was incorporated into the vaccine.

Effects of adjuvants on antibody production. Adjuvants have frequently been used to increase the immunizing efficiency of various vaccines including the toxoids and bacterin-toxoids of S. aureus. Minnet (1939) reported that one dose of alum precipitated S. aureus toxoid gave better immunity in sheep than two doses of plain toxoid. Derbyshire (1960a), while immunizing the goats against S. aureus experimental infections, compared different types of vaccines with and without the adjuvant and concluded that cell toxoid with aluminum hydroxide adjuvant produced highest antibody titers of all types of vaccines used and gave best protection against the challenge doses. Alum precipitated
vaccines have been used by Farrell and Kitching (1938),
Richou (1943, 1945), Richou et al. (1941), Ramon et al.
(1953), Pearson (1959), and Blobel and Berman (1962) for
increasing the immunity against \textit{S. aureus} infections in
cows and other animals. Other commonly employed adjuvants
are aluminum phosphate (Tasman et al., 1952) and aluminum
hydroxide (Ramon et al., 1952a; Spencer et al., 1956; and
Derbyshire, 1960a). Ramon et al. (1952b, c, 1953) recom-
mended the incorporation of killed streptococci, staphylo-
cocci, or \textit{Escherichia coli} suspensions in the \textit{S. aureus}
toxoids, either alone or with potassium alum to increase
the level of immunity.

To enhance the antigenicity of coagulase prepara-
tions for rabbit immunization, mineral oil (Blobel et al.,
1960), alum sulphate (Lominski et al., 1962), and Freund
adjuvant (Borchardt et al., 1963) have been used success-
fully.
MATERIALS AND EXPERIMENTAL METHODS

Staphylococcus aureus strains. During the entire study period, six strains isolated from well established cases of bovine mastitis were used. Stock cultures were maintained in frozen state as 18 hour broth cultures with an equal amount of sterile 20% glycerine. The strains represented different phage types, produced alpha and beta hemolysins, and were coagulase positive.

Strains 7, 15, 570, and Ger were used for vaccine preparations, strains 10 and 570 for challenging the cows, strains 7 and 1531 for challenging the rabbits, and strains 570 and 1531 for challenging the mice. Strain 570 was used in the studies of toxin production.

Experimental animals. Holstein-Fresian first calf heifers usually during their first to third months of lactation were used. The udders of all cows were free from bacterial infections at the time of challenge. Albino rabbits of both sexes about 8 to 10 weeks old and weighing between 1.5 and 2.0 kilograms were used. The source of the supply was the same during the entire period of the study. Animals with natural staphylococcal agglutinins of 1:32 or over were not used for immunological studies. Commercially available male and female albino mice weighing about 20 grams were used after acclimatization. For each particular experiment, mice from the same source were used.
**Media.** Extract broth (Difco*), extract agar (Difco*), veal infusion broth (Difco*), heart infusion broth (Difco*), and trypticase soy broth (BBL**) were prepared according to the recommendations of the makers of the media. For N.Z. amine broth (Sheffield Chemical, Norwich, New York) the constituents were used in the following proportions.

N.Z. amine type A (Enzymic digest of casein) ........................................ 2.25%
NaCl .................................................. 0.5%
KH₂PO₄* ........................................... 0.1%
K₂HPO₄* ........................................... 0.1%
MgSO₄ ............................................. 0.01%
Thiamine (0.0005% solution) ........................................... 0.1%
Nicotinic acid (0.05% solution) ........................................ 0.24%
Ammonium lactate ........................................ 0.5%
Distilled water to ........................................ 100%

N.Z. amine and salts were dissolved in distilled water, brought to boiling temperature, and then the pH was adjusted to 7.1. The vitamins and ammonium lactate solutions were added and the medium was autoclaved for 15 minutes at 1.5 lbs. steam pressure (121 C).

**Media employed for toxin production.** One hundred and fifty ml medium of extract broth, trypticase soy broth, veal infusion broth, heart infusion broth, and N.Z. amine broth were sterilized in separate 500 ml Erlenmeyer flasks. The content of each flask was inoculated with 10 ml 18 hour broth culture of strain 570. One set of all the five media

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*Difco Laboratories, Detroit, Michigan.

**Baltimore Biological Laboratories, Baltimore, Maryland.*
was incubated under static conditions, the second set on a shaker (Incubater shaker - Model G26, New Brunswick Scientific Co. Inc., New Jersey) with 70-80 revolutions per minute, and the third set on the shaker in an atmosphere of 50% O₂ and 50% CO₂. Three sets of the five media comprising 15 flasks were incubated at 37°C, and a second and third batch of similar 15 flasks were incubated at 34°C and 40°C respectively. Three flasks of heart infusion broth with 1.5% NaCl concentration were incubated only at 37°C i.e., one flask under each of the three aeration variables. Ten ml samples from all the culture flasks were collected at 4 hour, 8 hour, 12 hour, 16 hour, 24 hour, 48 hour, and 72 hour incubation periods. The oxygen and CO₂ gases were replaced at each sampling time. The optical density (OD) of each sample with appropriate controls was recorded. The culture samples were then centrifuged for ten minutes at 4000 revolutions per minute (RPM)* and the supernatants were kept under frozen conditions until they were examined for alpha and beta hemolysins and coagulase titers.

**Vaccines.** All the vaccines used in this study contained either cellular antigens or both cellular and diffusible antigens.

**Vaccines V-1 and V-2.** The growth from 18 hour nutrient agar cultures was washed off with normal saline. The density was adjusted to McFarland opacity tube 10.

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* International centrifuge, universal model UV manufactured by International Equipment Co., Needham Hts., Mass. was used in the entire work.
To one portion of the cell suspension, 0.5% formalin was added and to another similar suspension, 0.75% BPL. The inactivation was carried out at 37°C. It took about two days to inactivate the formalinized suspension, while the inactivation of BPL treated cells was complete within four to five hours. The pH of the latter vaccine dropped to about 3.8 during the inactivation processes and was returned to 7.0 with NaOH. The sterility of vaccines was tested in thioglycolate broth and on blood agar plates, and they were then stored at 4°C refrigeration temperature except when in use.

**Vaccine V-3.** The bacterial suspension was obtained as mentioned under vaccines V-1 and V-2. The rest of the technique was the same as described by Greenberg and Cooper (1960). Five-tenths per cent phenol was added in the bacterial suspension which was then killed at 56°C for one and one half hours. After sterility testing, 100,000 units of Dornase* were added to every 500 ml of the vaccine suspension. The flask was incubated at 37°C for cell lysis, which took about three days. The vaccine was centrifuged at 3000 RPM for half an hour to spin down the few intact cells left. The supernatant was collected and stored at 4°C refrigeration temperature after sterility testing on blood agar plates and in thioglycolate broth. This may be referred to as phenolysed, heat killed, dornase treated vaccine.

* Dornavac from Merck, Sharp, and Dohme, West Point, Pennsylvania.
Vaccine V-4. Washed 18 hour cell suspensions were centrifuged at 4000 RPM for 15 minutes. Five-tenths gram of sedimented cells were taken into each of the two stainless steel cylinders of Nossel's disintegrator which already contained ten grams of sterilized glass beads. About 10 ml of 0.9% KCl solution was then added into each of the cylinders. The cells were disrupted for about four and one half minutes on alternate cycles of 30 seconds each. Between the two operation cycles, the cylinders were alternately kept cold in ice water. At different time intervals, disruption of the cells was examined microscopically with stained preparations. At the end of four and one half minutes of operation time, about 80 to 90% of the cells were found to be disrupted. Two-tenths per cent formalin was then added to ensure sterility and preservation of the vaccine. After a sterility test, the vaccine was kept in a refrigerator at 4 C until used.

Vaccine V-5. One ml of a 6 hour broth culture of the vaccine strain and 1 ml of S2 phage having 10^{-6} titer as routine test dilution were inoculated into 100 ml trypti-case soy broth in a 250 ml flask. The medium was incubated for six hours at 37 C and over night at room temperature (25 C). The liquid was then centrifuged at 4000 RPM for 15 minutes and the supernatant was formalinized to give a final strength of 0.2%. After sterility testing, the vaccine was stored under refrigeration at 4 C until used.
**Vaccines V-6 and V-7.** Veal infusion broth cultures of strains 7, 15, 570, and Ger grown in an atmosphere of 50% CO₂ and 50% O₂ on a shaker were harvested after 48 hours of incubation. The cultures of all four strains were mixed, resulting in 4 x 10⁹ viable cell count per ml. After separating the cells from the toxins by centrifugation, the cells were disintegrated by Nossal's disintegrator, and the disintegrated material then added to the original toxin. One half of the disrupted cell toxin mixture was inactivated with 0.5% formalin (vaccine V-6) and the other half with 0.75% BPL (vaccine V-7). The vaccines were stored in a refrigerator at 4°C after sterility testing.

**Vaccines V-8 to V-12.** These vaccines were prepared as follows: vaccine V-8 contained formalin inactivated 48 hour whole broth culture of strains 7, 15, 570, and Ger in equal proportions; vaccine V-9 was similar to vaccine V-8 except that inactivation was done with beta-propiolactone; vaccine V-10 consisted of beta-propiolactone detoxified toxins and formalin inactivated 18 hour nutrient agar culture suspensions of all four vaccine strains; vaccine V-11 was prepared similar to vaccine V-10 but contained high beta toxin and low coagulase levels; vaccine V-12 was prepared similar to vaccine V-10 but contained high coagulase and low beta toxin levels. Vaccines V-8 to V-12 contained 4.2 billion cells/ml. The potencies of alpha and beta toxins and coagulase are shown in table 1.
Vaccines MV-A and MV-B. These were formalinized monovalent bacterin-toxoid vaccines obtained from a commercial company.* There was no difference between the two vaccines except that MV-B contained aluminum hydroxide as adjuvant.

Vaccines PV-A and PV-B.** All the four vaccine strains were grown separately in veal infusion broth on shaker under 50% CO₂ and 50% O₂. After 48 hours of incubation, the cultures were harvested and detoxified separately by 1% BPL. The whole broth cultures of the four strains were mixed in equal proportions and the pH was adjusted to 5.5 with NaCl. The culture was then divided equally into two flasks.

For preparing vaccine PV-A, one part of sterile aluminum hydroxide gel was added to every 11 parts of inactivated broth culture. After allowing it to stand for two hours, the pH was adjusted to 6.0. Merthiolate was then added to obtain a final concentration of 1:10,000.

For vaccine PV-B, to every four parts of the inactivated broth culture one part of aluminum hydroxide was added. The mixture was thoroughly shaken and then allowed to settle in a measuring cylinder. After settling, the mixture was re-shaken and allowed to settle again.

* American Cyanamid Co., Princeton, New Jersey.

** Kindly prepared by Dr. Clara H. Bartley. Dept. of Microbiology, Univ. of New Hampshire, Durham, N.H. and used to vaccinate dairy cattle.
After the second settling, three-fifths of the liquid from the top was removed retaining the two-fifths concentrated liquid at the bottom. The pH was adjusted to 6.0. Merthiolate 1:10,000 strength was added as preservative. Vaccines PV-A and PV-B were tested for sterility and stored under refrigeration at 4°C.

Vaccine PV-C. This was prepared the same way as vaccine PV-B except that N.Z. amine broth medium was used instead of veal infusion broth and formalin in 0.6% concentration was used as the inactivating agent. The vaccine was tested for sterility and preserved with 1:10,000 merthiolate before being stored under refrigeration. Vaccines PV-A, PV-B, and PV-C contained \( 4 \times 10^9 \) billion cells per ml before inactivation.

Vaccines V-1 to V-5, MV-A and MV-B were prepared from strain 7. Other vaccines were polyvalent in character and contained strains 7, 15, 570, and Ger.

The titers of coagulase and hemolysins in cell toxoid vaccines before inactivation are shown in table 1.

Procedure of immunization. In rabbits the vaccination was carried out intravenously for three weeks, three to four consecutive days per week. The order of doses was 0.1 ml, 0.2 ml, 0.3 ml, and 0.4 ml in the first week; 0.4 ml, 0.6 ml, 0.8 ml, and 1 ml in the second week; 1 ml, 1.2 ml, and 1.5 ml in the third week. The doses of vaccines V-3, V-4, and V-5 were determined on the basis of their protein content which was calculated to be 1.0, 3.5, and 10.0 mgs
Table 1

Potencies of coagulase and hemolysins in various cell toxoid vaccines.

<table>
<thead>
<tr>
<th>Vaccines</th>
<th>Reciprocal of the titers/ml</th>
<th>Coagulase</th>
<th>Alpha hemolysin</th>
<th>Beta hemolysin</th>
</tr>
</thead>
<tbody>
<tr>
<td>PV-6</td>
<td>512</td>
<td>1152</td>
<td>1280</td>
<td></td>
</tr>
<tr>
<td>PV-7</td>
<td>512</td>
<td>1152</td>
<td>1280</td>
<td></td>
</tr>
<tr>
<td>PV-8</td>
<td>2112</td>
<td>2624</td>
<td>1024</td>
<td></td>
</tr>
<tr>
<td>PV-9</td>
<td>2112</td>
<td>2624</td>
<td>1024</td>
<td></td>
</tr>
<tr>
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<td>2112</td>
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<td></td>
</tr>
<tr>
<td>PV-11</td>
<td>128</td>
<td>1536</td>
<td>16635</td>
<td></td>
</tr>
<tr>
<td>PV-12</td>
<td>16384</td>
<td>1707</td>
<td>341</td>
<td></td>
</tr>
<tr>
<td>PV-A</td>
<td>512</td>
<td>1152</td>
<td>1280</td>
<td></td>
</tr>
<tr>
<td>PV-B</td>
<td>512*</td>
<td>1152*</td>
<td>1280*</td>
<td></td>
</tr>
<tr>
<td>PV-C</td>
<td>3072*</td>
<td>900*</td>
<td>1824*</td>
<td></td>
</tr>
</tbody>
</table>

* Indicates calculation before concentration.

respectively. For vaccines V-3 and V-4, 12 to 13 mgs, and for vaccine V-5, 50 mgs of protein was injected into each rabbit. Blood was collected for serum separation one week after the last injection.

In cows, vaccines were injected intramuscularly.**

One 10 ml dose of vaccine MV-A and MV-B followed by a second 10 ml dose after four weeks was given to two cows separately. Blood sera were collected at weekly intervals to be used

*** The vaccination was carried out by Dr. F.E. Allen, Dept. of Microbiology, Univ. of New Hampshire, Durham, N. H.
for antibody titrations. Each of the vaccines PV-A, PV-B, and PV-C were injected separately into a group of five cows. All cows received two doses of 5 ml each at an interval of two weeks. Blood samples were collected at suitable time intervals and also at the time of intramammary challenge. Sera from control cows were also tested for antibodies at the time of challenge. All animals were tested for normal antibodies before immunization.

Collection and preservation of blood sera. In rabbits bleeding was done from the marginal ear vein. About 20 ml blood was collected from each rabbit in a large wide mouth 50 ml test tube. The clotted blood was stored over night at 4 C refrigeration temperature. The serum from each blood sample was collected aseptically and then inactivated at 56 C for 30 minutes. After the addition of merthiolate in a final concentration of 1:10,000, the sera was frozen until used. The experimental and control cows were bled through the jugular vein. The separation, inactivation, and preservation of sera were done according to the procedures mentioned for rabbit sera.

Procedures of challenge. Rabbits were challenged nine days after the last injection. All experimental and control rabbits were challenged intravenously with 1 ml of 18 hour trypticase soy broth cultures of strains 7 and 1531. The challenge doses were determined by injecting 1 ml diluted and undiluted 18 hour trypticase soy broth cultures into control rabbits. Diluted cultures did not
kill all rabbits whereas undiluted cultures killed nearly all the injected rabbits in about two weeks. The number of viable cells per ml was $3.1 \times 10^9$ and $3.4 \times 10^9$ in the challenge doses of strains 7 and 1531 respectively. Mortality was observed from two to four weeks in different experiments.

Fifteen experimental and five control cows were challenged through the teat canals.* After two weeks of the second injection, right rear (RR) and left front (LF) quarters of all 20 cows were challenged with strain 10 (heterologous) and strain 570 (homologous) respectively. The average number of organisms in each inoculum (in 1 ml skim milk) of strain 10 were 79 and of strain 570 were 29. The details of preparing the inoculum and the injections were the same as has been described by Slanetz et al. (1963).

Optical density (OD). The optical density of cultures for the measurement of cell growth was determined with the Bausch and Lomb Spectronic-20 Colorimeter** at a wavelength of 590 m/λ. The instrument was set at zero OD with appropriate control samples before recording the OD of the cultures.

Protein measurement. Protein determination was done by the Folin-ciocalteau phenol reaction modified by Lowry et al. (1951). The optical density read at 660 m/λ

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* The challenge doses were administered by Dr. F.E. Allen.

** Bausch and Lomb Optical Co., Rochester, New York.
in a Bausch and Lomb Spectronic-20. Bovine plasma albumin was used as reference standard.

**Titration of alpha hemolysin.** Using normal saline as diluent, toxin samples in 1 ml quantity were diluted serially two fold. An equal amount of 1% washed rabbit red blood cells was added to each dilution. One control tube having 1 ml saline solution and 1 ml 1% rabbit cells was also set up. The test specimens were incubated for one hour in a water bath at 37 °C and then left over night at 4 °C. Complete hemolysis was considered the end point of the titers.

**Titration of beta hemolysin.** The procedure followed was the same as mentioned for alpha hemolysin except that 1% washed sheep red blood cells were used in place of rabbit red blood cells.

**Titration of free coagulase.** In 13 x 100 mm test tubes, two fold serial dilutions of the toxins were prepared. The quantity of the diluted toxin in each test tube was 1 ml and the diluent used was 1% sterile peptone water containing 1:5000 merthiolate as a preservative. One ml of 20% human plasma (diluted in normal saline solution) was then added into each toxin dilution, and the tubes were incubated at 37 °C water bath for four hours and over night at room temperature. One control tube containing 1 ml 20% plasma and 1 ml saline was run with the test. The clotting was determined by examining the titers with gentle tapping in indirect light against a black background. The degrees of
clots were assigned 4+ (complete solid clot), 3+ (clot with small amount of liquid on the top or the sides), 2+ (clotting about one-third to one-fourth of the plasma fluid leaving rest unclotted), and + (a very small but a definite and distinct clot). Dilutions showing up to + clot were considered the end point of the titers, which was referred to as one minimum clotting dose (M.C.D.).

Titration of alpha antitoxin. For the determination of the hemolytic test dose of the toxin (Lh dose), one unit of standard alpha antitoxin* contained in 0.5 ml was added to each 0.5 ml two fold serial dilution of alpha toxin. The mixtures were shaken and allowed to react at room temperature. After 20 minutes of reaction period, 1 ml 1% washed rabbit red blood cells were added to all the tubes and the mixture was incubated at 37°C water bath for one hour. The highest toxin dilution showing 50% hemolysis in the presence of one unit of standard antitoxin was the Lh dose of the toxin which was used in the antitoxin titration of the test serum. To a series of two fold 0.5 ml test serum dilutions 0.5 ml of standard toxin (Lh dose) was added. The mixture was shaken and allowed to react at room temperature for 20 minutes. One ml of 1% rabbit red blood cells were then added to each tube and incubated for one hour at 37°C water bath. The highest dilution showing 50% hemolysis was the end point of the

* Received from National Institute of Health, Bethesda, Maryland.
antitoxin titration. This dilution, when multiplied by a factor of two, gave antitoxin units per ml in the test serum. Two controls, one having 1 ml of 1% red blood cells and 1 ml of normal saline and the second with one unit (in 0.5 ml) of standard antitoxin, one Lh dose of the toxin (in 0.5 ml), and 1 ml of 1% rabbit red blood cells, were also set up.

Titration of beta antitoxin. The procedure was the same as has been mentioned for alpha antitoxin titration except that (1) one unit of standard beta antitoxin was used for determining the Lh dose of semi-purified beta toxin; (2) one percent sheep red blood cells were used instead of rabbit red blood cells; (3) the incubation period was one hour at 37 C water bath followed by overnight refrigeration temperature at 4 C.

Titration of anticoagulase. Antibodies inhibiting coagulase were measured by determining the highest dilution of the test serum which completely inhibited the clotting activity of two minimum clotting doses (M.C.D.). The method of determining the M.C.D. has already been described under coagulase titration. Five-tenths ml of the coagulase containing two M.C.D. was mixed with 0.5 ml of two fold test serum dilutions and incubated for 30 minutes at 37 C. Then 1 ml of 20% human plasma was added. The mixture was incubated for four hours at 37 C water bath and over night at room temperature. Highest serum dilution inhibiting the clot formation was considered the end point of coagulase
titration. One control having two M.C.D. of coagulase contained in 0.5 ml and 0.5 ml diluting fluid with 1 ml of 20% human plasma and the second consisting of 1 ml diluting fluid and 1 ml of plasma dilution were also included in the test. The highest dilution of the serum inhibiting the clot formation, when multiplied by a factor of 2, gave the titer per ml.

Hemagglutination test for determining the antileuco-
cidin titers. The hemagglutination test described by Markham (1961) was employed. Sheep red blood cells, previously stored for three days in Alsever solution, were washed thrice with normal saline solution. For tanning the sheep red blood cells, a 2% cell suspension was added to equal parts of 1:20,000 freshly prepared tannic acid solution. The mixture was incubated at 37°C for ten minutes, then washed with buffered saline solution with a pH of 7.2 (M/15 phosphate and sodium chloride solution). The cells were finally re-suspended in saline to make a 2% suspension.

Tanned sheep cells were sensitized by mixing equal volumes of cell suspension and a solution containing 4L+ units of fast or slow components* either separately or in a combination of both in buffered saline solution with pH 6.4. (L+ = smallest amount of leucocidin in 1 ml in the presence of 1 unit of antitoxin which will kill macrophages under the test conditions.) The sensitization was allowed

at room temperature for ten minutes. The excess of leuco-
cidin was then washed off at 2000 RPM for three minutes.
The cells were washed once with normal saline and once more
with saline with 0.5% normal rabbit serum. The cells were
stored in a refrigerator as 2% suspension in normal rabbit
serum saline solution.

Duplicate sets of two fold serial dilutions of test
sera were prepared in 5 x 1 cm tubes using normal rabbit
serum saline as diluent. Each tube contained 0.2 ml serum
dilution. To one row of the tubes, sheep cells sensitized
with Fast Component and to the second row, cells sensitized
with Slow Component were added in 0.02 ml quantity. When
the cells were sensitized with equal parts of Fast and Slow
Components (2L+ each), only one row of tubes was run. After
shaking the mixtures, the tubes remained at room temperature
over night. The results were recorded after observing the
patterns of the cells. Tubes showing a layer of uniformly
agglutinated cells covering the bottom were considered as
positive. The negative results were shown as compact,
sharply demarcated discs of sedimented cells in the center
of the bottom of the tube. Control tests consisted of
sensitized sheep cells (Fast and Slow) and tanned sheep
cells using diluting fluid in place of serum dilutions.
Normal cow or rabbit serum in various dilutions were also
run as controls using sensitized (F and S factors) and
tanned sheep cells separately.
Agglutination. The agglutination test was performed on a large glass plate with 1" x 1" squares made from enamel paint. Formalinized cell suspensions from 18 hour nutrient agar cultures of appropriate strains in 1½ saline solution were used as antigens after adjusting the opacity to McFarland tube #10. Two fold serum dilutions were prepared using 1½ saline solution and one drop of each dilution (starting from highest to lowest dilution) was put on one square of the glass plate. One drop of the antigen was then added on each serum dilution drop and mixed with a thick platinum loop. The plate was rotated a few times clockwise or anti-clockwise and then kept for 10 to 12 minutes at 37°C for agglutination reaction. The results were recorded against a black background using indirectly transmitted light. Bacterial cell suspensions used as antigens were checked for auto-agglutination. Four serum samples were tested at a time against the same strain.

Immunodiffusion test. This test was performed in plates as well as on microscopic slides. For plates the method of Ouchterlony (1949) was followed with slight modification. Eight-tenths per cent Ion agar (oxoid) was used in a solution containing 0.6% sodium chloride and a 1:10,000 concentration of merthiolate. In scrupulously clean flat bottomed petri dishes of four inch diameter, one central and five or six peripheral stainless steel cylinders (8 mm diameter) were arranged in such a way that the distance from central to peripheral cylinders was 8 mm and between
two adjoining peripheral cylinders about 1 cm. Fifteen ml of melted agar was then poured into each plate and allowed to cool. When the agar was hardened, the cylinders were removed and the bottoms of the cups were sealed with melted agar. Either the sera were added in the central and the antigens in the peripheral wells or an alternative arrangement was followed. The reaction was allowed to occur for 24 hours at room temperature and 48 hours at refrigeration temperature (4°C) before recording the results. For slide immunodiffusion test the method described by Ouchterlony (1962) was followed.

For better differentiation of the precipitation lines, the slides or the plates were dried and stained with azocarmine (5 mg in 100 ml of 2% acetic acid). After staining, they were rinsed in 2% acetic acid and dried.

Mouse protection test. For testing the protection power of the normal and immune sera against challenge doses of S. aureus in mice, various four fold dilutions were made. Each dilution was injected into a batch of five mice. The dose was 0.5 ml by intraperitoneal route. After 18 hours, a trypticase soy broth culture of the challenge strain grown for 18 hours was injected intraperitonially in 0.5 ml doses. Broth cultures of strain 7 were diluted to 1:3 and that of strain 1531 to 1:2 before inoculations. These inoculums generally killed the control mice. The rate of mortality was recorded up to five days and the 50% protection power of the sera was calculated by the method of Reed
and Muench (1938) (see appendix). The protection power of the normal sera was tested for comparison.
RESULTS

A. FACTORS AFFECTING THE PRODUCTION OF ALPHA AND BETA TOXINS AND COAGULASE

Three variables, namely temperature, aeration, and medium were used to determine the optimal conditions for the production of alpha and beta hemolysins and coagulase by *S. aureus*. One strain of *S. aureus* (570) was utilized for these studies. The culture samples were withdrawn for the titrations of the toxins every 4 hours for the first 16 hours and at 24 hours, 48 hours, and 72 hours of incubation periods.

1. Maximum yield alpha toxin—conditions for. As is indicated in fig. 1, generally maximum growth was obtained in all media within 72 hours by aeration of the culture medium by means of constant shaking. Of the three aeration systems, the static condition produced the least amount of growth. On the other hand, alpha and beta hemolysin production was found to be maximum in the cultures grown on the shaker under 50% CO₂ and 50% O₂. Figure 2 shows the relationship of growth and production of hemolysins and coagulase. It is evident that the production of alpha and beta hemolysins lagged behind the growth. During the log phase of growth, hemolysins were produced at a faster rate and continued to increase after the maximum growth was attained. In cultures growing under stationary conditions the production of alpha hemolysin was at a minimum.
Fig. 1. Growth curves at 37°C in different media under various aerations.

- ○ Static cultures.
- △△ Cultures on shaker.
- □□ Cultures on shaker with 50% CO₂ and 50% oxygen.
Fig. 2. Relationship of growth, coagulase and alpha and beta hemolysins in N.Z. Amine broth culture grown under 50% CO$_2$ and 50% oxygen.
It is evident from table 2 that with few exceptions, the maximum yield of alpha hemolysin occurred within 48 hours. At 40°C the optimal incubation period was from 24 to 48 hours.

With the exception of trypticase soy broth, all other media tested were found quite suitable for the production of alpha hemolysin. Increasing sodium chloride concentration to 1.5% in the heart infusion broth produced an adverse affect on alpha hemolysin production without having any ill effect on growth (fig. 3).

Out of the three incubation temperatures investigated, 37°C was found to give the highest alpha toxin titers under increased aeration and CO₂ (fig. 4). Veal infusion and heart infusion broths produced higher toxin yields at 34°C than at 40°C.

2. Maximum yield beta toxin—conditions for. The relationship of bacterial growth to the production of beta hemolysin by *S. aureus* strain 570 is shown in fig. 2. During the early part of the log phase, the production of beta hemolysin was slower than the growth. The toxin production subsequently increased at a faster rate and continued after growth had stopped. The influence of different aeration, temperatures, and incubation periods on the production of beta hemolysin is illustrated in table 3. The results obtained were similar to those obtained for the production of alpha hemolysin.

The maximum yield of beta hemolysin was obtained
Table 2

Optimal conditions for the production of maximum
alpha hemolysin titers.

<table>
<thead>
<tr>
<th>Incubation temperature</th>
<th>Aeration</th>
<th>Medium (**)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ext. broth</td>
<td>T.S.B.</td>
</tr>
<tr>
<td>37 °C</td>
<td>stationary</td>
<td>512</td>
</tr>
<tr>
<td></td>
<td>on shaker</td>
<td>1024</td>
</tr>
<tr>
<td></td>
<td>on shaker with 50% CO₂ &amp; 50% O₂</td>
<td>4096</td>
</tr>
<tr>
<td>40 °C</td>
<td>stationary</td>
<td>128</td>
</tr>
<tr>
<td></td>
<td>on shaker</td>
<td>1024</td>
</tr>
<tr>
<td></td>
<td>on shaker with 50% CO₂ &amp; 50% O₂</td>
<td>2048</td>
</tr>
<tr>
<td>34 °C</td>
<td>stationary</td>
<td>128</td>
</tr>
<tr>
<td></td>
<td>on shaker</td>
<td>512</td>
</tr>
<tr>
<td></td>
<td>on shaker with 50% CO₂ &amp; 50% O₂</td>
<td>2048</td>
</tr>
</tbody>
</table>

T.S.B. = trypticase soy broth.
V.I.B. = veal infusion broth.
H.I.B. = heart infusion broth.
N.Z.A. = N.Z. amine broth.
* Expressed as reciprocal of the highest dilution showing complete hemolysis of 1% rabbit red blood cells.
** Incubation time in hours showing maximum yield of alpha hemolysin.
**Fig. 3. Effect of increased NaCl concentration on alpha hemolysin.**

**Explanation:**
- **Heart Infusion Broth (0.5% NaCl):**
  - Culture under static condition
  - Culture on shaker
  - Culture on shaker under 50% CO₂ and 50% Oxygen

- **Heart Infusion Broth (1.5% NaCl):**
  - Culture under static condition
  - Culture on shaker
  - Culture on shaker under 50% CO₂ and 50% Oxygen
Fig. 4. Alpha hemolysin at 37 C on shaker under 50% CO₂ and 50% oxygen.
Table 3

Optimal conditions for the production of maximum beta hemolysin titers.

<table>
<thead>
<tr>
<th>Incubation temperature</th>
<th>Aeration</th>
<th>Medium (**)</th>
<th>Ext. broth</th>
<th>T.S.B.</th>
<th>V.I.B.</th>
<th>H.I.B.</th>
<th>N.Z.A.</th>
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</thead>
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<tr>
<td>37°C</td>
<td>stationary</td>
<td>64(\frac{1}{72})</td>
<td>16(\frac{1}{72})</td>
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<td>64(\frac{1}{72})</td>
<td>64(\frac{1}{72})</td>
<td></td>
</tr>
<tr>
<td></td>
<td>on shaker</td>
<td>128(\frac{1}{48-72})</td>
<td>64(\frac{1}{48-72})</td>
<td>256(\frac{1}{72})</td>
<td>256(\frac{1}{72})</td>
<td>512(\frac{1}{72})</td>
<td></td>
</tr>
<tr>
<td></td>
<td>with 50%</td>
<td>1024(\frac{1}{72})</td>
<td>64(\frac{1}{72})</td>
<td>1024(\frac{1}{72})</td>
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<tr>
<td></td>
<td>CO(_2) &amp; 50% O(_2)</td>
<td>72(\frac{1}{24-72})</td>
<td>24(\frac{1}{48-72})</td>
<td>72(\frac{1}{48-72})</td>
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<td></td>
<td>on shaker</td>
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<td>with 50%</td>
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<td>512(\frac{1}{48-72})</td>
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<tr>
<td></td>
<td>CO(_2) &amp; 50% O(_2)</td>
<td>72(\frac{1}{24-72})</td>
<td>24(\frac{1}{48-72})</td>
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<td>32(\frac{1}{72})</td>
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<td>on shaker</td>
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<td>64(\frac{24-72}{48-72})</td>
<td>128(\frac{24-72}{48-72})</td>
<td>64(\frac{24-72}{48-72})</td>
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<td>256(\frac{24-72}{48-72})</td>
<td>128(\frac{24-72}{48-72})</td>
<td>512(\frac{24-72}{48-72})</td>
<td>512(\frac{24-72}{48-72})</td>
<td>512(\frac{24-72}{48-72})</td>
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<tr>
<td></td>
<td>CO(_2) &amp; 50% O(_2)</td>
<td>72(\frac{24-72}{48-72})</td>
<td>24(\frac{24-72}{48-72})</td>
<td>72(\frac{24-72}{48-72})</td>
<td>72(\frac{24-72}{48-72})</td>
<td>72(\frac{24-72}{48-72})</td>
<td></td>
</tr>
</tbody>
</table>

T.S.B. = trypsinase soy broth.
V.I.B. = veal infusion broth.
H.I.B. = heart infusion broth.
N.Z.A. = N.Z. amine broth.
* = Expressed as reciprocal of the highest dilution showing complete hemolysis of 1% sheep red blood cells.
** = Incubation time in hours showing maximum yield of beta hemolysin.
after 72 hours of incubation at 37 °C under continuous shaking conditions with 50% CO₂ and 50% O₂ (fig. 5).

3. **Maximum yield coagulase—conditions for.** The optimal conditions for the production of coagulase differed in certain respects from hemolysins. The relationship of coagulase production with growth is shown in fig. 2. The coagulase production has a lag phase similar to the growth lag phase. The yield decreased on prolonged incubation and depended largely upon the type of media employed and aeration conditions.

The relationship of coagulase titers with varying media, incubation periods, temperatures, and aeration conditions is shown in table 4 and fig. 6.

In general, the optimum period for coagulase production under static conditions was found to be 24 hours; on shaker 12 hours; and on shaker under 50% CO₂ and 50% O₂ only 8 hours. It is indicated that the variation of incubation temperatures did not affect the coagulase production. The highest coagulase titer i.e., 1:8192 was obtained at all the three incubation temperatures. With regard to the suitability of media, both extract broth and the N.Z. amine broth gave maximum coagulase yield. The other two media i.e., veal infusion broth and heart infusion broth were slightly inferior for coagulase production. With some exceptions in N.Z. amine medium, higher coagulase yields were obtained in the atmospheric gases.

A prolonged incubation period was found to have
Fig. 5. Beta hemolysin on shaker under 50% CO₂ and 50% oxygen at 37 C.
Table 4

Optimal conditions for the production of maximum coagulase titers.

<table>
<thead>
<tr>
<th>Incubation temperature</th>
<th>Aeration</th>
<th>Medium (**)</th>
<th>Ext. broth</th>
<th>V.I.B.</th>
<th>H.I.B.</th>
<th>N.Z.A.</th>
</tr>
</thead>
<tbody>
<tr>
<td>37 C</td>
<td>stationary</td>
<td>2048</td>
<td>1024</td>
<td>512</td>
<td>1024</td>
<td></td>
</tr>
<tr>
<td></td>
<td>on shaker</td>
<td>12-24</td>
<td>12-24</td>
<td>12-24</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td></td>
<td>on shaker with 50% CO₂ &amp; 50% O₂</td>
<td>8-12</td>
<td>8-16</td>
<td>8-12</td>
<td>2048</td>
<td></td>
</tr>
</tbody>
</table>

| 40 C                   | stationary     | 2048        | 1024       | 512    | 1024   |
|                        | on shaker      | 12-24       | 12-24      | 12-24  | 16     |
|                        | on shaker with 50% CO₂ & 50% O₂ | 8-12 | 8-16 | 8-12 | 2048 |

| 34 C                   | stationary     | 2048        | 1024       | 512    | 1024   |
|                        | on shaker      | 12-24       | 12-24      | 12-24  | 16     |
|                        | on shaker with 50% CO₂ & 50% O₂ | 8-12 | 8-16 | 8-12 | 2048 |

V.I.B. = veal infusion broth.
H.I.B. = heart infusion broth.
N.Z.A. = N.Z. amine broth.

* = Minimum clotting doses/ml expressed as the reciprocal of the highest dilution showing minimum amount of clot (+) of 20% human plasma.

** = Incubation time in hours showing maximum yield of coagulase.
Temperature of cultures (°C).

- ○ Extract broth.
- △ Veal infusion broth.
- □ Heart infusion broth.
- • N.Z.Amine broth.

Fig. 6. Coagulase production in different media under different aeration and temperature conditions.
deleterious effects on the total yield of coagulase (fig. 2). The destruction began at 16 to 24 hours of incubation on the shaker under 50% CO₂ and 50% O₂; at 24 to 48 hours on the shaker under atmospheric gases; and approximately at 72 hours in the cultures grown without shaking. The destruction was more marked at 4°C than at 37°C or 32°C.

The relationships concerning the production of coagulase, alpha and beta hemolysins at various incubation periods are illustrated in fig. 7.

B. INACTIVATING AGENTS

Formalin is frequently used as an inactivating agent for the preparation of various types of bacterial vaccines. A preliminary attempt was made to investigate whether this chemical in the strengths commonly used for vaccine preparations has any destructive effect on the antigens of staphylococci. *S. aureus* culture filtrates before and after detoxification were tested for antigenic analyses by using gel diffusion technique. The sera for precipitation reactions were available from two cows with experimentally produced staphylococcal mastitis infection. The gel precipitation results are illustrated in fig. 8.

The reaction shown in the left half of the photograph indicates that the number of precipitation lines between serum A and the culture filtrates 1 and 4 is greater at least by one than the lines depicted by their toxoids 5 and 6 respectively. The reactions between serum B and the
Fig. 7. Relationship of alpha and beta hemolysins and coagulase at 37 C, in N.Z. Amine broth culture grown on shaker under 50% CO₂ and 50% oxygen.
Fig. 8. Double diffusion analyses of *S. aureus* toxins before and after inactivation with formalin. Central wells A and B contained experimentally infected cow sera. In the left gel diffusion system peripheral wells 1 and 4 contained toxin before formalin treatment; well 5, concentrated toxoid from Cyanamid Co.; well 6, U.N.H. prepared concentrated toxoid and wells 2 and 3 filtrates from U.N.H. concentrated toxoid. In the right gel diffusion system wells 1 and 4 represented untreated toxin; wells 2 and 3 formalin inactivated concentrated toxoid and wells 5 and 6 filtrates from concentrated toxoid.
filtrates 1 and 4 on the one hand and their toxoids 2 and 3 on the other hand do not, however, reveal any difference in the number of precipitation lines.

**Inactivation of cells.** Various means of inactivating the bacterial cells were studied to find most suitable means of inactivation for vaccine preparation. **S. aureus** cells were treated in five different ways, namely with formalin, beta-propiolactone, lysed by dornase or bacteriophage, and mechanically disintegrated. The means of evaluation of the antisera were quantitative (by titrating the antibodies), qualitative (by immunodiffusion), and protection of the vaccinated animals against a lethal dose of challenge organisms.

The agglutinin and alpha antitoxin titers of the sera obtained from rabbits immunized against various vaccines are shown in table 5.

The agglutinin titers varied from animal to animal within the same group and the mean differed from one group to another. In general, disrupted and lysed cell vaccines gave the highest agglutinin titers. Disintegrated and phage lysed cell vaccines stimulated low alpha antitoxin titers, although former vaccine did not contain any amount of alpha toxin.

For qualitative antigenic analyses by immunodiffusion technique, all the five antisera were reacted against each of the three vaccines containing disrupted or lysed cell antigens. The reactions showing precipitation lines
Table 5

Agglutinin and alpha antitoxin titers in rabbits vaccinated against differently treated cell vaccines.

<table>
<thead>
<tr>
<th>Vaccines</th>
<th>Rabbit numbers</th>
<th>Alpha antitoxin titers (I.U./ml)</th>
<th>Reciprocal of homologous agglutinin reciprocal of titers against agglutinin strain</th>
</tr>
</thead>
<tbody>
<tr>
<td>V-1</td>
<td>1</td>
<td>1</td>
<td>256</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>2</td>
<td>512</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>-</td>
<td>512</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>-</td>
<td>256</td>
</tr>
<tr>
<td>V-2</td>
<td>5</td>
<td>-</td>
<td>512</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>-</td>
<td>256</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>-</td>
<td>256</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>-</td>
<td>256</td>
</tr>
<tr>
<td>V-3</td>
<td>9</td>
<td>-</td>
<td>1024</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>-</td>
<td>512</td>
</tr>
<tr>
<td>V-4</td>
<td>11</td>
<td>2</td>
<td>1024</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>4</td>
<td>512</td>
</tr>
<tr>
<td></td>
<td>13</td>
<td>2</td>
<td>1024</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>3</td>
<td>1024</td>
</tr>
<tr>
<td>V-5</td>
<td>15</td>
<td>5</td>
<td>512</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>4</td>
<td>512</td>
</tr>
</tbody>
</table>

V-1 = Formalinized cells.
V-2 = Beta-propiolactone treated cells.
V-3 = Phenolysed-heat killed domnase lysed cells.
V-4 = Disintegrated cells.
V-5 = Phage lysed cells.
are illustrated in fig. 9.

It is presumed that each precipitation line is represented by a separate antigenic determinant and antibody reaction. The reactions shown in fig. 9 are identified by different lines in fig. 10.

The disrupted and lysed cell antigens produced better response to precipitating antibodies than the whole cell antigens. The specificity of precipitation line 8 shown by phage lysed cells (V-5) and the serum against this vaccine (S-5) could not be ascertained with certainty. There is some possibility, however, that the lines 8 and 6 may be one and the same.

Ten days following the last injection of the vaccine preparations, all 16 vaccinated and 2 control rabbits were challenged intravenously with 1 ml of 18 hour trypticase soy broth culture of S. aureus strain 7 (3.1 x 10^9 cells/ml). The rabbits were observed up to two weeks for mortality. The results are recorded in table 6.

Only 3 out of 16 vaccinated rabbits survived the challenge dose. Although the number of rabbits used in the experiment was small and no definite conclusion can be derived from protection studies, formalinized whole cell vaccine gave better protection than the other vaccines tested.

Inactivation of toxin. The comparison of formalin and beta-propiolactone as toxoiding agents was made by vaccinating two groups of rabbits with formalinized cell toxoid (PV-6) and beta-propiolactone treated cell toxoid (PV-7)
Fig. 9. Photograph of drawings showing precipitation lines in antisera S-1 to S-5 against vaccine V-3, V-4, V-5 and V-5 by the Ouchterlonny gel agar plate method.
<table>
<thead>
<tr>
<th>Antiserum</th>
<th>Cell antigens represented in vaccines</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>V-3</td>
</tr>
<tr>
<td>S-1</td>
<td>1—</td>
</tr>
<tr>
<td></td>
<td>2—</td>
</tr>
<tr>
<td>S-2</td>
<td>1—</td>
</tr>
<tr>
<td></td>
<td>2—</td>
</tr>
<tr>
<td>S-3</td>
<td>1—</td>
</tr>
<tr>
<td></td>
<td>2—</td>
</tr>
<tr>
<td></td>
<td>3—</td>
</tr>
<tr>
<td></td>
<td>4—</td>
</tr>
<tr>
<td>S-4</td>
<td>1—</td>
</tr>
<tr>
<td></td>
<td>2—</td>
</tr>
<tr>
<td></td>
<td>3—</td>
</tr>
<tr>
<td></td>
<td>4—</td>
</tr>
<tr>
<td></td>
<td>5—</td>
</tr>
<tr>
<td></td>
<td>7—</td>
</tr>
<tr>
<td>S-5</td>
<td>1—</td>
</tr>
<tr>
<td></td>
<td>2—</td>
</tr>
<tr>
<td></td>
<td>3—</td>
</tr>
<tr>
<td></td>
<td>4—</td>
</tr>
<tr>
<td></td>
<td>5—</td>
</tr>
</tbody>
</table>

The key of the vaccines V-3, V-4, and V-5 is the same as explained in Table 5. S-1, S-2, S-3, S-4, and S-5 are antisera against vaccines V-1, V-2, V-3, V-4, and V-5 respectively.

Figure 10. Results of comparative precipitation reaction by Ouchterlony method when 5 different treated S. parasus cell antigens were separately reacted with 5 rabbit antisera.
Table 6  
Rabbits showing mortality after the challenge dose of 1 ml 18 hour trypsinase soy broth culture.

<table>
<thead>
<tr>
<th>Vaccine</th>
<th>No. of rabbits</th>
<th>Days to death</th>
<th>Survival/No. challenged</th>
</tr>
</thead>
<tbody>
<tr>
<td>V-1</td>
<td>4</td>
<td>2,6</td>
<td>2/4</td>
</tr>
<tr>
<td>V-2</td>
<td>4</td>
<td>1,1,1,4</td>
<td>0/4</td>
</tr>
<tr>
<td>V-3</td>
<td>2</td>
<td>1</td>
<td>1/2</td>
</tr>
<tr>
<td>V-4</td>
<td>4</td>
<td>1,1,3,3,</td>
<td>0/4</td>
</tr>
<tr>
<td>V-5</td>
<td>2</td>
<td>4,5</td>
<td>0/2</td>
</tr>
<tr>
<td>control</td>
<td>2</td>
<td>1,4</td>
<td>0/2</td>
</tr>
</tbody>
</table>

The key to vaccines V-1 to V-5 is the same as shown in Table 5.

Vaccines. Blood sera were collected at weekly intervals from these animals and titrated for agglutinins and alpha and beta antitoxins. Figure 11 illustrates the mean values obtained for alpha and beta antitoxins. Beta-propiolactone treated cell toxoid stimulated significantly higher alpha antitoxin than formalinized cell toxoid. There was no appreciable difference in the beta antitoxin levels. With beta-propiolactone treated cell toxoid, highest alpha and beta antitoxin levels were reached in about three weeks. Beta antitoxin dropped slightly during the fourth and fifth weeks and considerably so during the sixth week. The reduction in the titers of alpha antitoxin was very significant during all three i.e., the fourth, fifth, and sixth
Fig. 11. Average units of alpha and beta antitoxins in rabbits immunized with formalinized and beta propiolactone treated disintegrated cell toxoids.
weeks. In the case of formalinized cell toxoid, the increase of antitoxins was very gradual reaching maximum at the end of five weeks. The drop during the sixth week was very sudden and sharp. At the end of four weeks, the reciprocal of the means of agglutination titers against formalinized and beta-propiolactone treated cell toxoids were 717 and 704 respectively.

All vaccinated and two control rabbits were challenged intravenously \((3.1 \times 10^9\) cells) six weeks after the first injection. There was no protection and none of the rabbits was able to resist this challenge dose. All rabbits died within six days of the challenge injection.

C. IMMUNE AND ANTIBODY RESPONSE IN RABBITS AND COWS TO VACCINES CONTAINING S. AUREUS CELL TOXOIDS

1. Vaccination in rabbits. Five groups of six rabbits each were vaccinated with polyvalent vaccines PV-8, PV-9, PV-10, PV-11, and PV-12. Vaccines PV-8 and PV-9 were prepared by treating the whole broth cultures with formalin and beta-propiolactone respectively. The cells of vaccines PV-10, PV-11, and PV-12 were inactivated with formalin and the culture filtrates with beta-propiolactone. Vaccine PV-11 contained low coagulase and high beta toxins, whereas in vaccine PV-12 the potency of beta toxin was low and that of coagulase was high. All vaccines contained about the same concentration of alpha toxin. Blood sera were collected one week after the last injection and titrated for agglutinins, alpha and beta antitoxins, anticoagulase, and anti P.V.
Figure 12 illustrates the mean values of various types of antibodies in the sera prepared against different vaccines. In all groups the highest agglutinin titers were obtained against strain Ger, followed by strains 7, 570, and 15 in that order. Strain 15 stimulates extremely low agglutinin titers in all the animals.

With the exception of antileucocidin, highest antibodies were obtained in antiserum PS-9 (serum against vaccine PV-9). Antiserum PS-12 (serum against vaccine PV-12) gave highest antileucocidin titers. Beta antitoxin and anticoagulase titers were not significantly different in the vaccinated animals. Higher antitoxin in the rabbits against beta-propiolactone treated vaccine (PV-9) than against formalinized vaccine (PV-8) confirmed the results of previous experiments. Rabbits belonging to various vaccine groups showed considerable difference in alpha antitoxin and antileucocidin (table 7).

All the experimental rabbits were challenged on the tenth day following the last injection of the vaccine preparations. Three animals from each group were challenged with one of the homologous strains (strain 7) and the other three with one heterologous strain (strain 1531). Three control rabbits were also challenged with each strain. The animals were observed for a period of four weeks. The results are recorded in table 8.

According to the results shown in table 8, vaccine
### Fig. 12. The means of various types of antibodies in different group sera.

<table>
<thead>
<tr>
<th>Anti Serum</th>
<th>Anti α hemolysin I.U./ml.</th>
<th>Anti β hemolysin I.U./ml.</th>
</tr>
</thead>
<tbody>
<tr>
<td>PS-8</td>
<td>3925</td>
<td>24</td>
</tr>
<tr>
<td>PS-9</td>
<td>6827</td>
<td>144</td>
</tr>
<tr>
<td>PS-10</td>
<td>8533</td>
<td>293</td>
</tr>
<tr>
<td>PS-11</td>
<td>9517</td>
<td>275</td>
</tr>
<tr>
<td>PS-12</td>
<td>10923</td>
<td>341</td>
</tr>
</tbody>
</table>

A, B, C, and D indicate titers against strains 7, 15, 570 and Ger respectively.
Table 7

Rabbits vaccinated with various vaccines showing the mean, standard deviation (S.D.), and the range of alpha antitoxin and antileucocidin.

<table>
<thead>
<tr>
<th>Rabbits in vaccine group</th>
<th>Alpha antitoxin I.U./ml</th>
<th>Reciprocal of antileucocidin titers/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>S.D.</td>
</tr>
<tr>
<td>PV-8</td>
<td>5.2</td>
<td>5.4</td>
</tr>
<tr>
<td>PV-9</td>
<td>9.7</td>
<td>14.4</td>
</tr>
<tr>
<td>PV-10</td>
<td>5.2</td>
<td>9.4</td>
</tr>
<tr>
<td>PV-11</td>
<td>2.8</td>
<td>2.8</td>
</tr>
<tr>
<td>PV-12</td>
<td>5.5</td>
<td>4.0</td>
</tr>
</tbody>
</table>

PV-12 gave the best protection against both organisms. It appears from the protection results that antibody against leucocidin plays an important part in the protection. Other types of antibodies may be helping.

2. Vaccination in cows. In a group of four experimental cows, an attempt was made to demonstrate the effect of adjuvant in the vaccine. Two cows were vaccinated with an adjuvant cell toxoid vaccine (MV-B). The other two cows served as controls and received injections of the same vaccine without any adjuvant (MV-A). The first and the second injections of 10 ml each were given at an interval of four weeks and the serum samples were collected every week for antibody titrations. The antibody response
Table 8

Rabbit's survival after intravenous challenge with homologous and heterologous strains.

<table>
<thead>
<tr>
<th>Vaccine</th>
<th>Days to death</th>
<th>Survival/No.challenged</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Homologous strain</td>
<td>Heterologous strain</td>
</tr>
<tr>
<td>PV-8</td>
<td>5,16,22*</td>
<td>4,11</td>
</tr>
<tr>
<td>PV-9</td>
<td>21; 21;*</td>
<td>3,16,21;*</td>
</tr>
<tr>
<td>PV-10</td>
<td>10,22*</td>
<td>22*</td>
</tr>
<tr>
<td>PV-11</td>
<td>2,12</td>
<td>21;<em>, 21;</em></td>
</tr>
<tr>
<td>PV-12</td>
<td>5</td>
<td>22*</td>
</tr>
<tr>
<td>control</td>
<td>6,12,24</td>
<td>2,4</td>
</tr>
</tbody>
</table>

* Rabbits were sacrificed because of high emaciation and arthritis.

indicates that from the second week onward, the level of alpha and beta antitoxins** was considerably higher in the cows vaccinated with adjuvant vaccine (fig. 13). The titers of antileuococcidin were also much higher during the third and the fourth weeks of the test. The booster injection of the adjuvant vaccine gave greater response of beta antitoxin than the plain vaccine. At the end of four weeks, the mean of the agglutinin titer in the cows immunized with adjuvant vaccine was found to be 1:3000 in comparison to only 1:480 titer shown by the other group. This is a significant

** Alpha and beta antitoxins were kindly determined by Dr. Clara H. Bartley.
Weeks after vaccination

<table>
<thead>
<tr>
<th>Weeks after vaccination</th>
<th>Vaccine without adjuvant</th>
<th>Vaccine with adjuvant</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

I.U. of Alpha and Beta antitoxins and reciprocal of Antileucocidin titer.

Fig. 13. Cows showing the means of Alpha and Beta antitoxins and Antileucocidin in response to vaccines with adjuvant and without adjuvant.
difference.

In another experiment, 15 cows were equally distributed into three groups and vaccinated with three types of polyvalent vaccines, each group receiving one type of vaccine.* The vaccines used were beta-propiolactone treated veal infusion whole broth culture (PV-A), beta-propiolactone treated concentrated veal infusion whole broth culture (PV-B), and formalin treated concentrated N.Z. amine whole broth culture (PV-C). All cows received two injections of 5 ml each at an interval of two weeks. Blood sera were collected on the 10th and 30th day after the first injection and titrated for agglutinins, alpha and beta antitoxins,** anticoagulase, anti P.V. leucocidin, and mouse protection tests.

The antibody responses are listed in table 9, which shows that different antibodies varied from one animal to another within the same group and also from one group to another.

The agglutinin titers in the blood of each cow is shown in fig. 14. Strain 15 again stimulated far less agglutinin titers than the other three vaccine strains. Some animals gave poor antibody response to all vaccine strains. The means of the agglutinin titers in the cows

* The vaccination was done by Dr. F.E. Allen.

** Alpha and beta antitoxins were kindly determined by Dr. Clara H. Bartley.
Table 9

Antibody titers in cows before and after vaccination.

<table>
<thead>
<tr>
<th>Cows</th>
<th>Alpha anti-A. toxin I.U./ml</th>
<th>Beta anti-B. toxin I.U./ml</th>
<th>Antileucocidin/ml*</th>
<th>Anticoag- ulase/ml**</th>
<th>F factor</th>
<th>S factor</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>30</td>
<td>0</td>
<td>30</td>
<td>0</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>day</td>
<td>day</td>
<td>day</td>
<td>days</td>
<td>day</td>
<td>days</td>
</tr>
<tr>
<td>Vaccine</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PV-A</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clyde</td>
<td>0</td>
<td>18</td>
<td>0</td>
<td>18</td>
<td>16</td>
<td>256</td>
</tr>
<tr>
<td>Ida</td>
<td>12</td>
<td>24</td>
<td>0</td>
<td>48</td>
<td>64</td>
<td>96</td>
</tr>
<tr>
<td>Sweetie</td>
<td>8</td>
<td>50</td>
<td>6</td>
<td>20</td>
<td>2</td>
<td>48</td>
</tr>
<tr>
<td>Sue</td>
<td>4</td>
<td>24</td>
<td>0</td>
<td>56</td>
<td>3</td>
<td>64</td>
</tr>
<tr>
<td>Kay</td>
<td>1</td>
<td>6</td>
<td>4</td>
<td>24</td>
<td>3</td>
<td>32</td>
</tr>
<tr>
<td>Means</td>
<td>5</td>
<td>30.4</td>
<td>2</td>
<td>36.2</td>
<td>17.6</td>
<td>99.2</td>
</tr>
<tr>
<td>Vaccine</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PV-B</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Opal</td>
<td>1</td>
<td>24</td>
<td>0</td>
<td>24</td>
<td>-</td>
<td>64</td>
</tr>
<tr>
<td>Maud</td>
<td>3</td>
<td>24</td>
<td>0</td>
<td>48</td>
<td>96</td>
<td>128</td>
</tr>
<tr>
<td>Celia</td>
<td>3</td>
<td>48</td>
<td>0</td>
<td>14</td>
<td>32</td>
<td>32</td>
</tr>
<tr>
<td>Lucy</td>
<td>12</td>
<td>24</td>
<td>0</td>
<td>50</td>
<td>4</td>
<td>512</td>
</tr>
<tr>
<td>Grace</td>
<td>0</td>
<td>24</td>
<td>0</td>
<td>50</td>
<td>-</td>
<td>128</td>
</tr>
<tr>
<td>Means</td>
<td>3.8</td>
<td>28.8</td>
<td>0</td>
<td>37.2</td>
<td>44</td>
<td>173</td>
</tr>
<tr>
<td>Vaccine</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PV-C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hazel</td>
<td>0</td>
<td>30</td>
<td>0</td>
<td>50</td>
<td>3</td>
<td>64</td>
</tr>
<tr>
<td>Quid</td>
<td>8</td>
<td>96</td>
<td>12</td>
<td>40</td>
<td>128</td>
<td>2048</td>
</tr>
<tr>
<td>Rose</td>
<td>0</td>
<td>50</td>
<td>4</td>
<td>96</td>
<td>2</td>
<td>4096</td>
</tr>
<tr>
<td>Judy</td>
<td>12</td>
<td>96</td>
<td>6</td>
<td>180</td>
<td>3</td>
<td>1024</td>
</tr>
<tr>
<td>Pat</td>
<td>0</td>
<td>96</td>
<td>0</td>
<td>48</td>
<td>1</td>
<td>768</td>
</tr>
<tr>
<td>Means</td>
<td>4</td>
<td>74</td>
<td>4.4</td>
<td>83</td>
<td>27</td>
<td>1600</td>
</tr>
</tbody>
</table>

* Reciprocal of highest serum dilution inhibiting 2 M.C.D.

** Reciprocal of highest serum dilution inhibiting 4 L+ dose of leucocidin.
Normal agglutinins □ Agglutinins in response to vaccination.

Fig. 1. Cows showing comparative agglutinin titers against different S. aureus strains included in the vaccines.

1, 2, 3, and 4 indicate reactions with strains 7, 15, 570 and Ger respectively.
of all the three vaccine groups are listed in table 10. The cows inoculated with vaccine PV-C produced higher agglutinin titers than other groups.

**Table 10**

Cows showing the means of agglutinin titers with different vaccine strains.

<table>
<thead>
<tr>
<th>Vaccine groups</th>
<th>Means of agglutinin titers*</th>
<th>Means of the means</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Strain 7</td>
<td>Strain 15</td>
</tr>
<tr>
<td>PV-A</td>
<td>998.2</td>
<td>134.4</td>
</tr>
<tr>
<td>PV-B</td>
<td>780.8</td>
<td>320</td>
</tr>
<tr>
<td>PV-C</td>
<td>1177.6</td>
<td>147.2</td>
</tr>
</tbody>
</table>

* All titers are expressed as reciprocal of the serum dilutions.

It is evident from the results shown in table 9 that the level of anticoagulase, in general, is poor in most of the cows. The response of alpha and beta antitoxins at different intervals is illustrated in fig. 15. The cows vaccinated with vaccine PV-C had considerably higher response of both types of antitoxins. The animals of the other two vaccine groups did not demonstrate much difference in either of the two antitoxin titers. The beta antitoxin levels were somewhat greater than alpha antitoxin levels.

Among various types of antibodies, anti F and S leucocidin (fig. 16) gave strikingly high titers. Their response was very high in the cows of vaccine group PV-C.
I. U. of Alpha and Beta antitoxins per ml.

- Alpha antitoxin against PV-A
- Beta antitoxin against PV-A
- Alpha antitoxin against PV-B
- Beta antitoxin against PV-B
- Alpha antitoxin against PV-C
- Beta antitoxin against PV-C

First and second injections

Fig 15. Means of alpha and beta antitoxin levels in the serum of cows injected with Vaccines PV-A, PV-B, and PV-C.
Fig. 16. Tanned sheep RBC sensitized with leucocidin (F and S components) showing hemagglutination sedimentation pattern in the sera of vaccinated cows.

Top row: S component absorbed; serial dilutions of a cow serum increasing towards the right.
Second row: F component absorbed; serial dilutions of the serum from the same cow increasing towards the right.
Third row: S component absorbed; serial dilutions of serum from a second cow increasing towards the right.
Bottom row: F component absorbed; serial dilutions of serum from the second cow increasing towards the right.

Last two tubes on the right of the bottom row are tanned RBC absorbed with F and S components showing negative reaction with normal cow serum.
and comparatively poor in PV-A and PV-B groups. It is evident from the results that prevaccination antileucocidin titers were higher in two cows (Quid and Maud). It can also be noticed from the results that the response of anti S leucocidin was always higher than anti F leucocidin.

The relationship of various types of antibodies within and outside the various vaccine groups is depicted by fig. 17. The illustration shows that the titers of alpha and beta antitoxins and anti F and S leucocidins were considerably higher in the cows vaccinated with vaccine PV-C than other groups.

The results of mouse protection test listed in table 11 shows considerable difference in the protection capacity of various sera against a lethal challenge dose. The results were calculated (see appendix) in terms of 50% protection following the Reed and Muench (1938) method. The results indicate that the sera from the cows vaccinated with vaccine PV-C showed better mouse protecting power followed by the cows of vaccine groups PV-A and PV-B respectively. The cows in the PV-C vaccine group also showed higher levels of other types of antibodies indicating some correlation. The difference is particularly significant for antileucocidin levels suggesting some mouse protective role of this antibody. It is also evident from the results that the serum of certain cows before vaccination showed some mouse protection power. One cow in particular belonging to vaccine group PV-A considerably raised the mean value
Fig. 17. Vaccinated cows showing the means of various types of antibodies before intramammary challenge.
Table 11

Mouse protecting antibody in the sera of cows before and after vaccination.

<table>
<thead>
<tr>
<th>Cows</th>
<th>Mouse protecting antibody*</th>
<th>Homologous strain</th>
<th>Heterologous strain</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0 day 15 days**</td>
<td>0 day 15 days</td>
</tr>
<tr>
<td>Vaccine PV-A</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clyde</td>
<td>3</td>
<td>194</td>
<td>4</td>
</tr>
<tr>
<td>Ida</td>
<td>128</td>
<td>194</td>
<td>49</td>
</tr>
<tr>
<td>Sweetie</td>
<td>11</td>
<td>64</td>
<td>8</td>
</tr>
<tr>
<td>Sue</td>
<td>8</td>
<td>64</td>
<td>12</td>
</tr>
<tr>
<td>Kay</td>
<td>8</td>
<td>97</td>
<td>8</td>
</tr>
<tr>
<td>Means</td>
<td>32</td>
<td>123</td>
<td>16</td>
</tr>
<tr>
<td>Vaccine PV-B</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Opal</td>
<td>8</td>
<td>49</td>
<td>8</td>
</tr>
<tr>
<td>Maud</td>
<td>4</td>
<td>64</td>
<td>0</td>
</tr>
<tr>
<td>Celia</td>
<td>16</td>
<td>64</td>
<td>2</td>
</tr>
<tr>
<td>Lucy</td>
<td>8</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>Grace</td>
<td>4</td>
<td>8</td>
<td>2</td>
</tr>
<tr>
<td>Means</td>
<td>8</td>
<td>39</td>
<td>2</td>
</tr>
<tr>
<td>Vaccine PV-C</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hazel</td>
<td>4</td>
<td>16</td>
<td>4</td>
</tr>
<tr>
<td>Quid</td>
<td>8</td>
<td>388</td>
<td>4</td>
</tr>
<tr>
<td>Rose</td>
<td>4</td>
<td>21</td>
<td>0</td>
</tr>
<tr>
<td>Judy</td>
<td>21</td>
<td>388</td>
<td>0</td>
</tr>
<tr>
<td>Pat</td>
<td>4</td>
<td>64</td>
<td>4</td>
</tr>
<tr>
<td>Means</td>
<td>8</td>
<td>175</td>
<td>2</td>
</tr>
</tbody>
</table>

* Reciprocal of the highest serum dilution in 0.5 ml protecting 50% of the mice against challenge.

** Serum tested 15 days following the second injection of vaccine.
of prevaccination sera of this group. The protection was slightly higher against homologous than heterologous strain, which may have been due to some antigenic difference between these strains.

About two weeks after the second vaccine injection, the cows were challenged with strain 570 (homologous) and strain 10 (heterologous). Each strain was injected into one quarter of all the experimental and five control cows. The challenge results are shown in Table 12.*

Table 12

Immune response in cows following intramammary challenge.

<table>
<thead>
<tr>
<th>Cows vaccinated with</th>
<th>Strain 570</th>
<th>Strain 10</th>
<th>Strain 570</th>
<th>Strain 10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vaccine PV-A</td>
<td>4/5</td>
<td>4/5</td>
<td>1 severe</td>
<td>1 moderate</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1 moderate</td>
<td>___</td>
</tr>
<tr>
<td>Vaccine PV-B</td>
<td>5/5</td>
<td>4/5</td>
<td>1 moderate</td>
<td>1 slight</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2 slight</td>
<td></td>
</tr>
<tr>
<td>Vaccine PV-C</td>
<td>3/5</td>
<td>2/5</td>
<td>1 slight</td>
<td>1 slight</td>
</tr>
<tr>
<td>Control cows</td>
<td>5/5</td>
<td>5/5</td>
<td>3 severe***</td>
<td>2 slight</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1 moderate</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1 slight</td>
<td></td>
</tr>
</tbody>
</table>

** Numerator indicates the number of quarters infected and denominator number of quarters challenged.

*** Two animals developed severe gangrenous mastitis and had to be sacrificed.

* Reproduced through the courtesy of Dr. L.W. Slanetz from the talk which he presented on Dec. 4 & 5, 1963 in the mastitis research workers conference in Chicago.
The table shows that vaccine PV-C gave better protection both in the number of infected quarters and clinical reactions. It is evident from fig. 17 that vaccine PV-C stimulated higher antibody response. The difference is particularly striking with regard to alpha and beta antitoxins and antileucocidin. By analysing the results statistically (see appendix), there was no significant difference in the three groups in the titers of alpha and beta antitoxins. On the other hand, the response of anti F and S leucocidins was significantly higher in the cows vaccinated with vaccine PV-C than in the animals of the other two groups. A good correlation between the intramammary challenge results and the mouse protection power of cow sera was observed among the three vaccine groups.

All the serum samples from the experimental cows were tested with purified F and S factors of leucocidin by the slide immunodiffusion test (fig. 18). It was found that only those sera which had titers of 1:500 or over were able to show precipitation lines. Most of these cows belonged to vaccine group PV-C.
Fig. 18. Immunodiffusion reaction in cow and rabbit sera against F and S components of leucocidin. The center wells 1 and 3 contained F component and 2 and 4 contained S components. The peripheral wells a, b, and c contained cow sera vaccinated against vaccines PV-A, PV-B, and PV-C respectively. Rabbit sera d, e, and f were against vaccines PV-9, PV-10, and PV-11 respectively.
DISCUSSION

Different *S. aureus* strains may have different optimum incubation temperatures for toxin and coagulase production. In the present study using strain 570, temperature did not affect the yield of coagulase; however, the titers of alpha and beta toxins were highest in the cultures grown at 37 °C. Davies (1951) obtained maximum coagulase yield at 35 °C. Bungay (1961) was unable to find any difference in the yield of alpha toxin at 37 and 41 °C temperatures.

There was no direct correlation between the cell growth based on optical density and the yield of toxins and coagulase. Maximum yield of alpha and beta toxins were obtained on the shaker under 50% CO₂ and 50% O₂. Walbum (1921, 1922), while investigating the conditions suitable for toxin production, reported that slightly acid reaction of the medium was most suitable for toxin production. Present studies showed similar results. Under increased aeration and CO₂, the cultures maintained a slightly acidic reaction. This was perhaps due to the buffering action of CO₂. Cultures grown under similar conditions, but without increased CO₂, showed an alkaline reaction due to ammonia production which was unsuitable for high toxin production.

Trypticase soy broth medium supported good growth of *S. aureus* but poor yields of alpha and beta toxins were obtained in this medium. This medium may be deficient or inadequate in certain minerals, vitamins, or amino acids.
essential for toxin production. Gladstone (1938) produced alpha toxin in a chemically defined medium which contained a mixture of 8 to 10 amino acids including arginine and glycine. The author reported that \textit{S. aureus} needed larger amounts of these amino acids (arginine and glycine) for toxin production than was needed for growth. In tryptcase soy broth medium these amino acids may be inadequate, resulting in poor toxin yield.

The production of coagulase by \textit{S. aureus} during the early phase of cell growth may be due to the availability of certain nutrients or precursors which may be exhausted in the later part of the log phase and the stationary phase. It is also possible that certain inhibitory substances or some repressive mechanism stops the production. It has been pointed out earlier that coagulase may be inactivated in the culture medium on prolonged incubation. The destructive effect may be due to the shaking or the substance may be oxygen labile as has been observed in the case of leucocidin (Valentine, 1936). The destruction is minimal under static conditions of culture.

\textit{S. aureus} cells disrupted by various means were compared with formalin and BPL treated cells for immune and antibody response in rabbits. Both quantitatively and qualitatively, disrupted cell vaccines gave higher antibody response than whole cell vaccines. On the other hand, against a challenge dose, best protection was afforded by formalin-ized whole cell vaccines. These results then suggest that
agglutinating or precipitating antibodies and the protecting antibodies may not be the same.

During the process of cell lysis or disintegration, the surface antigenic structure is disorganized. The antibodies formed against such antigens are less specific to the cell surface antigens and are inadequate to give effective protection against a challenge dose. Greenberg and Cooper (1960) claimed to have protected rabbits vaccinated with phenolysed heat killed dornase lysed cells. In their opinion, intracellular antigens play a greater role in immunity than the antibodies to surface and extracellular antigens. Koenig (1962), on the other hand, reported that specific antibodies against surface or capsular antigens were required for phagocytosis of certain virulent \textit{S. aureus} strains.

In rabbits BPL treated cell toxoid vaccines produced higher alpha and beta antitoxin titers than formalinized cell toxoids, although neither gave complete protection against the challenge dose. These results in rabbits are in conformity with the results reported by other workers (Orlans and Jones, 1958 and Flaming, 1960, 1962). Fisher (1957) reported that the antigenicity of the protecting factor present in \textit{S. aureus} toxin is reduced during the process of formalin inactivation.

Different species of animals seem to have different immune and antibody response against vaccines inactivated by various means. In cows higher antibody titers were
obtained against formalin treated cell toxoids. The levels of alpha and beta antitoxins and antileucocidin were considerably lower with BPL treated vaccines. The cattle vaccinated with formalinized vaccine also showed greater resistance to challenge doses. The use of BPL treated vaccines in cows also gave discouraging results in the hands of other workers (Schultze et al., 1963).

More work is still needed to determine the efficiency of formalin and BPL as inactivating agents for vaccines. The drawback against the former is that it takes a long period to detoxify the toxins, which increases the chances of antigen denaturation. Other means of toxoiding like papain treatment deserve attention (Ramon and Richou, 1963).

It has been found that the immune and antibody response in vaccinated animals varied with different strains of *S. aureus*. Different animals of the same species also showed considerable variation in the immune and antibody response to vaccine preparations. In this study no correlation was obtained between the results of protection against challenge organisms and the alpha and beta antitoxin titers. Some rabbits showing good response of alpha antitoxin demonstrated lower resistance against the challenge dose of *S. aureus* than the animals having low alpha antitoxin titers. Rabbits injected with vaccine PV-12 had higher response of antileucocidin. These animals also showed greater resistance to challenge dose. This suggests
that antileucocidin may have some correlation with immunity.

Vaccination trials in cows confirmed some of the observations made from rabbit studies. Out of the three vaccines (PV-A, PV-B, and PV-C) cows vaccinated with PV-C (formalinized N.Z. amine whole broth culture) stimulated higher levels of antibodies including alpha antitoxin and antileucocidin. These animals also showed considerably better protection against challenge dose of organisms than the cows of other groups. The difference in protection can only be attributed to the high protecting power of antileucocidin as the level of this antibody is significantly higher than the animals of other vaccine groups. It is expected that high antitoxins in the cows of this group helped in decreasing the severity of the infection.

The importance of coagulase in immunity has been reported by Boake (1956), Blobel and Berman (1961), and Lominski et al. (1962). In the present studies in cows there was virtually no increase of anticoagulase titers in response to vaccination. Derbyshire (1960a, 1961a) in two of his experiments in goats was unable to detect any anticoagulase titer. However, in the subsequent work (Derbyshire and Helliwell, 1962) some response was obtained with concentrated undetoxified preparations. Its role in immunity was reported insignificant (Derbyshire, 1963).

The results of the present studies are in conformity with the results of Johanovsky (1958a) who found a very high correlation between the resistance and the titers of anti-
leucocidin rather than that of alpha antitoxin. Sebek et al. (1959) reduced the incidence of *S. aureus* infection in humans by seven fold after using the toxoids containing high leucocidin. The correlation between the resistance to infection and the levels of antitoxins is reported low and irregular (Conner and McKie, 1933; Blair and Hallman, 1936; Dolman, 1937; Hite et al., 1938; and Pearson, 1959). Derbyshire (1961a) was unable to protect vaccinated goats against challenge with strain BB. None of these goats showed antileucocidin response. In a subsequent experiment (Derbyshire and Helliwell, 1962), the goats were immunized with a preparation containing measured amounts of alpha lyso, coagulase, and leucocidin. These goats showed much better protection on challenge against the same strain (strain BB). The better protection is attributed to be due to the increased response of antileucocidin. The levels of alpha antitoxin in both the experiments remained more or less the same.

The value of alpha antitoxin is undoubtedly in the reduction of the severity of the infection by neutralization of *S. aureus* alpha toxin. It is, however, questionable whether antitoxin will prevent or eliminate the existing infection. On the other hand, the antileucocidins prevent damage to the leucocytes and the body's defensive mechanism of phagocytosis is effective against the staphylococci. In certain cases for successful phagocytosis specific antibody (opsonin) may be needed (Koenig, 1962).
SUMMARY AND CONCLUSIONS

The maximum yield of alpha and beta toxins was obtained at 37°C on the shaker under 50% CO₂ and 50% O₂. Except for trypticase soy broth, all other media under investigation were found suitable for toxin production. An adverse effect on toxin yield was demonstrated by increasing the sodium chloride concentration from 0.5% to 1.5% in heart infusion broth. For maximum alpha and beta toxin production optimum incubation period was found to be 48 hours and 72 hours respectively.

The production of coagulase was not affected by any of the three incubation temperatures used in this study. Highest coagulase yield was obtained in extract broth and N.Z. amine broth media. Cultures grown under increased aeration and CO₂ concentrations showed destructive effect on coagulase during a prolonged incubation period. In general, the optimum period for coagulase production was found to be 24 hours in the cultures grown under static conditions, 12 hours in the cultures on the shaker, and 8 hours in the cultures on the shaker under 50% CO₂ and 50% O₂.

Antisera from rabbits injected with disrupted or lysed cell vaccines gave higher agglutinin titers and more precipitation lines than the whole cell vaccines. However, on challenge, better protection was shown by the rabbits vaccinated with formalinized whole cells rather than disrupted cells or beta-propiolactone treated cell vaccines.
Beta-propiolactone treated vaccines produced higher alpha and beta antitoxin response in the rabbits than the formalinized vaccines. The levels of these and other types of antibodies were higher in cows injected with formalinized vaccines than with beta-propiolactone inactivated vaccines.

Five vaccines differing in either the potency of certain diffusible products or in the manner of inactivation were used for immunization of rabbits. There was no correlation in vaccinated rabbits between the levels of alpha or beta antitoxins and the protection against a challenge dose. However, the rabbits showing higher antileucocidin titers showed better protection against challenge doses of \textit{S. aureus}.

Cows vaccinated with vaccine PV-C (polyvalent N.Z. amine whole broth culture treated with formalin) showed greater resistance to intramammary challenge than the cows vaccinated with two other vaccines. Vaccine PV-C stimulated higher levels of antileucocidin in these cows than in cows vaccinated with other vaccines. This suggests that antileucocidin may be important in resistance to staphylococcal infection. Antisera from the cows vaccinated with PV-C vaccine also demonstrated higher mouse protecting power following challenge with strains of \textit{S. aureus}.

The importance of adjuvants in the vaccines has been confirmed.
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APPENDIX

Calculation of standard deviation (S.D.) of alpha antitoxin and antileucocidin in the sera of rabbits vaccinated with vaccines PV-8, PV-9, PV-10, PV-11, and PV-12.

\[
S.D. = \sqrt{\frac{\text{Sum } x^2 - \frac{(\text{Sum } x)^2}{n}}{n - 1}}
\]

**Alpha antitoxin in response to vaccine PV-8:**

<table>
<thead>
<tr>
<th>Rabbit</th>
<th>( x )</th>
<th>( x^2 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4</td>
<td>16</td>
</tr>
<tr>
<td>2</td>
<td>3</td>
<td>9</td>
</tr>
<tr>
<td>3</td>
<td>4</td>
<td>16</td>
</tr>
<tr>
<td>4</td>
<td>16</td>
<td>256</td>
</tr>
<tr>
<td>5</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>6</td>
<td>3</td>
<td>9</td>
</tr>
</tbody>
</table>

\[
\sqrt{\frac{307 - \frac{(31)^2}{6}}{5}} = 5
\]

\[
\sqrt{\frac{147}{5}} = 5.4
\]

By following the method shown above the standard deviations of remaining alpha antitoxin and antileucocidin were calculated.
Calculation of 50% mouse protection power of pre and post vaccination cow sera against vaccines PV-A, PV-B, and PV-C by Reed and Muench (1938) method.

Before testing the mouse protection power of different serum samples, a 100% lethal dose against one homologous (strain 570) and one heterologous strain (strain 1531) was determined. A group of five mice were injected with 0.5 ml dose of various dilutions of the culture. In testing serum samples as far as possible an attempt was made to use such serum dilutions which gave a mortality range of 0 to 100% against the challenge dose.

Fifty per cent mouse protection power of serum obtained from cow Clyde after vaccination with vaccine PV-A.

<table>
<thead>
<tr>
<th>Serum dilutions</th>
<th>1:32</th>
<th>1:128</th>
<th>1:512</th>
</tr>
</thead>
<tbody>
<tr>
<td>log 10</td>
<td>-1.5051</td>
<td>-2.1072</td>
<td>-2.7177</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Mortality rate</th>
<th>0/5</th>
<th>4/5</th>
<th>5/5</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. died</td>
<td>0</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>No. Survived</td>
<td>5</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Accumulation totals</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Died</td>
<td>9</td>
<td>9</td>
<td>5</td>
</tr>
<tr>
<td>Survived</td>
<td>5</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Mortality rate</td>
<td>9/14</td>
<td>9/15</td>
<td>5/11</td>
</tr>
<tr>
<td>% mortality</td>
<td>43.3%</td>
<td>60%</td>
<td>45.5%</td>
</tr>
</tbody>
</table>

↑ 50% protection
Proportionate distance = \frac{50\% - \% mortality next below 50\%}{\% mortality next above 50\% - \% mortality next below 50\%}

\[
= \frac{50 - 45.5}{60 - 45.5} = \frac{4.5}{14.5} = 0.310 = 0.3
\]

\[\text{ld 50 end point} = \text{log lower dilution} \times \text{proportionate distance} \times \text{log dil. factor} \]
\[= -2.1072 \times 0.3 \times 0.6012 = -0.1806 \]

\[\text{Sum} = -2.2878\]

a) 50\% end point = 10
b) ld 50 log = 10
c) 50\% neutralization end point = 2.2878
d) antilog of 50\% neutralization end point = 194

By following the method shown above the 50\% protection power of other serum samples against the challenge dose of homologous and heterologous strains was calculated.
Differences of alpha and beta antitoxins and antileucocidins in the sera of cows vaccinated with vaccines PV-A, PV-B, and PV-C — Analysis of variance by F values.

**Alpha antitoxin.**

<table>
<thead>
<tr>
<th>Calculation from the columns</th>
<th>Vaccines</th>
<th>Calculation from the rows</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reciprocal of titers</td>
<td>PV-A</td>
<td>PV-B</td>
</tr>
<tr>
<td>( x ) (log)</td>
<td>( x^2 )</td>
<td>( x ) (log)</td>
</tr>
<tr>
<td>48</td>
<td>1.68</td>
<td>2.80</td>
</tr>
<tr>
<td>24</td>
<td>1.38</td>
<td>1.90</td>
</tr>
<tr>
<td>50</td>
<td>1.70</td>
<td>2.90</td>
</tr>
<tr>
<td>24</td>
<td>1.38</td>
<td>1.90</td>
</tr>
<tr>
<td>6</td>
<td>0.78</td>
<td>0.61</td>
</tr>
</tbody>
</table>

\[ \sum x = 6.92 \quad 7.20 \quad 9.12 \quad \frac{\sum \sum x}{2} = 23.24 \]

\[ \bar{x} = 1.38 \quad 1.44 \quad 1.82 \quad \bar{x} = 1.55 \]

\[ \sum (x)^2 = 10.11 \quad 10.40 \quad 16.80 \quad \sum (\sum x)^2 = 37.31 \]

\[ (\sum x)^2 = 47.88 \quad 51.84 \quad 83.17 \quad (\sum (x)^2) = 182.89 \]

**Difference within vaccine groups:**

\[ (x)^2 = \frac{1}{15} [(5(37.31) - 182.89)] = 0.73 \]

Mean square (variance) = \( \frac{0.73}{12} = 0.06 \) i.e. \( F = 1 \)

**No significant difference**

**Difference between vaccine groups:**

\[ (x)^2 = \frac{1}{15} [(3(182.89) - 23.24)^2] = 0.57 \]

Mean square (variance) = \( \frac{0.57}{2} = 0.29 \) i.e. \( F = 1 \)

**No significant difference**
### Beta antitoxin.

<table>
<thead>
<tr>
<th>Calculation from the columns</th>
<th>PV-A</th>
<th></th>
<th>PV-B</th>
<th></th>
<th>PV-C</th>
<th></th>
<th>Calculation from the rows</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reciprocal of titer (log)</td>
<td>( x )</td>
<td>( x^2 )</td>
<td>Reciprocal of titer (log)</td>
<td>( x )</td>
<td>( x^2 )</td>
<td>Reciprocal of titer (log)</td>
<td>( x )</td>
</tr>
<tr>
<td>48</td>
<td>1.68</td>
<td>2.80</td>
<td>24</td>
<td>1.38</td>
<td>1.90</td>
<td>50</td>
<td>1.70</td>
</tr>
<tr>
<td>48</td>
<td>1.68</td>
<td>2.80</td>
<td>48</td>
<td>1.68</td>
<td>2.80</td>
<td>40</td>
<td>1.60</td>
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<tr>
<td>20</td>
<td>1.30</td>
<td>1.69</td>
<td>14</td>
<td>1.14</td>
<td>1.30</td>
<td>96</td>
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<tr>
<td>56</td>
<td>1.75</td>
<td>3.05</td>
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<td>1.70</td>
<td>2.90</td>
<td>180</td>
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<tr>
<td>24</td>
<td>1.38</td>
<td>1.90</td>
<td>50</td>
<td>1.70</td>
<td>2.90</td>
<td>48</td>
<td>1.68</td>
</tr>
</tbody>
</table>

\[
\Sigma x = 7.79 \quad 7.60 \quad 9.22 \quad \Sigma \Sigma x = 24.61
\]
\[
\bar{x} = 1.56 \quad 1.52 \quad 1.84 \quad \bar{x} = 1.64
\]
\[
\Sigma (x)^2 = 12.24 \quad 11.80 \quad 17.26 \quad \Sigma \Sigma (x)^2 = 41.30
\]
\[
\Sigma x^2 = 60.68 \quad 57.76 \quad 85.00 \quad \Sigma (\Sigma x)^2 = 203.44
\]

**Difference within vaccine groups:**

\[
\frac{(x)^2}{12} = \frac{1}{5} \left[ 5(41.30) - 203.44 \right] = 0.61
\]

Mean square (variance) = \( 0.61 / 0.05 = 12 \) i.e. \( F = 1 \)

**Difference between vaccine groups:**

\[
\frac{(x)^2}{15} = \frac{1}{3} \left[ 3(203.44) - 24.61 \right] = 0.31
\]

Mean square (variance) = \( 0.31 / 0.16 = 1 \) i.e. \( F = 1 \)

No significant difference
### Anti S leucocidin

#### Vaccines

<table>
<thead>
<tr>
<th></th>
<th>PV-A</th>
<th></th>
<th>PV-B</th>
<th></th>
<th>PV-C</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Calculation</td>
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<td>Calculation</td>
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</tr>
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<td>Reciprocal of</td>
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<td>Reciprocal of</td>
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<td>Reciprocal of</td>
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<td>Reciprocal of</td>
</tr>
<tr>
<td>titers (log)</td>
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<td>titers (log)</td>
<td></td>
<td>titers (log)</td>
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<td>titers (log)</td>
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</tr>
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<td>8192</td>
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<td>15.30</td>
</tr>
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<td>2.71</td>
<td>512</td>
<td>2.71</td>
<td>8192</td>
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<td>15.30</td>
</tr>
<tr>
<td>128</td>
<td>2.11</td>
<td>1024</td>
<td>3.01</td>
<td>4096</td>
<td>3.61</td>
<td>13.00</td>
</tr>
<tr>
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<td>2.41</td>
<td>128</td>
<td>2.11</td>
<td>3072</td>
<td>3.49</td>
<td>12.20</td>
</tr>
</tbody>
</table>

\[
\begin{align*}
\Sigma x &= 12.35 & 12.65 & 17.33 & \Sigma \Sigma x = 42.33 \\
\bar{x} &= \frac{\Sigma x}{5} = 2.47 & 2.53 & 3.47 & \bar{x} = 2.82 \\
\Sigma (x)^2 &= 30.73 & 33.15 & 61.60 & \Sigma \Sigma x^2 = 125.48 \\
(\Sigma x)^2 &= 152.52 & 160.02 & 300.33 & (\Sigma x)^2 = 612.87
\end{align*}
\]

**Difference within vaccine groups:**

\[
\text{Sum of squares} = (\Sigma x)^2 = 1/5 \left[ 5(125.48) - 612.87 \right] = 2.96
\]

Mean square (variance) = \(\frac{2.96}{12} = 0.246\) (12 is the degree of freedom)

**Difference between vaccine groups:**

\[
\text{Sum of squares} = (\Sigma x)^2 = 1/15 \left[ 3(612.87) - (42.33)^2 \right] = 3.12
\]

Mean square (variance) = \(\frac{3.12}{2} = 1.56\) (2 is the degree of freedom)

The variance ratio i.e. \(F = \frac{\text{Mean square between vaccine groups}}{\text{Mean square within vaccine groups}} = \frac{1.56}{0.246} = 6.34\)

\(P = \frac{f_1}{f_2} (\text{degrees of freedom for greater mean square}) = \frac{2}{12} = <.05 \& >.01\)

\(P < .05\) significant difference in sera against vaccine PV-C versus PV-A and PV-B.

---

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### Anti F leucocidin.

<table>
<thead>
<tr>
<th></th>
<th>PV-A</th>
<th></th>
<th>PV-B</th>
<th></th>
<th>PV-C</th>
<th></th>
<th>Calculation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Reciprocal of (log)</td>
<td>x²</td>
<td>Reciprocal of (log)</td>
<td>x²</td>
<td>Reciprocal of (log)</td>
<td>x²</td>
<td>from the rows</td>
</tr>
<tr>
<td></td>
<td>titers</td>
<td></td>
<td>titers</td>
<td></td>
<td>titers</td>
<td></td>
<td></td>
</tr>
<tr>
<td>256</td>
<td>2.14</td>
<td>5.80</td>
<td>64</td>
<td>1.81</td>
<td>3.26</td>
<td>64</td>
<td>1.81</td>
</tr>
<tr>
<td>96</td>
<td>1.98</td>
<td>3.90</td>
<td>128</td>
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<td>4.45</td>
<td>2048</td>
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<tr>
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<td>2.11</td>
<td>4.45</td>
<td>768</td>
<td>2.89</td>
</tr>
</tbody>
</table>

\[
\begin{align*}
\bar{x} &= \frac{\sum x}{5} \\
\sum x &= 9.39 \\
\sum x^2 &= 86.00 \\
(\sum x)^2 &= 88.00
\end{align*}
\]

\[
\bar{x} = \frac{\sum x}{5} = 1.88
\]

\[
\sum x^2 = 21.78
\]

\[
(\sum x)^2 = 44.56
\]

\[
\sum (\sum x)^2 = 404.50
\]

#### Difference within vaccine groups:

- Sum of squares = \( \sum x^2 = \frac{1}{5} \left[ 9.39^2 + 10.25^2 + 14.63^2 + 34.27^2 \right] = 3.48 \)
- Mean square (variance) = \( \frac{3.48}{12} = 0.29 \)

#### Difference between vaccine groups:

- Sum of squares = \( (\bar{x} x)^2 = \frac{1}{15} \left[ 3(404.50) - (34.27)^2 \right] = 2.60 \)
- Mean square (variance) = \( \frac{2.60}{2} = 1.30 \) (2 is the degree of freedom)

The variance ratio i.e. \( F = \frac{1.30}{0.29} = 4.49 \)

\[
P = \frac{f_1}{f_2} = \frac{2}{12} \approx 0.05 \& > 0.01 \) (from table 10.5.3 p. 246 of Snedecor, 1956)

**Significant difference in sera against vaccine PV-C versus PV-A and PV-B.**