# ANTIBODY PRODUCTION IN THE BOVINE MAMMARY GLAND

by

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# INTRODUCTION

The ability of the living animal body to produce antibodies in response to the stimulation by antigens is adequately established. Techniques for the measurement of antibodies are standardized and accepted.

Immunological protection of the bovine mammary gland from mastitis is a phase of immunology which has not been fully investigated. There has been a recent revival of interest in the prophylactic vaccination of cows against the etiological agents of mastitis, but most of the observations along this line have been of a clinical nature. Passive immunization of human beings against the effects of their pathogens by the drinking of milk containing homologous antibodies produced in bovine mammary glands has recently been proposed.

The purpose of this study was to make observations which would help determine possibilities regarding the protection of the bovine mammary gland by active immunization, and of the protection of man against his pathogens by the drinking of milk containing antibodies produced in the bovine udder in response to antigens introduced through the teat orifice. To evaluate such procedures, it was necessary to determine the antigenic response of the bovine mammary gland to the stimulation by various antigens. The

desirability of these practices depends upon the level of antibodies produced, and the distribution of the antibodies within the body of the host. This study included some determinations of these facts. Rapidity of production of antibodies was determined, as an indication of the mechanism of antibody production in the bovine mammary gland, and as an indication of the usefulness of this response in the alleviation of infectious processes already progressing.

In order to determine whether it would be feasible to produce immunity by the introduction of antigens into the milk spaces of the teats and the mammary glands, it is necessary to determine the effects of such inoculated substances on the gland itself, and on its secretions. Observations regarding these results were made and recorded.

# REVIEW OF LITERATURE

The literature contains few reports regarding the effects of inoculation of bovine mammary glands with antigens introduced through the teat canals.

Campbell et al. (2) refer to observations regarding antibody production. By using a live culture of Salmonella pullorum, they stimulated the production of antibodies which could be detected within 4 hours after inoculation. In the work cited, the inoculum was placed in the mouths of calves, which inoculated lactating cows during the act of nursing. Diathelic immunization is the term these investigators suggest for this phenomenon.

Mitchell et al. (10) inoculated viable Newcastle Disease virus into bovine mammary glands and demonstrated the production of antibodies. These investigators extended their experimentation to the inoculation of influenza virus into bovine mammary glands. Following the inoculation of this virus, homologous antibodies were produced. In a later report, Mitchell et al. (12) discussed the failure of the mammary gland to produce antibodies following its inoculation with live poliomyelitis virus.

Numerous references are available to indicate the infection of unnatural hosts with the virus of Newcastle Disease. Evans (5) reported observations following several

such infections in man. These were primarily infections of the conjunctiva, but the virus had also been recovered from nasal secretions, saliva, rarely from the blood, and on one occasion from the urine. Respiratory involvements were slight to absent. He reported that in most cases the antibody response in serum from such patients was minimal to absent, as measured by neutralization and hemagglutination-inhibition tests. Burnet (1) suggests that Newcastle Disease virus might on occasion find opportunity for human passage and by the appearance and selective survival of mutants give rise to a new type of influenza.

Spencer and Angevine (13) reported the development of hypersensitivity in streptococcic infections in cows and rabbits. Hypersensitive cows responded to the intramammary injection of killed streptococci, or of streptococcic polysaccharide with a rapid inflammatory reaction characterized by swelling, tenderness, and abnormal milk.

The antigenic stimulation by staphylococcus toxin has been more fully studied following natural infections and subcutaneous inoculation of the toxin. Dolman (4) reported on the antigenic properties of staphylococcus toxin and toxoid following subcutaneous inoculation of rabbits and horses.

Minett (9) reported on antitoxin titers in blood and milk of cows and other animals following natural infections

(mastitis) and subcutaneous inoculations of toxins. This investigator reported a slow development of antibodies following either stimulation.

Miller and Heishman (8) reported on levels of staphylococcus antitoxin in the blood, milk, and colostrum of cows with staphylococcic mastitis. They postulate that antitoxin is found as a constituent of a blood protein only, and may be found in mammary gland secretions provided tissue damage in the gland permits the escape of blood serum to the milk-collecting spaces.

# METHODS OF PROCEDURE

The substances used as antigens are classified as bacterins, a virus, a pharmaceutical preparation, and a toxin (or its toxoid). The procedures used for each class are discussed separately.

## Bacterins as Antigens

Salmonella choleraesuis var. kunzendorf, Brucella abortus, and Escherichia coli were used to prepare bacterins and for the preparation of plate and tube antigens. The Sal. choleraesuis strain was isolated by the Department of Veterinary Hygiene at Iowa State College from a case of salmonellosis in a herd of 60 pound pigs. The Br. abortus strain was taken from a vial of lyophilized Strain 19 vaccine prepared by the Norden Laboratories, Lincoln, Nebraska. The E. coli strain was isolated by the Department of Veterinary Hygiene from a sample of milk collected at the Iowa State College dairy farm.

Organisms for the preparation of bacterins, plate antigens, and tube antigens were propagated on nutrient agar in Roux flasks. The inoculum consisted of 2 ml. of a 24 hour culture of organisms in nutrient broth. They were incubated aerobically at 37° C. The Sal. choleraesuis and E. coli were harvested after 48 hours of incubation. Br. abortus

organisms were harvested after 72 hours of incubation.

Identity of organisms from each flask was checked biochemically at the end of the incubation period. The growth was harvested by washing colonies from the agar surface with sterile physiological saline. Organisms, following harvesting, were washed twice with sterile physiological saline. The organisms were heat killed by exposure to 65° C. for 30 minutes in a water bath. The completeness of this step was checked by inoculation of a tube of Thiol medium, and one of nutrient broth.

Heat-killed organisms were suspended in sterile physiological saline and standardized to tube No. 2 of the McFarland Nephelometer to use as a bacterin for inoculation. Another suspension with a turbidity comparable to tube No. 7 of the McFarland Nephelometer was prepared as a rapid plate antigen. Dyes were not used to color the organisms. For tube antigen, a suspension was prepared with a turbidity equal to that of the standard Br. abortus tube antigen as prepared by the Agricultural Research Service, United States Department of Agriculture. A Coleman Photo-Nephelometer was used to compare turbidity. Physiological saline containing 0.5% phenol was used to suspend the organisms.

Before inoculating the cows, milk samples were collected and cultured to assure the freedom of the cows from mastitis. Cows with infections in the mammary glands were

not used. No other discernible infectious processes were present in the cows used.

Bacterins were inoculated through the teat orifice of one quarter of lactating cows. The inoculum consisted of 2 ml. of bacterin, followed by 2 ml. of sterile physiological saline. Inoculations of Sal. choleraesuis were repeated in the same quarter 15 days and 26 days following the first inoculation. Br. abortus bacterin was inoculated into the same cow that received Sal. choleraesuis bacterin, using a quarter not previously inoculated. An interval of 28 days separated this inoculation, and the last one of Sal. choleraesuis. E. coli bacterin was inoculated into 2 cows.

Venous blood and individual milk samples from all quarters were collected prior to inoculation and at regular intervals afterward. Serum was harvested from the blood and tested for homologous agglutinating antibodies, using standard rapid plate and tube agglutination techniques. Milk serum was collected by centrifugation of milk samples after adding 0.1% of a commercial rennet preparation and incubating at 37° C. for 1 hour. Milk serum was tested for agglutinating antibodies by the same techniques as were used with blood serum. When testing for agglutinating antibodies for Br. abortus, antigens prepared in the laboratory, and by the Agricultural Research Service, were used.

## Newcastle Disease Virus as Antigen

Work with Newcastle Disease Virus (Tortor furens) was done with Strain 174, isolated and identified by Dr. M. S. Hofstad of the Veterinary Medical Research Institute, Ames, Iowa. The virus was propagated by inoculating 0.1 ml. of harvested allantoic fluid into the allantoic cavity of eggs containing 10-day embryos. When death of the embryos occurred, the allantoic fluid was harvested. This harvest titered to a $LD_{50}$ of $10^{8.25}$. The right rear quarter of each of 2 cows was inoculated with 1 ml. of this virus suspension. Cow No. 14 was in production; milking of Cow No. 13 had recently ceased, but a secretion was available for collection.

Venous blood and individual milk samples from all quarters were collected prior to inoculation, and at regular intervals thereafter.

Embryonated eggs (10-day) were inoculated via the allantoic cavity with 10-fold serial dilutions of milk obtained from the inoculated quarters, and with undiluted blood serum and undiluted milk from an uninoculated quarter of each cow, to determine the length of time the virus was recoverable. Embryonated eggs were inoculated in the allantoic cavity with 0.1 ml. of the milk dilutions.

Serum neutralization tests were performed using milk from the inoculated quarter of Cow No. 13, according to the procedure of Cunningham (3).

Hemagglutination-inhibition tests, using the beta procedure of Merchant and Packer (7) were performed on blood serum, milk from inoculated quarters, and milk from an uninoculated quarter of each cow. Chicken erythrocytes are agglutinated by normal bovine milk. To avoid this interference, 0.5 ml. of milk or blood serum to be tested were mixed with 2 ml. of 4% chicken erythrocytes. After incubating at 37° C. for 20 minutes, the erythrocytes were sedimented by low speed centrifugation. The supernatant was used without further dilution in tubes 1 and 2 in the hemagglutination-inhibition test.

In order to determine if the virus were still virulent for chickens, virus recovered from milk by embryo inoculation was inoculated intranasally into 3-week-old chickens.

## Intramammary Infusion Ointment as Antigen

A tube of a commercial intramammary infusion ointment[1] was instilled into the right rear quarter of each of 9 cows in various stages of lactation. Inoculations were repeated after intervals of 7 and 14 days. These quarters were previously determined, by culture and physical examination,

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[1]This pharmaceutical preparation contained 500,000 units of procaine penicillin G, 100 mg. of dihydrostreptomycin, 500 mg. of phthalylsulfacetamide, 500 mg. of sulfathiazole, and 50 mg. of crystalline papain in a diffusible base.

to be free of mastitis. Milk samples were collected from all four quarters just prior to inoculation and for 2 regular milkings afterward. Examinations were made as to gross appearance of the mammary gland and of the secretions at each milking. Whiteside, catalase, and Babcock tests were performed on each collection. Weight of milk produced was recorded throughout the experiment.

## Staphylococcus Toxin and Toxoid as Antigens

For this study, the hemotoxins of Staphylococcus aureus were employed, using five strains of the organism. They were designated Johnson, Howland, 3722, 3883, and 4031. All were isolated by the Department of Veterinary Hygiene of Iowa State College from severe cases of bovine mastitis. Toxin was obtained by inoculating Staphylococcus Toxin Media, described by Merchant and Packer (7), in Kolle flasks. The inoculum consisted of 2 ml. of a 24 hour culture in tryptose broth. The flasks were incubated for 48 hours at 37° C. in an atmosphere containing 30% $CO_2$, then aerobically for 48 hours at 5° C. Toxin was recovered by adding 1 ml. of sterile physiological saline per 10 ml. of medium, breaking the agar and filtering through 4 layers of cheesecloth, centrifuging, and saving the supernatant containing the hemotoxin.

The potency of each lot of toxin was determined

according to the procedure outlined in Table 1. In making this test, the dilutions were continued beyond the end-point of hemolytic activity of the hemotoxin in each case.

Table 1. Staphylococcus hemotoxin potency test

| Tube | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
|---|---|---|---|---|---|---|---|
| Toxin, ml. | 0.5 | Starting with tube No. 1, mix and transfer 0.5 ml. to the next tube | | | | | |
| Saline, ml. | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 |
| 1% washed bovine erythrocytes, ml. | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 |

In the above procedure, the erythrocytes were added after preparing doubling dilutions of the toxin in saline and discarding 0.5 ml. from tube No. 7. The mixtures were placed in a constant temperature water bath at 37° C. for 1 hour. After reading the results, the test was refrigerated at 5° C. for 12 hours, following which a final reading for complete hemolysis was made.

After testing the potency of each lot of toxin, equal amounts of toxin from the 5 strains were combined. A bacteriologically sterile toxin-containing product was obtained by filtering through a Seitz EK filter. This step reduced the hemotoxin titer from 1:128 to 1:64. For this study, a unit of Staph. aureus hemotoxin is defined as the

smallest amount of hemotoxin contained in 0.5 ml. of diluent that will completely hemolyze the freshly prepared bovine erythrocytes in 0.5 ml. of a 1% suspension.

Staph. aureus hemotoxin was rendered non-hemolytic by incubating it aerobically for 8 days at 37° C. after adding 0.2% formalin, or by boiling for 18 minutes.

Three young lactating cows, Nos. 4010, 4054, and 4178, were inoculated with equal portions of the same lot of the toxin, or its toxoid. These were selected because they had no history of mastitis, were free of mastitis organisms as determined by culture, and contained no detectable antibodies to the hemotoxin of Staph. aureus in the milk and blood. The right front quarter was injected in each case. Cow No. 4010 was inoculated with 384 hemolytic units of the toxin. Formalinized toxin (toxoid) was inoculated into Cow No. 4054, and the boiled toxin was inoculated into Cow No. 4178. Cow No. 4010 and Cow No. 4054 received inoculations, identical to their first, 15 days later. At regular intervals after each inoculation, milk was collected from inoculated and uninoculated quarters and tested for antibodies to the hemotoxin of Staph. aureus, according to the procedure given in Table 2. Results were compared with those obtained when using milk and blood collected before inoculation, and with a standard staphylococcus antitoxin obtained from the National Institute of Health. Using the

same procedure, tests were performed on milk and blood from Cow No. 3565, which was chronically infected with Staph. aureus, and on milk and blood from Cow No. 4122, which was a fresh heifer with a staphylococcic mastitis of 5 days duration.

Table 2. Procedure for testing milk or blood for antitoxin

| Tube | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
|---|---|---|---|---|---|---|---|
| Milk, ml. | 0.5 | 0.5 | Starting with tube No. 2 mix and transfer 0.5 ml. to the next tube | | | | |
| Units toxin in 0.5 ml. | | 2 | 1 | 1 | 1 | 1 | 1 |
| 1% washed bovine erythrocytes, ml. | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 |

In the above procedure, milk or blood was diluted in toxin in tubes 2 through 6. In order that the toxin content would be constant, 2 units were placed in tube No. 2. After the mixing in tube No. 6, 0.5 ml. were discarded. Erythrocytes were added last to all tubes. Since tube No. 1 contains no toxin, it serves as a check on the compatibility of the erythrocytes and the milk. Tube No. 7 is a check on hemolytic activity of the toxin. In some instances, in order to reach an end-point, it was necessary to use additional dilutions beyond those shown in Table 2.

After preparing the test, the mixtures were placed in a

constant temperature water bath at 37° C. for 1 hour. Results were read and the test refrigerated at 5° C. for 12 hours, after which a final reading for complete hemolysis was made.

For this study, a unit of antitoxin to staphylococcus hemotoxin is defined as the smallest amount of antitoxin which will completely inhibit the hemolytic activity of 1 unit of staphylococcus hemotoxin. Therefore, the titer of a milk or serum sample is the reciprocal of the greatest dilution which completely inhibits the hemolytic activity of 1 hemolytic unit of staphylococcus hemotoxin.

# RESULTS

## Bacterins as Antigens

Local signs of inflammation, swelling, heat, and sensitiveness, were evident by 4 hours after the first inoculation of bacterins in bovine mammary glands. This reaction was seen only in the quarters inoculated. Secretions became serous in appearance and consistency. They contained clots resembling those present in the early stages of mastitis. In cows producing less than 10 pounds of milk daily, increased secretion by the inoculated quarters was evidenced for 12 hours after inoculation. Symptoms of inflammation were subsiding by 24 hours after inoculation, and were nearly absent after a lapse of another 24 hours. By this time, milk production was reduced. After inoculations of Sal. choleraesuis bacterin were repeated, the signs of local inflammation were less evident, however the milk production was decreased more noticeably, and the animal appeared depressed. Each inoculation caused alteration of the appearance of milk secretions. Milk serum, collected after coagulation of milk proteins by the action of rennet, was more cloudy when obtained from milk produced after the inoculation. This change became more pronounced after the repeated inoculations.

Antibody levels produced as a result of inoculations of

Sal. choleraesuis bacterin are shown in Figure 1. Agglutinating antibodies in the milk from the quarter inoculated with Sal. choleraesuis bacterin were demonstrable at a low level in milk collected 2 hours after inoculation. The appearance of antibodies in the inoculated quarter preceded the appearance in the other quarters and the blood. The highest titer was obtained about 50 days after the first inoculation. In this animal, the titer reached a higher level in the inoculated quarter, but the antibody level of all quarters and the blood tended to approach equality after 65 days post inoculation.

Figure 2 graphically illustrates the titers determined following the inoculation of Br. abortus bacterin. Antibody levels developed following the inoculation could be measured first in the milk from the inoculated quarter. Titers in milk from uninoculated quarters developed later. A demonstrable titer in blood serum was found about 72 hours post inoculation. Agglutination titers were determined for 11 days after the date of inoculation. During this time, the titers were rising in the milk from all quarters, and in the blood. Results were the same using antigen produced in the laboratory and by the Agricultural Research Service. Inoculation of the Br. abortus bacterin resulted in signs of acute local inflammation in the quarter inoculated. The changes in quality and amount of secretions of this quarter

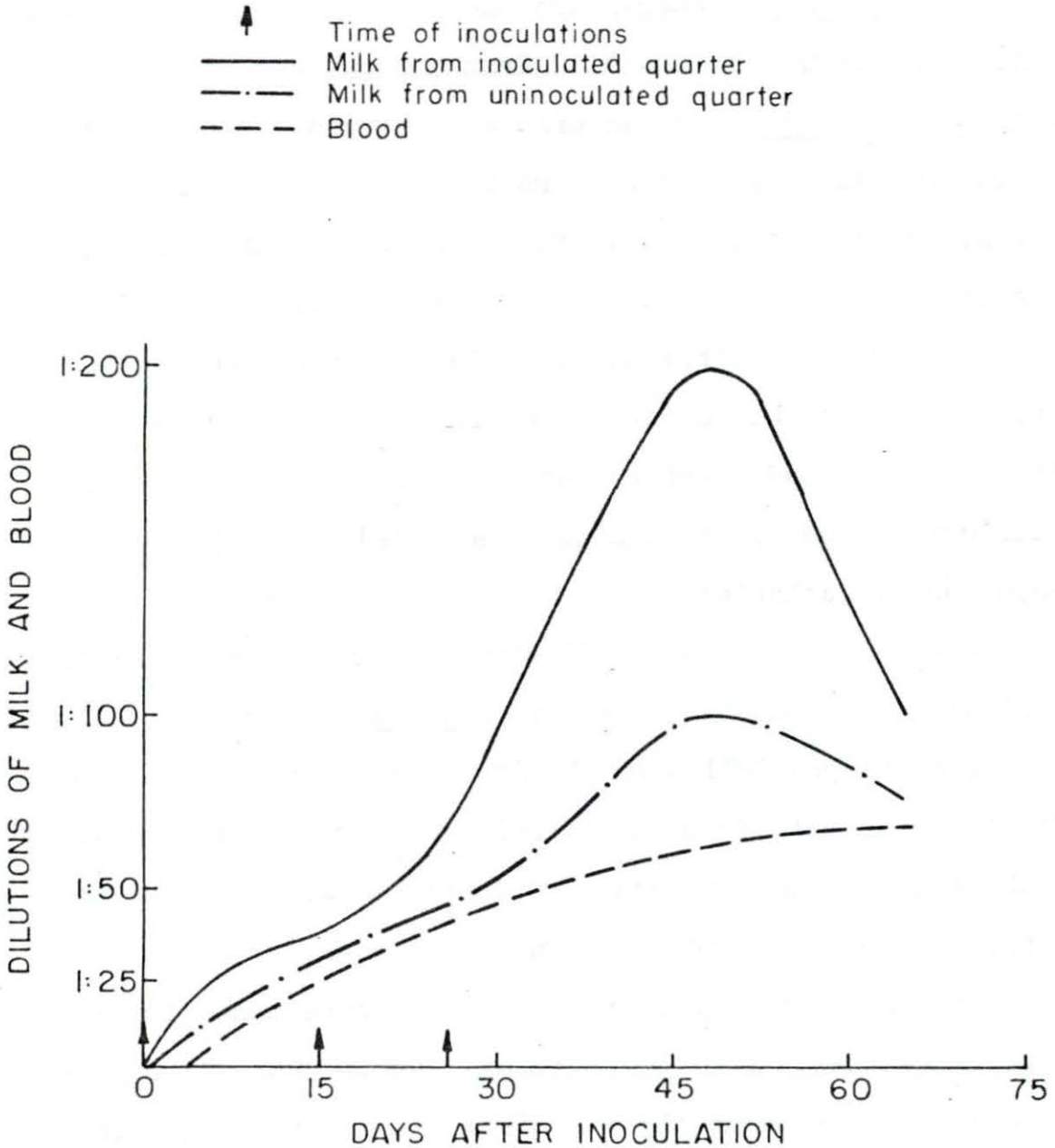


Figure 1. Antibody titers following intramammary inoculation of Sal. choleraesuis bacterin

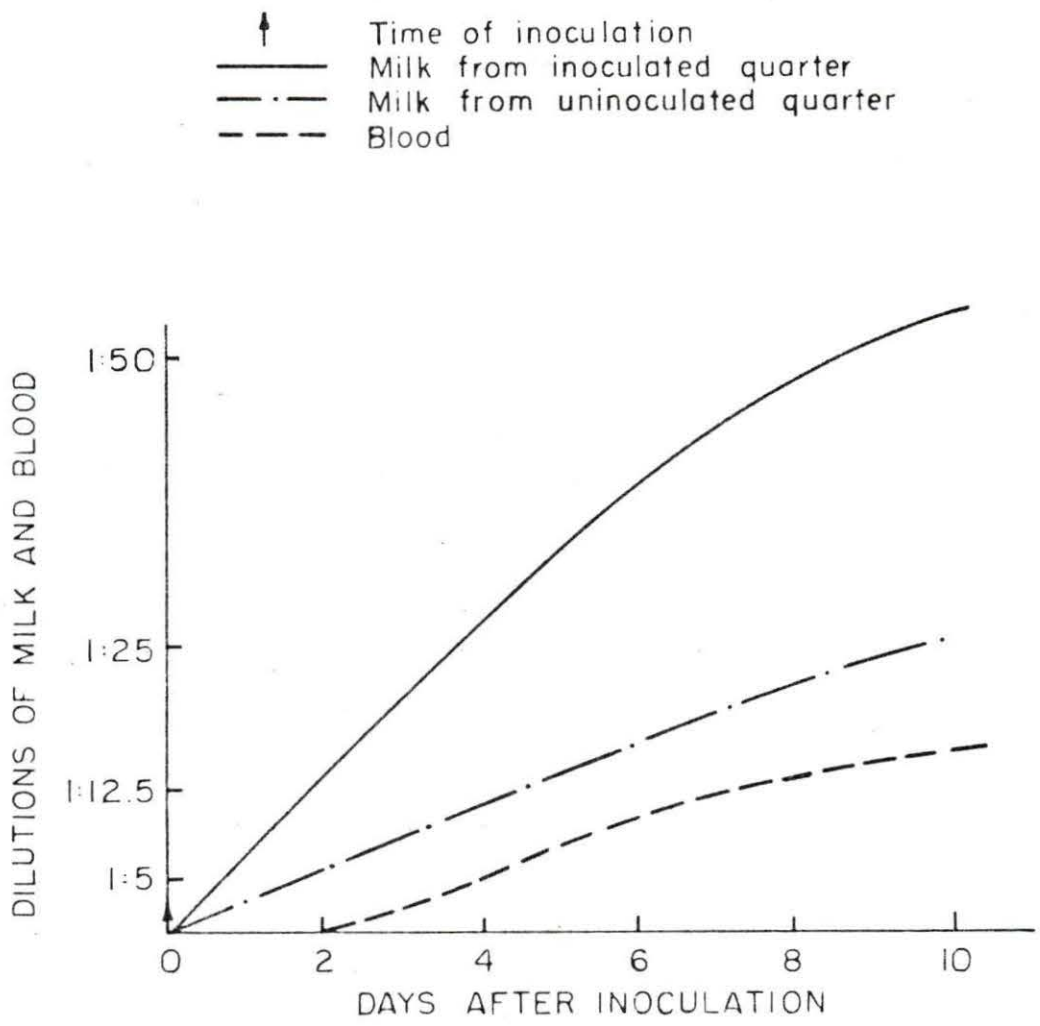


Figure 2. Antibody titers following intramammary inoculation of Br. abortus bacterin

after inoculation were similar to those following inoculation of Sal. choleraesuis bacterin.

Following the inoculation of E. coli bacterin into the mammary glands of 2 cows, inflammatory reactions, similar to those mentioned following inoculation of other bacterins, were observed. The alteration in appearance and the effect on quantity of secretion in Cow No. 14, which was producing less than 4 pounds of milk per day, did not differ from observations following the inoculation of other bacterins. Cow No. 3 was producing 35 pounds of milk per day. Abnormalities in secretion were less noticeable in her, and less effect on the amount of production was observed, however a decrease in production was noted.

Figure 3 and Figure 4 show the antibody titers developed after inoculation of E. coli bacterin in Cow No. 3 and Cow No. 14, respectively.

A titer of 1:12.5 to E. coli was present in the blood of both cows at the time they were inoculated with the bacterin. This blood titer increased rapidly in both cows, resulting in pronounced agglutination at 1:400 by 10 days after inoculation. Agglutinating titers rose slowly in milk from all quarters of the cow in good production. Milk from all quarters of the cow in low production showed a rapid rise in agglutinating antibodies. In both animals, the blood titers rose more rapidly than those of milk, and the titers in milk

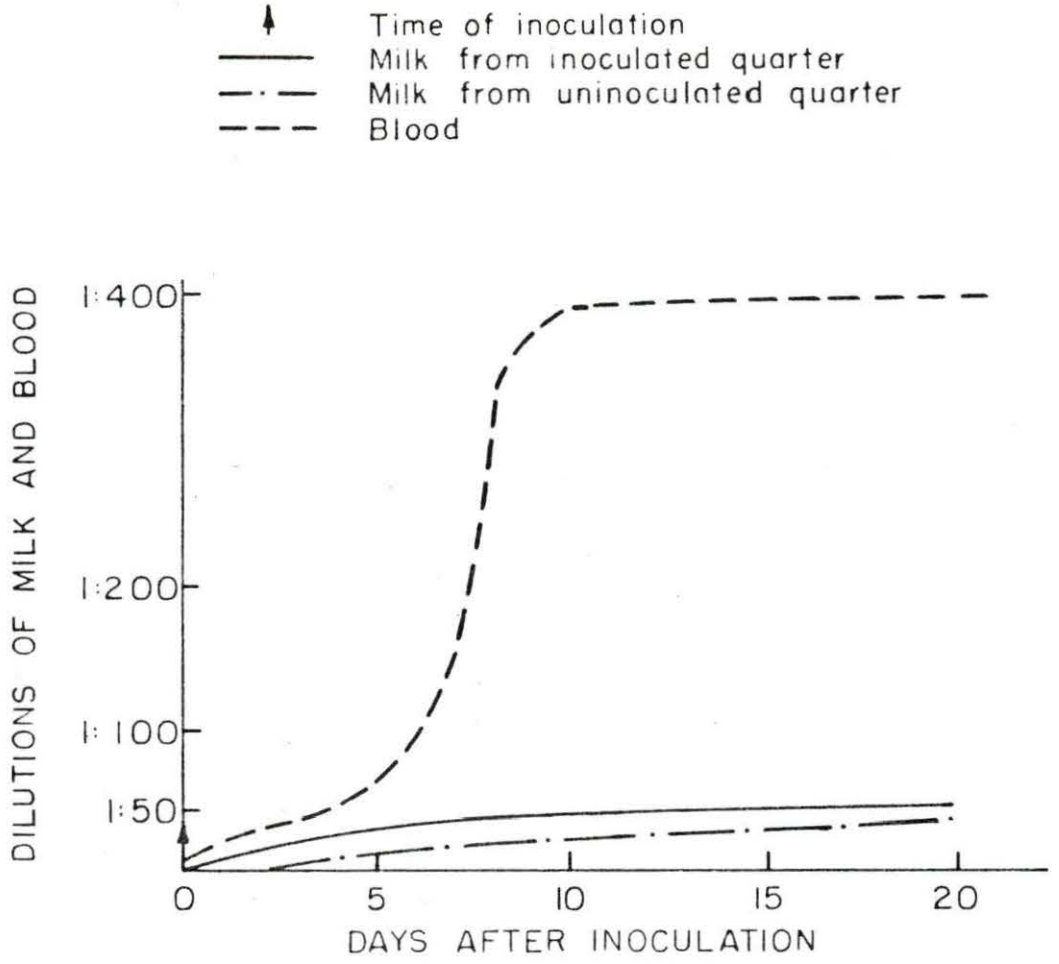


Figure 3. Antibody titers of Cow No. 3 following intramammary inoculation with E. coli bacterin

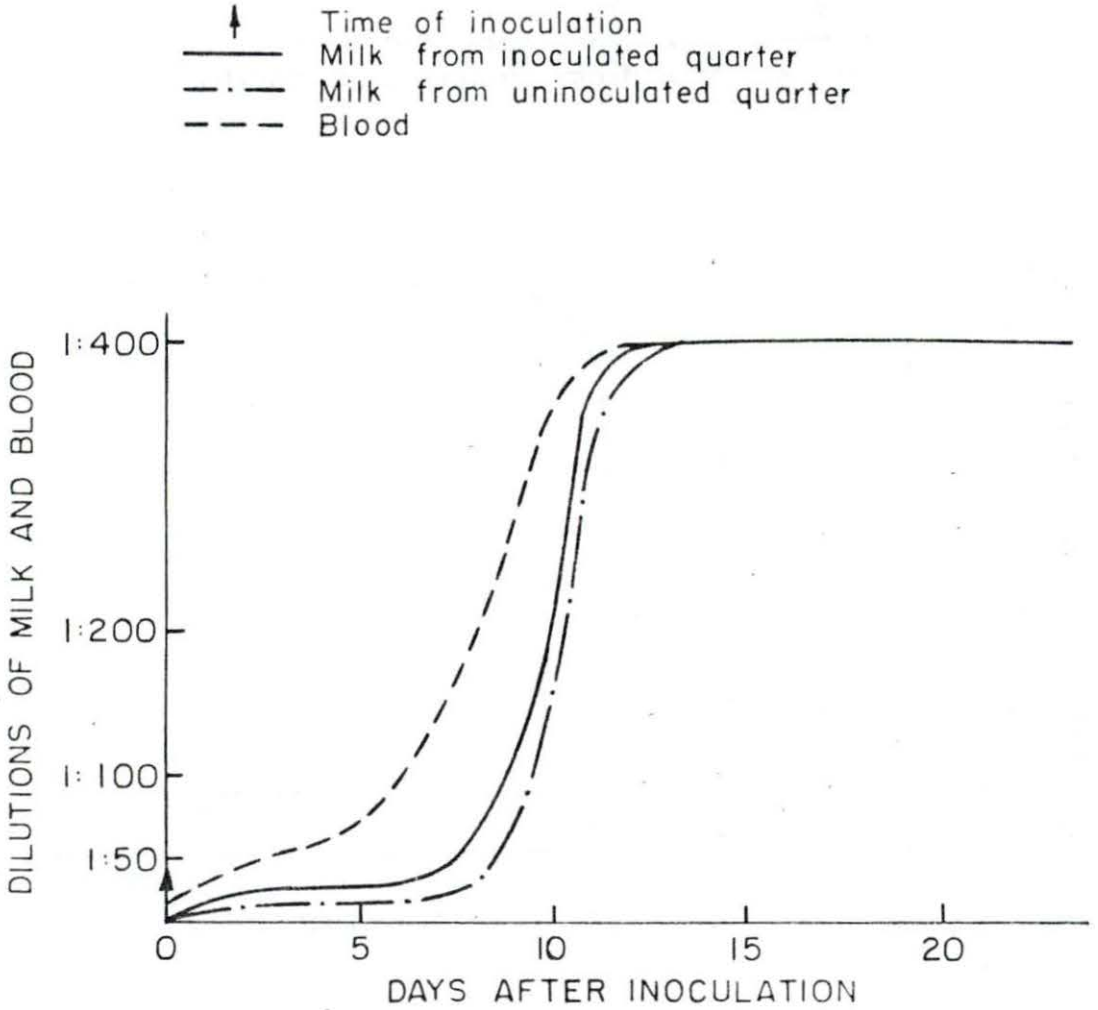


Figure 4. Antibody titers of Cow No. 14 following intramammary inoculation of E. coli bacterin

from the inoculated quarters rose ahead of those of milk from uninoculated quarters.

## Newcastle Disease Virus as Antigen

Inoculation of live Newcastle Disease virus into the mammary glands of two cows was followed by local clinical manifestations of inflammation which did not differ from those which followed bacterin inoculation in cows at a low level of production. In both animals, these symptoms subsided within 48 hours, as was the case with those inoculated with bacterins, even though this agent was alive and was recoverable for nearly 10 days from one animal. No generalized symptoms were noted at any time.

The results of the recovery trials are shown in Figure 5. It will be noted that the virus was recovered for as long as 216 hours from the inoculated quarter of Cow No. 13, from which milking had recently ceased. The virus could be recovered for only 24 hours, from the inoculated quarter of Cow No. 14, which was at a stage of low production. It was observed that the virus concentration decreased rapidly in the samples collected during this period.

The virus recovered 8 hours after inoculation produced typical Newcastle Disease symptoms when administered intranasally in 3-week-old chickens.

Results of serum neutralization tests on the milk from

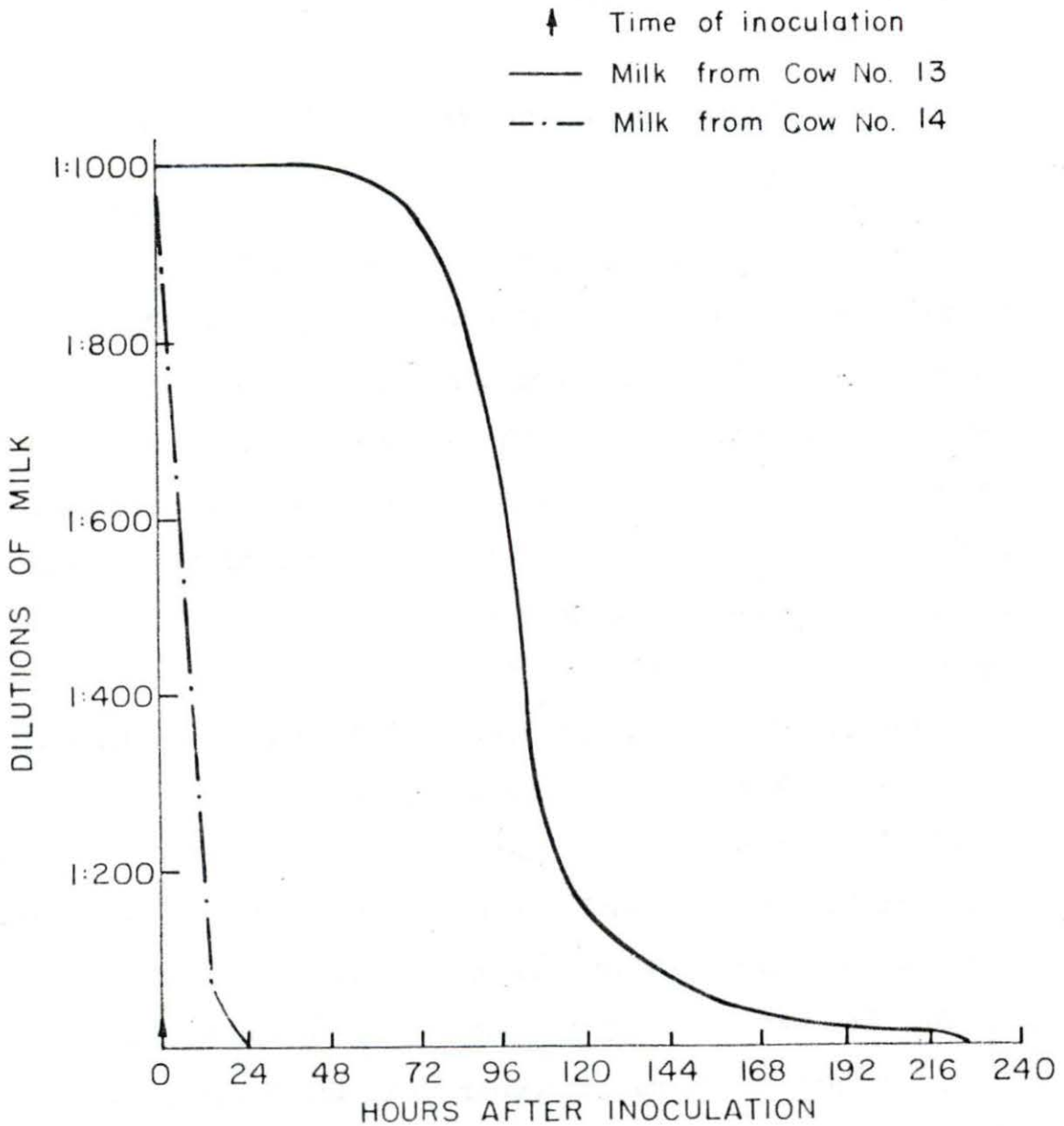


Figure 5. Greatest dilution of milk from which Newcastle Disease virus was recovered at various time intervals following inoculation

the inoculated quarter of Cow No. 13 are presented in Figure 6. These tests, employing embryonated eggs for inoculations, showed that neutralizing antibodies were detected in the milk from the inoculated quarter of Cow No. 13 at a very low level 11 days after inoculation, and the neutralization index rose rapidly after the 20th day post inoculation.

Hemagglutination-inhibition test results are shown in Figure 7. Hemagglutination-inhibiting antibodies were demonstrated 10 days post inoculation in Cow No. 13. They appeared first in the milk from the inoculated quarter, and soon thereafter in uninoculated quarters. Titers in the blood were detected still later. The hemagglutination-inhibition titers developed during the 24 days of post inoculation testing rose in this same order.

No hemagglutination-inhibiting antibodies were detected in the blood, milk from the inoculated quarter, or milk from the uninoculated quarters of Cow No. 14.

## Intramammary Infusion Ointment as Antigen

Total production and the results of Whiteside tests, catalase tests, and butterfat determinations, following each of the 3 administrations, are shown in Figures 8, 9, 10, and 11, respectively.

Severe local inflammatory symptoms followed administration of intramammary infusion ointment into mammary glands,

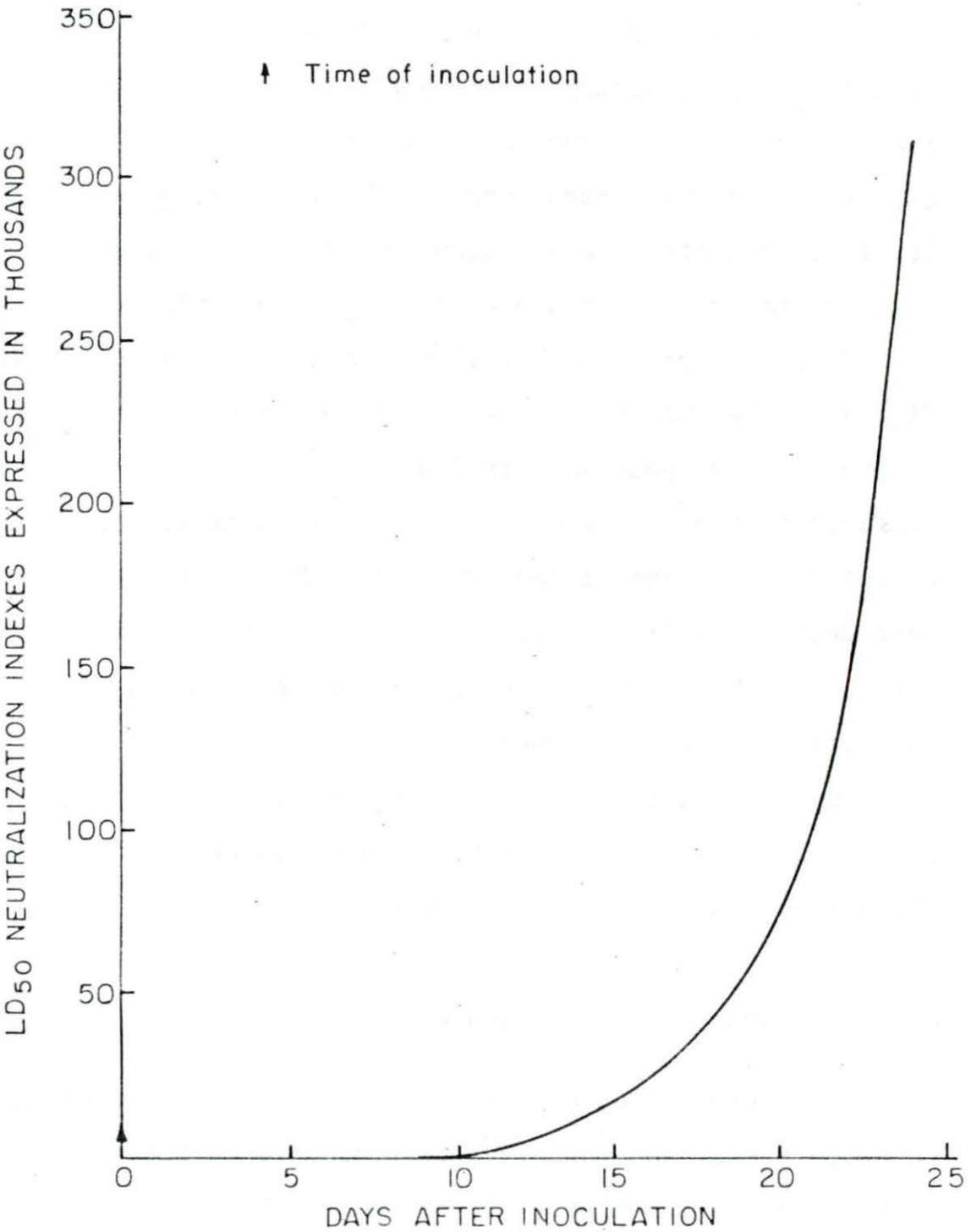


Figure 6. Serum neutralization indexes of milk of Cow No. 13 after inoculation of Newcastle Disease virus

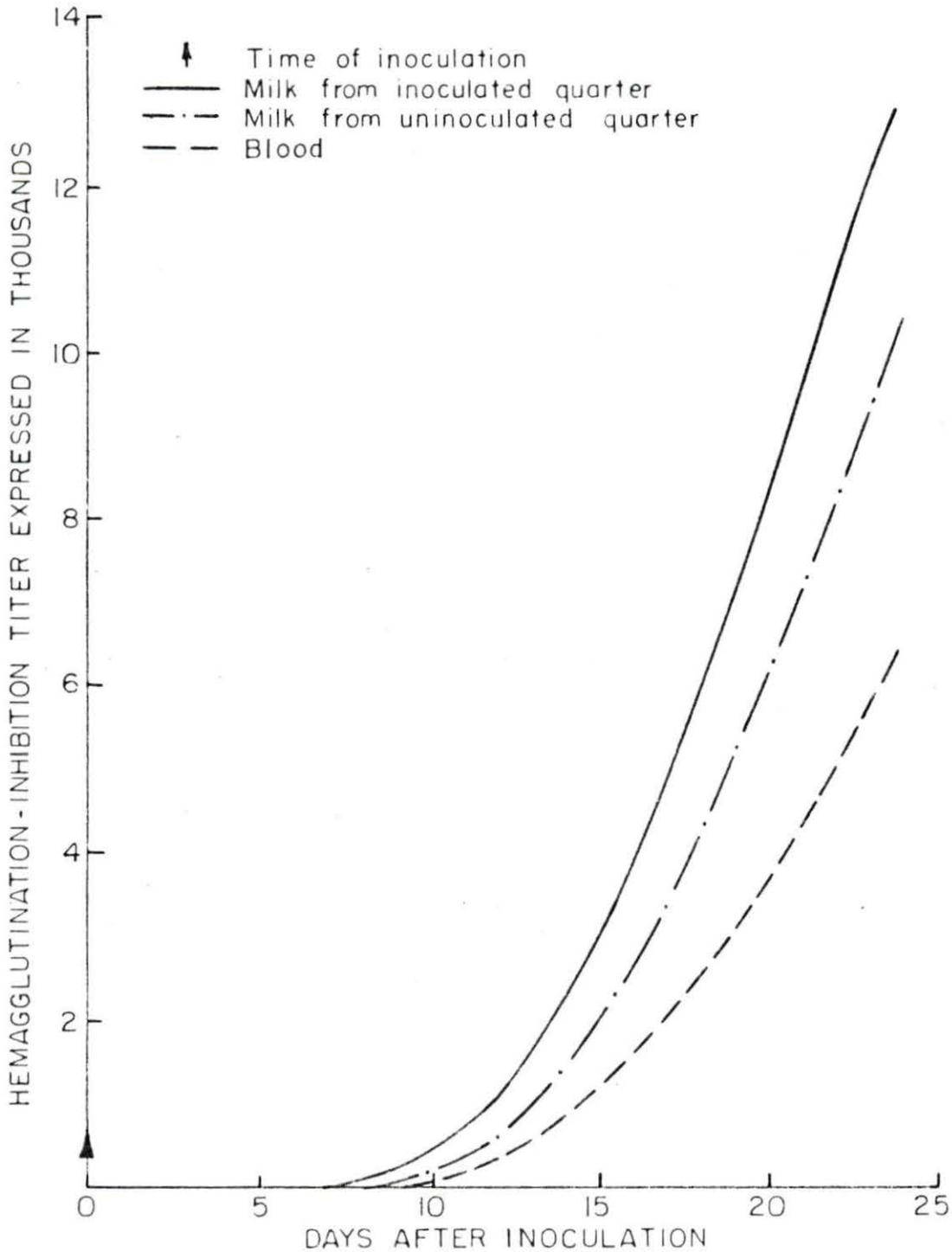


Figure 7. Hemagglutination-inhibition titers of milk and blood of Cow No. 13 following Newcastle Disease virus inoculation

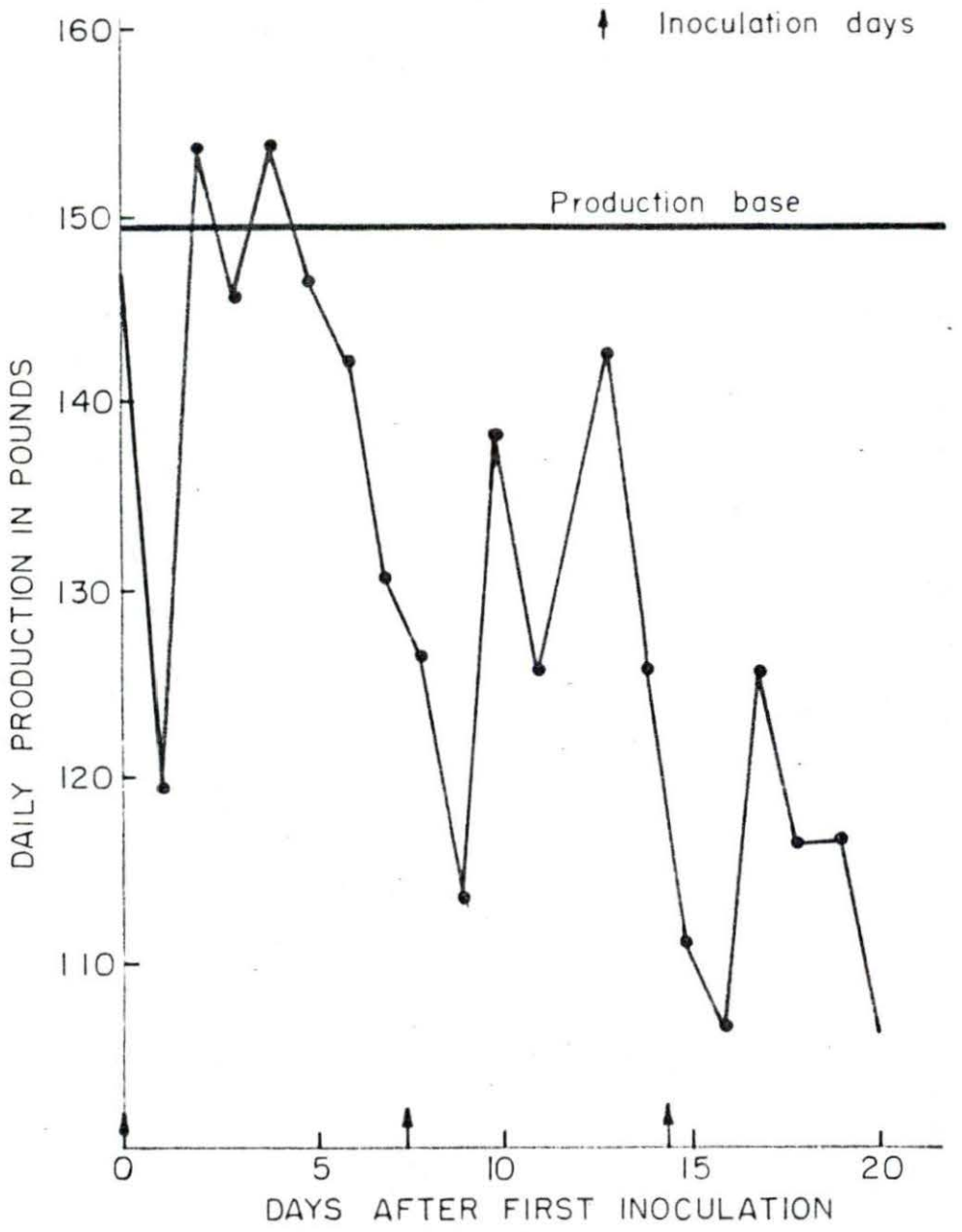


Figure 8. Total daily production of 9 cows after inoculation of an intramammary infusion ointment

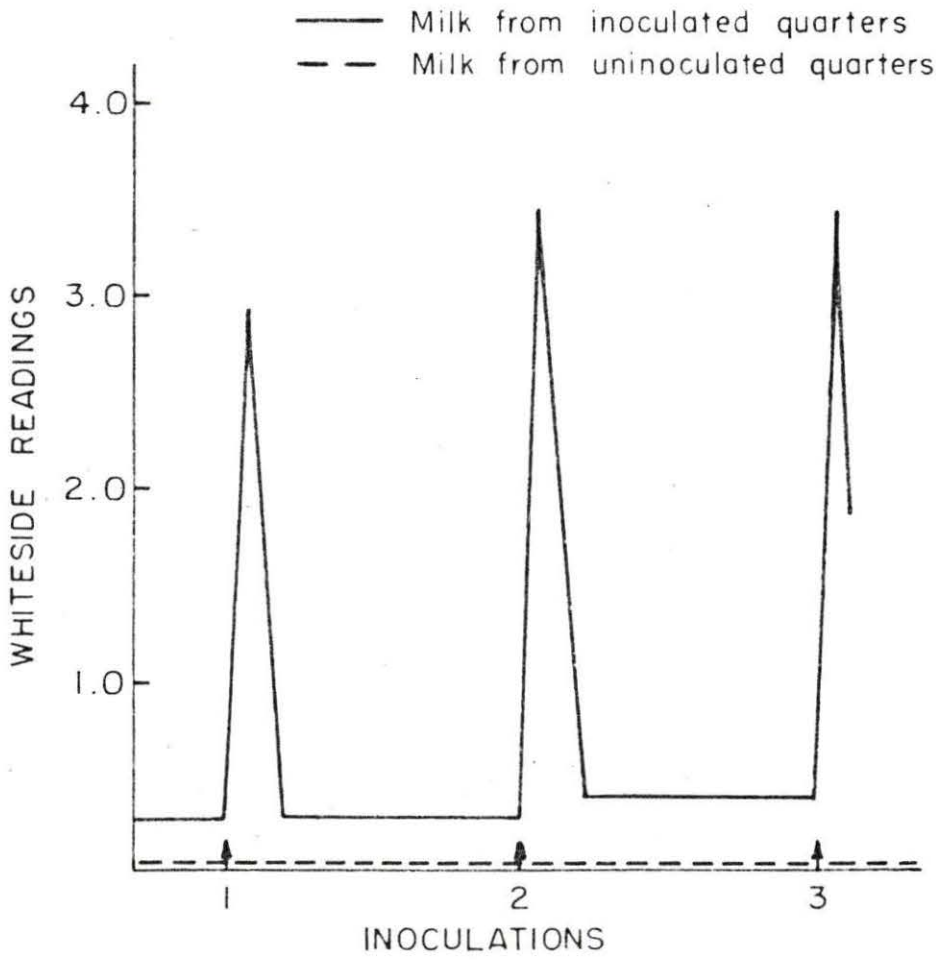


Figure 9. Average Whiteside test readings on milk after administration of intramammary infusion ointment

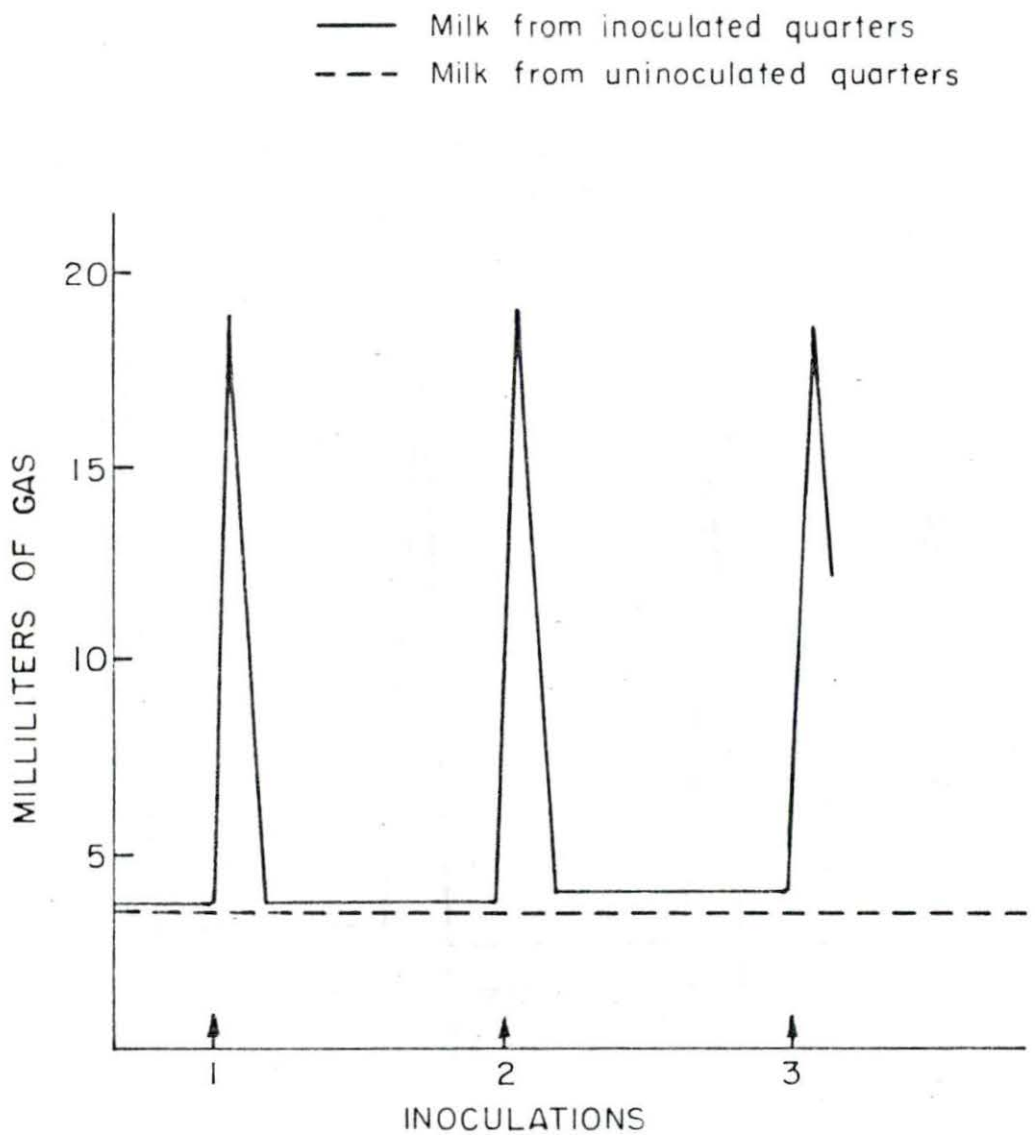


Figure 10. Average catalase test readings on milk after administration of intramammary infusion ointment

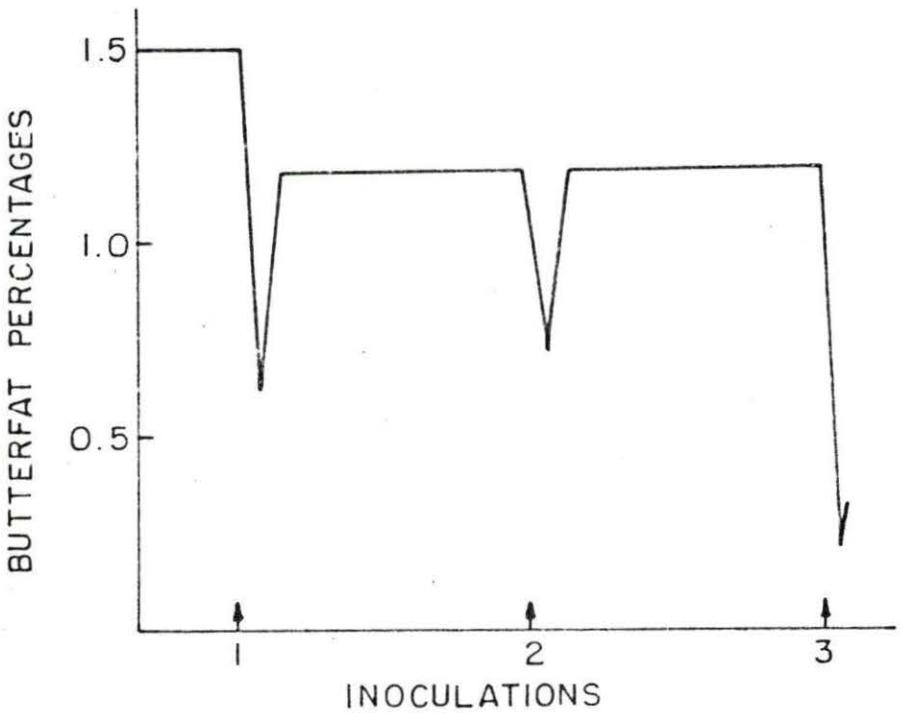


Figure 11. Average butterfat percentages of foremilk from inoculated quarters after administration of intramammary infusion ointment

especially in cows at a low level of production. The swelling and sensitiveness were greater in these animals than in those inoculated with bacterins and a live virus (Newcastle Disease Virus). Secretions were immediately increased in amount in low-producing animals, but decreased in those producing over 10 pounds of milk daily. Milk quality was lowered by the time of the first milking after inoculation. Secretions became serous, containing clots. Butterfat percentages were lowered in all instances, and Whiteside and catalase test readings rose immediately. Symptoms were less evident 24 hours after inoculation, and had disappeared after the lapse of another 24 hours.

After repeated inoculations of the same product, the inflammatory symptoms were less severe, but the grossly observed effects on secretions were quite similar to those following the first inoculation. The results of Whiteside and catalase tests were practically the same as those obtained after the first inoculation. Following each succeeding inoculation, the total production and butterfat percentages dropped.

## Staphylococcus Toxin and Toxoid as Antigens

Results of tests for antitoxin in the milk of inoculated quarters are given in Figure 12. It will be noted that the unaltered toxin was the most antigenic, and that no

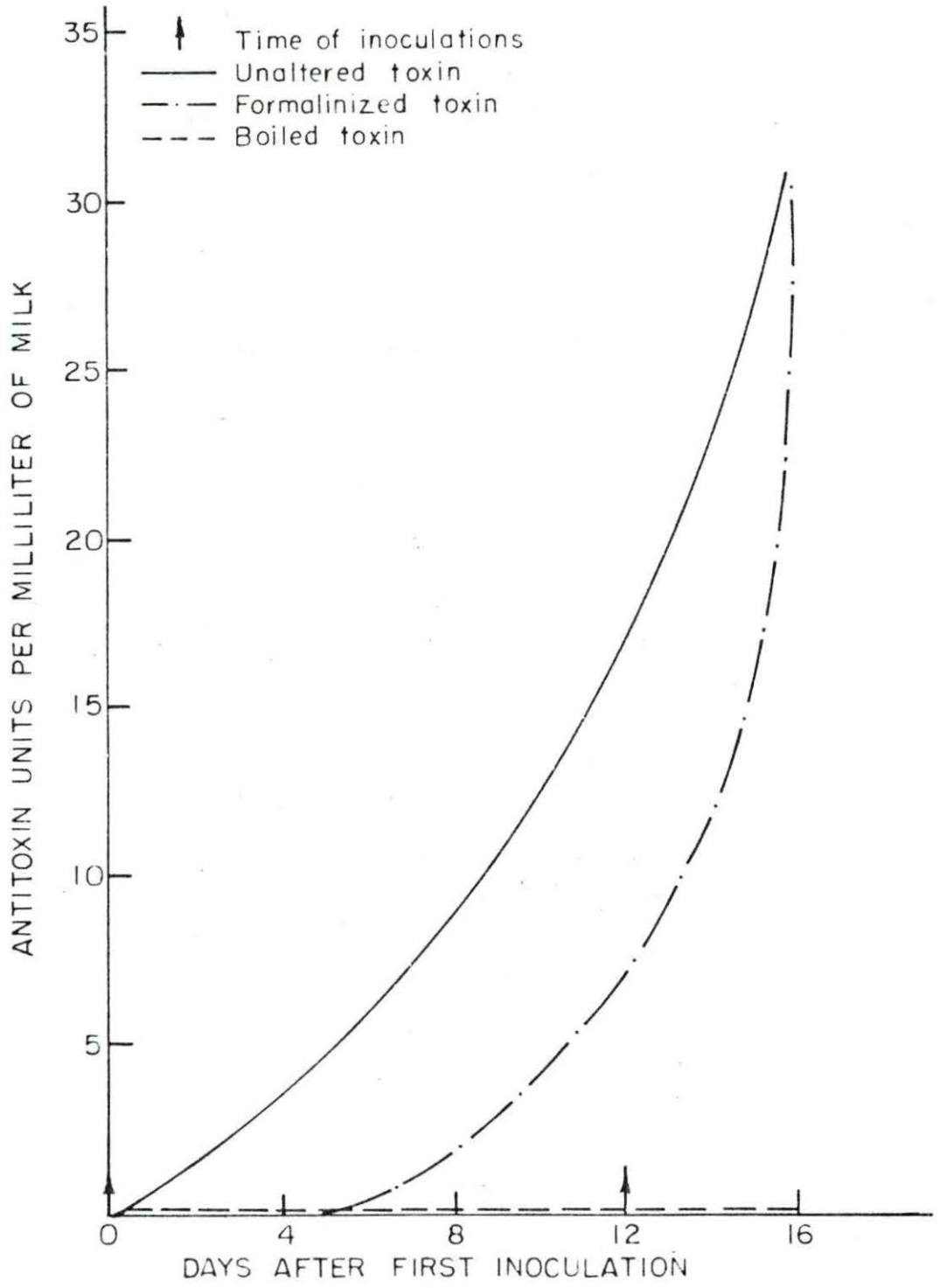


Figure 12. Levels of antitoxin in milk from quarters inoculated with staphylococcus toxin and detoxified toxin

antitoxin was detected in the quarter inoculated with boiled toxin. Antitoxin was demonstrated in uninoculated quarters of the cows receiving unaltered toxin and formalinized toxin, however in these quarters the antitoxin appeared later and titers rose more slowly. No antitoxin was demonstrated in milk from uninoculated quarters of the cow which received boiled toxin. Milk from infected and non-infected quarters of the chronic case of staphylococcic mastitis contained much higher titers of antitoxin than milk from inoculated animals. The same was noted in milk from the heifer which recently contracted staphylococcic mastitis.

Figure 13 shows a comparison of antitoxin titers in milk from the inoculated quarter, milk from an uninoculated quarter, and blood of Cow No. 4010, which was inoculated with unaltered toxin. It will be noted that the antitoxin titer in the blood was much higher than in the milk. With Cow No. 4054, which was inoculated with formalinized toxin, these comparisons are similar.

Table 3 gives a comparison of antitoxin levels in the blood of the 3 inoculated cows and of the 2 cows with staphylococcic mastitis. Antitoxin titers are higher in the blood of infected animals, even though the infection was of short duration in one, than in animals inoculated with toxin or toxoid. Antitoxin was demonstrated in the blood of Cow No. 4178, which was inoculated with boiled toxin, even

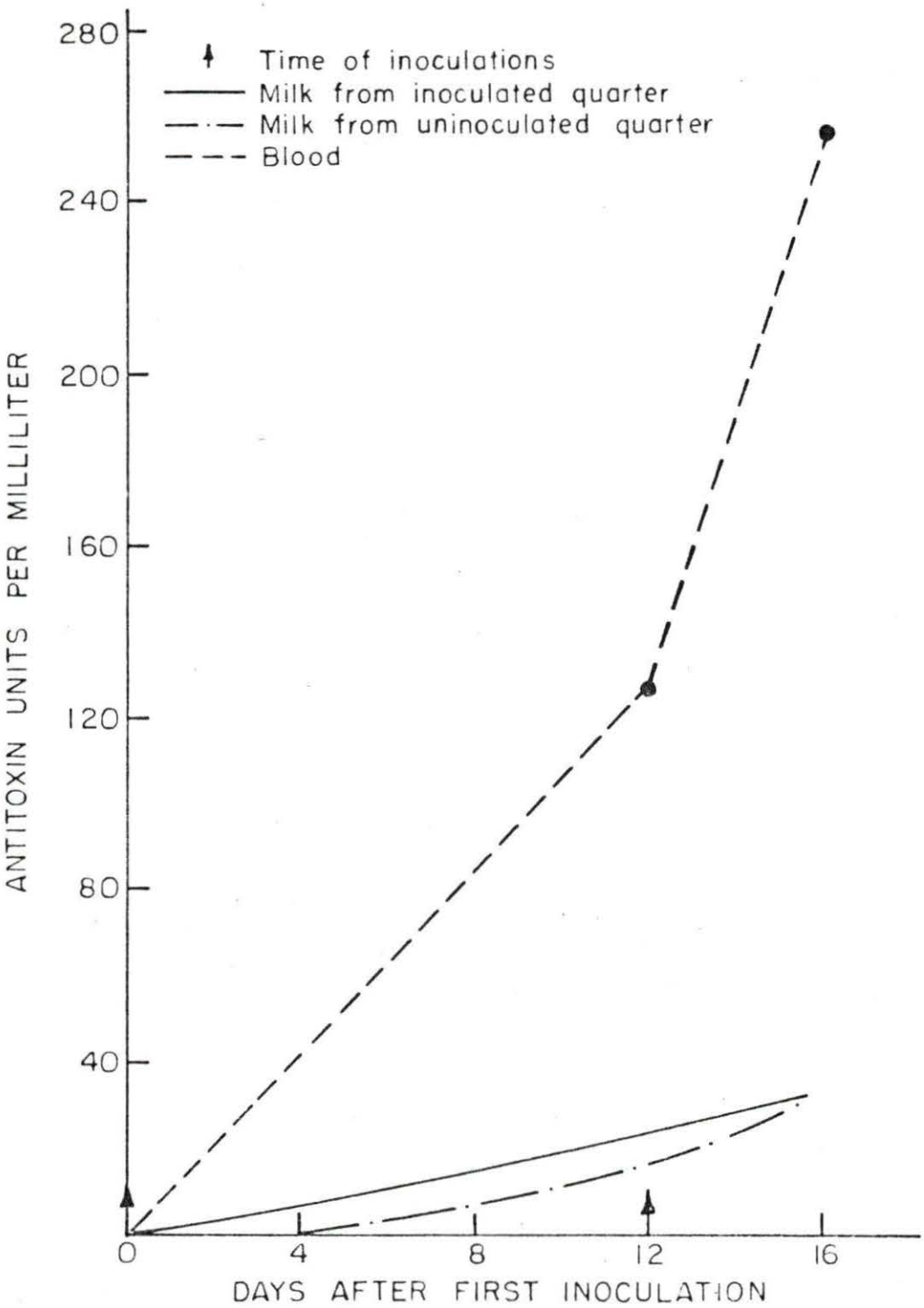


Figure 13. Units of antitoxin per milliliter following intramammary inoculation of staphylococcus toxin

though none could be detected in the milk from any of her quarters. The standard staphylococcus antitoxin supplied by the National Institute of Health contained 1024 units of staphylococcus antitoxin per ml., as determined by the same test.

Table 3. Staphylococcus antitoxin units per ml. of blood serum

| Cow No. | Infection status | Units at initial exam. | Units post inoc. 12 da. | Units post inoc. 16 da. |
|---|---|---|---|---|
| 4010 | Non-infected | 0 | 128 | 256 |
| 4054 | Non-infected | 0 | 128 | 128 |
| 4178 | Non-infected | 0 | 64 | 64 |
| 3565 | Chronic infection | 4096 | | |
| 4122 | Recent infection | 1024 | | |

Local inflammatory reactions followed inoculation of the staphylococcus toxin and toxoid. No differences were noticed between the effects of the 3 products used. Reactions were comparable to those described following the intramammary inoculation of bacterins. In the 2 cows which were reinoculated, the production of milk dropped noticeably after the second administration.

# DISCUSSION

## Bacterins as Antigens

Results obtained indicate that bacterins inoculated into the bovine mammary gland provide sufficient stimulation for the elaboration of antibodies which are measurable in the milk of all lactating quarters and blood of the inoculated animal.

After bacterins produced from Sal. choleraesuis and Br. abortus were injected into mammary glands, antibodies were demonstrated first in milk of the inoculated quarters, next in uninoculated quarters, and later in the blood, with the indication that the levels approached equality after the cessation of localized stimulation. This appears to be the sequence of developments in cows with no previous stimulation by a particular antigen.

The immediate development of antibodies indicates that they are produced by tissues whose functions include the rapid production of large quantities of proteins, especially of lacto-globulins. The sequence of appearance of the antibodies indicates that this production is a function of tissues within the mammary gland itself, probably the structures whose functions include the synthesis of milk proteins. This sequence of appearance of antibodies in body fluids, and the rapidity of appearance in a structure which

synthesizes large amounts of proteins, are consistent with the theories that antigens stimulate alterations of subsequently produced globulins, rather than changes in previously synthesized globulin molecules.

Following intramammary inoculation of bacterins, titers in both milk and blood reached a level comparable with those usually obtained after parenteral inoculation of bacterins at other sites. These levels indicate the possibility of protective properties of the antibodies produced in mammary glands, either by active or passive immunization, in those infections where therapeutic and prophylactic properties of antibodies have been shown.

The E. coli bacterin was inoculated into 2 cows whose blood sera showed a titer to that organism of 1:12.5 at the time of inoculation. The rise in titer in the blood, as shown in Figures 3 and 4, was more rapid than the increase measured after inoculation of Sal. choleraesuis and Br. abortus bacterins. The rise also appeared after a shorter period of time following inoculation, and titers rose higher. In view of the fact that a titer to the organism was present at the time of inoculation, it appears that a natural stimulation by this organism may have occurred previously, and these rapid responses were partially due to an anamnestic reaction. Differences in titers which developed in the milk of Cows No. 3 and No. 4 may be due to the level of

production of these 2 animals. The effect of this factor should be determined by controlled experiments.

Spencer and Angevine (13) reported evidence that hypersensitivity of the bovine and rabbit mammary glands is responsible for some of the manifestations in infections due to Streptococcus agalactiae. Results of tests recorded herein indicate that a similar condition develops following bacterin inoculation, as evidenced by an increasing severity of the reactions after repeated inoculations. If the material inoculated were merely toxic, then the first injection would produce as much reaction as the second and third. The manifestations of hypersensitivity in these experimentally produced cases are similar to those symptoms seen in cases diagnosed clinically as mastitis but yield no recognized pathogens upon culture. It appeared to the author that such cases occur most frequently during rainy seasons when husbandry and sanitation fail to prevent natural exposure to saprophytes and accidental inoculation with them. If such exposure occurs and saprophytic bacteria do enter in these conditions, it would be reasonable to expect that a hypersensitivity to them would be produced after the first exposure and symptoms of mastitis would develop after repeated exposures. This points to the practical necessity of cleanliness as an aid in reducing the incidence of accidental entry of any bacteria into the bovine mammary gland.

## Newcastle Disease Virus as Antigen

Recovery of the virus of Newcastle Disease for 9 days after its inoculation indicates that multiplication of the virus occurred. Complete milking of the secretions at the time of each collection would have diluted the virus beyond the end-point of recovery. Also, in one cow, No. 14, the virus could not be recovered more than 24 hours after inoculation, which indicates that in this instance the virus did not multiply and that which had been injected was milked out. The failure of the Newcastle Disease virus to become established in this cow is not explained, however it does not appear that active immunity was responsible, since no antibodies in her milk or blood could be demonstrated by the hemagglutination-inhibition test.

The fact that no virus was recovered from milk from quarters adjacent to the inoculated one, or from the blood, appears significant, especially in connection with the high level of hemagglutination-inhibiting antibodies in these fluids. Persistence of virus in the inoculated quarter agrees satisfactorily with the results of Mitchell et al. (10), who reported recovery for 15 days after inoculation.

Antibodies, both hemagglutination-inhibiting and neutralizing, were first detected in the milk from the inoculated quarter of Cow No. 13 soon after disappearance of the virus from the milk. The hemagglutination-inhibiting

antibodies were soon thereafter detected in milk from uninoculated quarters, which differs from the work of Mitchell et al. (11), who reported no detectable antibodies in uninoculated quarters. In a later report, these investigators reported antibodies appearing in the uninoculated quarters of cows following inoculation of one quarter. They also reported that antibodies developed in the milk of the inoculated mammary gland and blood of a goat, but only a trace was shown at any time in the other mammary gland. However, they were working with a different strain of Newcastle Disease virus, the Twiss strain. It is also noted that they did not remove the non-specific agglutinins in bovine milk for chicken erythrocytes, when performing the test for hemagglutination-inhibiting antibodies.

That the bovine mammary gland developed high titers of antibodies to a virus which apparently survived and multiplied for a period of 9 days after inoculation, even though it is not normally considered a pathogen of bovines, is significant as evidence of the response of a bovine mammary gland to antigenic stimulation.

No hemagglutination-inhibiting antibodies were detected at any time from either of the mammary glands or from the blood of Cow No. 14, from which the virus was recoverable for only 24 hours. This finding is similar to that reported by Mitchell et al. (11) when chemically inactivated

Newcastle Disease virus was injected into a bovine mammary gland. Mitchell et al. (12) also reported that when the virus of poliomyelitis was injected into a mammary gland, propagation did not take place and neutralizing antibody was not found in the milk or blood serum. These facts make it appear that virus multiplication may be necessary in order to stimulate antibody production in the bovine mammary gland.

Following the inoculation of Newcastle Disease virus, antibodies were first demonstrated 11 days after inoculation. This differs greatly from the rapid response after the inoculation of bacterins into the bovine mammary glands. The difference is not explained, but it may be due to the fact that the virus remained virulent for 9 days. Milk dilutions which were made to determine the amount of virus shed in the milk, indicated that during the first few days after inoculation a constant amount of virus was present in the milk of the inoculated quarter. This indicates a balance between virus multiplication and antibody production, which prevented the in vitro and in vivo demonstration of antibodies. After continued stimulation, the antibody production increased proportionately faster than virus multiplication, which resulted in virus elimination from the gland and free antibodies to react in hemagglutination-inhibition and serum neutralization tests.

Local inflammatory symptoms were evident in both cows

which were inoculated with Newcastle Disease virus. The symptoms and milk alterations resembled those described as occurring following bacterin inoculation. Since the virus apparently failed to infect the mammary gland of one cow, it appears that in her case, at least, the inflammatory reaction was due to other inoculated materials, probably some component of the allantoic fluid. No measure of the immunological response to such components was attempted.

## Intramammary Infusion Ointment as Antigen

The inoculation of intramammary infusion ointment into healthy mammary glands produced an inflammatory reaction following each injection as evidenced by the Whiteside and the catalase tests. These tests did not indicate any significant difference in degree of severity following the 3 injections. Grossly, the mammary glands appeared to be less affected by succeeding inoculations. However, during the period of observation, a progressive lowering of production followed each administration. These observations point toward an allergic response by the mammary glands, since the reaction became progressively more severe.

The development of allergic conditions by inoculation of intramammary infusion ointments into a gland which is reactive to introduced antigens, indicates the advisability of avoiding such states of sensitization.

## Staphylococcus Toxin and Toxoid as Antigens

The results obtained indicate that the bovine mammary gland responds to the antigenic stimulation of staphylococcus toxin inoculated into its milk-collecting spaces. The sequence of appearance of antibodies appears to be in the milk of the inoculated quarters, in uninoculated quarters, and then in blood. Blood titers were much higher than those of milk, however these were cows in good production. It is believed that the continuous secretion of cows in high production diluted the antitoxin and kept titers low.

In this experiment, unaltered toxin was the most antigenic, and boiled toxin was the least antigenic. Boiled toxin possessed some antigenicity for, as shown in Figure 13, antitoxin was demonstrated in the blood following stimulation by this product. An explanation for the absence of antitoxin in the milk of this same animal is lacking.

Antitoxin titers, developed as a result of inoculation, rose more slowly than titers in a first calf heifer with staphylococcic mastitis. The continued stimulation in the gland with mastitis may have resulted in a more rapid response. It appears that titers develop much higher in animals with staphylococcic mastitis than in animals inoculated with toxin or toxoid. This raises doubt as to the practical value of such inoculations in the prevention of

staphylococcic mastitis.

As has been noted with inoculations of other materials, the inflammatory reactions followed each administration, and marked drops in production were a consequence of repeated inoculations. The significance of these observations is important.

## Implications of the Results Observed

The ability of the bovine mammary gland to respond to antigenic stimulation and to produce antibodies rapidly and in large amounts in the milk, indicates the possibility of the use of this phenomenon for the passive immunization of those human beings or animals that consume milk frequently and regularly. This utilization may be the result of a planned program, or may function without deliberate planning, especially in situations where sanitation is poor. Work reported by Campbell et al. (2) demonstrated that the nursing young may inoculate the mammary gland of the lactating mother during feeding. When the organisms which are introduced are pathogens of the offspring, homologous antibodies would soon be consumed in the milk. However, the question might be raised as to whether or not antibodies produced in the milk of one species would be absorbed into the blood of another species after oral administration. The possibility of digestion of ingested antibodies must also be

considered. Light is shed on these questions by the findings of Gruskay and Cooke (6), who reported on the gastrointestinal absorption of unaltered proteins in infants recovering from diarrhea, and in infants convalescing from diseases unrelated to the gastrointestinal tract. Quantitative determinations for the absorption of egg albumin in blood drawn 1 and 2 hours after administration by gavage, demonstrated that egg albumin could be absorbed by both groups, but the concentration was significantly higher in patients recovering from diarrhea. This gastrointestinal absorption without digestion is of even greater significance when consideration is given to the fact that the protein was from a different species. These investigators suggested that this absorption could stimulate the production of a state of hypersensitivity to proteins ingested at such times. The absorption of unaltered antibodies from milk would be desirable if the inoculation of antigens caused no unfavorable effects on the mammary gland of the cow, and the absorption produced no hypersensitivity in the consumer.

Production of antibodies by a mammary gland, in response to the stimulation of organisms which are pathogens of this gland, or of products of their metabolism, indicates possibilities of influencing later natural infections by the same organisms. It was noted that antibody titers produced by this method did not rise as fast as in cases of natural

infections. Perhaps they do not rise as high. The possibility exists that the hypersensitivity developed may be of greater importance than the protective value of antibodies present. Experimentation to determine the relative importance of these reactions is needed for a conclusive evaluation.

The immediate inflammatory reaction which follows intramammary inoculations is sufficiently severe to question the advisability of such inoculations. Acceptance by the dairyman seems unlikely, especially if reinoculations in hypersensitive mammary glands, which cause a marked drop in production, are required for adequate levels of antibodies in the milk. During this study, observations indicated that intramammary inoculations of any substance are undesirable.

The survival of Newcastle Disease virus in an unnatural host indicates a possibility for altering pathogenicity of this virus for both the natural and the unnatural hosts, and the development of variant strains, following experimental or natural transfers, but this was not investigated.

Since bovine mammary glands respond to antigenic stimulation, and hypersensitivity is demonstrated, substances which are instilled into mammary glands as medicaments should be examined to determine their antigenicity. It appears that hypersensitivity resulting in lowered production and abnormal secretions is obtained along with

beneficial effects of bacterial inhibition. Observations strongly indicated the desirability of other routes of administration of medication, if comparable therapeutic effects are obtained.

Development of hypersensitivity in the bovine mammary gland appears to occur as a result of inoculation of organisms which usually are not considered pathogenic for this organ. This points to the desirability of strict sanitation to prevent accidental inoculations. Since repeated inoculations may lead to loss of milk production, prevention of hypersensitivity gives added significance to regulations for sanitary physical facilities and procedures. The health and functioning of the mammary gland are affected by these procedures. It appears that if antibodies are desired in milk, stimulation by inoculation by some other route is preferred. It also seems desirable, if antibodies are proven to be of value in the treatment of mastitis cases, to administer them passively using antiserum with very high titers, rather than depend on active immunization which sensitizes the bovine mammary gland. The value of such antitoxin is doubted, unless titers are extremely high. The writer found it difficult to find mature cows without antitoxin, however resistance to staphylococcic infection seemed to be low.

# CONCLUSIONS

1. Bacterins of Sal. choleraesuis, Br. abortus, and E. coli, when instilled into the bovine mammary gland by way of the teat orifice, stimulate the production of agglutinating antibodies which can be demonstrated in the milk of inoculated and uninoculated quarters and in the blood.
2. The antibodies produced as a result of stimulation by bacterins inoculated into the bovine mammary gland appear within 2 hours following inoculation in the milk of the inoculated quarters. Later they are detectable in the milk of uninoculated quarters, and still later titers are measurable in blood serum. This sequence of appearance also follows the intramammary inoculation of viable Newcastle Disease virus, however the response was delayed.
3. In most instances, antibody titers following intramammary inoculations reach a high level.
4. Newcastle Disease virus can become established for a period of time up to 9 days in the bovine mammary gland. Hemagglutination-inhibiting and neutralizing antibodies are produced as a result of this stimulation. They are present in the milk of inoculated and uninoculated quarters and in the blood, however antibodies are not demonstrable until the virus is eliminated from the milk.

5. Antibody titers developed in milk, following stimulation by bacterins and viruses inoculated into the bovine mammary glands, approach those developed in circulating blood and persist with continued regular milking.
6. The bovine mammary gland shows allergic response to substances contained in intramammary infusion ointments. This development is expressed most clearly by a progressive decline of production and lowering of the quality of milk.
7. Antitoxin to staphylococcus hemotoxin is produced following the inoculation of staphylococcus toxin or toxoid into the bovine mammary glands. The sequence of appearance of the antitoxin is in the milk of the inoculated quarter, in the milk of the uninoculated quarter, and in the blood serum. Titers develop more slowly than in cases of staphylococcic mastitis.
8. The extent of the reactions produced by the inoculation of antigenic substances into the bovine mammary gland prevents the justification of such inoculations if the desired production of antibodies can be developed to a comparable titer following some other route of administration which causes no unfavorable reactions.

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