JULY, 1952 RESEARCH BULLETIN 500

UNIVERSITY OF MISSOURI COLLEGE OF AGRICULTURE

AGRICULTURAL EXPERIMENT STATION

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A Study of Some Causative Mechanisms In Streptococcus Agalactiae Infections Of the Bovine Mammary Gland

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(Publication authorized June 9, 1952)

COLUMBIA, MISSOURI

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ABSTRACT

Microscopic examination of streptococcal mastitic milk and broth cultures of *Streptococcus agalactiae* indicated that the morphological form of *Streptococcus agalactiae,* as found in the udder, apparently differs from that found in many laboratory cultures. Casein-acetate-lactose broth was found to favor the maintenance of morphological characteristics which were similar to those of the organisms in unincubated streptococcal mastitic milk. Contaminant bacterial cell-forms in the complex constituents of some media interfered with morphological observations of cultures grown on those particular media. "Motility" was exhibited by *Streptococcus agalactiae* cells. In three instances, cell multiplication was observed following a brief period of rapid, progressive movement by pairs of diplococci.

The effect of *Streptococcus agalactiae* filtrates upon the *in vitro* anaerobic glycolysis of bovine mammary tissue slices was studied by means of the Warburg technique using sodium acetate as the metabolic substrate. A total of 135 determinations were made on tissue from seven cows. The Lancefield group B organisms were isolated from acute cases of streptococcal mastitis and grown in a casein-acetate-lactose broth. Eighteen-hour cultures were rendered sterile by means of Selas No. 03 porcelain filters and the comparative effect of these filtrates and uninoculated medium determined manometrically. Short-chain *Streptococcus agalactiae* filtrates caused a marked decrease in the rate of anaerobic glycolysis compared to the slight inhibitory effect of long-chain *Streptococcus agalactiae* filtrates and uninoculated medium.

A total of 122 intramammary infusions of *Streptococcus agalactiae* filtrates and uninoculated casein-acetate-lactose broth were made into individual quarters of eight cows. Infusions of short-chain *Streptococcus agalactiae* filtrates produced hardness and swelling of the infused quarters within one to two hours, followed by rectal temperature increases of three to five degrees Fahrenheit reaching a peak six to nine hours after infusion. The rectal temperatures returned to approximately normal at 12 to 14 hours although production of physically abnormal milk persisted for two to three days. Subsequent daily infusions of the filtrate in the same quarter resulted

in continued production of abnormal milk; however, the response of the quarter in the form of swelling gradually decreased with each consecutive infusion. Temperature response to continued infusions varied from no effect to a gradual decline in temperature of three to five degrees Fahrenheit. Subsequent infusions of filtrate in different quarters of the same cow caused marked physical and temperature responses similar to those induced by the initial infusion. Infusions of the uninoculated medium and long-chain *Streptococcus agalactiae* filtrates produced only minor symptoms of irritation in the infused quarters although the milk obtained at the first post-infusion milking period was physically abnormal.

INTRODUCTION

The numerous references in the literature to streptococcal mastitis graphically illustrates the importance of this disease to the dairy industry. Yet the large volume of literature on the subject also serves to indicate the complex nature of mastitis and the difficulties involved in making a study of this udder infection.

Streptococcus agalactiae has been designated by many research workers as the principal causative organism in chronic bovine mastitis. Although basic information on the actual pathogenic mechanisms of *Streptococcus agalactiae* is very limited, there can be little question of its pathogenicity. The spread of this infection through a herd from an infected animal, with the subsequent decreased milk production and the toxic symptoms displayed by animals suffering from a flare-up of this disease, readily indicates that under certain conditions the organism is definitely virulent in its effect on the bovine mammary gland. However, attempts to produce mastitis experimentally with cultures of *Streptococcus agalactiae* have given variable results. These results have sometimes been attributed to resistance of the experimental animals but more often to a variation in the virulence of the particular cultures employed. The factors responsible for the virulence of *Streptococcus agalactiae* are not definitely known; however, there is an apparent association of virulence with certain biochemical and morphological characteristics of the organisms. Thus, morphological variations may indicate more profound changes in the physiology of *Streptococcus agalactiae.* Since these variations would tend to obscure the causative mechanisms involved in streptococcal mastitis, a study of the means by which *Streptococcus agalactiae* induce the mastitic symptoms should include consideration of streptococcal variation.

Streptococcus agalactiae infections of the bovine mammary gland often induce general systemic reactions in addition to the ef-

fects localized in the udder. The systemic reactions are usually seen only in acute flare-ups of the disease, whereas the effects localized in the mammary gland may be detected in all forms of mastitis. The localized effects are reflected by swelling of the quarter and in the secretion of abnormal milk. The various changes in composition of the milk would seem to indicate that the enzymes involved in milk secretion are adversely affected by products of *Streptococcus agalactiae*. A consideration of this latter problem would seem to be of prime importance in determining the actual means by which *Streptococcus agalactiae* causes bovine mastitis.

This study was undertaken in an effort to gain a more specific knowledge of the means by which *Streptococcus agalactiae* causes the pathological changes in bovine mammary tissue, as well as to provide a basis for further studies on the physiology of this organism as related to bovine mastitis.

REVIEW OF LITERATURE

Bovine mastitis has been described as any inflammation of the udder resulting from a variety of etiological factors such as physical injuries, chemical, thermal, and infectious agents (Little and Plastridge, 1946). Regardless of the contributory agent, mastitis may be characterized as acute, subclinical, or chronic depending upon the severity of the condition. In the acute form, the entire udder or only individual quarters may be involved with the affected portion showing a severe reaction in addition to a general systemic disturbance of the animal. The subclinical form is usually so mild that only bacteriological or biochemical tests will detect the abnormal condition in the udder or its secretion. The most common form of mastitis is that of the chronic type which progresses slowly with only occasional flare-ups; thus, there is a gradual replacement of secretory tissue by fibrotic tissue with an accompanying decrease in milk production in addition to physical and chemical changes in the secretion itself.

Numerous causative agents have been found to be associated with infectious mastitis such as streptococci, staphylococci, corynebacteria, coliform, and viruses (Little and Plastridge, 1946). The streptococcal group of organisms seem to be primarily associated with the chronic form of the disease; whereas, the staphylococci, corynebacteria, and coliform organisms, although often associated with chronic mastitis, are the predominating contributory bacteria in the cases of acute mastitis. The relation of viruses to bovine mastitis is still the subject of considerable controversy.

Broadhurst, *et al.* (1939) isolated a filtrable virus from the milk

and blood of cows infected with mastitis, and they were able to propagate the virus in tissue cultures for 32 serial transplants. The transplantation of infected tissue cultures into mice caused an inflammatory response in the lymph nodes and mammary glands from which the virus could be reisolated. Cellular inclusion bodies which are characteristic of viruses were demonstrated in the inoculated mice as well as in the tissue cultures and milk. Similar results were reported by Baker and Little (1946) who isolated a virus-like agent from mastitic milk. Four to seven days after the agent had been transferred to guinea pigs a fever response was obtained which lasted for a period of three days. The virus could also be propagated in rabbits, mice, and the chorioallantoic membrane of embryonic hen eggs. Subcutaneous inoculation of the virus in the neck of two lactating cows produced a response similar to the natural infection; the virus could be removed from the blood of both cows and the milk of one. The formation of neutralizing antibodies for the virus was found to occur in laboratory animals as well as in cows and calves. Peterson, *et al.* (1938) failed to find inclusion bodies in cases of nonspecific mastitis, but their experimental results seemed to indicate a virus as being the most probable etiological agent in nonspecific mastitis. Nyiridy (1943) and Stuart and Lancaster (1949) failed to induce mastitis in cows by intramammary injections of bacteriologically sterile filtrates of mastitis milk. The contradictory findings of the foregoing reports illustrates the lack of agreement on the specific relationship between virus agents and bovine mastitis.

The Morphology and Physiology of Streptococci

Bacteria belonging to the genus Streptococcus are considered to be the major contributory agents in chronic bovine mastitis; however, the predominating type of bacteria may vary at different times in any particular herd (Little and Plastridge, 1946). The classification and characterization of the numerous species of streptococci still presents many problems. Some difficulties connected with the use of strictly morphological and biochemical classification systems have been resolved through the classical work of Lancefield (1933) on group serological classification of hemolytic streptococci.

As a result of the work of Lancefield and other investigators, many of the streptococci have been divided into a number of serological groups, each of which is characterized by a group specific carbohydrate. Most of the strains of streptococci pathogenic for humans have been found to belong to Lancefield group A, while those organisms in group B are commonly associated with bovine mastitis. Some strains belonging to group C have also been found to be infrequently associated with mastitis. Lancefield (1934a) studied 21 strains

of group B streptococci and was able to differentiate them into three specific types through the use of precipitin tests. Chemical analysis of the type-specific substances showed that they were polysaccharide in nature as contrasted to the protein substances responsible for type-specificity among strains belonging to group A. The group-specific substance was also found to be of a polysaccharide nature. Plastridge and Hartsell (1937) studied the biochemical and serological characteristics of streptococci isolated from bovine sources and concluded that the biochemical tests were useful in classifying bovine streptococci into broad groups, but the precipitin test was necessary for final identification of the organisms.

Morphologically, streptococci belonging to Lancefield group B, *Streptococcus agalactiae,* when cultured on bacteriological media have been characterized as spherical or ovoid cells with a diameter of 0.4 to 1.2 microns. The organisms usually occur in chains of seldom less than four cells in length and frequently appear to be composed of paired cocci (Breed *et al.,* 1948). Rosell (1931) however, found that when infected milk sediment was examined for mastitis streptococci, the organisms frequently occurred in the form of isolated individuals, diplococci, or in small masses of diplococci and micrococci without showing the extreme chaining often associated with pure laboratory cultures. Also, spherical forms were found among the individuals composing the chains or masses of streptococci and these were thought to be "hypertrophic forms or a phase of predivision." Subsequent work by Dawson, *et al.* (1938) indicated that *Streptococcus agalactiae* has at least three distinct cultural phases which are expressed by different morphological characteristics. The organisms appear as diplococci in the mucoid phase, short chains in the smooth phase, and as long tangled chains frequently composed of large and coarse cells in the rough phase. The morphology of the rough phase was further characterized by variation in cell size and shape with a marked tendency for the cell members of the chain to have flattened opposing surfaces. These cells are apparently similar to the giant streptococci of Mellon's giant-coccus rough culture phase (Mellon, 1948) wherein the individual cells in the chain appear to be elongated with the long axis transverse to the length of the chain. Similar forms have been reported by Rosell (1931) who also recognized an apparent line of division transverse to the long axis of the cocci and parallel with the chain length. This he attributed to a tendency for the cocci to divide in a second direction of space although the division did not occur after chain formation.

Mellon (1920) studied pure cultures of diphtheroids isolated from a single bacterial cell and observed a transformation of these

diphtheroids to diplococci having characteristics essentially those of streptococci. This transformation was confirmed by his subsequent observations on a smooth phase culture of group A hemolytic streptococcus (Mellon, 1948; Mellon and Cooper, 1938). The organisms underwent repeated spontaneous transformations to a typical nonhemolytic diphtheroid which in turn redissociated to a group C, animal type, streptococcus. Thus the diphtheroid appeared to constitute a phase in a cyclic dissociation pattern of streptococci. Mellon also cited the suggestive evidence recorded by Dorner for the rapid *in vivo* conversion of hemolytic streptococci to diphtheroids in cows recovering from mastitis. In contrast to this, Peterson, *et al.* (1938) could find little or no reason for considering the diphtheroids to be etiological agents in nonspecific mastitis.

In a study on transmutation of group B streptococci, Beck (1950) was able to demonstrate a change of group B carbohydrate antigen to group A when dissociated group B streptococci were cultured in the presence of heat-killed group A streptococci. He found that fresh human blood has little effect on the transmutation of group B streptococci to another group, but under the condition of his experiments, it was directly responsible for the reversion of rough phase group B to smooth phase group B. A study on the dissociation of group A beta-hemolytic streptococci by Solotorovsky and Buchbinder (1941) demonstrated a mathematically precise rate of dissociation with regard to the degree of hemolysis on sheep's blood agar. The dissociation appeared to be correlated with changes in virulence; however, there was no apparent change in antigenicity according to type and group specific reactions. Similar results were reported by Lancefield (1934b) from observations on a variant from a culture of group B hemolytic streptococci which lost the powers of hemolysis and pigment production although retaining the virulence of the original culture as well as the same antigenic and serological specificity. Also, differences in the ability of group B variants to hemolyze blood and attack salicin and lactose have been found by Sherman, *et al.* (1941). Dawson, *et al.* (1938) reported an apparent gradual loss of capsule formation, virulence, and type specificity for group B streptococci accompanying changes from the mucoid phase to the smooth and rough phases. Hadley and Wetzel (1943) concluded that the potential virulence of a strain of streptococci isolated from a case of subacute endocarditis was determined exclusively by the dissociative culture phase of the organism at the time of the test, but that the actual virulence was controlled by the effect of environmental influences on a single culture phase. Their subsequent work (Hadley and Wetzel, 1947) on the same strain of organisms indicated that a flocculent

sediment type of growth was characteristic of a lack of virulence or weak virulence, in contrast to the diffuse cloudy type of growth almost invariably exhibited by cultures of moderate to high virulence. However, both forms of growth could occur in the same tube. Dawson, *et al.* (1938) also found that diffuse growth was characteristic of the more virulent mucoid organisms in the case of group B streptococci, while a flocculent growth denoted organisms in the avirulent rough phase. Young streptococci in the logarithmic growth phase have been found to be apparently more invasive (Felty and Bloomfield, 1924) and better able to withstand the bactericidal power of human blood (Hare, 1929) in addition to showing a more homogeneous growth of diplococci and short-chains (Loewenthal, 1938) than those cultures in the declining phase of growth. Results of a study on the distribution of the various Lancefield groups of streptococci in human infections (Rantz and Keefer, 1941) indicated that though over 95 per cent of the infections were due to group A streptococci, organisms belonging to groups B and C occasionally cause serious human infections. Fry (1938) reported on three fatal human infections due to group B streptococci, and the review by Lancefield (1940) includes a record of 20 human fatalities resulting from group B infections. These reports tend to substantiate the statement "—that streptococci belonging to groups B, C, and G may not be so innocuous to man as was originally thought" (Anonymous, 1942). Group B streptococci have also been found to be pathogenic for the chorioallantoic membrane of embryonic chicks in the studies of Sherwood, *et al.* (1949). Using goats as the experimental animal, Holman, *et al.* (1950) found that the mouse virulence of *Streptococcus agalactiae* was increased when the organisms were reisolated from the goat mammary gland.

The Relation of Enzymes to Streptococcal Virulence

Several conflicting reports regarding the importance of enzymes in streptococcal virulence have been presented. Wenner, *et al.* (1951) studied the hyaluronidase enzymes produced by Lancefield groups A, B, and C and found them to differ serologically. The hyaluronidase production and capsulation of group B streptococci was studied by McClean (1941) who found that the capsules of group B are not composed of hyaluronic acid, in contrast to groups A and C. Crowley (1944) found that all of the hyaluronidase producing strains studied in groups A, C, and G were noncapsulated but that the lack of capsule formation was not necessarily indicative of hyaluronidase production. There was little apparent correlation between hyaluronidase production and virulence. In contrast to this, Sallman and Birkeland (1950) concluded that hyaluronidase is an important fac-

tor in the pathogenicity of hemolytic streptococci and that a relationship does exist between hyaluronidase production by streptococcal strains and their virulence for humans. Work by Rothbard (1948) on the hyaluronic acid capsule of group A streptococci indicates that the capsule is only a minor factor in mouse virulence of these organisms. Other investigators have failed to find any correlation of hyaluronic acid or hyaluronidase production by group A streptococci with the mouse virulence (Pike, 1948, 1950). Similar results were obtained by Russell and Sherwood (1949) when the virulence of streptococci belonging to groups A, B, and C for the chorioallantoic membrane of the embryonic chick could not be directly related to the hyaluronidase production of the streptococci. Gochanauer and Wilson (1951) were unable to correlate the *in vitro* hyaluronidase production of streptococci isolated from cases of bovine mastitis with the degree of inflammation observed in the mastitic quarters. Their results also indicated that hyaluronidase did not increase the effectiveness of antibiotics used in treating mastitis caused by *Streptococcus agalactiae* and *Streptococcus dysgalactiae.*

The production of the enzymes ribonuclease and desoxyribonuclease by various groups of streptococci has been studied by several investigators. McCarty (1948) found that all of the strains of group A hemolytic streptococci tested produced both nucleases which were released into the culture medium. Brown (1950) made a survey of the ribonuclease and desoxyribonuclease production of 267 cultures representing 24 groups or species of streptococci. Of the strains tested, all those belonging to group A and 20 of 73 classified as group B produced both nucleases; whereas, most strains of group C and some strains belonging to groups F, G, and L produced only the desoxyribonuclease. Neither type of nuclease was found to be produced by any of the strains belonging to the other streptococcal species which were studied. Evidence obtained by Bernheimer and Ruffier (1951) indicates that desoxyribonuclease production by streptococci occurs in the resting cell system rather than during growth of the organisms. Partial inhibition of desoxyribonuclease from strains of group A streptococci was caused by sonic extracts of groups A, B, C, and D cocci. However, groups B and C desoxyribonuclease was not inhibited by extracts of homologous strains or strains belonging to other groups. Thus, the results would seem to indicate a variation in the type of desoxyribonuclease produced by various streptococci.

Nutritive Factors Affecting Virulence

A limited correlation between the virulence of group A hemolytic streptococci and their need for the presence of glutamine in the

growth medium has been demonstrated by McIlwain, *et al.* (1939) and Hale and McIlwain (1947). In the latter report, it was concluded that the need for glutamine may be only one of many factors associated with virulence or that virulence and glutamine-need may represent parallel but independent adaptations to the environment of the host. The specific requirement of glutamine to obtain good growth of *Streptococcus hemolyticus* was emphasized by Landy (1939), McIlwain (1939) and McIlwain, *et al.* (1948); however, group B hemolytic streptococci have been indicated as differing from group A streptococci in that they are able to grow without the presence of glutamine in the nutrient media (Fildes, 1939). Similar results were obtained by Niven (1943) who found that the addition of glutamine to the nutrient media did not alter the growth of eight strains of group B streptococci. The exact role of glutamine in the growth and virulence of streptococci is as yet unknown although the breakdown or utilization of glutamine by beta-hemolytic streptococci has been demonstrated only in the presence of glucose (McIlwain, 1946b; McIlwain, *et al.* 1948). During the reaction, the glucose is largely converted to lactic acid (McIlwain, 1946a). McIlwain (1946a) suggests that glutamine participates in streptococcal glycolysis by means of an ammonia transference. A more definite correlation of streptococcal virulence with a component of the nutrient media has been reported by McIlroy, *et al.* (1948). In their studies on Lancefield group C hemolytic streptococci, the presence of sodium acetate in the culture medium was necessary to maintain the virulence of the organism when cultured *in vitro*. After 64 serial daily transfers, the group C streptococci cultured on media containing sodium acetate possessed a virulence 600,000 times greater than that of organisms which had been grown on the same basal medium without sodium acetate.

Immunity and Streptococcal Mastitis

Studies on immunity to streptococcal mastitis and vaccination against this disease have received considerable attention although the results present a rather confused picture of the immunological relationships involved. Jones and Little (1928) found that the inoculation of scarlet fever streptococci into one quarter of the bovine udder apparently conferred some immunity to the other quarters of the udder. The immunity was indicated by the milder course of infection upon the subsequent inoculations of noninfected quarters with the same strain of streptococci. Hadley, *et al.* (1930) however, could find no evidence that one infection produced immunity against later attacks of streptococcal mastitis. Contradictory results have also been obtained with regard to vaccination for streptococcal mas-

titis. Rosell (1931) used a polyvalent autovaccine in treating 168 infected cows and found this vaccine to be 77 per cent effective when given in conjunction with weak formalin-milk injections. In contrast to this report, Hucker and Hansen (1937) concluded that vaccines prepared with stock and freshly isolated strains of *Streptococcus agalactiae* gave no indication of causing increased resistance to mastitis in dairy cattle. More recent studies by Pattison (1948) indicate that mice and guinea pigs can be immunized against group B streptococci by intraperitoneal inoculations of the homologous strain. The serum of goats tested after experimental inoculation with *Streptococcus agalactiae* was found to give increased protection against test doses of the streptococci in mice (Pattison, *et al.,* 1950).

Agglutinins for *Streptococcus agalactiae* have been detected in the blood serum and whey of infected cows (Plastridge and Cunningham, 1942). Graham, *et al.* (1932) did not observe any local reactions following the intradermal injection of *Streptococcus epidemicus* filtrate into cows. However, additional evidence on the immunological relationships of streptococcal mastitis has been contributed by Spencer and Angevine (1950). In their work, rabbits and cows infected with *Streptococcus agalactiae* gave an inflammatory response to intradermal injections of streptococcal antigen, thus showing antibody formation. The results on cattle indicated that hypersensitivity may be an important factor in bovine streptococcal mastitis.

Experimental Production of Mastitis

By means of intramammary infusions, experimental production of bovine mastitis has been successful with a number of bacterial strains and species belonging to the genus Streptococcus. Various strains of hemolytic streptococci of human origin were found to cause mastitis when injected into the bovine mammary gland via the teat canal (Bendixen and Minett, 1938; Davis and Capps, 1914; Hadley, *et al.,* 1930; Jones and Little, 1928). Hadley and Frost (1933), however, found a species difference in the ability of various hemolytic streptococci to produce mastitic symptoms in the bovine mammary gland. Beta-hemolytic strains of *Streptococcus infrequens* and *-equi* failed to produce clinical symptoms of mastitis although *Streptococcus epidemicus* caused a severe mastitis condition. Variable results were obtained with strains of alpha-hemolytic *Streptococcus mitis;* some inoculations resulted in severe mastitis and others caused only temporary symptomatic changes. *Streptococcus mastitidis* was found to be capable of causing a severe and more or less chronic type of mastitis. Carpenter (1922) was able to establish a transient infection by introducing hemolytic streptococci into the udders of

lactating cows. Other studies (Foley and Byrne, 1949) have shown that the rapid progressive growth of streptococci within infected quarters is inhibited by the inflammatory changes in milk. The ease of producing bovine mammary infection with *Streptococcus agalactiae* has been shown to be correlated with the dilution of the culture employed (Little, 1937) and number of inoculations when small numbers of organisms were used (Jones and Little, 1934). However, Taylor, *et al.* (1950) could find no correlation between the degree or type of reaction and the number of living *Streptococcus agalactiae* inoculated into the teat canal of goats.

The intramammary inoculations of *Streptococcus epidemicus* (Hadley and Frost, 1933) and *Streptococcus agalactiae* (Miller, 1934) in cows have been demonstrated to cause an increase in the body temperature of the animals. Maximum body temperatures of 104.6 degrees Fahrenheit to 106 degrees Fahrenheit resulted within a day after the inoculation of cows with *Streptococcus epidemicus,* but the temperatures returned to approximately normal during the following two-day period. Temperature response to *Streptococcus agalactiae* inoculations was less pronounced, the maximum being 103.4 degrees Fahrenheit. However, in studies using goats as the experimental animal, Pattison, *et al.* (1950) found that intramammary infusions of living *Streptococcus agalactiae* produced sharp systemic reactions lasting two to three days with a temperature rise to four degrees Fahrenheit above normal within the first 24 hours. The infusion of heat-killed streptococci into the goat mammary gland caused a sharp increase in body temperature during the first 24-hour post-inoculation period but no marked general systemic disturbance occurred. Temperatures returned to normal on the second day and remained normal during the rest of the observation period. No marked response was obtained to the infusion of ten milliliters of Seitz EK filtrate of whole broth culture; however, the slight changes recorded were similar in direction to those caused by the infusion of living or dead streptococci. Stuart and Lancaster (1949) found that one milliliter infusion of Seitz EK filtrates of a bacteriologically sterile mastitic secretion into bovine mammary glands failed to cause mastitis. Similar results were obtained with bovine intramammary infusions by Dawdy and Petersen (1947) using Seitz filtrates of *Streptococcus agalactiae* cultures in 2.5 to 10 milliliters quantity. The infusion of whey resulting from the *in vitro* action of *Streptococcus agalactiae* on milk into both lactating and non-lactating bovine udders in quantities of 50 to 1650 milliliters produced symptoms of acute streptococcal mastitis (Pounden and Zehner, 1941).

The Effects of Bacterial Products Upon Tissue Metabolism

Rosell's studies on intramammary and intramuscular injections in cows and guinea pigs indicated that mastitis streptococci have a specific pathogenic effect for the glandular tissues of the udder (Rosell, 1931). Peskett and Folley (1933) were of the opinion that mastitis caused pathological rather than physiological changes in the secretory cell membranes thus permitting blood constituents to pass into the milk. Pathologic alterations of the bovine mammary gland in acute mastitis observed by Spencer and McNutt (1950) suggested that the causative agent spreads rapidly in the infected quarter by means of the duct system. The inflammatory agent was postulated as being lactic acid, endotoxins, an exotoxin, or a combination of these factors. Using several carbohydrates and amino acids as substrates, Frei and Witschard (1950) studied the effect of bacterial metabolic products upon the dehydrogenase activity of guinea pig liver extracts. The products of *Streptococcus agalactiae* stimulated the dehydrogenation of pyroracemic acid, glutamic acid, and alanine. Variable effects were obtained with glucose, lactic acid, malic acid, leucine, and histidine, while a definite inhibitory influence was exerted upon the dehydrogenation of succinic acid and oxalacetic acid. In contrast, products of *Streptococcus hemolyticus* had a uniform acceleratory action upon the dehydrogenation of all substrates tested. Other studies of the effect of bacterial toxins upon enzymatic processes indicate that glycogen synthesis is inhibited by diphtheria toxin (Cross and Holmes, 1937) and meningococcal or Salmonella endotoxin (Kun, 1948a, 1948b; Kun and Miller, 1948). These endotoxins inhibited the succinic dehydrogenase activity of both muscle and liver; whereas, cytochrome oxidase activity was not affected (Kun and Miller, 1948). Work by Reineke, *et al.* (1941) indicates that mastitis streptococci retard the *in vitro* oxidation of ascorbic acid.

Utilization of Acetate by the Mammary Gland

Relatively little work has been done on the enzymatic processes involved in milk secretion; however, *in vitro* studies were made by Folley and French (1949) on the intermediary metabolism of the mammary glands from several mammalian species. In the presence of glucose, the mammary tissue from the mouse, rat, guinea pig, and rabbits had a respiratory quotient of well above 1.0 in contrast to a respiratory quotient of less than 1.0 for mammary tissue from ruminants such as the goat and cow. Thus, the results indicate that the short-chain fatty acids of ruminant milk fat are not synthesized from carbohydrate in the udder. Subsequent studies were made on milk fat synthesis of both ruminants and nonruminants with acetate,

glucose, or a combination of the two as substrates (Folley and French, 1950). Mammary tissue from ruminants, including sheep, goats, and cows, was shown to utilize acetate although it was practically inert with respect to glucose. The utilization of acetate by mammary tissue from the rat, rabbit, and sheep was found to be stimulated by the presence of glucose.

Perfusion studies on an isolated lactating bovine udder using heparinized blood containing radioactive sodium acetate has shown that the acetate is utilized by the mammary gland for the synthesis of milk fat (Cowie, *et al.,* 1951). Similar results were obtained by means of intravenous injections of radioactive sodium acetate into a lactating goat (Popjak, 1951). Half of the radioactivity which remained in the body was found in the milk fat, and fractionation of the fat revealed that the short-chain group of fatty acids had a higher radiological activity than the long-chain fatty acids. Thus, the short-chain fatty acids appeared to be synthesized from acetate rather than formed by the breakdown of long-chain fatty acids. The studies also indicated synthesis of the milk fatty acids in the udder rather than transfer of fatty acids from the blood into the milk.

MATERIALS AND METHODS

Cultural:

The *Streptococcus agalactiae* cultures used in this study were obtained from animals in the Missouri Experiment Station dairy herd. All cultures were isolated from blood agar plates containing five per cent defibrinated bovine blood and two per cent sodium acetate. These plates were incubated at 37.5°C. for 24 hours. Individual, sub-surface, beta-hemolytic colonies were picked into tubes containing ten milliliters of casein-acetate-lactose broth medium; the medium being a modification of that developed by McIlroy, *et al.* (1948).

CASEIN-ACETATE-LACTOSE BROTH
Basal Medium

Casein hydrolyzate	30.0 g.
Sodium acetate	20.0 g.
Lactose	40.0 g.
K_2HPO_4	.5 g.
KH_2PO_4	.5 g.
$MgSO_4.7H_2O$	200.0 mg.
$FeSO_4.7H_2O$	10.0 mg.
$MnSO_4.2H_2O$	6.0 mg.
NaCl	10.0 mg.
$CuSO_4.5H_2O$	1.0 mg.
$ZnSO_4.7H_2O$	1.0 mg.
$CaCl_2.2H_2O$	10.0 mg.
l-cystine	100.0 mg.
l-tryptophane	20.0 mg.
Uracil	10.0 mg.
Adenine	10.0 mg.
Nicotinic acid	1.0 mg.
Pyridoxine hydrochloride	1.0 mg.
Calcium pantothenate	4.0 mg.

Thiamine hydrochloride	1.0 mg.
Folic acid	1.0 mg.
Riboflavin	.5 mg.
Biotin	.0002 mg.
Vitamin B_{12}	5.0 gamma
Distilled Water	900.0 ml.

Addition Products

Sodium bicarbonate	2.0 g.
Glutamine	200.0 mg.
Thioglycollic acid	130.0 mg.

This medium, without the addition products, is adjusted to a pH of 6.5 with ten per cent sodium hydroxide, filtered, tubed in nine milliliter aliquots, and then autoclaved for 15 minutes at 15 pounds pressure. Immediately prior to inoculation of the media, .5 milliliter of glutamine solution and .5 milliliter of sodium bicarbonate-thioglycollic acid solution is added aseptically to each tube containing nine milliliters of casein-acetate-lactose broth. The glutamine solution is prepared by adding 400 milligrams of glutamine to 100 milliliters of sterile distilled water, filtering through a sintered glass bacteriological filter, and then storing aseptically at 0 to 4°C. The individual components of the sodium bicarbonate-thioglycollic acid solution are prepared as follows: 200 milligram portions of sodium bicarbonate are weighed into individual screw-cap test tubes and sterilized in the autoclave. The thioglycollic acid stock solution is prepared by adding one milliliter (1.3 grams) to nine milliliters of sterile distilled water and then boiling in a water bath for ten minutes as described by Adams and Roe (1945). Just before making the additions to the broth tubes, ten milliliters of sterile distilled water are added to a tube containing the sodium bicarbonate and then 0.2 milliliter of the ten per cent thioglycollic acid solution is added to the bicarbonate solution to give the final sodium bicarbonate-thioglycollic acid addition mixture.

The Lancefield group of all streptococcal cultures was determined with the use of capillary pipettes for conducting the precipitin reaction as described by Swift, *et al.* (1943). Neopeptone phosphate broth was used as the basal growth medium for preparation of the streptococcal extracts. This broth is a modification of tryptose phosphate broth (Difco Lab. Inc., 1948) and is composed of 20 grams neopeptone, two grams glucose, five grams sodium chloride, 2½ grams disodium phosphate (anhydrous) and one liter of distilled water. The constituents are dissolved and boiled for ten minutes, then the pH is adjusted to 7.6, followed by filtration through Whatman No. 40 filter paper using a Buchner filter. The medium is tubed in 40 milliliter aliquots and autoclaved at 15 pounds pressure for 20 minutes. Prior to inoculation, two milliliters of sterile, filtered "V-8 juice" is added to each tube. The "V-8 juice" is a commercial vegetable ex-

tract of unknown composition which provides additional growth factors for the streptococcal organisms, thus giving a heavier cell growth to be used in the preparation of the bacterial extract for the Lancefield group classification. To date, no adverse effects of this vegetable extract have been noted on the capillary group precipitin reactions.

The bacteria-free filtrates of *Streptococcus agalactiae* used in this study were prepared by filtering 18 hour cultures of the organisms (grown on casein-acetate-lactose broth) through sterile Selas No. 03 porcelain filters. The pH of this filtrate was then adjusted to 7.4 with sterile ten per cent sodium hydroxide.

Manometric:

The manometric studies were made using a rectangular Warburg Apparatus accommodating 14 reaction flasks per experimental run. Prior to the manometric determinations, all manometers and reaction flasks were simultaneously gassed with a 95 per cent nitrogen and five per cent carbon dioxide gas mixture using a water vacuum pump and manifold gassing arrangement. The reaction flasks and manometers were alternately evacuated and filled with the gas mixture a minimum of ten times to insure replacement of the original atmosphere by the nitrogen-carbon dioxide mixture. The composition and preparation of the Krebs-Ringer bicarbonate buffer was that given by Umbreit, *et al.* (1949) with the addition of 0.02 molar sodium acetate as used by Folley and French (1950). The manometric results have been expressed on a dry-weight basis using a 24 hour acetone extraction of the tissue followed by a 24 hour heating of the slices at 100 degrees Centigrade prior to weighing.

The mammary tissue used in the manometric studies was obtained from cows which had been culled from the station dairy herd because of low production, reproductive difficulties, or other conditions not attributable to mastitis. All animals were negative for mastitis at the time of slaughter according to the results of Hotis tests and microscopic examinations of incubated milk samples. The animals were slaughtered at a local slaughter house and the udder removed immediately after death of the animal. The udder was brought to the laboratory and portions of the secretory tissue removed and sliced by means of a Stadie-Riggs Tissue Slicer into sections approximately one centimeter in diameter and one-half millimeter thick. These tissue slices were then placed in the reaction flasks containing Krebs-Ringer bicarbonate buffer at pH 7.4 and were gassed with the nitrogen-carbon dioxide gas mixture. The manometric determinations immediately followed this gassing procedure. An average of 30 minutes elapsed between death of the

animal and suspension of the tissue slices in the buffering medium. If the tissue slices were to be stored before use, they were placed in a storage solution consisting of ten per cent bovine blood serum and 90 per cent modified Tyrode's solution containing 0.02 molar sodium acetate. Each slice of tissue was placed in a sterile 125 milliliter Erlenmeyer flask containing three milliliters of the storage solution. Then the flasks were tightly sealed with a rubber stopper. These flasks were stored at four to five degrees Centigrade until the tissue was used. No tissue was stored longer than three days before use.

Modified Tyrode's Solution

Sodium acetate	1.64 g.
NaCl	8.00 g.
KCl	.20 g.
$CaCl_2$ (anhydrous)	.20 g.
$MgCl_2$	.10 g.
NaH_2PO_4	.05 g.
$NaHCO_3$	1.00 g.
Redistilled water	1100.00 ml.

In the order listed, each of the first six compounds are weighed and added separately (stirring until each is dissolved before adding the next one) to 950 milliliters of redistilled water in a two-liter Erlenmeyer flask. The sodium bicarbonate is prepared separately in a 250 milliliter Erlenmeyer flask by adding .7 gram of sodium bicarbonate to 150 milliliters of redistilled water. Both solutions are then autoclaved at 15 pounds pressure for 30 minutes and allowed to cool in the autoclave. After cooling, enough of the sodium bicarbonate solution (approximately 100 milliliters) is added to the salt solution to give a pH of 7.4. The flask containing the final solution is sealed with a sterile rubber stopper and paper cap and stored at zero to 5°C. as described by Cameron (1950). The bovine blood serum was added just prior to use of the storage solution.

Intramammary Infusions:

The intramammary infusions were made with 18-hour *Streptococcus agalactiae* culture filtrates prepared in the same manner as those used in the manometric studies. Prior to the infusions, the udder and teats were washed with a clean cloth and chlorine water, and then the teats were immersed in 70 per cent alcohol. Sterile hypodermic syringes and teat cannulas were used to infuse the material through the teat canal. Following the infusion, the quarter was massaged in an attempt to disperse the material throughout the quarter.

Hotis tests and microscopic examinations of incubated milk samples were made daily on the fore-milk from each quarter of all cows in the study. The rectal temperatures of all animals were determined at regular intervals throughout the experiment.

OBSERVATIONS AND RESULTS

Bacterial:

Microscopic examination of unincubated fore-milk samples from cows infected with *Streptococcus agalactiae* indicated that very few long-chain streptococci were present; however, many organisms were present in the form of diplococci, short-chains, and masses of cells. Also present were an extremely large number of minute "granules." The small size of these "granules" even at a magnification of 1000x, in addition to possible staining artifacts, prevented the determination of granule shape and staining reactions. After the milk samples had been incubated at 37.5°C. for 24 hours, the predominate form of bacteria present was characteristic, long-chain streptococci. The number of diplococci, short-chains, minute "granules," and masses of individual cells was markedly reduced, and in some instances, no forms other than the long-chain streptococci were observed.

Pure cultures of beta-hemolytic *Streptococcus agalactiae* (Lancefield group B) revealed even more extensive morphological variations when grown on various laboratory culture media. When casein-ace-

Fig. 1.—Diplococcoid and short-chain forms of *Streptococcus agalactiae*. Twenty-four hours casein-acetate-lactose medium. Methylene blue. 1666x.

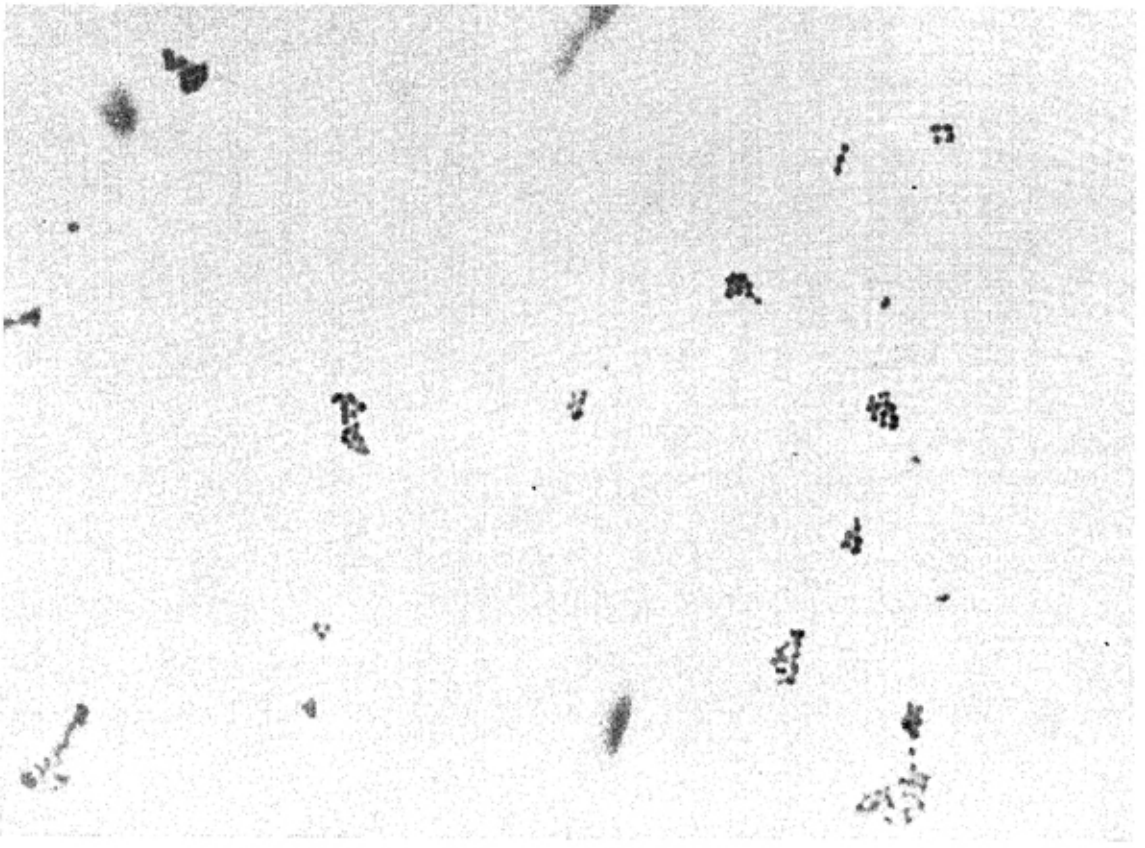

Fig. 2.—Masses of *Streptococcus agalactiae* cells in an 8-hour culture. Casein-acetate-lactose medium. Methylene blue. 1666x.

tate-lactose broth was used as the culture medium, the morphological form of the *Streptococcus agalactiae* was primarily that of diplococci and short-chains, although a few long-chain forms were usually present (Fig. 1). Occasionally the long-chain forms of *Streptococcus agalactiae* would predominate, but upon subsequent transfer, they would usually appear in the minority. Daily serial transfers were necessary for the continued propagation of *Streptococcus agalactiae* upon casein-acetate-lactose broth. A lapse of 36 to 48 hours between transfers resulted in apparent death of the culture. After 18 hours incubation at 37.5°C. of the viable culture, the microscopic appearance of the streptococci upon this medium was a diffuse cloudy growth with a compact sediment in the bottom of the culture tube. The culture tubes retained this general appearance for several weeks after the cultures became nonviable. When long-chains were the predominating morphological type, most of the cells settled out forming a granular sediment. Microscopic examination of the cultures which had been grown on the casein-acetate-lactose broth for a period of four to eight hours showed the presence of masses of cells (Fig. 2)

and minute granules, as well as diplococci, short-chains and occasional long-chain forms of *Streptococcus agalactiae,* similar to those which were observed in the unincubated milk samples.

Cultures of *Streptococcus agalactiae* grown in phosphate broths containing "V-8 juice" and either neopeptone or tryptose exhibited a flocculent type of growth with the organisms settling to the bottom of the culture tube in a fluffy sediment. Microscopic examination of cultures grown on these two media was rendered difficult by the presence of bacterial cell-forms in some constituents of the media (Figs. 3a and b). The morphological appearance of *Streptococcus agalac-*

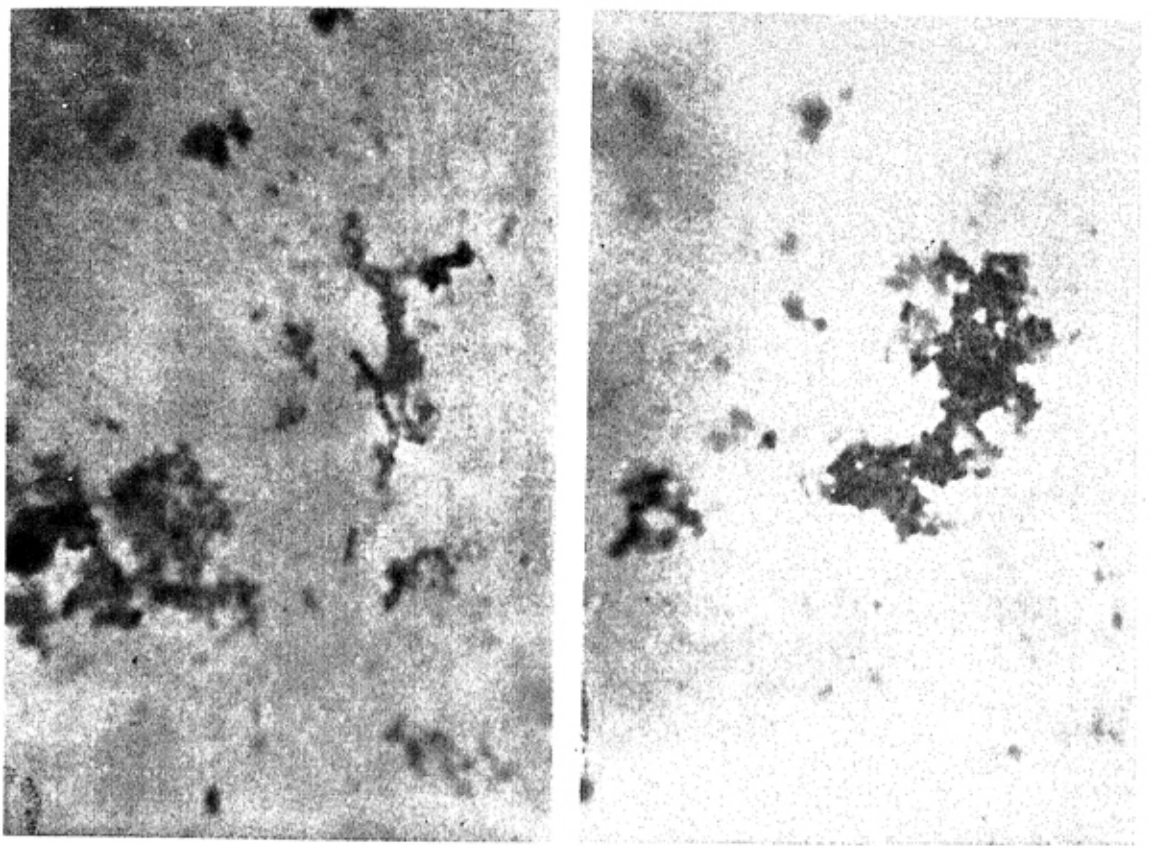

Fig. 3A-B.—Contaminant bacterial forms in protein constituents of some media. Aseptic sample of material as received from manufacturer, dissolved in sterile double-distilled water. Methylene blue. 1222x.

tiae grown on these media for 24 hours at 37.5°C. was that of extremely long-chains composed of paired cocci (Figs. 4a and b). The individual cells show a tendency to be elongated in a direction transverse to the length of the chain. After 48 to 144 hours incubation, the elongation of the individual cells usually becomes more pronounced, as shown in Figs. 5a and b. At various times during the incubation of these cultures, observations were made of long-chain forms of *Streptococcus agalactiae* in which only the cell outline was

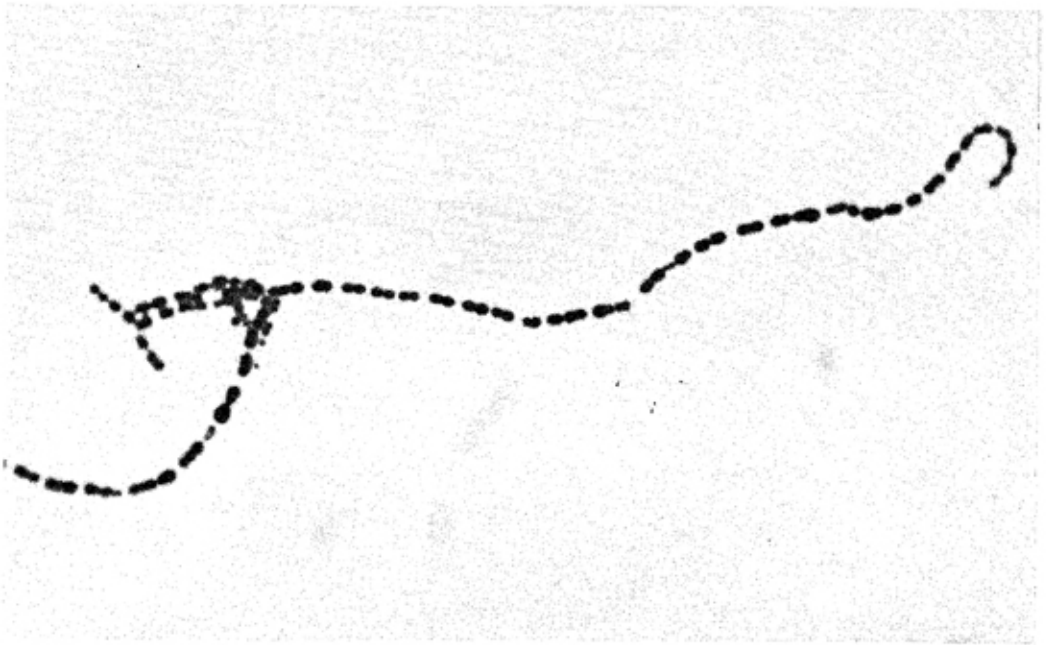

Fig. 4A.—Long-chain *Streptococcus agalactiae.* Twenty-four hours neopeptone-phosphate broth. Methylene blue. 1250x.

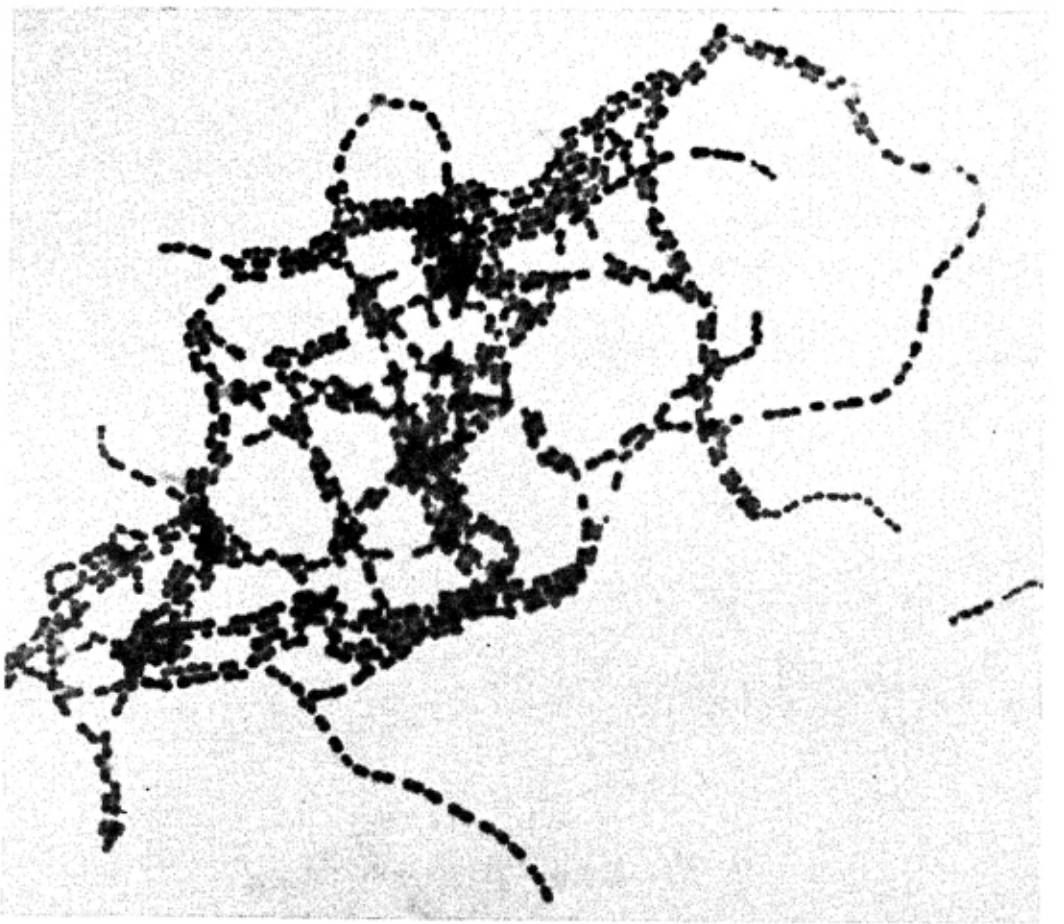

Fig. 4B. Long-chain *Streptococcus agalactiae.* Twenty-four hours tryptose-phosphate broth. Methylene blue. 1129x.

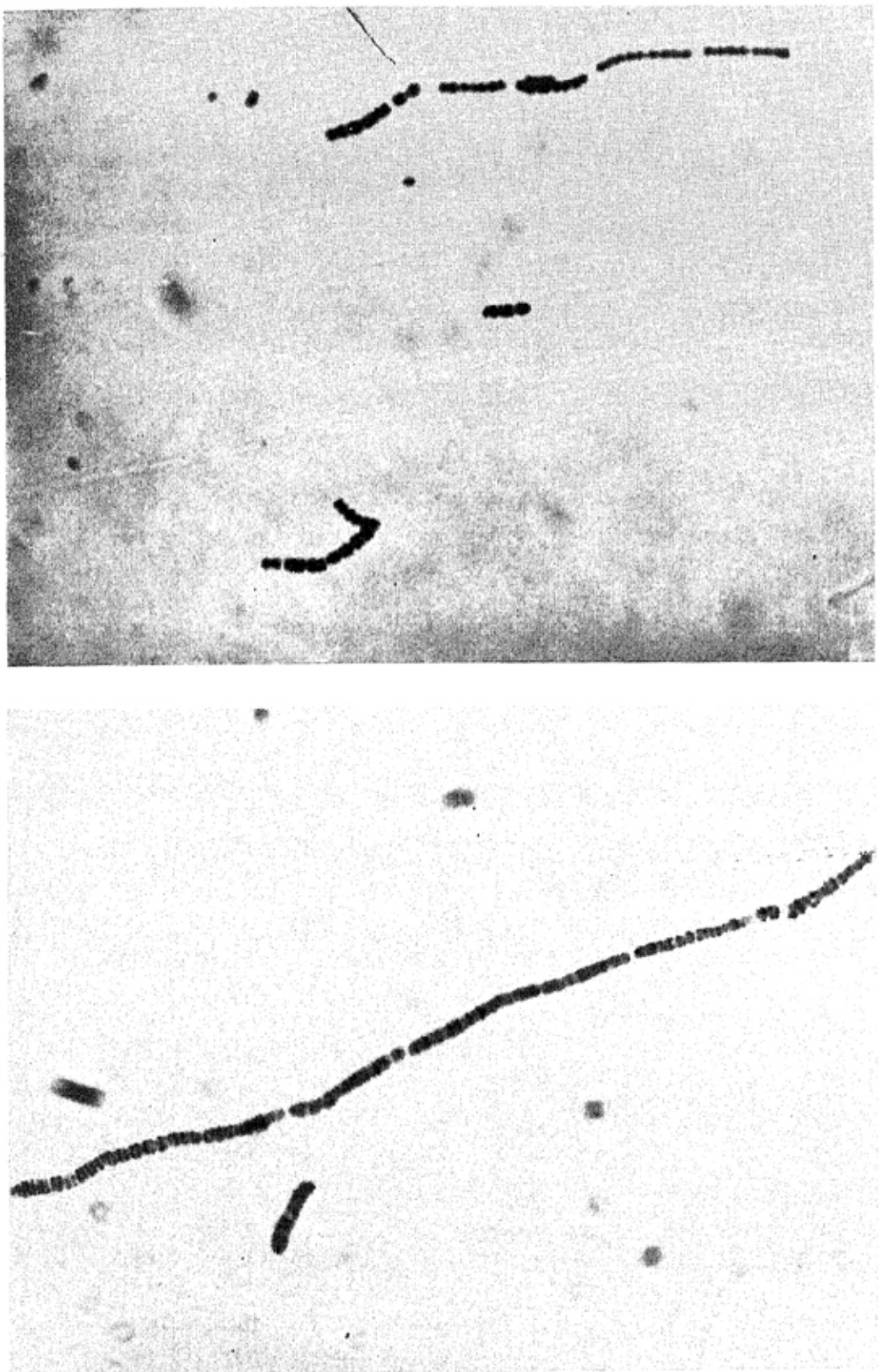

Fig. 5A-B.—Long-chain *Streptococcus agalactiae*. Tryptose-phosphate broth, 144 hours. Note elongation of individual cell members transverse to chain length. Methylene blue. 1250x.

discernible for some individual members of the chain (Fig. 6). Frequently these "ghost-cells" were surrounded by several minute "granules."

The *Streptococcus agalactiae* organisms gave the characteristic Lancefield group B precipitin reaction when grown on neopeptone- or tryptose-phosphate broth; however, the same organisms grown on casein-acetate-lactose broth did not show a precipitin reaction with either Groups A, B, or C antisera.

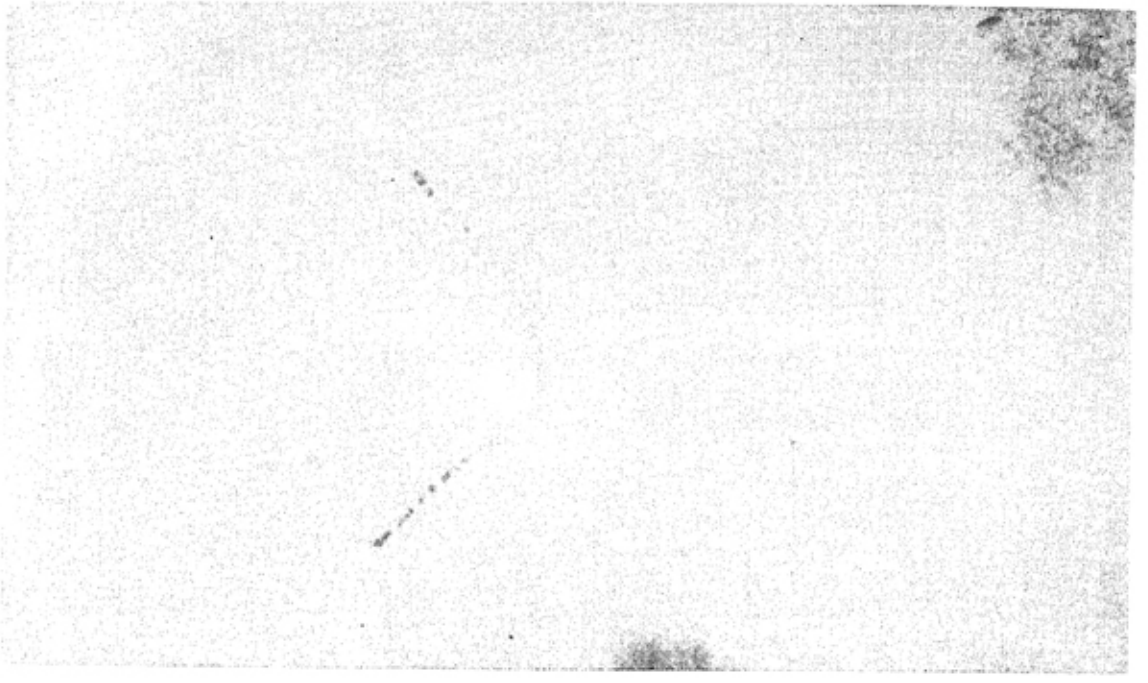

Fig. 6.—Long-chain *Streptococcus agalactiae* showing presence of "ghost cells" in chain. Tryptose-phosphate broth, 144 hours. Methylene blue. 1283x.

Sterile milk was used as one of the culture media for the growth of various strains of *Streptococcus agalactiae*. Most strains used caused coagulation of the milk within 24 hours after inoculation, although some strains also brought about peptonization of the milk. While there was no strict correlation, strains isolated from a flare-up of *Streptococcus agalactiae* mastitis more commonly caused the peptonization of milk than did strains isolated from subclinical chronic cases of streptococcal mastitis. In addition to the apparent difference in peptonization, a further possible difference between those strains from chronic and subclinical streptococcal mastitis was considered in a limited number of observations on hyaluronidase production by these organisms. The particular method used involved intradermal diffusion of Evan's Blue dye in rabbits. However, no evidence of hyaluronidase production could be conclusively demonstrated with the strains tested.

Warm-stage microscopic examination at a magnification of 1000x of *Streptococcus agalactiae* broth cultures in the logarithmic growth phase revealed that these organisms exhibit a type of "motility" during their growth and multiplication. This type of "motility" was most frequently observed in the case of diplococci. Initially, a diplococcoid pair of cells floating free in the medium would undergo a series of vibratory movements distinct from the usual Brownian movement. This would be followed by a *rapid* progressive movement by the cell-pair lasting from two to ten seconds. The speed of movement was sufficiently rapid to cause difficulty in keeping the cell-pair in view at this magnification. The path of movement was essentially that of a straight line with deviations occurring as the result of deflections off other cells in the path of motion. Changes in direction also occurred after the cells started revolving erratically and their forward progress diminished, for this movement was followed by the rapid straight-line motion in a new direction. After the *rapid* progressive movement ceased, the cells would continue revolving slowly. During this latter type of motion, an increase in cell number was observed on three different occasions. In each instance, no other

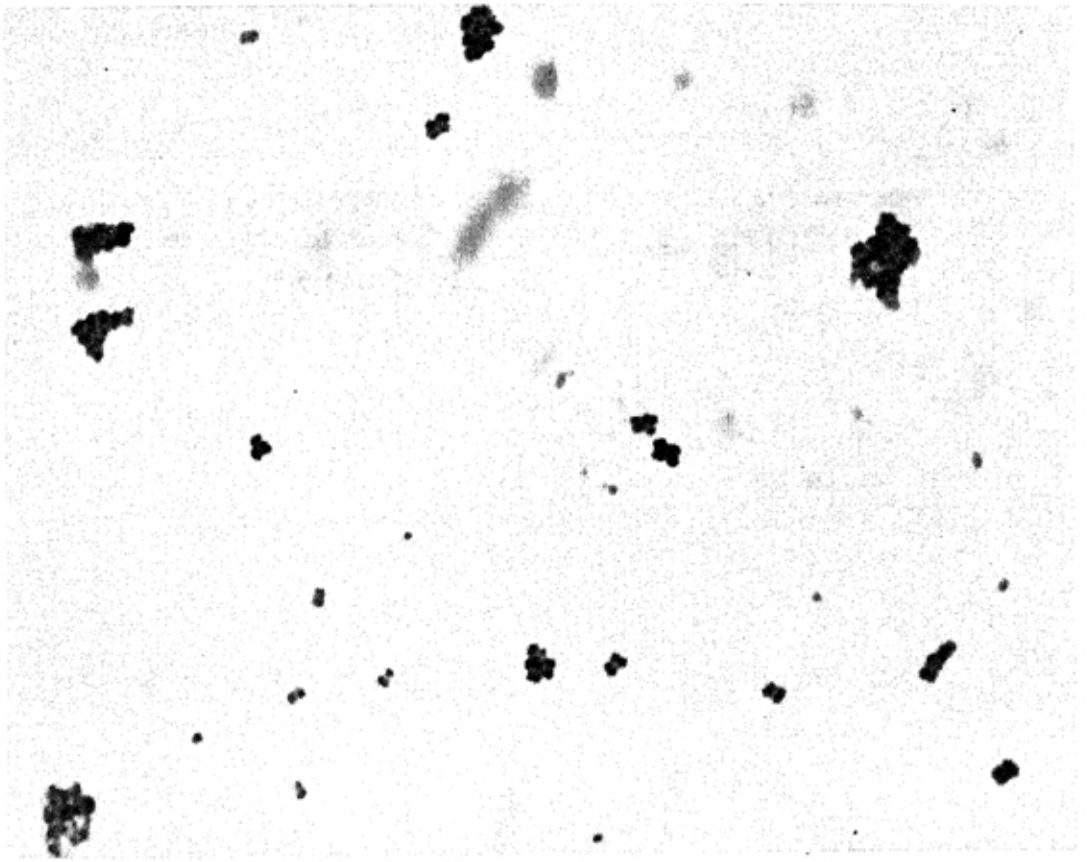

Fig. 7.--*Streptococcus agalactiae* cells from blood-acetate-agar. Methylene blue. 1521x.

bacterial cells were near the pair under observation, but first one extra cell appeared and then a second, thus forming a tetrad-like arrangement. The mode of cell multiplication could not be definitely determined due to the revolving motion of the cells under observation. In general appearance, the individual cells resembled those in Figure 7 which were from a beta-hemolytic colony of *Streptococcus agalactiae* grown on agar containing five per cent defibrinated blood and two per cent sodium acetate.

A type of "motility" was also observed in a few instances during numerous warm-stage microscopic examinations of broth cultures of the long-chain form of *Streptococcus agalactiae*. In these cases, a tangled mass of long-chains would begin vibrating violently and suddenly one or more chains of cells would leave the tangled mass in an extremely rapid spear-like motion. Short-chains and diplococci in the path of motion were usually forced violently to one side without altering the direction of the moving chain. However, long-chains or masses of cells in the path of movement frequently deflected the moving chain or entirely stopped its forward progress. No increase of cell number was observed in any of the chains after the movement stopped although some were kept under continuous observation for two hours. However, this does not preclude the possibility of cell multiplication having occurred, since it was difficult to keep the entire chain in focus throughout the observation period.

Manometric:

The comparative effects of 18-hour *Streptococcus agalactiae* culture filtrates and culture medium upon the anaerobic metabolism of bovine mammary tissue slices with sodium acetate as the metabolic substrate were studied with 135 individual determinations on tissue from seven lactating cows. The *Streptococcus agalactiae* cultures were isolated from 16 different cows showing a flare-up of chronic mastitis. Some of the tissue slices were stored at 4 to 5°C. for up to 72 hours prior to use in the determinations. Under the conditions employed for storage, there appeared to be a gradual decrease in the rate of anaerobic glycolysis of the tissue slices with increasing storage time, but due to individual variations and the small number of samples stored for an extended period of time, no definite correlation could be demonstrated. The variation in absolute values obtained in the manometric determinations (as a result of the use of tissue from cows in different stages of lactation and different lengths of storage time prior to use) was largely offset by the fact that the first 30-minute period of each determination constituted an internal control for the results obtained in that particular determination. Thus, all results could be used for comparative purposes as shown

in Figure 8. The rate of anaerobic glycolysis as expressed by the microliters of carbon dioxide released from the buffer-substrate per unit tissue per unit time was found to decline gradually throughout

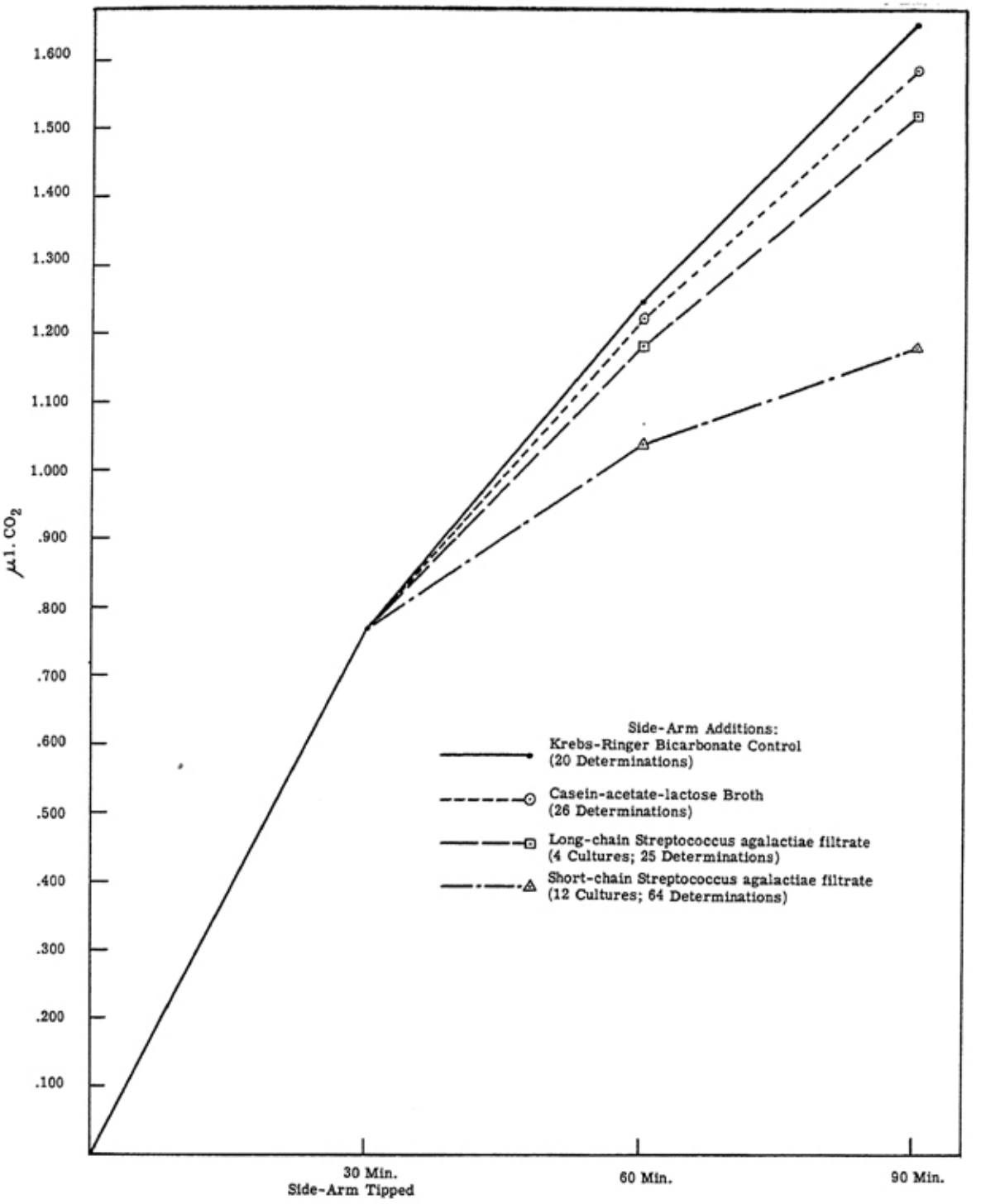

Fig. 8.—Effect of culture medium (casein-acetate-lactose broth) and *Streptococcus agalactiae* filtrates upon the anaerobic glycolysis of bovine mammary tissue slices, with .02M sodium acetate used as substrate. Glycolysis expressed as μ 1. CO_2 released from buffer per mg. (dry wt.) tissue.

the 90-minute determination regardless of the type of material added at the end of the first 30-minute control period. The addition of one-half milliliter of buffer or casein-acetate-lactose broth to the reaction chamber containing two and one-half milliliters of buffer and tissue slices caused the slowest rate of decline in carbon dioxide release. The addition of one-half milliliter sterile filtrate of the long-chain form of *Streptococcus agalactiae* (Figure 9) induced a slightly great-

Fig. 9.—Long-chain *Streptococcus agalactiae,* 18 hours casein-acetate-lactose medium. Methylene blue. 1250x.

er inhibitory effect although not significantly different from that due to the broth medium addition. The most striking inhibition of the rate of anaerobic glycolysis was induced by the addition of one-half milliliter sterile filtrate of the diplococcoid and short-chain forms of *Streptococcus agalactiae* (Figure 10). In general, the inhibitory

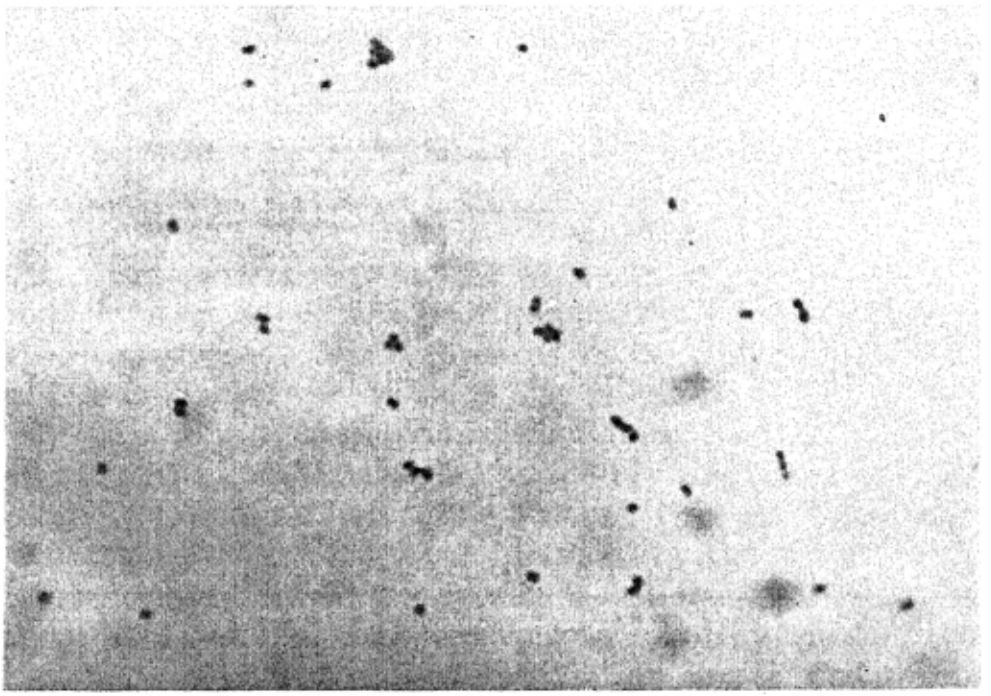

Fig. 10.—Short-chain and diplococcoid forms of *Streptococcus agalactiae,* 18 hours casein-acetate-lactose medium. Methylene blue. 1363x.

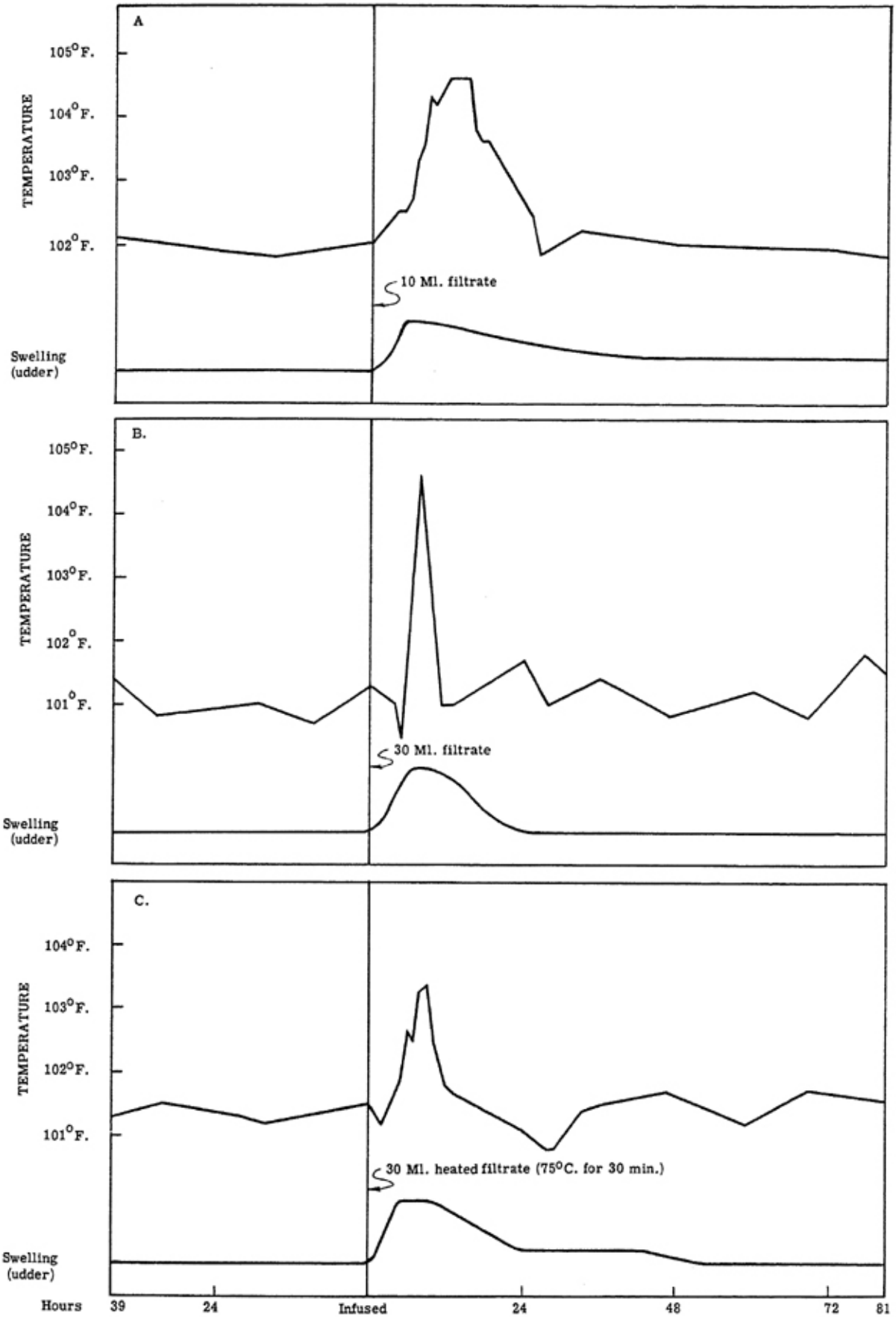

Fig. 11.—Single intramammary infusions with short-chain *Streptococcus agalactiae* filtrates.

effect of this latter type of filtrate was greatest on those tissues showing the highest rate of anaerobic glycolysis. However, in no instance did the inhibition presumably due to the broth medium or long-chain *Streptococcus agalactiae* filtrate equal that induced by the short-chain *Streptococcus agalactiae* filtrate regardless of the glycolytic rate of the tissue employed.

Intramammary Infusions:

A total of 122 infusions were given to eight cows with the amount of material used varying from five to 50 milliliters. This total includes both single and serial infusions.

The effects of single intramammary infusions of culture medium and short-chain *Streptococcus agalactiae* filtrate into one quarter of the bovine udder are shown in Figures 11A-C. In order to illustrate the variation shown by the different animals, individual responses have been given rather than an average of all responses. The rectal temperature reactions induced by the infusions varied both in the maximum temperature increase obtained and in the duration of this elevated temperature. Figure 11A shows the most prolonged temperature response observed when only a single infusion of the filtrate was used. The majority of the responses resembled those shown in Figure 11B in that the temperature increase started two to four hours after the infusion with a maximum at six to nine hours post-infusion. Then the temperature declined sharply to approximately normal 12 to 14 hours after the infusion. Figure 11C shows a typical reaction obtained by the infusion of *Streptococcus agalactiae* culture filtrate which had been heated for 30 minutes at 75°C. The temperature reactions resulting from infusions of heated filtrate were as severe as comparable infusions of unheated filtrate. In this comparison, both types of filtrate were infused into quarters of animals having received a number of previous intramammary infusions.

The maximum temperature response to the infusion of the casein-acetate-lactose broth medium is shown in Figure 12B. All other infusions of the broth medium resulted in temperature increases of less than one degree Fahrenheit and frequently no apparent temperature response could be noted. The infusions of long-chain *Streptococcus agalactiae* filtrates (Figure 12A) induced a response similar to that of the broth medium.

The response by individual quarters, in the form of swelling, to these intramammary infusions are also shown in Figures 11A-C and 12A-B, but they are necessarily only comparative in nature. In general, the swelling resulting from the infusion of the broth medium and long-chain filtrates was small and of a short duration (Figures 12A-B). Infusions of both the heated and unheated short-chain

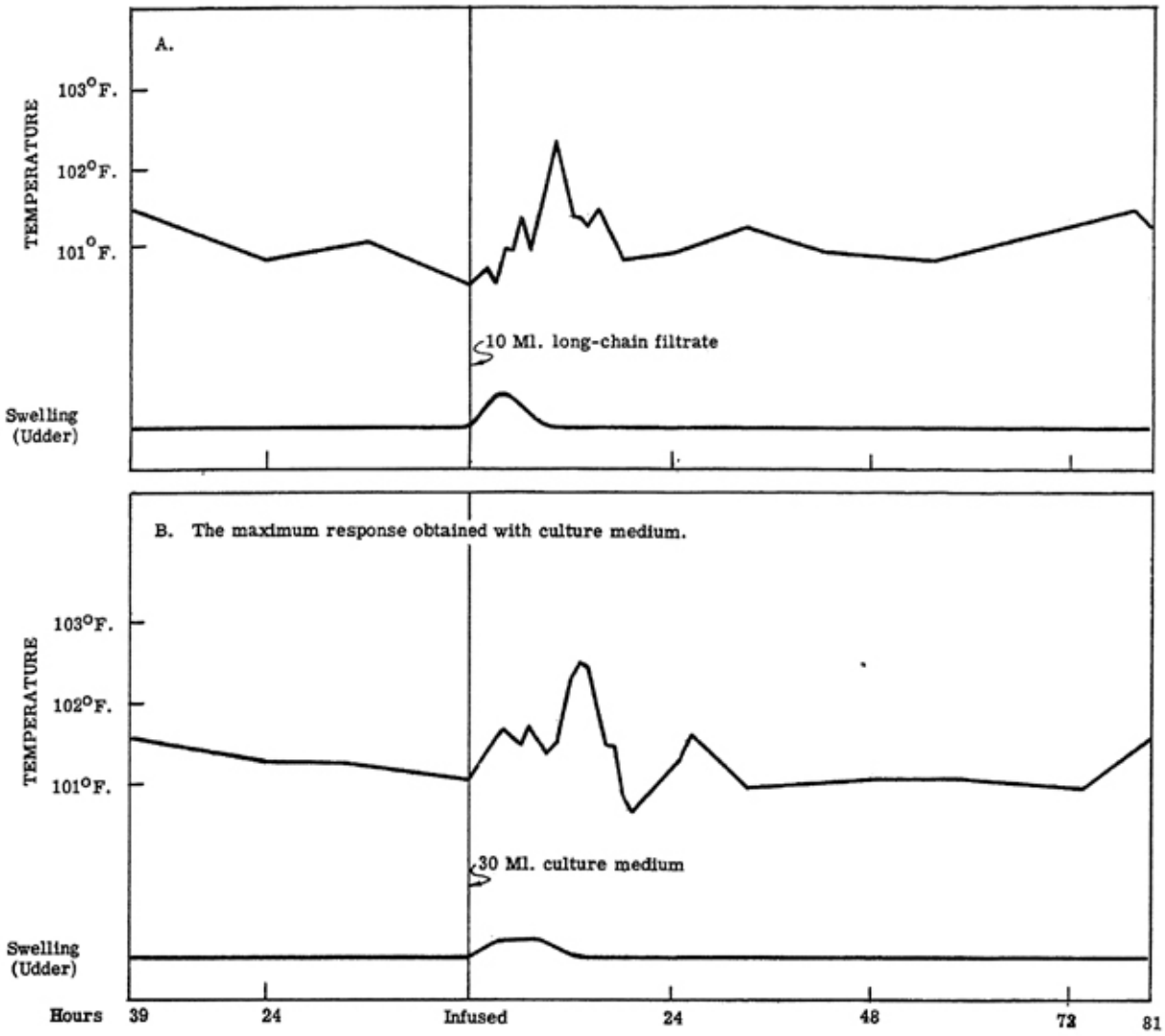

Fig. 12—Single intramammary infusions with long-chain *Streptococcus agalactiae* filtrate and culture medium (casein-acetate-lactose broth).

filtrates (Figures 11A-C) induced a rapid and relatively severe swelling in the treated quarters. The swelling was noticeable within one to two hours after infusion and was very pronounced (the quarter being markedly enlarged and hard) by four to five hours. The quarters remained in this condition for an additional four to six hours before the swelling of the quarter gradually subsided. In some instances, a small amount of swelling persisted for two to three days. The response shown by rectal temperature changes and swelling of the infused quarters appeared to be indirectly related to the amount of material used since there seemed to be a threshold level which varied between cows and between quarters of the same cow. In some quarters, the infusion of ten milliliters of short-chain *Streptococcus agalactiae* filtrate resulted in a sharp response, while in others the same amount would induce only a moderate response. Infusion of

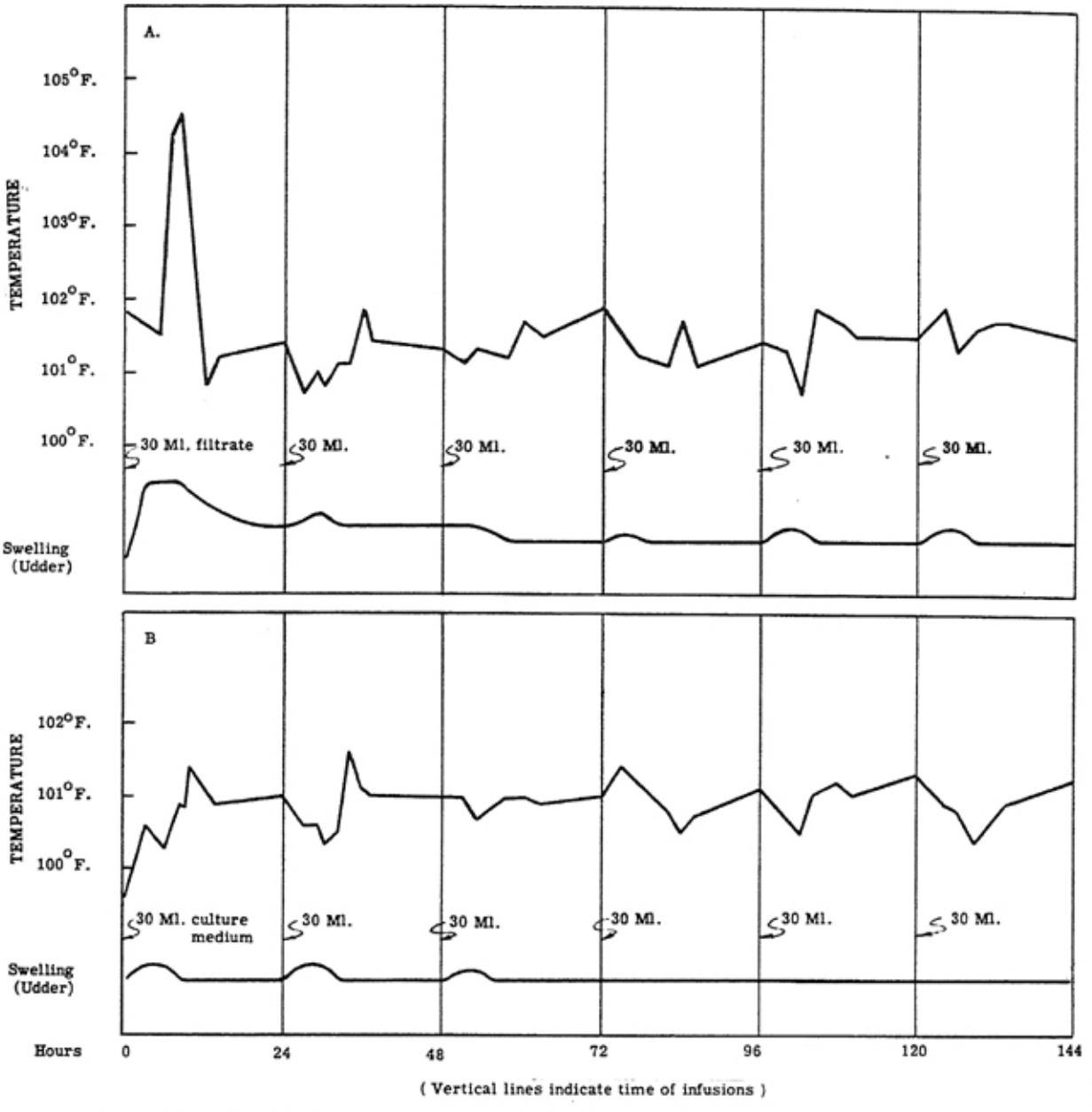

Fig. 13.—Serial intramammary infusions with short-chain *Streptococcus agalactiae* filtrates and culture medium (casein-acetate-lactose broth).

five milliliters of the filtrate gave a response similar to that of the infusions of the casein-acetate-lactose broth medium.

The effect of prolonged administration of short-chain *Streptococcus agalactiae* filtrate was studied by making a series of daily infusions in the same quarter. A similar type of infusion sequence was used to study the effect of consecutive injections in different quarters. Figure 13B illustrates the response to infusions with the control medium. Figure 13A shows a typical temperature and quarter swelling response obtained by repeated infusions of 30 milliliters of *Streptococcus agalactiae* filtrate in the same quarter. The first infusion of each series was characterized by a marked temperature

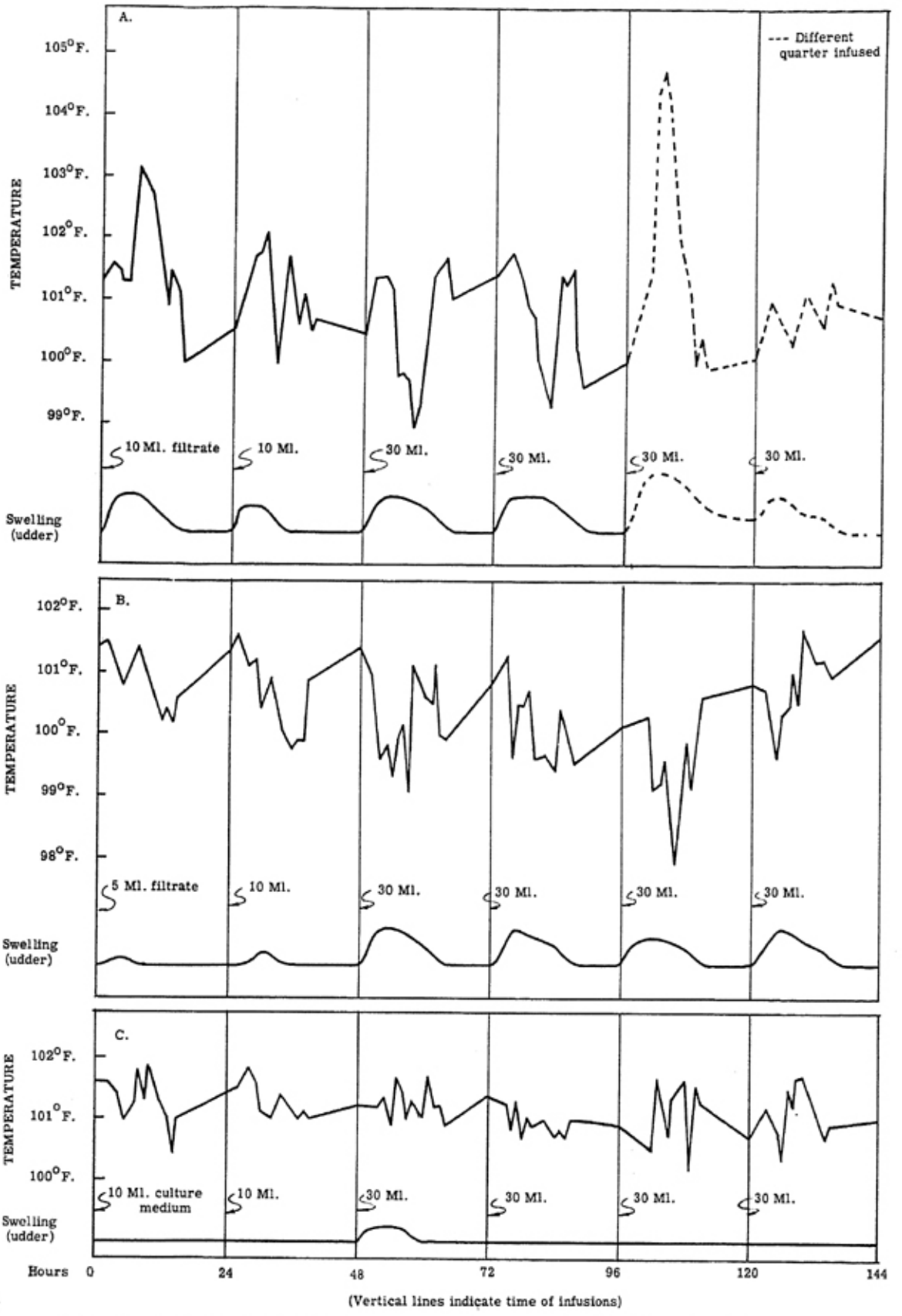

Fig. 14.—Serial intramammary infusions with short-chain *Streptococcus agalactiae* filtrates and culture medium (casein-acetate-lactose broth) during period of low environmental temperature.

increase and severe swelling of the experimental quarter. Subsequent infusions in the same quarter at 24-hour intervals did not induce any marked temperature reaction and the amount of swelling due to each infusion in the experimental quarter gradually diminished. If a rest period of two to three days was given between the infusions the second infusion induced considerable response in temperature as well as quarter swelling, but the response was not as marked as with the initial infusions.

Figures 14A and B illustrate the reactions of three animals receiving a series of short-chain *Streptococcus agalactiae* filtrate infusions during a period of low environmental temperatures. Barn air temperatures were not recorded; however, outside air temperatures of 0°F. and less were noted for this six-day experimental period. Two animals received the series of filtrate infusions shown in Figure 14B, while the other experimental animal received the amounts shown in Figure 14A. With each succeeding infusion of the filtrate of the short-chain form of *Streptococcus agalactiae* in the same quarter, all animals showed a tendency for a successively lower minimum rectal temperature. The one animal not shown in graphs for this period apparently had a minimum temperature of 96.5°F. after the fifth infusion; however, a second temperature measurement five minutes later showed a one-degree rise. Such a rapid temperature change seemed questionable, consequently the data concerning this animal has not been presented. All experimental animals exhibited extreme shivering and lethargy at the time of lowered body temperatures. Figure 14C shows the reaction of the control animal which received infusions of the casein-acetate-lactose broth medium during this period of low environmental temperatures. Similar experiments on these animals two weeks later with warmer weather gave results as indicated in Figure 13A for the three experimental animals. The reaction of the control animal did not seem to be influenced by environmental temperature changes.

After the experimental trials with *Streptococcus agalactiae* filtrate were completed, three cows were given single intramammary infusions of 30 milliliter quantities of 18-hour *Streptococcus agalactiae* culture. The marked response was of a similar duration to the filtrate infusions although more severe. One animal showed a maximum rectal temperature of 106.8°F. when the culture was infused into a quarter which had received only casein-acetate-lactose broth infusions during the preceding two weeks. The other two cows showed maximum temperatures of 104.1 and 103.5°F. when the culture was infused into quarters which had received seven consecutive daily infusions of the short-chain *Streptococcus agalactiae* filtrate.

Although in all three animals the temperature had returned to approximately normal within 14 hours after the initial culture infusion and remained normal for the following two days, the swelling in each infused quarter persisted at a relatively high level throughout the observation period. These animals were sold for slaughter on the third day after the initial culture infusion.

The milk from quarters receiving an infusion of casein-acetate-lactose broth, *Streptococcus agalactiae* filtrate, or culture was visibly abnormal after the infusion. The milk from quarters that had received the casein-acetate-lactose broth was only mildly abnormal as evidenced by the presence of a few flakes or clots in the fore-milk; in the milk from quarters receiving the short-chain filtrate, the abnormalities seemed to be roughly correlated with the degree of swelling for that particular quarter. In quarters receiving the *Streptococcus agalactiae* culture, the milk remained extremely abnormal throughout the observation period.

Samples of milk from each quarter of four cows in this study were taken twice daily for the first three weeks of the experimental period. These samples were analyzed for lactose, total solids, solids-not-fat, and chloride content, as well as for specific gravity and number of leucocytes present. The results of these tests showed a large variation from day to day between cows and between quarters of the same cow. In general, maximum deviations from normal occurred after the intramammary infusions, but the variation was so pronounced that these tests were not used in the balance of the experiments.

Hotis and microscopic examinations for the presence of *Streptococcus agalactiae* were made daily on the milk from all quarters of the eight cows in this study. These tests remained negative until three cows were infused with the living culture of *Streptococcus agalactiae,* and then only the quarters infused with the organisms became positive; they remained in that condition for the rest of the experimental period.

In addition to udder swelling and rectal temperature changes, some general clinical symptoms observed in animals receiving intramammary infusions of short-chain *Streptococcus agalactiae* filtrate or culture included loss of appetite, shivering, and nervous irritability frequently followed by general lethargy when the rectal temperature was extremely abnormal. These symptoms were very evident after infusions of the filtrate, but they were even more extreme when the culture was used.

DISCUSSION

The causative mechanisms involved in bovine mammary gland infections with *Streptococcus agalactiae* are apparently extremely diverse in nature. The establishment of an active infection depends not only upon the resistance of the host animal but also upon the virulence of the organisms involved. Frequently the variable results obtained in the production of experimental mastitis with *Streptococcus agalactiae* has been at least partly attributed to physiological variations in the cultures used. All of these factors tend to obscure the actual processes responsible for the clinical symptoms which are observed after an infection is established.

Observations in this study as well as other work including that of Rosell (1931) indicate that the predominate forms of bacteria present in unincubated streptococcal mastitic milk are diplococci, short-chains, and masses of individual cells with only a few long-chains in evidence. However, after incubation of these milk samples the long-chains, characteristic of streptococci in certain growth phases, become the predominating morphological form, while there is a marked reduction in the numbers of other forms. Thus, the morphological forms of *Streptococcus agalactiae* causing bovine mastitis are apparently different from many of the forms obtained by laboratory culture of organisms isolated from streptococcal mastitis. The occurrence of large numbers of minute "granules" in unincubated samples of streptococcal mastitic milk, as well as in certain growth phases of *Streptococcus agalactiae* cultures would seem to indicate another morphological form of the organisms. These "granules" may be similar to the granule-like bodies within the cells that were observed by France and Hadro (1940) in an atypical strain of *Streptococcus salivarius*. Frequently, staining artifacts make it difficult to observe these "granules" during a routine microscopic examination for the presence of streptococci. The apparent reduction in numbers of "granules" upon incubation of milk from cows known to be infected with *Streptococcus agalactiae* together with a marked increase in characteristic streptococcal forms provides suggestive evidence concerning possible relationship of the "granules" to streptococci.

A wide variation in culture morphology of *Streptococcus agalactiae* is readily shown when different bacteriological media are used. Phosphate broth containing "V-8 juice" and either tryptose or neopeptone was found to favor rapid growth of cultures characterized by extremely long-chains of paired cocci. With continued incubation, the individual members of the chain show a tendency for swelling and elongation transverse to the chain length. Frequently, some

individual members of the chains can be seen only as cell outlines. These "ghost cells" are occasionally surrounded by minute "granules." The formation of long-chains of swollen cells has been previously reported by workers including Rosell (1931) and Dawson, *et al.* (1938). The latter concluded that such chains were characteristic of the rough, avirulent phase of *Streptococcus agalactiae.* Thus, as a result of these various studies, it would seem that the production of experimental streptococcal mastitis with this rough phase of *Streptococcus agalactiae* would depend to a large extent upon the resistance of the experimental animal. The use of casein-acetate-lactose broth as the culture medium has been found to favor the rapid growth of cultures composed primarily of diplococci and short-chains. They apparently belong to the growth phases designated as mucoid or smooth by Dawson, *et al.* (1938) and closely resemble the forms found in unincubated streptococcal mastitis milk. Such organisms possess a high or moderate virulence when compared to the rough phase of *Streptococcus agalactiae.* The various morphological forms of Lancefield group B organisms observed in this study show a close similarity to some of the forms reported by Mellon (1948) for streptococci belonging to Lancefield groups A and C. These observations on morphological variation when considered in relation to work reported by Mellon (1948) and Beck (1950) support the idea of a cyclic dissociation pattern for streptococci. Such a cyclic pattern together with possible unknown morphological and immunological phases may be related to some cases of nonspecific mastitis.

All the organisms used in this study, when grown on phosphate broth containing either neopeptone or tryptose, gave the characteristic precipitin reaction for Lancefield's group B. However, when casein-acetate-lactose broth was used as the culture medium, the organisms did not give a precipitin reaction with group B antisera. The failure of the *Streptococcus agalactiae* to give the characteristic Lancefield group B precipitin reaction when grown on casein-acetate-lactose broth may be due to lack of some specific substrates required for the development of these particular antigenic characteristics.

Cultures of *Streptococcus agalactiae* isolated from a flare-up of mastitis showed a greater tendency to cause peptonization of milk than did cultures isolated from subclinical cases. Thus, the *Streptococcus agalactiae* causing mastitis flare-ups may possess certain enzymatic activities which are not apparent in the organisms from subclinical mastitis. However, such a difference could not be demonstrated in the hyaluronidase production of *Streptococcus agalactiae* from subclinical cases and flare-ups of chronic mastitis.

Since the culture medium constitutes such an important factor

in the growth and maintenance of any particular phase or even strain of *Streptococcus agalactiae,* bacterial contamination of the medium constituents presents a major problem. This type of contamination has been found to occur in some of the more complex compounds used in the preparation of various bacteriological media. The use of such media for any critical bacteriological experiment may lead to erroneous results. The contaminant bacterial forms would interfere with morphological observations and possible changes in the media composition would make the interpretation of bacterial nutrition studies very difficult. Also, there may be an effect upon immunological studies; for example, Beck (1950) found that the addition of heat-killed group A streptococci to the nutrient medium was intimately related to the transmutation of group B carbohydrate antigen to Group A. Residual compounds of the contaminant bacteria could conceivably induce a similar effect. Thus, the need for bacteriological media of known chemical composition prepared under strictly aseptic conditions assumes great importance for research investigations of this nature.

The significance of the apparent "motility" exhibited by *Streptococcus agalactiae* is not well understood. But if, as these studies indicate, the "motility" is a characteristic of the organisms, and is not due to localized convection currents, this phenomenon may prove of value in studying the mode of cell multiplication and the energy relationships involved. The work of Martin (1950) suggests that a type of "motility" is characteristic of many different groups of streptococcal organisms. Using both ordinary light microscopic and dark-field microscopic techniques, he observed "motility" in numerous strains of streptococci which had been isolated from human infections.

The results obtained by intramammary infusions of *Streptococcus agalactiae* filtrate and by manometric studies of the effect of the filtrate upon the anaerobic glycolysis of bovine mammary tissue slices show that *Streptococcus agalactiae* produces or brings about the formation of component(s) in its nutrient medium which are toxic for bovine mammary tissue both *in vivo* and *in vitro.* The increased toxity of diplococcoid and short-chain forms of *Streptococcus agalactiae* when compared to that of the long-chains indicates that morphological variations are often associated with more profound physiological changes in the organisms. Virulence or toxicity may not be directly related to the morphological characteristics, for the apparent association of these characters may be the result of independent adaptations to a common environment.

In the manometric determinations, the effect of the *Streptococ-*

cus agalactiae filtrate upon the sum total of the glycolytic reaction was studied using sodium acetate as the metabolic substrate. The reduction in the rate of glycolysis induced by the *Streptococcus agalactiae* filtrates is probably due to various effects upon the enzymatic constituents of the metabolizing cells. Studies on isolated enzymes of the mammary gland would be necessary to determine whether the inhibitory effect of the *Streptococcus agalactiae* filtrates is specific for certain enzymes, groups of enzymes, or is of a general nature affecting many different types of enzymes involved in the secretion of milk. Various workers including Folley and French (1950) have found that acetate is utilized in the synthesis of ruminant milk fat. This fact considered with the decreased butterfat content of streptococcal mastitic milk seems to support the results obtained in the manometric determinations of this study which indicate that the component(s) produced by *Streptococcus agalactiae* are toxic for the enzymes of the secretory tissue in the bovine mammary gland.

The marked increases in rectal temperature and severe swelling of experimental quarters induced by intramammary infusions of short-chain *Streptococcus agalactiae* filtrates supports the manometric results for toxicity of *Streptococcus agalactiae*. In the intramammary infusions, the general systemic effect of the filtrate as shown by the temperature increases and attendant clinical symptoms of decreased appetite, shivering, and general lethargy indicates a multiple effect of the toxic component(s). The tendency for a decline in the response to continued infusions of the material suggests either the development of some short-term immunity to the toxic component(s) or a transitory desensitization of the tissue to the effects of the filtrate. The brief or transitory nature of immunity or desensitization of an experimental quarter is indicated by marked responses to infusions separated by three- to five-day rest periods. These responses appeared to be similar to those of the initial infusions.

One series of intramammary infusions was made during a period of low environmental temperature; the results obtained, although interesting, are difficult to interpret. The decline in the minimum rectal temperature of the experimental animals with consecutive infusions in the same quarter could possibly be attributed to the low environmental temperature. However, this interpretation would necessarily imply that the infusions into one quarter of the udder interferes with the animal's ability to control the body temperature rather than inducing either a temperature increase or decrease. Such an interpretation is further complicated by the marked rectal temperature increase shown by one of the experimental animals when a different quarter was infused. Thus, the *Streptococcus agalactiae* fil-

trates infused into the mammary gland induce a variety of effects both local and systemic in nature.

Heating the *Streptococcus agalactiae* filtrate for 30 minutes at 75°C. prior to the intramammary infusions did not cause a reduction in the post-infusion response when compared to similar infusions of the unheated filtrate. This is in apparent agreement with the results of Pounden and Zehner (1941), who found that the infusion of whey resulting from the action of mastitis streptococci upon milk caused symptoms of acute streptococcal mastitis even though the whey had been boiled prior to the infusion. Therefore, the toxic component(s) produced by *Streptococcus agalactiae* are apparently heat-stable.

The degree of response could not be directly related to the amount of *Streptococcus agalactiae* filtrate infused, for each quarter appeared to have an individual threshold level below which the infusion induced little response. The initial infusions of small amounts of filtrate was found to have little effect upon the amount of swelling resulting from subsequent infusions. However, at the small dosage levels even those filtrate infusions not causing an initial response did inhibit or suppress the rectal temperature reaction induced by subsequent daily infusions of larger amounts of the filtrate in the same quarter. Thus, it would appear that even subcritical amounts of the filtrate stimulates a transitory desensitization or brief immunity to the apparent pyrogenic activity of the filtrate.

SUMMARY AND CONCLUSIONS

Diplococci, short-chains, masses of individual cells, and minute "granules" were the predominating morphological forms found in microscopic examination of unincubated streptococcal mastitic milk. However, the long-chains, which are characteristic of certain growth phases of streptococci, predominated after incubation of the milk samples. In broth cultures of *Streptococcus agalactiae,* a diffuse growth of diplococci and short-chains occurred with casein-acetate-lactose medium. A flocculent growth of long-chains composed of paired cocci and swollen cells was found with neopeptone- or tryptose-phosphate broth.

It is indicated that *Streptococcus agalactiae* organisms causing mastitis may be different morphologically from those found in many laboratory cultures. The morphological variations indicate a cyclic dissociation pattern for streptococci, and the "granules" observed may constitute a morphological form of *Streptococcus agalactiae.* Smooth or mucoid characteristics of Lancefield group B streptococci were maintained by casein-acetate-lactose broth medium.

Streptococcus agalactiae grown on neopeptone- or tryptose-phosphate broth gave characteristic Lancefield group B precipitin reactions although when grown on casein-acetate-lactose broth, no precipitin reaction could be demonstrated. Thus, casein-acetate-lactose broth may lack specific substrates needed for development of this antigenic character.

Bacterial contamination was found in some complex media constituents. This contamination does interfere with morphological observations, and may affect the results of nutritional and imological experiments.

Streptococcus agalactiae isolated from flare-ups of mastitis more frequently caused peptonization of milk than did the organisms isolated from subclinical cases of streptococcal mastitis.

A type of "motility" was exhibited by *Streptococcus agalactiae* which was most frequently associated with diplococci. In three cases, cell multiplication following "motility" was observed.

The effect of *Streptococcus agalactiae* filtrate on the anaerobic glycolysis of the mammary tissue slices was studied with sodium acetate as the metabolic substrate. One hundred and thirty-five manometric determinations were made on tissue from seven cows. Filtrates of cultures isolated from 16 cows showing flare-ups of streptococcal mastitis were prepared using Selas #03 porcelain filters. Filtrates of the short-chain form of *Streptococcus agalactiae* caused marked inhibition of anaerobic glycolysis. Long-chain filtrates and uninoculated medium caused only slight inhibition of anaerobic glycolysis.

Streptococcus agalactiae organisms produce or bring about the formation of component(s) in the nutrient media which are toxic to bovine mammary tissue. The enzymes affected are apparently those involved in the utilization of acetate by the mammary gland. Maximum toxicity was associated with the diplococcoid and short-chain form of *Streptococcus agalactiae.*

One hundred and twenty-two intramammary infusions of *Streptococcus agalactiae* filtrate were made into the mammary glands of eight cows. Infusions of short-chain *Streptococcus agalactiae* filtrates caused symptoms similar to those of acute streptococcal mastitis. Infusions of long-chain *Streptococcus agalactiae* filtrates and of uninoculated casein-acetate-lactose broth induced only minor symptoms of irritation. When the short-chain filtrates were heated (75°C. for 30 minutes) prior to infusion, the response was essentially the same as that obtained with infusions of unheated filtrate.

The toxicity of *Streptococcus agalactiae* component(s) and their association with certain phases of streptococci is indicated by both

intramammary infusions and manometric determinations. The component(s) produce general systemic effects in addition to those effects localized in the mammary gland. The component(s) are apparently heat-stable. Temporary desensitization or immunity to the toxic component(s) may occur in the mammary gland.

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