EVALUATION OF CITRIC ACID AS A POSSIBLE TREATMENT FOR STREPTOCOCCIC MASTITIS

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ABSTRACT

EVALUATION OF CITRIC ACID AS A POSSIBLE TREATMENT FOR STREPTOCOCCIC MASTITIS

by Dineshwar Prasad Sinha

Since it was shown by Huddleson that citric acid in blood prevented the growth of Streptococcus agalactiae, a study was undertaken to determine if citric acid would also exert a growth inhibitory action to Streptococcus agalactiae in milk.

Initially a modified skim milk was used for in vitro work, but as the study advanced an attempt was made to obtain milk resembling as closely as practical the natural product. A technique was devised for collecting the milk aseptically and dispensing it into sterile test tubes without contamination. This gave a satisfactory quality of milk for in vitro work.

The growth rate of Streptococcus agalactiae was studied in skim milk, heat sterilized milk and nonsterilized milk in the presence of sodium citrate, citric acid, and a mixture of both.

There was no significant growth inhibition of Streptococcus agalactiae in milk when sodium citrate was added. When citric acid was added to the milk in a concentration of 0.8% or more, the pH of the milk dropped from 6.64 to

4.08 or less. Two experiments were set up to determine if the growth inhibition of Streptococcus agalactiae in citrated milk (milk containing sodium citrate or citric acid) was due to the absence of magnesium ions, required for growth, or due to the low pH (acidity) of the media. For the first experiment excessive amounts of magnesium chloride were added to the citrated milk to provide sufficient magnesium ions for the growth of Streptococcus agalactiae. The addition of magnesium chloride to the citrated milk did not accelerate the growth rate of Streptococcus agalactiae. In the second experiment, mineral acid was added to the milk to lower the pH and it was found that the growth rate of Streptococcus agalactiae was similar to that in the milk which had its pH lowered by adding citric acid.

It has been demonstrated that growth inhibition of Streptococcus agalactiae in milk containing citric acid was due to low pH of the media. Streptococcus agalactiae may grow in the absence of free magnesium ions in the milk or else it is impossible to chelate total magnesium ions in the milk by using citrate as a chelating agent.

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by

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Respectfully dedicated to my mother

Lalo

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I. INTRODUCTION

Mastitis is one of the most important diseases of the dairy cow. It may be mild and undetectable or it may be severe enough to cause a decrease in both the quality and the quantity of the milk, even terminating in the complete cessation of milk production or in death of the animal.

Van Houweling (1957) pointed out that milk losses from mastitis in the United States during the year 1956 were estimated at about 5 billion pounds with a value of approximately 200 million dollars. This loss represents 4% of the total milk production. Total losses including cost of replacement of mastitic cows and losses due to death were more than 245 million dollars for 1956.

The dairy cow appears to be becoming more and more susceptible to mastitis, due in part to man's continual effort to develop her mammary glands far beyond the natural requirement of the newborn calf. In the age of antibiotics and sulfonamide drugs, it probably seems paradoxical that citric acid might be thought of for the treatment of mastitis. Nevertheless, this line of thinking is not without foundation since Huddleson (1959) has shown that citric acid is effective in preventing *in vitro* growth of *Steptococcus agalactiae*, the organism most prevalent in cases of mastitis (Drury *et al*, 1961).

Webb (1953) stated that the absence of an adequate supply of ionic magnesium in the culture medium affects adversely certain aspects of the nitrogen metabolism of gram-positive bacteria.

By removing the ionic magnesium from the udder milk, it might be possible to inhibit the growth of the gram-positive bacteria. Citric acid, which is one of the constituents of normal milk (139-210 mg.%) appears to be one of the effective agents available for the binding of ionic magnesium in udder milk. If a solution of citric acid can be used as an intramammary injection to bind magnesium ions, a cure might be effected. If citric acid is effective in preventing the growth of Streptococcus agalactiae, a logical follow-up study would be to determine its effect on udder tissue.

This study was designed to determine the in vitro effect of citric acid and sodium citrate on Streptococcus agalactiae in milk.

II. REVIEW OF LITERATURE

Extensive work has been done on mastitis, but still there is a vast area remaining for mastitis research. A survey of the literature reveals nothing whereby citric acid has been evaluated for the treatment of mastitis (streptococcic). There were only a few supportive articles on which this project is based. In addition, the literature on the streptococcic mastitis, composition of cows' milk, magnesium required for growth of bacteria and the influence of feed on mastitis was reviewed.

1. Streptococcic mastitis

Laing, et al (1956) stated that in western Scotland the average incidence of mastitis in the year 1956 was 37% of all cows. In about 60% of the cases specific pathogens were present in the milk (specific mastitis) and in the remaining 40% there were no organisms either in milk or tissue (nonspecific mastitis). Of the specific infections, about 75% were due to staphylococci (in small proportion mixed staphylococcal and streptococcal infection) the rest were chiefly due to streptococci.

Little, et al (1946) stated that up until approximately 1940, all forms of bovine mastitis were considered to be an incurable disease.

In 1939 a mastitis survey of the Lansing area was

done by Bryan (reviewed by Drury, 1961) who found that 86% of the herds and 26% of the cows in these herds had mastitis. He also determined that 98% of the mastitic infections were associated with streptococci.

In the Fall of 1959, a mastitis survey consisting of 25 herds involving 380 milking cows in the Lansing area was done by Drury, et al (1961). He found that 59% of the cows showed infection in one or more quarters. Of the infected quarters, 58.8% were streptococcus, 39.4% were staphylococcus and 1.8% were other organisms.

Carpenter (1922) observed that age of the cow and the level of milk production had an influence on the effectiveness of mastitis producing organisms. He mentioned that nonlactating cows showed no ill effects from the intramammary injection of organisms but clinical symptoms of mastitis were observed in the same animals a few months after parturition, when organisms were introduced by intramammary infusion. Young cows were more susceptible than older cows.

Spencer, et al (1950) reported that roughening of the surface of the epithelium of the milk-duct and the occlusion of ducts by clots of fibrin are probably important in the pathogenesis of bovine mastitis.

It has been mentioned in the book Diseases of Cattle (2nd ed.) that, "Str. agalactiae enters the gland through the teat opening and apparently resides mainly in the lumen of ducts and on the lining surface of ducts and alveoli."

The action of the streptococci on the tissue is through an irritant which forms in the milk.

Little (1937) reported that the intact duct and sphincter of the teat may act as a natural barrier to the passage of pathogens into the udder.

2. Composition of Milk

a. Normal milk:

Field (1960) stated that magnesium is a constituent of milk with a concentration of 4, 12, 16, and 31 mg./100ml. in human, cow, goat and rat whole milk respectively. Only about 20% of the magnesium in cow's milk is in the ionic form.

Robertson, et al (1960) have found that the concentration of magnesium in milk can remain constant over a wide range of serum magnesium levels.

Kon and Cowie (1961) reported that there is insignificant variation in magnesium content of milk of different breeds of cows.

Stewart, et al (1956) have reported a large seasonal variation in the magnesium content of herbage with the lowest content being in April and May when magnesium tetany is most prevalent. He further suggested that higher magnesium content of the herbage in April and May produced by magnesium limestone dressing considerably reduced the degree of hypomagnesemia in a herd.

Vankreveld, et al (1955) mentioned that calcium and

magnesium in milk occur in different forms. They are bound partly to the protein and partly to phosphate or citrate and thirdly there are free Ca and Mg ions. This last fraction is probably the rate determining factor in the various coagulation processes.

Kemp, et al (1960) reported that 33% of the calcium, 33% of the phosphate, 75% of the magnesium, and 90% of the citrate of milk are present in the dissolved state.

There is a gradual increase in the magnesium content of milk from the beginning of lactation to the end as reported by Vanschoubrock, et al (1957). This increase, however, was less in the second half than in the first half of lactation.

Gueguen, et al (1960) observed variations in the P, Ca, K, Na, and Mg content of milk with 10 cows of 3 different breeds which calved at the beginning of the winter and had the same diet. He found that in the course of lactation, the K content diminishes regularly whereas the Na content increases, especially at the end of lactation. Calcium and phosphorus content were at their peak at the beginning and end of lactation. Mineral composition of milk is not affected if the animals are put out to pasture.

Rook and Storry (1962) published an article on the nutritional aspect of magnesium in farm animals. In their literature review it was found that there is a small variation in magnesium content of milk with change in diet. There

was no fall of magnesium level of milk even though the intake of feed or magnesium was reduced. There was no significant fall in milk magnesium in the case of a hypomagnesemic cow.

Todd, et al (1962) injected parathyroid extract into parathyroidectomized lactating Jersey cows and observed that parathyroid treatment caused an increase in milk magnesium with a corresponding decrease in urine magnesium whereas the citric acid content of urine and plasma increased, but remained constant in the milk.

Davidov, et al (1960) studied variations in the citric acid content of milk on the basis of bulk milk received in a Moscow dairy and milk produced at one farm. The average monthly level of citric acid over a 2-year period was 108 to 210 mg.% for bulk milk and 139 to 210 mg.% for the farm milk. In a feeding experiment he found the average citric acid content of milk decreased from 177 mg.% in December-March to 163 mg.% in May and rose to 185 mg.% during the first few days after the transfer of the cows to pasture.

Anagama, et al (1961) determined the citric acid (by Marier and Boulet method) in 180 samples of bulk Holstein Friesian milk taken between May, 1960, and April, 1961. The average was 192 mg.%. The monthly average ranged from 137 to 134 mg.% in July and August to 221 mg.% in March. He suggested that the decrease of citric acid content was due to the high temperatures of summer.

Nickerson (1960) pointed out that milk from various areas differed significantly in their content for all constituents except soluble organic phosphoros, citric acid and magnesium.

b. Mastitic milk (streptococcic):

Redaelli, et al (1957) have studied the behavior of inorganic substances in mastitic milk. In the course of streptococcic infections there was a remarkable increase of the chlorine and sodium values and a diminution of calcium and potassium content. There was no significant variation for magnesium.

Little, et al (1933) stated that the udder capillaries under severe inflammatory conditions are permeable to blood protein. Under these conditions the same structure may permit the passage of blood alkali. In many cases of bacterial invasion, reaction of milk is usually altered towards the alkaline side.

3. Magnesium Required for Growth of Bacteria

Webb (1953) stated that an inadequate supply of ionic magnesium in the culture medium adversely affects certain aspects of the nitrogen metabolism of gram-positive bacteria. Webb (1951) found that the requirement of magnesium for growth of gram-positive and gram-negative bacteria depends upon the organic composition of the medium. In general, gram-positive bacteria failed to grow when the magnesium content was less than 0.6 ppm., whereas this concentration is almost sufficient

to maintain maximum growth of gram-negative species. Webb (1949) reported that the concentration of magnesium necessary to support optimal growth of gram-positive bacteria, some 20 to 30 ppm., is about ten times greater than that required by the gram-negative organism under the same conditions.

Huddleson (1959) reported that Staph. aureus and Str. agalactiae failed to grow in blood in the presence of citric acid due to the binding of the particular metal ion Mg, which is essential for growth. He also observed that citrate even in low concentration in blood inhibited the growth of Str. agalactiae. Higher concentrations inactivated an agent or agents of the anti-microbial system that suppressed growth of gram-negative bacteria.

Abelson, et al (1950) has demonstrated the influence of magnesium on the toxicity of Ni, Cd, Zn, and Mn, on the growth of E. coli, Aerobacter aerogenes, Aspergillus niger, and Torulopsis utilis. The toxicity of these cations is lowered in the presence of much magnesium. If magnesium is not present in the media these elements are toxic at very low levels for all four organisms. Higher levels of magnesium diminish the amount of nickel and cobalt bound by the cell.

Bienvenu, et al (1963) found that the brucellacidal mechanism in nonvaccinated unbred heifers was dependent upon the level of ionized magnesium in their blood. After injecting

100,000 I.U. of human chorionic gonadotrophin into unbred heifers, brucellacidal activity and serum magnesium was sharply decreased by lowering renal threshold for the mineral.

4. Influence of feed on mastitis

Pounden (1952 & 1957) observed two groups of eight cows in the same herd. One group was fed a ration of alfalfa hay, forage crops, silage and moderate quantities of grain. The other group received grass hay, mostly timothy of relatively poor quality, and a more liberal feeding of grain. Both groups were observed for 16 weeks. In the first group mastitis (clinical) was evidenced in one quarter of two cows and the second group had clinical mastitis in eight quarters in 5 cows. Streptococcus agalactiae, or unidentified streptococci or staphylococci were the causative organisms. Pounden (1952) stated that the resistance for Streptococcus agalactiae in the milk was lessened as lactation progressed. Pounden, et al (1956) observed that the inhibitory action of milk to the growth of Streptococcus agalactiae was lowered for a few days when corn silage was added to the hay and grain ration which had been fed continuously for a year and a half. Inhibitory action was retained or improved slightly when cows which had been on pasture all summer were fed forage crop silage as a part of the ration in the fall. Pounden, et al (1958) mentioned that the number of mastitis cases increased in a large herd each spring

when fresh green feed was added to the ration of hay and grain.

Care (1960) reported that a daily supplement of 2 ounces of magnesium oxide in feed per head of cattle maintains the level of plasma magnesium within the normal concentration. He concluded that the daily supplementation of the diet of adult cattle up to the extent of 4 ounces of magnesium oxide per head for a period of six weeks is unlikely to be attended by a loss of condition or to produce undesirable plasma levels of magnesium, calcium and inorganic phosphate.

Rook and Balch (1958) reported that when dairy cows were changed from winter ration to cut young grass, there was an immediate fall in urinary magnesium excretion and progressive development of hypomagnesemia was noticed. When more mature herbage was fed, less marked changes in urine and serum magnesium levels occurred for the first few days following the changes from the winter feed. Thereafter a gradual rise in serum magnesium, followed by urine magnesium, levels took place, which appeared to result from a slight increase in the intake of herbage magnesium in the first few days following the change of the diet.

Burt and Thomas (1961) studied dietary citrate and hypomagnesemia in the ruminant and suggested that the high citrate content of spring grass may be the cause of hypomagnesemia.

III. MATERIALS AND METHODS

1. Isolation of Streptococcus agalactiae

Streptococcus agalactiae was the test organism used in this study. It was obtained from one of the Michigan State University (MSU) Holstein cows affected with a clinical case of mastitis. Milk was aseptically collected and incubated at 37°C for 24 hours and the following tests were performed to identify the organism.

a. Newman's staining

b. Streaking of blood agar plate (5% bovine blood)

c. Inoculation of tryptose broth

d. Hotis test

e. Inoculation of sodium azide crystal violet agar

f. Streaking on Edward's medium

The isolated strain of Streptococcus agalactiae was maintained by monthly transfer on thick tryptose agar plate and stored at 5C. Actively growing 22-24 hours broth culture that previously had been transferred for several consecutive days were used for the citric acid testing procedure. Transfer of the organism tends to stimulate a constant uniform, rapid and more vigorous growth.

2. Milk used as media

The milk used to test the growth of Streptococcus agalactiae organisms was obtained from the following sources:

a. Commercial-Modified skim milk* sold by the M.S.U. Dairy was heat sterilized (autoclaved) at 115C and 10 pounds pressure for 15 minutes.

b. Fresh, hand milked, sterilized--A cow not receiving any antibiotics and showing zero (negative) reading on the California Mastitis Test (CMT) for all four quarters was selected for taking milk samples. The udder was washed with disinfectant and the cow was hand milked into a flask and then the milk transferred to a separatory bottle and placed in the refrigerator (7 C) for 12 hours after which the fat separated milk was dispensed into test tubes and autoclaved at 115 C and 10 pounds pressure for 15 minutes.

c. Fresh, aseptically hand milked, nonsterilized--A cow not receiving any antibiotics and showing zero (negative) reading on CMT was selected. The udder was washed 5 times with a warm aqueous solution of Novadine.** Special care was taken to wash the orifices of the teats. The cow was milked directly into a sterile flask with utmost practicable aseptic precaution. Then the milk was transferred to a sterile separatory bottle and placed in the refrigerator (7 C) for 12 hours. A special fitting burette (Fig. 1) was attached to the bottle to dispense the milk into sterile test tubes without contamination. Before using, the milk

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*Skim milk to which 1% dried skim milk and 2000 USP units of vitamin A and 400 USP units of vitamin D were added.

**1/2 oz. CLENESCO NOVADINE in 2 1/2 gallons of water, Cowles Chemical Company, Cleveland, Ohio.

was examined for sterility.

3. Materials used for plate counting

a. Tryptose agar (Difco)*--Tryptose agar was reconstituted and autoclaved at 121 C at 15 pounds pressure for 15 minutes, and stored at 10 C. Whenever required, agar was melted and poured into petri dishes (sterile and disposable). After solidification all petri dishes were incubated at 37 C for 24 hours and were only used if they were negative for bacterial growth.

b. Dilution blank--Composition:

Peptone	10 mg.
Sodium Chloride	500 mg.
Distilled Water	100 ml.

The dilution blank was dispensed in 99 ml. amounts into rubber stoppered bottles and autoclaved at 121 C at 15 pounds pressure for 30 minutes.

4. Precipitating point of normal milk with citric acid

Citric acid ranging in concentration from 0.2%-4.0% were added to milk. Degree of precipitation and pH at different concentrations of citric acid in milk was determined. Beckman's pH meter and Beckman's standard buffer were used.

5. Growth of Streptococcus agalactiae in milk in the presence of sodium citrate

Sterile solutions of sodium citrate** were added

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*Difco Laboratories, Detroit 1, Michigan.

**Sodium Citrate ($Na_3C_6H_5O_7 \cdot 2H_2O$):F.W. 294.111, J. T. Baker Chemical Co., Phillipsburg, New Jersey.

to the sterile test tubes containing 9 ml. of skimmed milk (obtained from M.S.U. dairy store). One milliliter of the appropriate concentration of sterile sodium citrate solution was added to 9 ml. of milk to make final concentrations varying from 0.1% to 4%. Thus in each instance the milk was diluted 9 to 1. All tubes having different concentrations of sodium citrate as well as control tubes were inoculated with the same number of Streptococcus agalactiae. In the control tube 1 ml. of 0.5% sodium chloride solution was added.

All tubes were incubated at 37 C and dilution plates were made at the end of 24 hours and 48 hours of incubation. Each tube was mixed on a Vortex* mixer for one minute to obtain uniformity of the mixture before sampling. Dilution plates were made by plating out different dilutions on 24 hour incubated tryptose agar plates. Tube contents were plated in original form as well as in different dilutions for which dilution blanks were used. Care was taken to place the plating materials on the center of agar plate and petri dishes were slowly rotated to obtain a uniform spread all over the surface. The petri dishes were set on a level surface until the plating materials were absorbed into the agar (approximately 2 hours). After 24 hours of incubation colonies were counted.

The same procedure was repeated with the fresh

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*Vortex Jr. Mixer, Scientific Industries, Inc., Queens Village, New York.

sterilized and nonsterilized milk. The pH was measured after 48 hours of incubation by using Beckman's pH meter.

6. Growth of Streptococcus agalactiae in milk in the presence of citric acid

The same procedure was adopted as in #5, the only difference being that citric acid was used as a source of citrate for binding Mg^{++} in the milk. A mixed solution of sodium citrate and citric acid was also tried which gave a higher pH than that of citric acid, and lower than sodium citrate when used alone. The pH was measured by Beckman's pH meter in the beginning and end of 48 hours of incubation.

To determine whether the growth inhibitory action of citric acid in milk on Streptococcus agalactiae was due to binding of Mg^{++} or to acidity of the medium, two methods were used:

a. Addition of magnesium chloride

b. Addition of hydrochloric acid to lower the pH of milk

A sufficient amount of magnesium chloride was added to the citrated milk to provide enough magnesium ions to satisfy the growth requirements of Streptococcus agalactiae. The growth of Streptococcus agalactiae in this milk was compared with its growth in citrated milk without the additional magnesium chloride.

The amount of hydrochloric acid required for a desired pH of 9 ml. of milk was determined. Then by adding

hydrochloric acid, the pH of milk was made to correspond to the pH of the citrated milk.

7. Study with another culture of Streptococcus agalactiae

Another culture of Streptococcus agalactiae was obtained from Dr. Huddleson* on which he worked in 1959. The dilution and cultural procedures were the same as previously described (Part 5).

*Department of Microbiology and Public Health, Michigan State University.

IV. RESULTS

1. Effect of sodium citrate on growth of Streptococcus agalactiae in milk

Results obtained from the experiments have been presented in Tables 1 through 6. No growth inhibitory effects on Streptococcus agalactiae in commercial skim milk occurred when sodium citrate concentrations varying from 0.1% to 4% were present in the milk.

Furthermore, there were no significant inhibitory effects on fresh milk (see Tables 4 to 6) when concentrations of 1% to 4% sodium citrate were present regardless of whether the milk had been sterilized or not. Growth of the organism, however, was more vigorous in the sterilized milk than it was in the nonsterilized milk (see Tables 5 & 6).

2. Precipitation of milk with citric acid

A small amount of precipitation occurred when the citric acid concentration in the milk was 0.2%. This lowered the pH of the milk from 6.65 to 5.64. With a citric acid concentration less than 0.2%, there was no gross precipitation of milk. Moreover, concentrations of citric acid higher than 0.3% in the milk produced a heavy precipitation (see Table 8).

3. Effect of citric acid on growth of Streptococcus agalactiae in milk

Growth of Streptococcus agalactiae was inhibited

when concentrations of 1%, 2%, and 4% citric acid were added to the milk. The minimum level capable of inhibiting the growth of Streptococcus agalactiae was found to be 0.8% (milk pH 4.08-4.12). However, at this concentration, results were variable, there being total inhibition on some trials and some growth on other trials. When the citric acid concentration was increased to 0.9%, there was complete inhibition of organism growth on all trials (Tables 10 to 14).

When, by the addition of hydrochloric acid, the pH of the milk was lowered to the same pH as that produced by 0.9% citric acid (3.90 to 4.10), inhibition of Streptococcus agalactiae likewise occurred (see Tables 11 to 14).

The addition of magnesium chloride to the 0.8% and 0.9% citric acid milk did not appreciably change the growth rate of Str. agalactiae; however, it did slightly increase the growth inhibitory action.

TABLE 1--Effect of Sodium Citrate on Growth of *Streptococcus agalactiae* in Milk (After 24 hours of incubation)

S. No.	Amount of milk*	Sodium citrate added	No. of organism inoculated	Growth** on plate dilution on tryptose agar		
				1:20	1:200	1:1000
1.	9ml.	0.1%	182	Inn.	Inn.	Inn.
2.	9ml.	0.2%	182	Inn.	Inn.	Inn.
3.	9ml.	0.3%	182	Inn.	Inn.	Inn.
4.	9ml.	0.4%	182	Inn.	Inn.	Inn.
5.	9ml.	0.5%	182	Inn.	Inn.	Inn.
6.	9ml.***	--	182	Inn.	Inn.	Inn.
7.	9ml.	--	None	None	None	None

*Commercial skim milk from M.S.U. Dairy Store.

**Approximate colony count.

***1ml. of 0.5% sodium chloride was added to 9ml. of milk.

Inn. = Innumerable colonies

TABLE 2--Effect of Sodium Citrate on Growth of *Streptococcus agalactiae* in Milk
(After 48 hours of incubation)

S. No.	Amount of milk*	Sodium citrate added	No. of organism inoculated	Growth** on plate dilution on tryptose agar		
				1:20	1:200	1:1000
1.	9ml.	0.1%	182	Inn.	Inn.	Inn.
2.	9ml.	0.2%	182	Inn.	Inn.	Inn.
3.	9ml.	0.3%	182	Inn.	Inn.	Inn.
4.	9ml.	0.4%	182	Inn.	Inn.	Inn.
5.	9ml.	0.5%	182	Inn.	Inn.	Inn.
6.	9ml.***	--	182	Inn.	Inn.	Inn.
7.	9ml.	--	None	None	None	None

*Commercial skim milk from M.S.U. Dairy Store.

**Approximate colony count.

***1ml. of 0.5% sodium chloride was added to 9ml. of milk.

Inn. = Innumerable colonies

TABLE 3--Effect of Sodium Citrate on Growth of *Streptococcus agalactiae* in Milk

S. No.	Amount of milk*	Sodium Citrate added	No. of organism inoculated	Growth** on plate dilution on tryptose agar					
				After 24 hours of incubation			After 48 hours of incubation		
				1:20	1:200	1:20T	1:20	1:200	1:20T
1.	9ml.	1%	80	PCG	Inn.	Inn.	PCG	Inn.	Inn.
2.	9ml.	2%	80	PCG	Inn.	Inn.	PCG	Inn.	Inn.
3.	9ml.	3%	80	PCG	Inn.	Inn.	PCG	Inn.	Inn.
4.	9ml.	4%	80	PCG	Inn.	Inn.	PCG	Inn.	Inn.
5.	9ml.***	--	80	PCG	PCG	PCG	PCG	PCG	PCG
6.	9ml.	--	--	None	None	None	None	None	None

*Commercial skim milk from M.S.U. Dairy Store

**Approximate colony count

***1 ml. of 0.5% sodium chloride was added to 9ml. of milk

Inn. = Innumerable colonies

PCG = Profuse, confluent growth

T. = Thousand

TABLE 4--Effect of Sodium Citrate on Growth of *Streptococcus agalactiae* in Milk

S. No.	Amount of milk*	Sodium Citrate added	No. of organism inoculated	Growth** on plate dilution on tryptose agar							
				After 24 hours of incubation				After 48 hours of incubation			
				1:10	1:200	1:20T	1:2000T	1:10	1:200	1:20T	1:2000T
1.	9ml.	1%	161	PCG	PCG	Inn.	147	PCG	PCG	Inn.	134
2.	9ml.	2%	161	PCG	PCG	Inn.	235	PCG	PCG	Inn.	339
3.	9ml.	3%	161	PCG	PCG	Inn.	144	PCG	PCG	Inn.	341
4.	9ml.	4%	161	PCG	PCG	Inn.	206	PCG	PCG	Inn.	459
5.	9ml.***	--	161	PCG	PCG	Inn.	157	PCG	PCG	Inn.	169
6.	9ml.	--	--	None	None	None	None	None	None	None	None

*Fresh, hand milked, fat separated and heat sterilized milk.

**Approximate colony count.

***1 ml. of 0.5% sodium chloride was added to the 9ml. of milk.

Inn. = Innumerable colonies

PCG = Profuse, confluent growth

T. = Thousand

TABLE 5--Effect of Sodium Citrate on Growth of *Streptococcus agalactiae* in Milk

S. No.	Amount of milk*	Sodium citrate added	No. of organism inoculated	Growth** on plate dilution on tryptose agar							
				After 24 hours of incubation				After 48 hours of incubation			
				1:20	1:200	1:20T	1:2000T	1:20	1:200	1:20T	1:2000T
1.	9ml.	0.8%	30	PCG	Inn.	...	...	PCG	PCG	Inn.	141
2.	9ml.	1.0%	30	PCG	Inn.	...	...	PCG	PCG	Inn.	162
3.	9ml.	2.0%	30	PCG	Inn.	...	...	PCG	PCG	Inn.	98
4.	9ml.	4.0%	30	PCG	Inn.	...	...	PCG	PCG	Inn.	59
5.	9ml.***	--	30	PCG	Inn.	...	...	PCG	PCG	Inn.	150
6.	9ml.	--	None	None	None			None	None	None	None

*Aseptically hand milked, fat separated, not sterilized milk (Cow #704, M.S.U., June 20, 1963).

**Approximate colony count.

***1 ml. of 0.5% sodium chloride solution was added to 9ml. of milk.

Inn. = Innumerable colonies

PCG = Profuse, confluent growth

T. = Thousand

TABLE 6--Effect of Sodium Citrate on Growth of *Streptococcus agalactiae* in Milk

S. No.	Amount of milk*	Sodium Citrate added	No. of organism inoculated	Growth** on plate dilution on tryptose agar							
				After 24 hours of incubation				After 48 hours of incubation			
				1:20	1:200	1:20T	1:2000T	1:20	1:200	1:20T	1:2000T
1.	9ml.	0.8%	30	PCG	PCG	Inn.	140	PCG	PCG	Inn.	149
2.	9ml.	1.0%	30	PCG	PCG	Inn.	113	PCG	PCG	Inn.	154
3.	9ml.	2.0%	30	PCG	PCG	Inn.	158	PCG	PCG	Inn.	220
4.	9ml.	4.0%	30	PCG	PCG	Inn.	208	PCG	PCG	Inn.	235
5.	9ml.***	--	30	PCG	PCG	Inn.	159	PCG	PCG	Inn.	185
6.	9ml.	--	None	None	None	None	None	None	None	None	None

*Aseptically milked, fat separated, heat sterilized milk (Cow #704, M.S.U., June 20, 1963).

**Approximate colony count.

***1 ml. of 0.5% of sodium chloride solution was added to 9ml. of milk.

Inn. = Innumerable colonies

PCG = Profuse, confluent growth

T. = Thousand

TABLE 7--Effect of Citric Acid on Growth of Streptococcus agalactiae in Milk

S. No.	Amount of milk*	Citric acid added	No. of organism inoculated	pH before inoculation	Growth** on plate dilution of tryptose agar After 24 hours of incubation					
					1:20	1:100	1:200	1:20T	1:1000T	1:2000T
1.	9ml.	1%	14	3.72	1	None	None	None	None	None
2.	9ml.	2%	14	3.20	None	None	None	None	None	None
3.	9ml.	4%	14	2.72	None	None	None	None	None	None
4.	9ml.***	--	14	6.64	PCG	PCG	PCG	Inn.	--	281
5.	9ml.	--	None	6.65	1	None	None	None	None	None

*Aseptically hand milked, not sterilized milk.

**Approximate colony count.

***1 ml. of 0.5% sodium chloride solution was added to 9ml. milk.

Inn. = Innumerable colonies

PCG = Profuse, confluent growth

T. = Thousand

TABLE 8--Precipitation of Milk with Citric Acid

% of Citric acid in milk*	pH	Degree of precipitation (ppt.)
0.0%	6.65	Normal milk
0.2%	5.64	Little ppt. started
0.3%	5.12	Heavy ppt.
0.4%	4.55	Heavy ppt.
1.0%	3.72	Heavy ppt.
2.0%	3.20	Heavy ppt.
4.0%	2.70	Heavy ppt.

*Fresh with fat (not sterilized)

TABLE 9a--Effect of Citric Acid and Sodium Citrate on Growth of *Streptococcus agalactiae* in Milk

(After 24 hrs. of incubation)

S. No.	Amount of milk*	Citric acid added	Sodium citrate added	pH	No. of organism inoculated	Growth** on plate dilution on tryptose agar				
						1:20	1:100	1:200	1:20T	1:2000T
1.	9ml.	0.3%	--	4.94	39	PCG	PCG	Inn.	Inn.	14
2.	9ml.	0.5%	--	4.25	39	Inn.	Inn.	Inn.	47	None
3.	9ml.	0.25%	0.35%	5.6	39	PCG	PCG	Inn.	508	8
4.	9ml.	0.5%	0.5%	4.7	39	Inn.	Inn.	Inn.	26	None
5.	9ml.***	--	--	6.72	39	Inn.	Inn.	Inn.	Inn.	9
6.	9ml.	--	--	6.72	None	PCG	Inn.	Inn.	482	--

*Aseptically milked, not sterilized milk (Cow #128, July 22, 1963).

**Approximate colony count.

***1 ml. of 0.5% sodium chloride solution was added to 9ml. of milk.

Inn. = Innumerable colonies

PCG = Profuse, confluent growth

T. = Thousand

TABLE 9b--Effect of Citric Acid and Sodium Citrate on Growth of *Streptococcus agalactiae* in Milk

(After 48 hrs. of incubation)

S. No.	Amount of milk*	Citric acid added	Sodium citrate added	pH	No. of organism inoculated	Growth** on plate dilution on tryptose agar				
						1:20	1:100	1:200	1:20T	1:2000T
1.	9ml.	0.3%	--	4.94	39	PCG	PCG	Inn.	Inn.	128
2.	9ml.	0.5%	--	4.25	39	PCG	Inn.	Inn.	Inn.	46
3.	9ml.	0.25%	0.35%	5.6	39	PCG	PCG	Inn	Inn.	192
4.	9ml.	0.5%	0.5%	4.7	39	PCG	Inn.	Inn.	Inn.	142
5.	9ml.***	--	--	6.72	39	PCG	PCG	--	Inn.	592
6.	9ml.	--	--	6.72	None	PCG	PCG	PCG	Inn.	46

*Aseptically milked, not sterilized milk (Cow #128, July 22, 1963).

**Approximate colony count.

***1 ml. of 0.5% sodium chloride solution was added to 9ml. of milk.

Inn. = Innumerable colonies

PCG = Profuse, confluent growth

T. = Thousand

TABLE 10a--Effect of Citric Acid on Growth of *Streptococcus agalactiae* in Milk
(After 24 hrs. of incubation)

S. No.	Amount of milk*	Amount of citric acid added	pH	No. of organism inoculated	Growth** on plate dilution on trybtosé agar				
					1:20	1:100	1:200	1:20T	1:2000T
1.	9ml.	0.1%	--	30	PCG	PCG	Inn.	Inn.	145
2.	9ml.	0.2%	5.64	30	PCG	PCG	Inn.	Inn.	69
3.	9ml.	0.3%	5.12	30	PCG	PCG	Inn.	Inn.	52
4.	9ml.	0.4%	4.55	30	PCG	PCG	Inn.	Inn.	23
5.	9ml.	0.5%	4.55	30	PCG	PCG	Inn.	Inn.	--
6.	9ml.	0.6%	--	30	PCG	PCG	Inn.	8	None
7.	9ml.	0.7%	--	30	PCG	PCG	Inn.	54	1
8.	9ml.	0.8%	4.12	30	1	None	None	None	None
9.	9ml.	0.9%	4.10	30	None	None	None	None	None
10.	9ml.***	--	--	30	PCG	PCG	PCG	Inn.	183
11.	9ml.	--	--	None	None	None	None	--	--

*Aseptically milked, not sterilized milk.

**Approximate colony count.

***1 ml. of 0.5% sodium chloride solution was added to 9ml. of milk.

Inn. = Innumerable colonies

PCG = Profuse, confluent growth

T. = Thousand

TABLE 10b--Effect of Citric Acid on Growth of Streptococcus agalactiae in Milk
(After 48 hrs. of incubation)

S. No.	Amount of milk*	Amount of citric acid added	pH	No. of organism inoculated	Growth** on plate dilution on tryptose agar				
					1:20	1:100	1:200	1:20T	1:2000T
1.	9ml.	0.1%	--	30	PCG	PCG	PCG	Inn.	133
2.	9ml.	0.2%	5.64	30	PCG	PCG	PCG	Inn.	100
3.	9ml.	0.3%	5.12	30	PCG	PCG	PCG	Inn.	93
4.	9ml.	0.4%	4.55	30	PCG	PCG	PCG	Inn.	65
5.	9ml.	0.5%	4.55	30	PCG	PCG	PCG	Inn.	70
6.	9ml.	0.6%	--	30	PCG	PCG	PCG	529	4
7.	9ml.	0.7%	--	30	PCG	PCG	PCG	Inn.	38
8.	9ml.	0.8%	4.12	30	None	None	None	None	None
9.	9ml.	0.9%	4.10	30	None	None	None	None	None
10.	9ml.***	--	--	30	PCG	PCG	PCG	Inn.	179
11.	9ml.	--	--	None	None	None	None	--	--

*Aseptically milked, not sterilized milk.

**Approximate colony count.

***1 ml. of 0.5% sodium chloride solution was added to 9ml. of milk.

Inn. = Innumerable colonies

PCG = Profuse, confluent growth

T. = Thousand

TABLE 11--Growth of *Streptococcus agalactiae* in Milk in Presence of Citric Acid, Hydrochloric Acid, Magnesium Chloride, and Sodium Citrate

(After 24 hours of incubation)

S. No.	Amount of milk*	Citric acid added	Hydro-chloric acid added	Mag-nesium chloride added	Sodium citrate added	pH	No. of organ-ism** inocu-lated	Growth*** on plate dilu-tion on tryptose agar				Physical appearance
								1:10	1:100	1:20T	1:2000T	
1.	9ml.	--	--	--	1.0%	7.15	32	PCG	Inn.	Inn.	15	Watery clear
2.	9ml.	--	--	--	2.5%	7.45	32	PCG	Inn.	Inn.	12	Watery clear
3.	9ml.	0.8%	--	--	--	4.08	32	PCG	Inn.	61	None	ppt.
4.	9ml.	0.9%	--	--	--	4.07	32	None	None	None	None	ppt.
5.	9ml.	0.9%	--	11.96mg.	--	4.04	32	None	None	None	None	ppt.
6.	9ml.	--	0.075ml. of 7N Hcl.	--	--	2.4	32	Inn.	Inn.	1	None	ppt.
7.	9ml.****	--	--	--	--	6.68	32	Inn.	Inn.	None	14	No ppt.
8.	9ml.	--	--	--	--	6.64	None	1	None	None	None	Clear

*Aseptically milked, not sterilized milk.

**Other culture obtained from Dr. Huddleson, Michigan State University.

***Approximate colony count.

****1 ml. of 0.5% sodium chloride was added to 9ml. of milk.

Inn. = Innumerable colonies

PCG = Profuse, confluent growth

T. = Thousand

TABLE 12--Growth of *Streptococcus agalactiae* in Milk in Presence of Citric Acid, Hydrochloric Acid, Magnesium Chloride, and Sodium Citrate

(After 48 hours of incubation)

S. No.	Amount of milk*	Citric acid added	Hydro-chloric acid added	Magnesium chloride added	Sodium citrate added	pH	No. of organism** inoculated	Growth*** on plate dilution on tryptose agar			
								1:10	1:100	1:20T	1:2000T
1.	9ml.	--	--	--	1.0%	7.15	32	PCG	Inn.	Inn.	8
2.	9ml.	--	--	--	2.5%	7.45	32	PCG	Inn.	331	1
3.	9ml.	0.8%	--	--	--	4.08	32	None	None	None	None
4.	9ml.	0.9%	--	--	--	4.07	32	None	None	None	None
5.	9ml.	0.9%	--	11.96mg.	--	4.04	32	None	None	None	None
6.	9ml.	--	0.075ml. of 7N Hcl.	--	--	2.4	32	None	None	None	None
7.	9ml.****	--	--	--	--	6.68	32	PCG	Inn.	Inn.	89
8.	9ml.	--	--	--	--	6.64	None	None	None	None	None

*Aseptically milked, not sterilized.

**Other culture obtained from Dr. Huddleson, Michigan State University.

***Approximate colony count.

****1 ml. of 0.5% sodium chloride solution was added to 9ml. of milk.

Inn. = Innumerable colonies

PCG = Profuse, confluent growth

T. = Thousand

TABLE 13--Growth of Streptococcus agalactiae in Milk in Presence of Citric Acid, Hydrochloric Acid, Magnesium Chloride, and Sodium Citrate

(After 24 hours of incubation)

S. No.	Amount of milk*	Citric acid added	Hydro-chloric acid added	Mag-nesium chloride added	Sodium citrate added	pH before inocu-lation	No. of organism** inoculated	Growth*** on plate dilution on tryptose agar			
								1:10	1:200	1:20T	1:2000T
1.	9ml.	0.8%	--	--	--	4.1	75	Inn.	25	None	None
2.	9ml.	0.9%	--	--	--	3.9	75	None	None	None	None
3.	9ml.	--	0.8ml. of .65N	--	--	4.1	75	8	None	None	None
4.	9ml.	0.8%	--	132.5mg.	--	3.7	75	None	None	None	None
5.	9ml.	0.9%	--	132.5mg.	--	3.7	75	None	None	None	None
6.	9ml.	--	--	--	1%	7.0	75	PCG	Inn.	Inn.	208
7.	9ml.****	--	--	--	--	6.51	75	PCG	PCG	Inn.	51
8.	9ml.	--	--	--	--	6.50	None	None	None	None	None

*Fresh, hand milked, fat separated and heat sterilized milk.

**Other culture obtained from Dr. Huddleson, Michigan State University.

***Approximate colony count.

****1 ml. of 0.5% sodium chloride solution was added to 9ml. of milk.

Inn. = Innumerable colonies

PCG = Profuse, confluent growth

T. = Thousand

TABLE 14--Growth of *Streptococcus agalactiae* in Milk in Presence of Citric Acid, Hydrochloric Acid, Magnesium Chloride, and Sodium Citrate

(After 48 hours of incubation)

S. No.	Amount of milk*	Citric acid added	Hcl added	$MgCl_2$ added	Sodium citrate added	pH before incubation	No. of organism** inoculated	Growth*** on plate dilution on tryptose agar				pH after 48 hrs. of incubation
								1:10	1:200	1:20T	1:2000T	
1.	9ml.	0.8%	--	--	--	4.1	75	PCG	Inn.	--	--	3.96
2.	9ml.	0.9%	--	--	--	3.9	75	None	None	None	None	3.82
3.	9ml.	--	0.8ml. of .65N	--	--	4.1	75	2	None	None	None	4.18
4.	9ml.	0.8%	--	132.5mg.	--	3.7	75	Inn.	16	None	None	3.68
5.	9ml.	0.9%	--	132.5mg.	--	3.6	75	None	None	None	None	3.65
6.	9ml.	--	--	--	1%	7.0	75	PCG	PCG	Inn.	None	4.63
7.	9ml.****	--	--	--	--	6.51	75	PCG	PCG	Inn.	62	4.74
8.	9ml.	--	--	--	--	6.50	None	None	None	None	None	6.45

*Fresh, hand milked, fat separated and heat sterilized milk.

**Other culture obtained from Dr. Huddleson, Michigan State University.

***Approximate colony count.

****1 ml. of 0.5% sodium chloride solution was added to 9ml. of milk.

Inn. = Innumerable colonies

PCG = Profuse, confluent growth

T. = Thousand

V. DISCUSSION

1. Selection of milk for media

Since there was a possibility that the procedure herein studied might have some practical applications, the milk used was of great importance. Initially a modified milk was used, but as the study advanced, milk resembling as closely as practical, the natural product, was used.

Since the presence of fat in milk interferes with uniform suspension of either the organism or the chemical, modified* skim milk was selected as a starting point even though it does not represent a completely natural condition for *in vivo* work. The modified skim milk was obtained from the M.S.U. Dairy Store.

Next, fresh milk from which the fat had been separated was heat sterilized and used as the medium. This also is not a natural product since heat treatment produces various changes in the milk.

After a careful investigation a technique was devised to collect the milk under aseptic condition and to continue this aseptic technique throughout the separation of fat and dispensing of milk into sterile test tubes. When this was

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*Vitamin A, 2000 USP units, and vitamin D, 400 USP units, had been added to each quart; also contained 1% dry skim milk.

done, a satisfactory quality of bacteria-free milk was obtained. At one time a heavy contamination of the milk occurred, but this was due to the animal's frequent kicking and tail switching. Milk from a young cow had less bacteria than that from an old cow.

Growth of Streptococcus agalactiae was more vigorous in heat sterilized milk than in nonsterilized milk. This was probably due to the inactivation of an anti-bacterial substance called "lactenin" during the heat sterilization process. Lactenin is inactivated by high temperatures, 80 C or more (Wilson, A. T.: 1952).

2. Effect of sodium citrate

By chemical equation the amount of sodium citrate required to chelate all magnesium and calcium ions present in milk was determined. Because of their chemical and physical relationship in milk, it was impossible to chelate total magnesium ions without chelating calcium since a masking agent was not used. Sodium citrate was also added in greater and less amounts than mentioned above. No growth inhibitory influence was noticed on Streptococcus agalactiae in milk. Sodium citrate had much less influence on the pH of milk than did citric acid (see Tables 10 & 11).

3. Effect of citric acid

In other experiments citric acid was added as a source of citrate to chelate magnesium and calcium ions in

the milk. The pH of the milk sharply dropped even with the addition of small quantities of citric acid (Table 8). Total growth inhibition of Streptococcus agalactiae was observed in the milk only after citric acid was added to the concentration of 0.8% or more. This lowered the pH of milk to 4.10 or lower (see Tables 10 & 7). At this point the question arose as to whether growth inhibition of Streptococcus agalactiae in the milk was due to the binding of all the magnesium ions by the citric acid, or to the low pH (acidity) of the media. Webb (1953) reported that the magnesium ions are essential for the nitrogen metabolism of gram-positive bacteria. Webb (1951) also mentioned that gram-positive bacteria fail to grow when the magnesium content of media was less than 0.6 ppm.

To clarify the above problem, two experiments were set up. The first was the addition of a mineral acid to the milk in order to reduce the pH of the milk to the pH of the milk containing 0.9% citric acid. Hydrochloric acid was used for this purpose. Results were similar to those obtained with citric acid.

The other experiment was addition of excessive amounts of magnesium chloride to the milk in order to provide free magnesium ions in the citrated milk. There was no growth response when this was done, but rather the growth inhibitory effect was accelerated probably due to the lowering of the pH by the addition of magnesium chloride to the citrated milk.

Huddleson (1959) reported that Streptococcus agalactiae failed to grow in blood containing 0.5% or higher concentrations of sodium citrate. He found that the growth inhibition was not due to the pH of the media, since when magnesium chloride was added to the citrated blood, growth of Streptococcus agalactiae was promoted. It was concluded that growth inhibition of Streptococcus agalactiae in the citrated blood was due to binding of magnesium ions.

However, similar results were not found with milk, perhaps because of the differences in composition of milk and blood. It may not be possible to chelate all magnesium ions present in the milk below the level required for the growth of Streptococcus agalactiae. On the other hand, Streptococcus agalactiae may grow in the milk, even in the absence of magnesium ions.

Since magnesium is one of the important factors influencing the growth of gram-positive bacteria, one might suppose that a high magnesium diet may influence the incidence of infectious mastitis caused by gram-positive bacteria. On reviewing the literature it has been found that there was only a small variation in the magnesium content of milk with a change of diet. There was no significant variation in magnesium content of streptococcic mastitic milk (Redaelli, et al, 1957). Higher levels (above normal) of ionic magnesium in the milk may influence the incidence rate of mastitis caused by gram-positive bacteria. To clarify this, a study on determination of ionic magnesium in the milk of different herds would be helpful.

FIGURE 1
Method for dispensing milk aseptically into test tubes

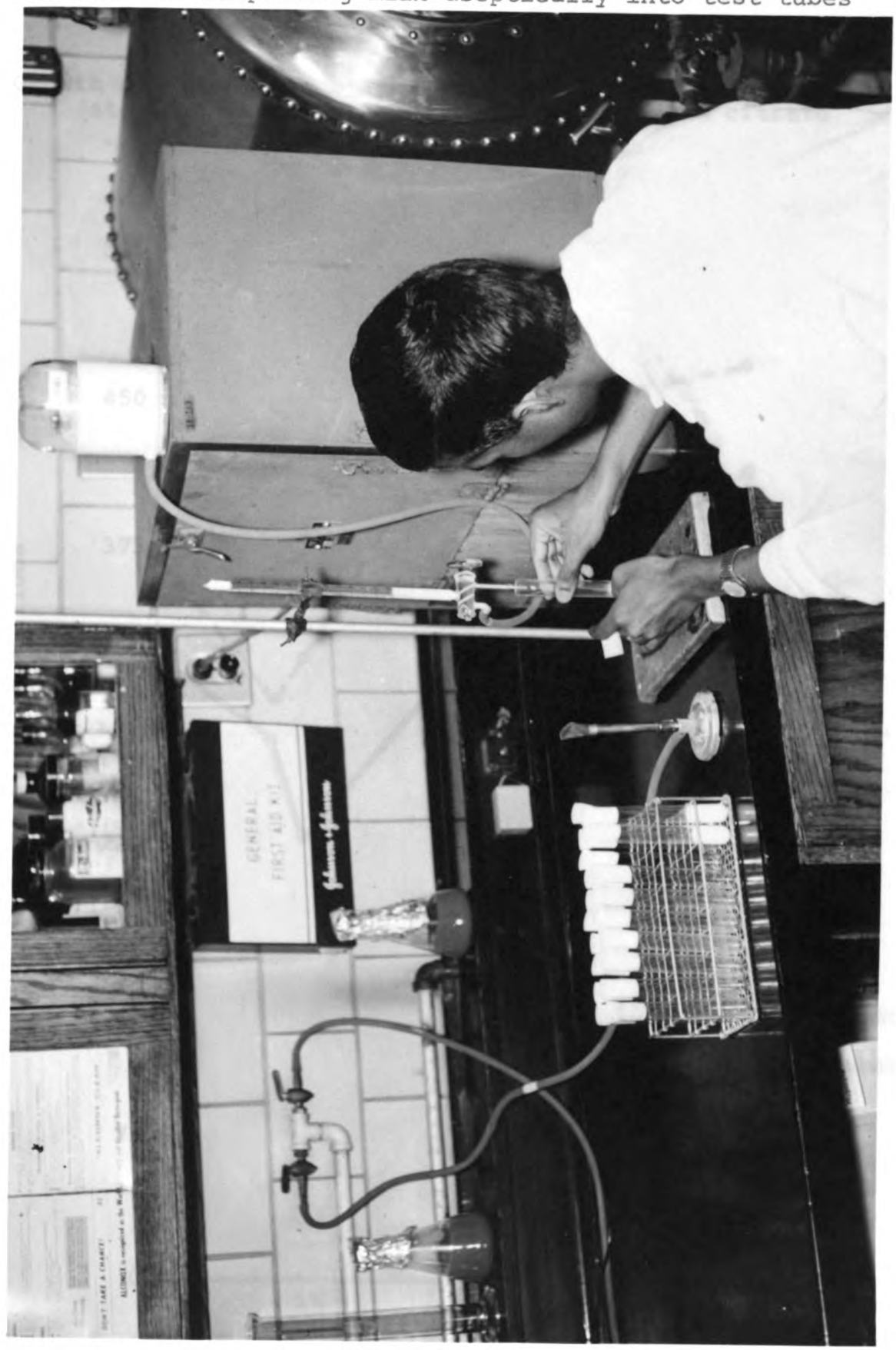


FIGURE 2

Growth of Streptococcus agalactiae in hand milked, heat sterilized milk in the presence of sodium citrate

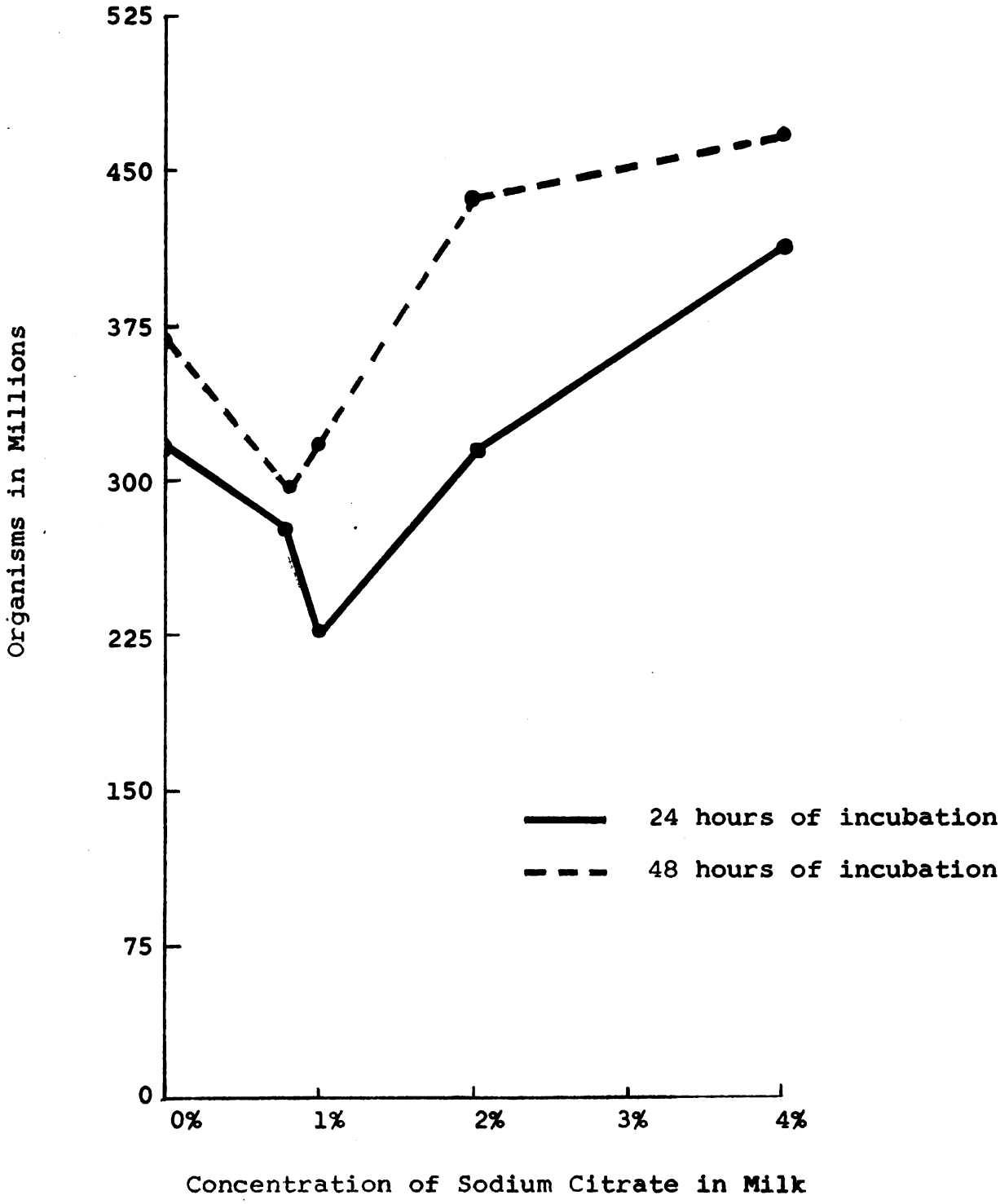


FIGURE 3

Comparison of growth rate of Streptococcus agalactiae between heat sterilized and nonsterilized milk in the presence of sodium citrate

(after 24 hours of incubation)

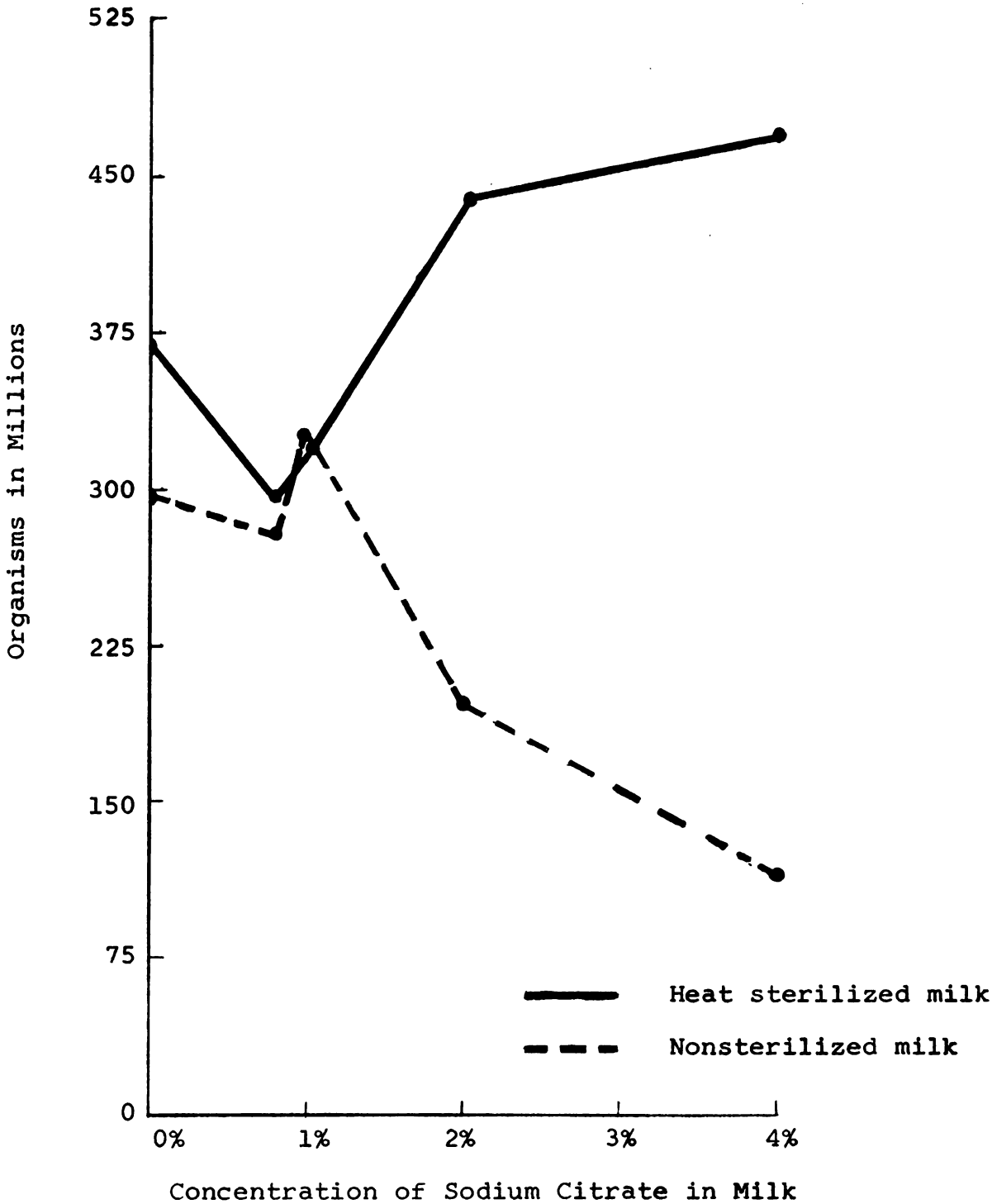
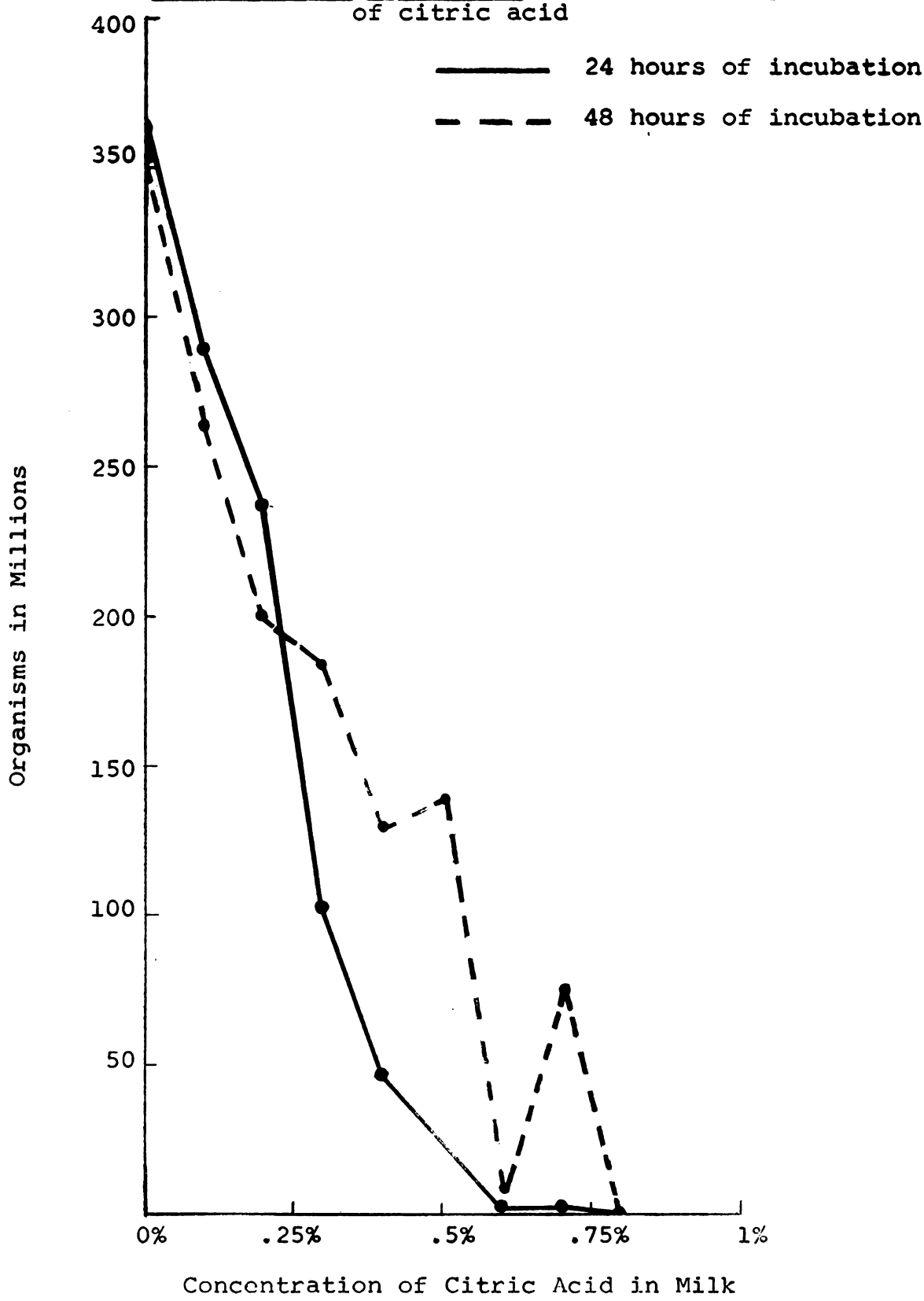


FIGURE 4

Growth of Streptococcus agalactiae in milk in the presence of citric acid



VI. SUMMARY AND CONCLUSIONS

1. When citric acid was added to the concentration of 0.3% or more, a sharp drop in the pH with a heavy precipitation of the milk occurred.

2. For *in vivo* work, satisfactory bacteria free milk was obtained by milking a young cow aseptically in a sterile vessel.

3. Growth of *Streptococcus agalactiae* was more vigorous in heat sterilized milk than in nonsterilized milk.

4. There were no growth inhibitory effects of sodium citrate on *Streptococcus agalactiae* in milk.

5. The addition of citric acid to the milk had a growth inhibitory influence on *Streptococcus agalactiae* in milk. This was due to lowering of the pH (acidity) of the media, but not due to the binding of magnesium ions in milk.

6. Either *Streptococcus agalactiae* can grow in the absence of free magnesium ions in the milk or it is impossible to chelate all free magnesium ions, in milk, since milk is complex in nature.

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