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To the Graduate Council:

I am submitting herewith a thesis written by Maheswaran Subrahmanyam entitled "Use of Hydrogen Peroxide as a Milk Preservative." I recommend that it be accepted for nine quarter hours of credit in partial fulfillment of the requirements for the degree of Master of Science, with a major in Dairying.

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USE OF HYDROGEN PEROXIDE
AS A MILK PRESERVATIVE

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The University of Tennessee

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Master of Science

by
Maheswaran Subrahmanyam

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CHAPTER I

INTRODUCTION

In many countries with hot or tropical climates there are numerous difficulties in handling of fresh milk, because milk remains sweet for a very short period at high temperatures. Very often, the milk has to be transported over long distances to reach the consumer. Therefore, great efforts have been made in different countries to develop their dairy industry and different methods have been tried to prolong the keeping quality. The most important of the methods tried are (i) cooling of milk on the farm and during transport by artificial means, (ii) pasteurization, and (iii) preservation or chemical treatment.

Even though the first is the ideal method, at present, it is very costly in many hot countries. The second method is practicable if milk depots exist. Therefore, preservation or chemical treatment has been tried in countries like Italy, France, India, South America, and South West Africa.

In the selection of a suitable preservative for milk, the following should be considered.

- 1) It must not react with any of the milk constituents.
- 2) It must be easily destructible before the milk is consumed.
- 3) The substances formed after destruction must be colorless, odorless, tasteless, and nonpoisonous.

At present, hydrogen peroxide is the most preferable one of all the preservatives. Hydrogen peroxide is looked upon as one of the "classical preservatives." This has caused considerable antipathy also against the

peroxide-catalase treatment of milk, although this does not mean the addition of preservatives, because the added hydrogen peroxide is entirely decomposed and removed by catalase action. It may therefore be considered more as a treatment rather than preservation. Hydrogen peroxide is considered to be one of the ideal preservatives because it can be destroyed easily, quickly, and completely through the addition of catalase, the enzyme which breaks down hydrogen peroxide. The break down products, water and oxygen, are not detectable and there is no residual poisonous effect after the hydrogen peroxide is destroyed.

Hydrogen peroxide has been found to be of interest in the dairy industry for the treatment of milk for two purposes: first as a short time treatment in the place of pasteurization, to reduce the total bacterial count, and secondly as a preservative to maintain and prolong the keeping quality of milk. In the first method hydrogen peroxide is present in the milk for about an hour whereas in the second method it remains for several hours or days. In the major part of all receiving depots in the world the milk arrives with a bacterial content that varies between a few hundred thousand and several millions. The peroxide-catalase treatment prevents the growth of bacteria and it increases the keeping quality of the milk. To prevent growth of bacteria after milking the addition of a few milliliter of electrolytically pure hydrogen peroxide per liter will be sufficient.

The purpose of this investigation was to find out the influence of hydrogen peroxide on total bacterial count in raw milk and on pure cultures of organisms so that the same can be applied for the preservation of raw milk for a period of four days.

CHAPTER II

REVIEW OF LITERATURE

History of Hydrogen Peroxide Preservation

The early experiments of preserving milk with hydrogen peroxide were made by Heidenhain (39), by adding 10 per cent and Jablin and Gonnett (44) by using 8 per cent while Chick (19) made use of 0.1 to 0.2 per cent, Renard (87) added 0.09 per cent, and Duclaux and Nicolle (24) used 0.06 per cent. Budde (18) combined hydrogen peroxide and low heat treatment (0.05 per cent hydrogen peroxide for 8-10 hours at 52° C. or 0.035 per cent hydrogen peroxide for 30 minutes at 48-50° C. and 2-3 hours at 52° C.). Curran, Evans, and Leviton (22) studied the sporicidal action of hydrogen peroxide and the use of crystalline catalase to dissipate the residual peroxide. It was difficult for a number of years to obtain hydrogen peroxide free of the heavier metals and sulfuric acid, but later electrolytic production of hydrogen peroxide gave a product of high purity. This method was used during the World War II period especially in Italy.

Since different units are used for the description of the peroxide concentration, Table I will be useful for easy calculation and comparison (53).

Chemistry of Hydrogen Peroxide Preservation

It has long been known that hydrogen peroxide is a strong oxidizing, bleaching and germicidally active agent. It decomposes to yield water and oxygen. The oxidizing effect of hydrogen peroxide is

TABLE I
CONCENTRATION EQUIVALENTS FOR HYDROGEN PEROXIDE
USED IN PRESERVATION OF MILK

Hydrogen peroxide concentration	Parts per million	Mole	Per cent by weight	Per cent of a 40 per cent solution	Per cent of a 130 volumes solution
0.01 mole	340	0.0100	0.034	0.085	0.086
100 parts per million	100	0.0029	0.010	0.025	0.025
0.1 per cent by weight	1000	0.0294	0.100	0.250	0.253
0.1 per cent (40 per cent)	400	0.0118	0.040	0.100	0.101
0.1 per cent (30 per cent)	300	0.0088	0.030	0.075	0.076
0.1 per cent (130 volumes)	395	0.0116	0.040	0.099	0.100

reduced in weak or dilute solutions. Of all the available preservatives used in the dairy industry, hydrogen peroxide, in concentrations used for preservation, appears to have very little influence on the constituents of milk, especially proteins (54,85). Highly concentrated hydrogen peroxide should be diluted with water before being added to milk (53). The influence of hydrogen peroxide on milk is intensified by an increase of the temperature and by prolonging the period of treatment.

The main disadvantage of making use of hydrogen peroxide in tropical countries is its instability and contaminations. The presence of 1 part of iron or 0.05 part of copper per million parts of hydrogen peroxide will cause rapid decomposition. At present very pure hydrogen peroxide is manufactured. The stability decreases as the pH increases. The decomposition is rapid at pH 10.0. By adding small amounts of organic or inorganic stabilizers the stability of hydrogen peroxide can be conserved (53).

For the use in dairy industry the hydrogen peroxide used must be of high purity and edible grade. For hygienic reasons and since the presence of heavier metals promote oxidation of milk, the hydrogen peroxide must be electrolytically pure. The milk containing hydrogen peroxide should be allowed to come into contact with only stainless steel or other corrosion resistant material. Copper, iron and tin should not be used since they are attacked by hydrogen peroxide chemically. The strength of hydrogen peroxide is often referred as "vols" (volumes). If the strength of hydrogen peroxide is 130 vols, this means that the decomposition of one liter of this peroxide will yield 130 liters of oxygen.

Improvement in the Keeping Quality of Milk

The increase in the keeping quality of milk obtained by treating milk with hydrogen peroxide has been pointed out by many authors as reviewed below.

The peroxide concentrations to be added depend on the climate, the quality of the milk, and the amount of preservation required. Budde (17) has described a method which consists in the addition of 0.9 gram of hydrogen peroxide per liter and heating rapidly to 55° C. or hydrogen peroxide may be added to milk previously heated to about 50° C., so that it will contain about 0.35 gram of hydrogen peroxide per liter. This method is known as the "Budde's Method" of sterilization of milk and is based on the action of nascent oxygen on the microorganisms in milk at a temperature above 40° C. Bandini (8) has stated that the addition of hydrogen peroxide in the proportion of 1:100 to 3:100 preserved milk for 156 days. Jablin and Gonnett (44) have found that, 1 milliliter of 8 per cent hydrogen peroxide preserved 1 liter of milk for 2 days, 2 milliliter for 4 days and 3 milliliter for 6 days. Vintika (110) has recommended 0.3 per cent of a 3 per cent solution of hydrogen peroxide. 0.075 per cent of 40 per cent hydrogen peroxide has been found effective by Nambudripad, Laxminarayana and Iya (75). Pien (81) found 0.1 to 0.2 per cent of a 40 per cent solution of hydrogen peroxide was effective and Satta (101) has recommended 0.2 to 0.3 per cent of a 40 per cent hydrogen peroxide solution. Because of the complete destruction of the hydrogen peroxide, addition of catalase is not necessary. Pasteurized milk, in which most of the catalase is destroyed by heat, can be preserved with less hydrogen peroxide than is required for raw milk.

Rosati (92,93) has stated that the treatment of milk for food purposes by adding 0.2 to 0.3 per cent of hydrogen peroxide (130 volumes) is useful in extending the period of freshness but cannot replace pasteurization.

Milk preserved with hydrogen peroxide must be pasteurized before using it for human consumption unless the peroxide concentration or the temperature of treatment is increased. Giolitti (31) agrees with the second method described by Rosati, and Nai et al (71) have stated that this has found practical application in Italy. From 0.25 to 0.6 per cent hydrogen peroxide (130 volumes) is added to raw milk at a small collecting center. After 8 hours contact the excess peroxide is eliminated by the addition of catalase, and the milk is bottled. Nemec (77) has found that the hydrogen peroxide-treated samples have a better keeping quality than pasteurized milk especially at 13° C. Preservation with peroxide concentrations higher than 1 per cent has also been employed by many workers. Romani (89) found that milk can be kept at 5° C. (41° F.) by the addition of 1, 2 or 3 per cent hydrogen peroxide (130 volumes) for 24 to 35 days, 32 to 40 days and up to 100-110 days respectively. Anselmi (4) has stated that the addition of 2 to 2.5 per cent hydrogen peroxide is not a satisfactory method for acceptance.

Romani (88) made comparison of the keeping quality of hydrogen peroxide treated milk in tightly stoppered bottles with that of samples in loosely stoppered ones, and has shown that pressures up to 2.5, 4, and 6 atmospheres were developed in the presence of 0.1, 0.2, and 0.3 per cent hydrogen peroxide (130 volumes) respectively at 28° C. The keeping quality under pressure of oxygen was better. An acidity of 0.21 per cent

was normally reached in the presence of 0.3 per cent hydrogen peroxide (130 volumes) in 1 day at 30° C. and in 4 or 5 days at 10° C. In the tightly stoppered bottles these time periods were 2 days and 30 to 40 days respectively. Roushdy (99) in his investigations has found that the addition of 0.03 and 0.05 per cent hydrogen peroxide to raw milk, effectively reduced the bacterial populations. There was a relationship between the catalase content of milk and the efficiency of hydrogen peroxide, the higher the catalase the greater was the destruction of bacteria. The keeping quality was influenced by the holding temperature. In lower concentration of 0.03 per cent hydrogen peroxide when added to milk at 36° C. effectively reduced the bacterial count and such treated milk could be kept without changes in color or flavor for 3 to 7 days (4.7 mean) at 20+ 2° C.

Mainardi (58) has studied the degree of the keeping properties of centrifuged milk as compared with whole milk, the efficiency of electrolytic hydrogen peroxide at 130 vols for the keeping of centrifuged milk, and the possibility to use centrifuged milk in the raising of calves. He has drawn the following conclusions.

- 1) centrifuged milk turns sour much more quickly than whole milk
- 2) electrolytic hydrogen peroxide at 130 vols used at the ratio of 2 parts per thousand is a practical and economical means for the storage of centrifuged milk and
- 3) centrifuged milk stored with hydrogen peroxide can well replace fresh centrifuged milk in the feeding of calves.

Roushdy (99) studied the effect of feeding treated milk on the growth rate of albino rats. The absence of any significant difference

in the growth rate of albino rats fed exclusively on treated milk (0.05 per cent hydrogen peroxide) diet and those fed on the normal stock ration for 42 days was observed.

Peroxide Treatment and Pasteurization

The treatment of milk by hydrogen peroxide has been reviewed by Minart (59,60) and Rosell (95). Hydrogen peroxide treatment as a method of retaining the properties of raw milk when making high quality Swiss cheese is recommended by Morris, Larsen, and Johnson (68,69). Hydrogen peroxide has been used in processing and canning whole sweet milk (115). Lewis (49) has used hydrogen peroxide in treating milk for human consumption.

In the manufacture of cheese edible grade hydrogen peroxide is added to the milk and, after the necessary period to kill the microorganisms present, the rest of the hydrogen peroxide is removed by the addition of catalase. Milk is treated with 0.2 per cent hydrogen peroxide (35 per cent) for 20-40 minutes at 120° - 130° F. (68). At this temperature the peroxide is not allowed to remain in the milk for more than one hour. If this treatment is continued for more than one hour the casein becomes gummy. The milk is then cooled to 100° F. and catalase at the rate of 0.5 gram per 2,000 pounds of milk is added (68).

The cheese made from milk which has been subjected to the above treatment was found to be superior in flavor, body and texture when compared with cheese from raw and pasteurized milk (68). Roundy (97) has shown that Swiss cheese and Cheddar cheese of excellent quality and uniformity can be produced from milk treated with 0.021-0.060 per cent

hydrogen peroxide and catalase. Treatment of milk at approximately 125° F. for about 25 seconds with 0.02 per cent peroxide destroys nearly all the coliform organisms and leaves intact more of the natural enzymes and beneficial organisms than accepted pasteurization procedures. Consequently cheese made from milk properly treated with a small amount of hydrogen peroxide and catalase ripens faster and usually has a finer flavor and body than pasteurized-milk cheese.

A similar peroxide treatment is being recommended for the manufacture of sterilized milk. The milk is treated with 0.1 to 0.2 per cent of a 35 per cent hydrogen peroxide solution at 120° F. for 30 minutes (115). After the removal of hydrogen peroxide with catalase (0.1-0.5 gram per 1,000 pounds of milk), the milk is homogenized, canned, and sterilized at a temperature above 212° F. The sterilized milk prepared in this way does not possess the cooked and scorched flavor common to heat sterilized milk. Winger (116) has also recommended pasteurization before peroxide treatment by the addition of only 0.015 per cent of 35 per cent hydrogen peroxide at 142°-144° F. for 30 to 35 minutes and no addition of catalase.

Patterson and Williams (80) have developed a new method of canning whole milk. It is done by adding a fraction of 1 per cent by weight of edible hydrogen peroxide to the milk at below pasteurization temperatures, allowing it to remain in the milk for 30 minutes before removal by an enzyme naturally present in milk, homogenizing, cooling and canning the milk, and placing it in a sterilizer for 30 minutes at a temperature above boiling. The resulting milk is said to be absolutely sterile.

Lewis (49) recommends the low heat-hydrogen peroxide-catalase process for the treatment of fresh milk in tropical countries. The effect of this method on Mycobacterium tuberculosis has not been investigated. Nai and Giolitti (72) state that at room temperature the period of treatment must be prolonged and higher hydrogen peroxide concentrations (12:1,000) must be used, in order to obtain the same destruction as with pasteurization.

Preservation of Whey and Cream by Hydrogen Peroxide

The widespread and sanctioned use of hydrogen peroxide as a milk preservative in some countries (94) has led into investigations as to the applicability of hydrogen peroxide as a preservative for liquid whey. Plottner (84) has made some studies in this area and concluded that the addition of 0.005 per cent hydrogen peroxide by weight produces a disagreeable taste and even the addition of 0.015 per cent and 0.03 per cent hydrogen peroxide by weight to raw sweet whey was not quite a successful one. The addition of 0.009 per cent hydrogen peroxide to raw whey only limits the extent of souring. Plottner (84) found that after the precipitation of the proteins by heating at 98° C. for 10 minutes or after the addition of small quantities of yeast, the taste of the hydrogen peroxide disappears. In contrast to the above results Yumus (117) reported that the addition of 0.1 per cent hydrogen peroxide has no preservative value for whey.

Jasewicz and Porges (45) have stated that concentrations of 0.02 per cent hydrogen peroxide in cheese whey exerted a preservative effect for as long as 10 days. According to the data, hydrogen peroxide addition

shortly after the separation of the whey from curd is recommended. 0.02 per cent peroxide addition to contaminated wheys of 28,000,000 micro-organisms per milliliter resulted in a 97 per cent bacterial kill within one hour.

The preservation of cream by hydrogen peroxide has been patented by Reichert, McAllister, and Hinegardner (86). In this the addition of hydrogen peroxide in amounts ranging from 0.01 to 0.09 per cent by weight and heating at a temperature of 61° to 63° C. for 15 to 30 minutes is recommended. Hobbs and Smith (42) have stated that 0.005 to 0.020 per cent hydrogen peroxide should be added for the control of infections in synthetic cream.

Influence of Hydrogen Peroxide on Milk Constituents

Taste. Unless too high a concentration of hydrogen peroxide is employed, the influence of hydrogen peroxide on the flavor of milk is transitory. The milk has an objectionable taste only if the presence of hydrogen peroxide is detectable by chemical tests. But if the hydrogen peroxide is destroyed by catalase the breakdown products of hydrogen peroxide are not detectable. Viotkevitch, Davidov, and Viotkevich (111) and Satta et al (102) have described the flavor of treated milk as "metallic." When hydrogen peroxide treated milk was heat processed after treatment, no undesirable flavor could be detected even after long period of storage. When high concentrations of hydrogen peroxide (more than 500 parts per million) are used in the case of milk having excessive bacterial counts, off flavor may develop. Nambudripad, Laxminarayana and Iya (75) have found that milk containing an appreciable

amount of undecomposed peroxide developed a slightly oxidized flavor after prolonged storage. These workers have also found that the development of off-flavors is influenced by the material of the containers; aluminum cans gave rise to a slight oxidized flavor, glass containers were the best, tinned brass, porcelain and aluminum containers coming next in order of preference. A smoky flavor has been caused by the transport of milk preserved on the farm with 0.2 per cent of 130 volumes hydrogen peroxide (88). Weinstein and Trout (113) have stated that the treatment of milk with hydrogen peroxide prior to pasteurization and homogenization can prevent the development of a typical solar-activated flavor in homogenized milk samples, whereas high temperature heat treatment (176° F. for 5 minutes) did not retard or prevent its development. The development of an oxidized flavor in milk during storage at -17° C. has been delayed by hydrogen peroxide treatment (12). Morris (67) found that oxidized flavor developed in 70 per cent of the pasteurized milk samples, but did not develop in samples treated with hydrogen peroxide (0.2 per cent hydrogen peroxide and addition of catalase). Except for a slight malty flavor the odor and flavor were satisfactory. The addition of hydrogen peroxide has retarded the development of a "tallowy" flavor during cold storage of milk (48).

Vitamins. By treatment with hydrogen peroxide very little damage is done to the vitamins in milk. Only vitamin C is seriously influenced. Since milk is not a good source of vitamin C this is not of great importance. The loss of ascorbic acid in milk held for 20 hours at 15°, 22°, 26° and 32° C., by the addition of 0.1 per cent hydrogen peroxide (130 volumes) was 54 per cent, 78 per cent, 85 per cent, and 92.5 per

cent respectively, whereas in the control the loss was 84 per cent (102).

The destruction was more at higher concentrations of peroxide and at higher temperatures. The effect of hydrogen peroxide treatment on the ascorbic acid content of milk has been studied by various workers (15, 9, 47). All the workers confirm the instability of ascorbic acid in milk containing peroxide. The ascorbic acid oxidation has been found to vary inversely with the quantity of hydrogen peroxide added to the milk (48). When the amount of the preservative added was in excess of that required to oxidize ascorbic acid completely, the reaction was retarded appreciably. Peroxidase has been found to be responsible for the loss of ascorbic acid (47) and destruction of this enzyme with higher concentrations of hydrogen peroxide may serve as an explanation for the inverse relationship between the retardation of ascorbic acid destruction and peroxide concentration.

The vitamin A content is decreased by 22 to 42 per cent by treatment with 0.1 per cent hydrogen peroxide (130 volumes) (34). Satta et al (102) have found that the addition of 0.3 per cent hydrogen peroxide (130 volumes) at 20° or 32° C. for 36 hours reduced the vitamin A content from 158 I. U. to 125 I. U. per 100 grams. The corresponding figures for the stored controls without the addition of hydrogen peroxide were 130-135 I. U. In the same sample the thiamin content was dropped down to 12 to 15 I. U. from 25-30 I. U., and in the controls it was 15-18 I. U. per 100 grams. In biological assays of vitamin D with rats, there was no detectable change in the milk treated up to 0.4 per cent hydrogen peroxide (130 volumes) and held at 22° C. for 36 hours. Nambudripad, Laxminarayana and Iya (75) have stated that none of the B-Complex

vitamins were found to be affected by peroxide treatment. The control milk was pasteurized at 63° C. for 1-2 hours and the milk used for the experiment was treated with 300 parts per million of hydrogen peroxide and then pasteurized as in the control. The thiamin, riboflavin, nicotinamide and vitamin B₁₂ content of both the samples were nearly the same. Banerjee (9) has stated that vitamin A is not affected but vitamin C is somewhat reduced.

Lactose and butterfat. Banerjee (9) found that the lactose was slightly reduced by treatment with hydrogen peroxide (16 grains per pint of milk). These results have been confirmed by Arnaudi and Treccani (7). Their results showed that the sugar content of untreated milk was 5.01 per cent, but after treatment with 0.025, 0.05, 0.1 or 0.2 per cent hydrogen peroxide (130 volumes) at 30° C. for 16 hours, the figures were 5.01, 4.95, 4.95, and 4.60 per cent respectively. However, Giolitti (34) found no change for lactose, fat, total nitrogen and pH by treating milk with 0.1 per cent peroxide. Not much information is available on the influence of hydrogen peroxide on the butterfat. In milk containing 0.1 per cent hydrogen peroxide there was no change in the butterfat (34). Nambudripad, Laxminarayana and Iya (75) have found that butter made from milk preserved with 300 parts per million of hydrogen peroxide did not differ much in quality from that made from fresh milk. No considerable differences were found in the yield, organoleptical properties and physico-chemical constants of butter made from milk containing 0.5 per cent hydrogen peroxide (130 volumes) (76).

Proteins and amino-acids. Even though hydrogen peroxide in higher concentrations oxidizes proteins, dilute solutions do not show this effect. Rondoni and Bassi (90) studied the influence of dilute hydrogen peroxide solutions, in concentration of 0.3 per cent of 40 per cent peroxide, on partly autolysed protein solutions like liver extract and serum. An aggregation of protein molecules and an increase of the trichloroacetic acid precipitable nitrogen resulted by the addition of hydrogen peroxide. Only pepsin, chymotrypsin, bovine fibrinogen, casein, mucin, and soya bean proteins were oxidized by hydrogen peroxide in the presence of the enzyme peroxidase (105). The amino acid methionine is very sensitive to hydrogen peroxide (61). Tyrosine and tryptophan can easily be oxidized. Addition of 0.03 per cent hydrogen peroxide by weight or 0.03 per cent hydrogen peroxide with 8×10^{-5} per cent copper had no effect on the sulfhydryl groups in skim milk after 20 hours at room temperature (118). The addition of even 0.25 per cent hydrogen peroxide (40 per cent) for one day at 30° C. or 20 minutes at 55° C. did not noticeably reduce the sulfhydryl content (54). Previtera (85) studied the amino acid behavior in milk treated with pure electrolytic 39 per cent hydrogen peroxide and found that the addition of 0.3 per cent hydrogen peroxide (39 per cent) to milk does not affect the amino acid content. Tepley, Derse and Price (108) have stated that no marked changes in the composition or nutritive value of milk treated with 0.1, 0.2 and 0.5 per cent hydrogen peroxide, or of the whey or cheese obtained from that milk. Giolitti (34) found that hydrogen peroxide treatment (0.1 per cent 130 volumes) had increased the albumin content from 0.042 to 0.061 (in nitrogen percentage) and

decreased the casein content from 0.367 to 0.317 (in nitrogen percentage). In the presence of peroxidase or metallic ions like copper and iron the oxidizing effect is catalytically accelerated. Hydrogen peroxide, with peroxidase obtained from haemoglobin, was able to deaminate the amino acid glycine, alanine, leucine, glutamic acid, and aspartic acid (107). Sizer (105, 106) has found that only tyrosine, tryptophan, lysine and possibly cystine were oxidized in a peroxidase-hydrogen peroxide system at 37° C. and pH 7.3. Generally peroxide has a softening action on casein. The addition of 0.2 per cent and more of hydrogen peroxide decreases the firmness of the curd. It has been observed that at the beginning, after the addition of sodium hypochlorite solution the carbon dioxide of peroxide treated casein (0.09 per cent hydrogen peroxide) is more intensified than in untreated samples (103). No difference in casein of treated and untreated samples was noticed by Luck and Joubert (54) with electrophoresis experiments. Particle size studies were made by means of an ultra centrifuge and electron microscope by Luck and Joubert (55). The experiments have shown that after 5-6 days preservation with 0.25 or 1 per cent hydrogen peroxide (39 per cent) at 4° C., a part of the casein has dissociated. The sedimentation constant of the fast sedimenting component was reduced from 6.2×10^{-13} in untreated milk to 3.8×10^{-13} in treated samples. The slowly sedimenting component (1.3×10^{-13}) was not changed. This effect seems to explain the longer period of time required for the coagulation by rennet observed in milk as well as in pure sodium caseinate solution. An examination under the electron microscope revealed that the size of the casein particles in milk was not changed significantly compared with that of untreated samples. The

alcohol concentration for the coagulation of treated milk was a little lower than for the same milk when untreated. Luck and Joubert (55) have concluded that the effect of hydrogen peroxide on casein is not serious since untreated milk kept at 4° C. for 11 days undergoes some alteration. Luck and Joubert (54) studied the effect of hydrogen peroxide on the sulfhydryl groups of whey proteins. The addition of 0.25 per cent hydrogen peroxide and storage of the milk 2 days at 30° C. or 35 minutes at 55° C. did not reduce the content of sulfhydryl groups of the corresponding whey proteins significantly. The use of higher concentrations of hydrogen peroxide (0.5-1.0 per cent) affected significant reductions within temperatures of 55° and 65° C. Electrophoretic studies made by the same authors (54, 56) show an influence of hydrogen peroxide on the whey proteins of treated milk. Spreading of the boundary is noticed and this is accompanied by a decrease in the electrophoretic mobility. Addition of 1 per cent hydrogen peroxide (40 per cent) for 6 days at 4° C. splits the betalactoglobulin into two components with considerably lower mobilities. A large number of minor components are observed.

Enzymes. The enzymes of the milk are not influenced to any great extent by the addition of hydrogen peroxide. The phosphatase test used for heated milk is applicable to milk preserved with hydrogen peroxide. The enzymes amylase, lipase, tryptase, and phosphatase were not affected by the addition of 0.2 to 0.3 per cent hydrogen peroxide (39 per cent) both at freezing temperature and at 20 - 30° C., but peroxidase, catalase, and reductase were nearly destroyed (20). If milk containing 0.06-0.15 per cent hydrogen peroxide is kept for a period of 10 days to 3 weeks at room temperature during the summer time, the activity of the

milk phosphatase is seriously inhibited (100). Luck and Joubert (55) have found that the delay of milk coagulation by rennet is caused by the influence of hydrogen peroxide on the casein and not on the rennet enzyme. Banerjee (9) found that 2 per cent of a 10 volumes hydrogen peroxide solution is required to destroy all peroxidase in milk; and smaller doses of peroxide allow some peroxidase to persist. Catalase and peroxidase are destroyed by hydrogen peroxide. If peroxidase persists, the danger of oxidation of proteins and amino acids would be much greater. If catalase persists, the hydrogen peroxide will be decomposed after a very short period of treatment and the preserving effect will be reduced. Molland (62) made intensive studies on the kinetics of the decomposition of hydrogen peroxide by bacteria and the inactivation of the catalase and found that the quantity of catalase inactivated was proportional to the quantity of hydrogen peroxide decomposed. Morgulis, Beber, and Rabkin (66) found that the inactivation of catalase by hydrogen peroxide is intensified by increasing temperature. Bersin and Logemann (13) have stated that papain is inactivated with M/500 hydrogen peroxide in 5 minutes at 40° C. The effect of hydrogen peroxide on urease was not greater than uncatalysed aeration (40). The following amounts of enzyme inactivation by hydrogen peroxide are given by Barron, Seki and Johnson (10): urease 0 per cent; yeast hexokinase 0 per cent; papain 40 per cent; yeast alcoholic dehydrogenase 23 per cent.

Tests on Hydrogen Peroxide

In the manufacture of dairy products like cheese from milk treated with hydrogen peroxide, the main problem is to make sure that no trace of

hydrogen peroxide is left in the milk. There are several tests being recommended for the detection of residual peroxide. Adam (1) has endeavored to determine whether milk free from hydrogen peroxide was previously treated with this substance. Storch's reagent consisting of paraphenylene diamine and Schardinger's reagent consisting of methylene blue and aldehyde were tested for this purpose. Raw fresh milk not previously treated with hydrogen peroxide is colored red by the addition of guaiac and hydrogen peroxide, and blue by paraphenylene diamine. The methylene blue is decolorized by the presence of an aldehyde. The same milk, when spoiled, no longer gives the first two reactions, but still decolorizes Schardinger's reagent. Raw milk containing hydrogen peroxide gives the color reaction with guaiac alone or with paraphenylene diamine alone and does not change the color of Schardinger's reagent. Raw milk previously treated with hydrogen peroxide but no longer containing this substance gives, upon again adding hydrogen peroxide, the same reactions as pure milk with guaiac and paraphenylene diamine, but unless putrified, does not decolorize methylene blue. Boiled milk shows none of the preceding reactions. The amount of undecomposed hydrogen peroxide in the milk was determined in the following manner by Barthel (11). 0.5 milliliter of dilute sulfuric acid (1:3) was added to 25 milliliter of milk to coagulate the protein; and to 5 milliliter of the clear filtrate 10 milliliter of a 10 per cent solution of potassium iodide and 5 milliliter of sulfuric acid (1:3) were added. The free iodine was then titrated back with a tenth normal thiosulfate solution, after having been left standing in a dark place for 4 hours and frequently shaken. It was found that an excess of only 0.05 gram of hydrogen peroxide per liter of milk

is plainly noticeable by taste. Huwart (43) found that the estimation of hydrogen peroxide in milk by the liberation and determination of iodine gave satisfactory results. Ovenston and Rees (78) and Patrick and Wagner (79) have used the following procedure for the quantitative determination of hydrogen peroxide in milk. 20 milliliter of the milk is mixed with trichloroacetic acid (4 milliliter of a 40 per cent solution) to coagulate the proteins. The solution is filtered and a few drops of sulfuric acid and potassium iodide are added to 10 milliliter of the filtrate. The free iodine is either titrated with sodium thiosulfate or determined spectrophotometrically. For qualitative analysis the precipitation of protein is not necessary. The color of the milk after the addition of some drops of sulfuric acid and some crystals of potassium iodide is compared with an untreated sample. If the treated sample becomes yellow, the hydrogen peroxide is not completely destroyed. With quantitative method 3 milligram of hydrogen peroxide can be detected. Pien, Desirant and Lafontaine (83) have stated that hypochlorites, giving the same color, are bound by the coagulated casein and are separated by filtration together with the casein. Pien, Desirant and Lafontaine (82) have found that the presence of formaldehyde and dichromate interfere. Rouquette (96) has stated that in milk containing dichromate, hydrogen peroxide can be determined by the blue color after shaking with ether and sulfuric acid. (2 milliliter of milk + 2 milliliter of ether + 5 drops of sulfuric acid). This test can be made only up to 2 hours after the dichromate has been added, because hydrogen peroxide reacts with dichromate at the pH of the milk. Andreae (3) has developed a sensitive method for the estimation of hydrogen peroxide in biological materials. In this test, hydrogen

peroxide is traceable through the reaction of skopoletin (6 methyl - 7 oxy - 1, 2 benzo pyrone) with hydrogen peroxide in the presence of peroxidase giving a decrease of the skopoletin fluorescence. This method is much more sensitive than any of the tests, since approximately 10^{-10} per cent hydrogen peroxide can be detected. But the presence of ascorbic acid, glutathione, and manganese ions interfere. Freytag (29) has modified the decolorization test of lead sulfide by hydrogen peroxide (fluorescence picture of the spotted lead sulfide test paper in ultra-violet light). Musha, Higashino, and Doi (70) made micro determination of hydrogen peroxide ferrous ferron reagent. The oxidation of Fe^{++} to Fe^{+++} by hydrogen peroxide takes place in this test and the Fe^{+++} forms a dark green complex.

Conditions of Maximum Preserving Effect with Hydrogen Peroxide

Milk contains catalase, normally present in milk and additionally from cell and bacteria. This enzyme decomposes hydrogen peroxide. Because of the presence of this enzyme in milk, one normally thinks that after a certain period of time all hydrogen peroxide will be destroyed by catalase and there will not be any preserving effect. But at the same time catalase is inactivated by hydrogen peroxide in the same way as hydrogen peroxide is decomposed by catalase. By this process some hydrogen peroxide is used up for the destruction of catalase. The remaining, undestroyed hydrogen peroxide, exerts the real preservative effect for a certain period. Both the speed of destruction of hydrogen peroxide by catalase and the destruction of catalase itself are increased at higher temperatures. Luck (51) found that the quantities of hydrogen peroxide (39 per cent)

decomposed, by milk which was highly contaminated with coliform bacteria, after a period of 24 hours at 4°, 13°, 20°, and 30° C. were 0.44, 0.31, 0.20 and 0.11 per cent respectively. According to these results it was found that the addition of 0.25 per cent hydrogen peroxide (39 per cent) would be able to preserve this milk at a temperature of 20° and 30° C. but not at 13° C. If surplus hydrogen peroxide has to be destroyed the catalase should be added when the milk is cold. At lower temperatures, more of catalase and longer time are required to decompose the same quantity of peroxide than at higher temperatures. The quantity of hydrogen peroxide destroyed at a constant temperature will be proportional to the amount of catalase present in milk. If maximum preservative effect is desired, the hydrogen peroxide has to be added after milking when the bacterial count is low and the milk is about 37° C. Again, the germicidal action of hydrogen peroxide is more at higher temperatures. Hydrogen peroxide has a better preserving effect on pasteurized milk than on raw milk because the catalase in pasteurized milk is destroyed by heat. Ferrara and Salerno (26) have found that "Abiol" (methyl p - oxybenzoate) 0.1 per cent together with hydrogen peroxide (130 volumes) 0.2 per cent was very satisfactory, as milk was preserved for a week at 15 - 20° C.

Dairy Products Made from Milk Preserved with Hydrogen Peroxide

Cheese: Cheese made from milk treated with hydrogen peroxide (0.2 per cent of a 35 per cent solution for 20-40 minutes at 120-140° F.) has been found to be superior in quality to that made from raw or pasteurized milk (98). Cheese made from milk preserved with hydrogen peroxide (0.2 per cent of a 40 per cent solution) for a longer period of 8-24 hours is

mostly of inferior quality, having a soft body and a pasty texture (67, 52). The occurrence of unsatisfactory dry cheese from milk preserved with hydrogen peroxide is due to two factors: (1) an inhibiting after effect on the lactic acid bacteria (6) and (2) the influence of hydrogen peroxide on the casein (55). To overcome these difficulties, the use of larger quantities of starter and cooking at a slightly higher temperature than usual, are necessary. Arnaudi, Carta segna, and Passani (6) have added three times the usual quantity of rennet to obtain a satisfactory firmness in the curd. Luck and Joubert (55) have stated that hydrogen peroxide prolongs the coagulation period by rennet and this is increased when a higher peroxide concentration and a longer preservation period are used. The longer the coagulation period necessary the less firm is the curd. According to Bandini (8) rennet acted the same in the case of milk preserved with hydrogen peroxide as upon normal milk. Luck (52) found that Gouda and cheddar cheese made from preserved milk (0.25 per cent hydrogen peroxide, 130 volumes for 24-30 hours at 26-30° C.) were not satisfactory, in spite of the addition of double the quantity of starter culture and calcium chloride. The body of the Gouda cheese was very weak; the cheese sagged, lost shape, had an unclean flavor, and on storage developed a bitter flavor. The cheddar cheese after 8 weeks of storage, developed a strong off flavor and the body was greasy and pliable. But experiments on cheese making with mixtures of preserved and untreated fresh milk, or with milk preserved with lower concentrations of hydrogen peroxide, gave good results. Preserved and fresh milk were mixed before pasteurization. The mixed milk was allowed to stand for $\frac{1}{2}$ - 1 hour, to enable the catalase of the fresh milk to decompose the remaining hydrogen

peroxide. No addition of any other catalase was required. The Gouda cheese made from 50 per cent preserved milk has a slight foreign flavor, but the cheddar cheese did not develop any off flavors. Cheese made out of raw milk mixed with 33 or 25 per cent of hydrogen peroxide treated milk were of better quality than controls, especially during the season when milk arrived over-acid at the factory and the body of the cheese had a tendency to become rough, crumbly and chalky. Arnaudi (5) and Treccani (109) have stated that preservation of milk with 0.05 - 0.10 per cent hydrogen peroxide (130 volumes) does not affect the technique of cheese making. Roundy (97) has shown that Swiss cheese and cheddar cheese of excellent quality and uniformity can be produced from milk properly treated with small quantities of hydrogen peroxide and catalase. Treatment of milk at approximately 125° F. for about 25 seconds with 0.02 per cent hydrogen peroxide destroys nearly all the coliform organisms and leaves intact more of the natural enzymes and beneficial organisms than accepted pasteurization procedures. Consequently cheese made from milk properly treated with a small amount of hydrogen peroxide and catalase ripens faster and has a finer flavor and body than the cheese made from pasteurized milk.

Butter. Not many experiments have been conducted in the making of butter from hydrogen peroxide treated milk. Nambudripad, Laxminarayana, and Iya (75) have shown that there was no appreciable difference in the quality of butter made from treated milk as compared to that of untreated milk. No differences in yield or flavor were observed in butter made from treated milk (76). Negretti (76) treated the milk with 0.5 per cent

hydrogen peroxide (130 volumes) and removed the surplus peroxide with catalase 8 hours later.

Casein Making. No real difference could be found in the casein manufacturing process from hydrogen peroxide treated (0.25 per cent hydrogen peroxide, 130 volumes, 24 hours at 25° C.) or untreated milk. The coagulation period of treated milk was prolonged and the acidity of the whey before cooking should be higher. Since casein manufactured from treated milk has a stronger tendency to become rubbery and plastic at higher temperatures, exact control of acidity is important. It can be prevented by adding some acid to the last wash water to get a pH of 4.6. Low acid curd from treated milk should not be left overnight in wash water without the addition of acid, since it may become so soft and milky by the next morning that it cannot be pressed and dried (52).

Influence of Hydrogen Peroxide on Bacteria

Reduction in total bacterial count. Hydrogen peroxide has been recognized as a bactericidal agent for a long time. The hydrogen peroxide treatment causes a higher bacterial reduction than pasteurization by heat. Lukin (57) conducted experimental investigations on the sterilization of milk with hydrogen peroxide and found that the number of bacteria in milk is of importance in sterilization with hydrogen peroxide. At room and incubator temperatures the quantity required increases with the bacterial content. At 52° C. this relation is no longer noticeable. Lukin used large quantity of hydrogen peroxide (0.07 to 1.50 per cent corresponding to 24.5 to 600 milliliter of the 3 per cent solution per liter) for the

sterilization of milk at lower temperatures. He concluded that 0.036 per cent of 12 milliliter of a 3 per cent solution per liter of milk is sufficient, except when the bacterial count is very high, then 0.05 per cent is required. Curran, Evans, and Leviton (22) have evaluated the germicidal activity of hydrogen peroxide and found that it is greatly facilitated by the use of purified, crystalline catalase which affords a rapid and efficacious means of dissipating residual peroxide. The logarithmic curve for survival of spores exposed to 1 per cent hydrogen peroxide (pH 6.9) indicates the rate of killing of most, and possibly all, of the spores is described by the unimolecular law for chemical reactions. Hydrogen-ion concentration affects the apparent sporicidal activity of hydrogen peroxide, largely through its influence upon peroxide stability. Thus, there is greater sporicidal activity in acid media than in neutral media and usually less in strongly alkaline than in neutral media. In 40 - 70° C. (pH 6.9) range the time required for the destruction of 50 per cent of the spores by 1 per cent hydrogen peroxide decreases from one-half to one-third for each 10° rise in temperature. The time required for total destruction of the spores through this range usually decreases with each 10° rise in temperature. As the temperature is raised from 30 to 40° C. the time required for the destruction of 50 per cent of the spores decreases from one-half to one-third. The time required for total destruction of the spores increases with this change in temperature. The time required for the destruction of spores by hydrogen peroxide increases as the concentration of spores is increased, and decreases as the concentration of peroxide is increased. Morris (67) has stated that anaerobic spore formers were entirely eliminated. Satta et al (102) have

determined the percentage reduction of the bacterial count in milk treated with 0.20, 0.25, and 0.30 per cent of 130 volumes hydrogen peroxide and held for 20 hours. The results were 99.82, 99.88, and 99.88 per cent respectively at 15-17° C.; 98.64, 99.76, and 99.76 per cent respectively at 20-22° C.; and 93.57, 99.53, and 99.96 per cent respectively at 32° C. Addition of 0.05 per cent of the peroxide solution arrested the multiplication of bacteria for more than 15 hours at 15 and 22° C. A reduction of the initial count for 24 hours was affected by 0.1 per cent. Onset of multiplication was delayed 36 hours over the control. Monaci (63) has found a reduction in total bacterial count varying from 74.3 to 96.3 per cent with 0.2 per cent hydrogen peroxide (130 volumes) for 14-24 hours at 20-22° C. and 28-30° C. Similar results have been obtained by Morandi (65). Nai and Giolitti (72) recommended contact with 0.7-1.0 per cent of hydrogen peroxide (130 volumes) for 8-10 hours, because addition of 0.25-0.3 per cent is said not to allow a sufficient safety margin. In winter the dose could be reduced to 0.4-0.6 per cent (36). Nambudripad, Laxminarayana, and Iya (75) have stated that the rate of bacterial reduction by hydrogen peroxide depends on the initial bacterial count of the milk. A short hydrogen peroxide treatment at higher temperatures (0.2 per cent of 35 per cent solution for 30 minutes at 120° F.) reduced the average bacterial count from 304,280 per milliliter in raw milk to 1,090 per milliliter in peroxide treated samples and to 11,485 per milliliter in pasteurized milk. The coliform counts were 4,339 per milliliter (raw milk), 4 per milliliter (treated milk), and 10 per milliliter (pasteurized milk). The aerobic spore formers were reduced from 1,730 per milliliter (raw milk) to 68 per milliliter in treated

milk, and to 88 per milliliter in pasteurized milk samples. Clostridium butyricum was greatly reduced in 11 hours and disappeared in 24 hours (68). Thermobacteria (Lactobacillus) and Streptococcus lactis are very sensitive to hydrogen peroxide (7). By experiments with xanthine oxidase Lipman and Owen (50) arrived at the conclusion that hydrogen peroxide is inhibitory to certain bacteria, mostly anaerobic and facultative anaerobic types devoid of catalase. Monaci and Scarpari (64) have found that the lowest counts were obtained when peroxide treated milk from receiving stations was allowed to stand for 9-12 hours in containers. The counts reported are, however, comparable with those obtained from samples of pasteurized milk in only a little more than half of the samples examined. Roushdy (99) made investigations into the effect of hydrogen peroxide on milk in connection with its bacterial action on microorganisms normally present in milk when kept at different temperatures. The mean colony counts per milliliter of untreated milk, (A) of milk treated with 0.03 per cent, and (B) with 0.05 per cent after 4 hours incubation at 30° C. were 249,380; 12,440; and 7,294 colonies and after 8 hours incubation the mean counts became 5,172,000; 7,464; and 4,150 respectively. After 24 hours incubation, the untreated sample had deteriorated, while the mean count had further decreased to 6,150 in A and 2,266 in B. In all the samples examined, the bacterial count was lower in batch A than in the untreated milk, and lower in B and in A; the activity of hydrogen peroxide continued up to 24 hours incubation at 30° C. and 18° C., except in milk treated with 0.03 per cent at room temperature (20± 2° C.) when kept at 30° C. The time temperature conditions, under which the treated milk was kept, had a profound effect upon the efficiency of hydrogen

peroxide. The initial temperature of the milk when treated was also a factor. The percentage reduction in the number of organisms was proportionately greater when hydrogen peroxide was added to the still warm milk ($\pm 36^{\circ}$ C.) than in milk treated at a temperature of $20 \pm 2^{\circ}$ C. Jasewicz and Porges (45) studied the preservation of whey by hydrogen peroxide and found that 0.02 per cent hydrogen peroxide concentration showed both a bactericidal and bacteriostatic action against 1,000 bacteria added per milliliter of whey, and inhibited bacterial growth for as long as 10 days. Bacterial plate counts were made of peroxide-preserved wheys of pH 4.9, 5.9, and 6.9, kept at 25, 37 and 50° C. According to the data, hydrogen peroxide addition is recommended shortly after separation of whey from curd. The bactericidal limitations of 0.02 per cent final concentration of hydrogen peroxide on milk souring types of organisms were investigated by Jasewicz and Porges (45) who found that within 1 hour, a 97 per cent kill of 28×10^7 bacteria was effected, a remarkable achievement considering that this number of bacteria represents a grossly contaminated material. Studies showing 100 per cent destruction of milk microorganisms have been reported by Nambudripad, Laxminarayana, and Iya (74).

Influence on certain types of bacteria. The bactericidal efficiency of hydrogen peroxide varies with different organisms, with bacterial count, the concentration of hydrogen peroxide, the period of time, and the temperature of treatment. Lukin (57) found that hay and coli bacilli and streptococci added to milk in large numbers are completely destroyed by treatment with 0.036 per cent at 52° C. Curran, Evans, and Leviton (22)

have stated that milk treated with hydrogen peroxide supports the growth of Escherichia coli, Pseudomonas aeruginosa, and Alcaligenes bookeri as well as milk treated by heat. Milk sterilized by hydrogen peroxide retards the growth of Streptococcus lactis, this is probably caused by the high oxidation-reduction potential of milk treated with peroxide. Molland (62) has investigated the catalase production of bacteria and their hydrogen peroxide tolerance. The strains which produced catalase are as a rule most resistant towards hydrogen peroxide. The aerobic strains (about 10^{10} cells per milliliter) were able to grow in a broth containing 0.0125 per cent hydrogen peroxide. Even bacteria which do not produce catalase or produce very little of it such as the pneumococci, streptococci, Salmonella typhosa, Bacillus anthracis, and Erysipelothrix rhusiopathiae could grow in a medium containing 0.0125 per cent hydrogen peroxide. The hydrogen peroxide concentrations tolerated by different strains of bacteria were as follows: Proteus species 0.2; Bacterium coli 0.05-0.1; Staphylococcus aureus strain I 0.0125-0.025; strain II 0.1-0.2; Brucella abortus 0.4; Streptococcus agalactiae 0.4; Streptococcus zymogenes 0.0125; and Neisseria catarrhalis 0.0125-0.025 per cent. Nambudripad, Ixaminarayana, and Iya (73, 74) have studied the bactericidal efficiency of hydrogen peroxide in relation to microorganisms isolated from milk. They have found that the Gram-negative bacteria (coliforms) are more susceptible to hydrogen peroxide than are Gram-positive organisms (spore-formers). The lactic acid bacteria were intermediate in susceptibility. The time required for total destruction after addition of 50, 300 and 500 parts per million by weight of hydrogen peroxide to nutrient broth at 37° C. was as follows: Streptococcus lactis 6 hours, 4 hours, and 2

hours 30 minutes; Streptococcus liquefaciens 16 hours, 7 hours, and 4 hours; Sarcina species 8 hours 10 minutes, 5 hours, and 2 hours 30 minutes; spores of Bacillus subtilis 36 hours, 24 hours, and 18 hours; spores of Bacillus cereus 24 hours, 14 hours, and 7 hours; spores of Bacillus megatherium not determinable, 24 hours, and 16 hours; Bacterium coli 5 hours, 45 minutes, and 45 minutes; Bacterium aerogenes 6 hours, 1 hour, and 30 minutes; Alcaligenes viscosus 2 hours, 1 hour, and 40 minutes; Torula species 24 hours, 7 hours, and 3 hours; Oidium species 26 hours, 8 hours, and 3 hours 30 minutes. The surviving cells of Streptococcus lactis exposed to 500 parts per million of hydrogen peroxide, plated and inoculated with an isolated colony into sterile skim milk showed a definite retardation in acid production after 3 days. The treated milk had 0.48 per cent acid expressed as lactic acid, whereas the untreated milk had 0.71 per cent. The ability of organisms to reduce methylene blue was not significantly changed. Nambudripad, Laxminarayana, and Iya (74) found 16 hours of exposure to 0.05 per cent hydrogen peroxide at 37° C. was necessary for full destruction of Bacillus megatherium in broth solutions. This is in contrast to the much shorter time of 30, 40, and 45 minutes required for 100 per cent killing of Aerobacter aerogenes, Alcaligenes viscosus, and Escherichia coli, respectively, all Gram-negative and peroxide-sensitive forms (45). Hewlett (41) made an experimental investigation of the "Budde" process (addition of 0.35 milliliter of hydrogen peroxide to a liter of milk heated to 50° C.) for the preservation of milk. Emulsions of cultures of various organisms were added to samples of milk treated by the Budde method. Samples were also subjected to the same amount of heat without the addition of hydrogen peroxide, and non-

inoculated samples were also used as controls. The organisms tested were Bacillus diphtheriae, Bacillus acidilactici, Bacterium coli, Bacillus typhosus, Paratyphoid bacillus, Bacillus dysenteriae, Cholera vibrio, Micrococcus pyogenes variety aureus, Bacillus tuberculosis, Bacillus anthracis with spores, Bacillus subtilis, Bacillus mycoides, and Penicillium glaucum. One to three experiments were made with each microorganism. The results showed that all the non-sporogenous organisms, both pathogenic and non-pathogenic, were destroyed by the Budde method. The spore forming organisms, while not destroyed, were greatly reduced in numbers. The reduction in numbers of organisms by heating alone was not so great as that by the Budde method. In milk obtained in the ordinary way, the number of microorganisms were reduced by the Budde method over 99.9 per cent. Gordon (37) also stated that typhoid bacilli and spores of Bacillus subtilis added to milk were not destroyed by the Budde method and quantities of 0.07 per cent or more of hydrogen peroxide gave more positive results. Evidence is presented by Fouassier (28) that the microorganisms Bacillus subtilis and Tyrothrix tenuis, which resist pasteurization are not only able to decompose hydrogen peroxide but hasten the development of the lactic ferments by furnishing them a more easily assimilable culture medium through the peptonizing action which they exert on the milk. The bacteriostatic and bactericidal actions of hydrogen peroxide have been studied by Giolitti (30). Serratia marcescens, Escherichia coli, Bacillus subtilis, and Eberthella dysenteriae were tested as test organisms for their differential high and low catalase and peroxidase contents. Forty-eight hour cultures in 1.5 per cent lactose peptone broth were treated with hydrogen peroxide, the cultures kept at

37° C., and the remaining hydrogen peroxide determined by vanadic acid. After 3-4 hours Serratia marcescens (with large catalase peroxidase activities) destroyed all the hydrogen peroxide and almost all of the bacteria survived. Escherichia coli (with considerable peroxidase and catalase activities) in large concentrations decomposed all the hydrogen peroxide in 6-7 hours and in concentrated cultures there was a high percentage of survival while in low concentrations there was little survival. The vegetative form of Bacillus subtilis (with low catalase and high peroxidase activity) was destroyed by hydrogen peroxide with little hydrogen peroxide splitting while the spores required a longer period of bactericidal action. Eberthella dysenteriae (lacking catalase and with little peroxidase) was rapidly killed with negligible peroxide loss. These data show the relation of catalase and peroxidase activity and susceptibility to hydrogen peroxide and the mechanism of bactericidal action of hydrogen peroxide. He has concluded a greater importance for the action of the hydrogen peroxide per se than for the oxygen produced from it. Haase (38) found that peroxides and oxygen in general showed low activity against Escherichia coli and Bacillus prodigiosus suspensions. Giolitti (33), by comparing the bactericidal activity of peroxidase, catalase, and hydrogen peroxide on the same strains of bacteria, is of opinion that the bactericidal effect of hydrogen peroxide cannot be accounted for on the basis of the liberation of molecular oxygen but probably depends on a direct oxidative action of the hydrogen peroxide molecule on the cell. Hydrogen peroxide treatment is more effective than pasteurization at 65° C. for 10 seconds in reducing the total bacterial content, especially coliforms, and the butyric acid bacteria

are reduced to within safe limits if the original infection is not heavy (112). Addition of 0.2 per cent of hydrogen peroxide (excess being removed by catalase), which diminished the average bacterial count from 423,000 per milliliter of milk to 15,000 per milliliter, gave cheese a firm body and typical flavor, but usually with a slight chemical after taste (104).

Effect of hydrogen peroxide on pathogenic organisms. Treatment with hydrogen peroxide reduces the bacterial counts and destroys most of the pathogenic organisms. But Mycobacterium tuberculosis is more resistant than other pathogenic organisms. It resisted hydrogen peroxide concentrations as high as 15-20:100 (130 volumes) and so it will be used only in milk free from Mycobacterium tuberculosis (32). Monaci (63) obtained positive results (guinea pig inoculation method) from two out of nine samples of milk, inoculated with suspensions of Mycobacterium tuberculosis variety bovis and treated with hydrogen peroxide. Complete destruction of Brucella abortus and Brucella melitensis has been obtained by treating milk with 0.2 per cent hydrogen peroxide (130 volumes) (14, 63, 102). Giolitti (35) has found that Brucella abortus and Brucella melitensis in milk (30 million per milliliter) were killed by adding 0.5-0.7 per cent hydrogen peroxide (130 volumes), leaving for 8 hours, and eliminating the excess hydrogen peroxide by catalase. Salmonella typhosa was killed in 8-9 hours by 0.2 per cent hydrogen peroxide (130 volumes) at 17-32° C. and in 4-5 hours by 0.25-0.30 per cent at the same temperature (102). Typhoid, cholera and dysentery bacilli added to milk sterilized by heating were destroyed in all cases by hydrogen peroxide in the proportion of 0.35:1000,

the milk being heated to 45-50° C.; typhoid bacilli were not all destroyed in the proportion of 0.18:1000 at 45° C. or 0.35:1000 at room temperature; tubercle bacilli were also destroyed in the proportion of 0.35:1000 and inoculation experiments with guinea pigs gave negative results. Monaci (63) isolated typhoid organisms from only one out of twenty milk samples, inoculated with Salmonella typhosa suspension and treated with 0.2 per cent hydrogen peroxide (130 volumes) for 14-24 hours at 20-22° C. and 28-30° C. Roushdy (99) found that Mycobacterium tuberculosis variety bovis in raw milk treated with 0.05 or 0.075 per cent hydrogen peroxide and held at 30° C. for 24 hours, was either partially destroyed or its virulence was lowered. He concluded that longer periods and higher concentrations would probably destroy them.

Hygienic Aspects of Hydrogen Peroxide Preservation of Milk

It has been found that the milk treated with hydrogen peroxide does not lose the nutritional value except for a small reduction of some vitamins like ascorbic acid. When hydrogen peroxide treated milk is to be used for human consumption, the residual hydrogen peroxide should be completely removed. The addition of catalase has been recommended for this purpose. Jensen ¹⁹⁵¹ et al (46), and Cleland ¹⁹⁴⁹ and Lotz (21) have found that hydrogen peroxide induces mutations in Neurospora species. Demerec, Bartani, and Flint (23) stated that hydrogen peroxide causes mutations in Bacterium coli. Hydrogen peroxide has been found to cause a decrease in the number of normal mitotic figures in the intestine of mouse (25). Rondoni and Cudkowicz (91) have put forward a hypothesis on the importance of undecomposed hydrogen peroxide in the tissues for carcinogenesis.

Therefore, care should be taken to see that the residual hydrogen peroxide is completely destroyed before the milk is used for human consumption. Under certain conditions hydrogen peroxide may be found naturally in dairy products. This is produced by anaerobic lactobacilli. Probably the inhibitor lactobacillin, produced by lactobacilli is identical with hydrogen peroxide (114).

CHAPTER III

EXPERIMENTAL METHODS

Three preliminary trials were made to determine the lowest concentration of hydrogen peroxide required to preserve raw milk for 4 days at 37° C. Samples of raw milk were taken in sterile flasks from the University of Tennessee Creamery and stored at 5° C. in the refrigerator for 2-3 hours until they were used for the experiment. The milk was distributed in four sterile screw capped bottles, 100 milliliter in each bottle. The samples in the four bottles were raised to 30° C. by placing them in water bath. Commercial hydrogen peroxide (3 per cent solution) was added to three of the samples at the rate of 3 milliliter for the first sample, 5 milliliter for the second sample and 10 milliliter for the third sample in order to obtain a final concentration of 0.09, 0.15, and 0.30 per cent hydrogen peroxide in the respective samples. To the fourth bottle of milk hydrogen peroxide was not added and it served as the control. The milk samples were shaken well. The milk from the control sample was plated according to Standard Methods for the Examination of Dairy Products (2) on milk protein hydrolysate (M.PH) agar for the total count. Dilutions of 1:10, 1:100 and 1:1000 were made for the total count. In order to avoid quantities of 1/10 milliliter and 1 milliliter of milk samples sticking to the plate or otherwise failing to mix properly in the agar, 1 milliliter of sterile water blank was placed in the plate prior to adding the milk sample and the milk was then added directly into the water. The plates were incubated at 32° C. for 48 hours and then counted for the number of colonies. The experimental samples were

kept at 37° C. throughout the experiment. From these samples sub-samples for counts were taken at an interval of 24, 48, 72, and 96 hours after the addition of hydrogen peroxide and plated on milk protein hydrolysate agar for the total count. Three series of trials were run in this way. The control sample was not plated on the second, third, fourth, and fifth days because it curdled usually on the second or third day, denoting that there is a large increase in the number of organisms.

To find out the influence of different concentrations of hydrogen peroxide on different types of bacteria the following procedure was adopted. 100 milliliter portions of skim milk were taken in four screw capped bottles, sterilized in the autoclave at 250° F. for 15 minutes at 15 pounds pressure and stored at 37° C. Twelve organisms were selected for the study. They were Aerobacter aerogenes, Escherichia coli, Streptococcus lactis, Streptococcus cremoris, Streptococcus liquefaciens, Streptococcus thermophilus, Lactobacillus acidophilus, Lactobacillus bulgaricus, Lactobacillus casei, Bacillus megaterium, Bacillus polymyxa, and Bacillus subtilis. These were selected because of their importance in the dairy industry. A pure culture of these organisms was grown in litmus milk for 48 hours at their optimum growth temperatures. The litmus milk containing each of these organisms was plated in different dilutions according to Standard Methods for the Examination of Dairy Products (2) on milk protein hydrolysate agar to determine the number of organisms per milliliter. This was then standardized to contain approximately 50,000 to 1,000,000 organisms per milliliter. To test the efficiency of hydrogen peroxide on each of these organisms a suitable quantity of the standardized culture of the organism was inoculated into each of the 100 milliliter

sterilized milk samples so that the concentration of the organisms in these milk samples was approximately 50,000 to 1,000,000 per milliliter. The samples were shaken well and one of the samples was plated on milk protein hydrolysate agar and incubated at 32° C. for 48 hours to determine the number of organisms added to these samples. Out of the 4 samples one was kept as a control without the addition of hydrogen peroxide. Commercial hydrogen peroxide (3 per cent) was added to each of the other three samples to get a final concentration of 0.03, 0.05, and 0.09 per cent hydrogen peroxide in the milk. The control as well as the experimental samples were stored at 37° C. and each sample of milk was plated on milk protein hydrolysate agar using various dilutions as outlined in Standard Methods for the Examination of Dairy Products (2). The plates were incubated at 32° C. for 48 hours to determine the number of viable organisms in each sample.

CHAPTER IV

RESULTS AND DISCUSSION

In tropical countries, the keeping quality of milk is generally very poor due to unorganized methods of production and distribution, and the adoption of refrigerated storage or transport of milk not possible for various economic reasons. So it has been found useful to explore the possibilities of adopting hydrogen peroxide treatment under controlled conditions as a means of preserving milk for long distance transport. Hydrogen peroxide is harmless and has bacteriostatic and bactericidal properties.

Influence of Hydrogen Peroxide on Total Bacterial Count in Raw Milk

Initially, the effect of adding different amounts of hydrogen peroxide to raw milk on its bacterial content and keeping quality at 37° C. was investigated. This was done to determine the concentration of hydrogen peroxide to be used for finding out the influence on pure cultures of organisms. The influence of 0.09, 0.15, and 0.30 per cent by weight of hydrogen peroxide on the total bacterial content of raw milk was determined. The results of the three trials are shown in Table II. The initial bacterial content of the raw milk in the trials varied from 28,300 to 65,000 per milliliter. By using 0.09 and 0.15 per cent peroxide there was found, uniformly in all the trials, a considerable reduction in the total count in 24 hours storage of the treated milk at 37° C. Thereafter, there was a gradual increase in the total count on the second, third and fourth days, but at a very slow rate. There was a

total destruction of bacteria in 1 milliliter quantities in the milk containing 0.30 per cent hydrogen peroxide. The destruction of bacteria with 0.09 per cent hydrogen peroxide in 24 hour period was 98.77, 97.31, and 96.40 per cent respectively in trial I, II, and III. With 0.15 per cent destruction was 99.85, 98.16, and 98.48 respectively for trial I, II, and III. The results obtained with 0.15 per cent hydrogen peroxide were close to the values obtained by Satta et al, (102) who found that 0.12 per cent peroxide brought about a destruction of 99.96 per cent at 32° C.

At the same time Nai and Giolitti (72) recommended contact with 0.28 to 0.40 per cent of the peroxide for 8-10 hours, because addition of 0.1 to 0.12 per cent did not allow a sufficient safety margin. Monaci (63) has found a reduction in the total bacterial count varying from 74.3 to 96.3 per cent with 0.08 per cent for 14-24 hours at 28°-30° C. A reduction of 99.6 per cent of bacterial count was reported by Morris, Larsen, and Johnson (68) by using a concentration of 0.2 per cent hydrogen peroxide in milk. But in this case the milk was heated and held at a temperature of 120° F. (49° C.) for 30 minutes before the addition of peroxide. The marked variations in the amounts of hydrogen peroxide used by several workers can be explained on the basis of the fact that the percentage reduction in the number of organisms in milk will depend upon time, temperature conditions under which the treated milk is kept, the temperature of the milk at the time of addition of peroxide, the initial bacterial count of the raw milk, the type of organisms present, and the catalase content of the milk (75, 99).

Effect of Hydrogen Peroxide on Pure Cultures of Organisms

In this work bacteriological analyses were made to determine the effect of hydrogen peroxide on pure cultures of representative species of organisms of common dairy importance. Table III and IV show the effect of different concentrations of hydrogen peroxide on Escherichia coli and Aerobacter aerogenes. Both these organisms belong to the group "coliforms." Both these organisms were completely destroyed in 1 milliliter samples by 0.03 per cent hydrogen peroxide within 24 hours at 37° C. The bacterial counts at the end of 48, 72, and 96 hours were less than one in 1 milliliter quantities. These results were in close agreement with those obtained by Nambudripad and co-workers (74) and Roundy (97, 98). Nambudripad and co-workers, in 1949, found that none of the Escherichia coli exposed to 0.05 per cent hydrogen peroxide for 10 minutes at 37° C. were able to survive. Only 6.77 per cent of them survived treatment with 0.03 per cent peroxide for 30 minutes. Aerobacter aerogenes was completely destroyed by 0.05 per cent in 30 minutes and only 55.89 per cent of them survived a peroxide treatment of 0.03 per cent for 30 minutes. The effect of various concentrations of peroxide on Streptococcus lactis, Streptococcus cremoris, Streptococcus liquefaciens and Streptococcus thermophilus are shown in Tables V, VI, VII and VIII respectively. It seemed that Streptococcus thermophilus was almost completely destroyed within 24 hours at 37° C. by 0.03 per cent peroxide treatment. Streptococcus lactis seemed to be next in sensitivity to hydrogen peroxide. Even though the counts on the first two days were less than one per milliliter there was a slight increase in the third and fourth days. The destruction of Streptococcus cremoris and Streptococcus liquefaciens on the first day

TABLE III
EFFECT OF DIFFERENT CONCENTRATIONS OF HYDROGEN PEROXIDE
ON ESCHERICHIA COLI IN STERILE MILK AT 37° C.

Concentration of H ₂ O ₂ by weight	Before the addition of H ₂ O ₂	After the addition of H ₂ O ₂			
		24 hours	48 hours	72 hours	96 hours
(%)		(count/mL.)			
0.00	920,000	> 300,000,000	> 300,000,000	> 300,000,000	115,000,000
0.03	920,000	*	*	*	*
0.05	920,000	*	*	*	*
0.09	920,000	*	*	*	*

* Less than one per milliliter.

TABLE IV
EFFECT OF DIFFERENT CONCENTRATIONS OF HYDROGEN PEROXIDE
ON AEROBACTER AEROGENES IN STERILE MILK AT 37° C.

Concentration of H ₂ O ₂ by weight	Before the addition of H ₂ O ₂	After the addition of H ₂ O ₂			
		24 hours	48 hours	72 hours	96 hours
(%)		(count/ml.)			
0.00	660,000	258,000,000	> 300,000,000	208,000,000	18,400,000
0.03	660,000	*	*	*	*
0.05	660,000	*	*	*	*
0.09	660,000	*	*	*	*

* Less than one per milliliter.

TABLE V
EFFECT OF DIFFERENT CONCENTRATIONS OF HYDROGEN PEROXIDE
ON STREPTOCOCCUS LACTIS IN STERILE MILK AT 37° C.

Concentration of H ₂ O ₂ by weight	Before the addition of H ₂ O ₂	After the addition of H ₂ O ₂			
		24 hours	48 hours	72 hours	96 hours
(%)		(count/ml.)			
0.00	470,000	> 300,000,000	> 300,000,000	39,000,000	5,700,000
0.03	470,000	*	*	34	7,500
0.05	470,000	*	*	*	*
0.09	470,000	*	*	*	*

* Less than one per milliliter.

TABLE VI
EFFECT OF DIFFERENT CONCENTRATIONS OF HYDROGEN PEROXIDE
ON STREPTOCOCCUS CREMORIS IN STERILE MILK AT 37° C.

Concentration of H ₂ O ₂ by weight	Before the addition of H ₂ O ₂	After the addition of H ₂ O ₂		
		24 hours	48 hours	72 hours
(%)		(count/ml.)		
0.00	1:10,000	105,000,000	18,600,000	384,000
0.03	1:10,000	1:1	350	6,200
0.05	1:10,000	*	*	*
0.09	1:10,000	*	*	*

* Less than one per milliliter.

TABLE VII
EFFECT OF DIFFERENT CONCENTRATIONS OF HYDROGEN PEROXIDE
ON STREPTOCOCCUS LIQUEFACIENS IN STERILE MILK AT 37° C.

Concentration of H ₂ O ₂ by weight	Before the addition of H ₂ O ₂	After the addition of H ₂ O ₂			
		24 hours	48 hours	72 hours	96 hours
(%)		(count/ml.)			
0.00	530,000	> 300,000,000	> 300,000,000	> 300,000,000	> 300,000,000
0.03	530,000	2,630	34,000	370,000	44,000
0.05	530,000	*	*	*	*
0.09	530,000	*	*	*	*

* Less than one per milliliter.

TABLE VIII

EFFECT OF DIFFERENT CONCENTRATIONS OF HYDROGEN PEROXIDE
ON STREPTOCOCCUS THERMOPHILUS IN STERILE MILK AT 37° C.

Concentration of H ₂ O ₂ by weight	Before the addition of H ₂ O ₂	After the addition of H ₂ O ₂		
		24 hours	48 hours	72 hours
				96 hours
(%)		(count/mL.)		
0.00	960,000	> 300,000,000	212,000,000	87,000,000
				126,000
0.03	960,000	*	*	*
0.05	960,000	*	*	*
0.09	960,000	*	*	*

* Less than one per milliliter.

were 99.99 and 99.50 per cent respectively. These two organisms multiplied very slowly on the second, third and fourth days but not to such a great extent as in the control. Streptococcus liquefaciens seemed to be a little more resistant than Streptococcus cremoris to 0.03 per cent peroxide treatment. All the four organisms are acid producing, Gram-positive, and aerobic in nature. From these it was found that the organisms of the genus Streptococcus seemed to be more resistant than coliforms to peroxide treatment. Nambudripad and co-workers (74) stated that Streptococcus lactis was considerably more resistant to destruction by peroxide than coliforms, and Gram-positive and catalase-negative organisms were more resistant to nascent oxygen from peroxide treatment. Roundy (98) also found that 0.06 per cent peroxide was required for 99.9 per cent reduction of Streptococcus lactis exposed to 130° F. Arnaudi and Treccani (7) recommended a concentration of 0.05 per cent peroxide for the lactic thermobacteria and Streptococcus lactis. Most of these investigations were in agreement with the observation made in this investigation with respect to the Streptococcus group. But Lukin (57) found that 0.036 per cent of peroxide killed Streptococci completely at 52° C. The higher temperature (52° C.) probably was responsible for the lower concentration being used in his study.

The influence of hydrogen peroxide on Lactobacillus group is shown in Tables IX, X, and XI. The three organisms taken for this study were Lactobacillus acidophilus, Lactobacillus bulgaricus, and Lactobacillus casei. There was complete destruction of the three species of bacteria in 24 hours by 0.03 per cent peroxide treatment. The susceptibility of these organisms to hydrogen peroxide treatment seemed to be great as in

TABLE IX
EFFECT OF DIFFERENT CONCENTRATIONS OF HYDROGEN PEROXIDE
ON LACTOBACILLUS ACIDOPHILUS IN STERILE MILK AT 37° C.

Concentration of H_2O_2 by weight	Before the addition of H_2O_2	After the addition of H_2O_2			
		24 hours	48 hours	72 hours	96 hours
(%)		(count/ml.)			
0.00	570,000	> 300,000,000	> 300,000,000	> 300,000,000	> 300,000,000
0.03	570,000	*	*	*	*
0.05	570,000	*	*	*	*
0.09	570,000	*	*	*	*

* Less than one per milliliter.

TABLE X
EFFECT OF DIFFERENT CONCENTRATIONS OF HYDROGEN PEROXIDE
ON LACTOBACILLUS BULGARICUS IN STERILE MILK AT 37° C.

Concentration of H ₂ O ₂ by weight	Before the addition of H ₂ O ₂	After the addition of H ₂ O ₂		
		24 hours	48 hours	72 hours
(%)				96 hours
0.00	320,000	820,000	110,000	7,200
0.03	320,000	*	*	273
0.05	320,000	*	*	*
0.09	320,000	*	*	*

* Less than one per milliliter.

TABLE XI
EFFECT OF DIFFERENT CONCENTRATIONS OF HYDROGEN PEROXIDE
ON LACTOBACILLUS CASEI IN STERILE MILK AT 37° C.

Concentration of H ₂ O ₂ by weight	Before the addition of H ₂ O ₂	After the addition of H ₂ O ₂		
		24 hours	48 hours	72 hours
				96 hours
(%)		(count./ml.)		
0.00	910,000	> 300,000,000	> 300,000,000	> 300,000,000
0.03	910,000	*	*	*
0.05	910,000	*	*	*
0.09	910,000	*	*	*

* Less than one per milliliter.

the case of coliform organisms. In the control sample to which Lactobacillus bulgaricus was added, there was an increase of $2\frac{1}{2}$ times the original number in a 24 hour period. From the second day onwards there seemed to be a steady reduction in the total number of organisms, which might have been due to the fastidious growth requirements of the organism. These organisms seemed to be the least resistant to destruction by peroxide like the coliforms, whereas Nambudripad and co-workers (74) found that Lactobacillus bulgaricus organisms were more resistant to destruction than coliform organisms for treatment with hydrogen peroxide. There was complete destruction of Lactobacillus bulgaricus with 0.03 per cent peroxide as against the 98.0 per cent destruction obtained by Roundy (98) with 0.06 per cent peroxide. These bacteria are mostly anaerobic to microaerophilic. The results obtained seemed to be in agreement with those of Lipman and Owen (50), who stated that anaerobic bacteria were mostly sensitive to hydrogen peroxide.

Tables XII, XIII, and XIV show the effect of the various concentrations of hydrogen peroxide on bacteria of the genus Bacillus. The specific organisms studied in this group were Bacillus subtilis, Bacillus megaterium, and Bacillus polymyxa. These organisms are Gram-positive and aerobic spore-formers. Of all the three species included in this study Bacillus subtilis seemed to be more resistant than the other two species. A very slow multiplication of the organism was noticed on the second day onwards in concentrations of 0.03 and 0.05 per cent of hydrogen peroxide. With 0.03 per cent hydrogen peroxide there was a destruction of 99.97 per cent of Bacillus megaterium on the first day, but the count was less than one per milliliter of milk on the subsequent days. Probably Bacillus

TABLE XII
EFFECT OF DIFFERENT CONCENTRATIONS OF HYDROGEN PEROXIDE
ON BACILLUS SUBTILIS IN STERILE MILK AT 37° C.

Concentration of H_2O_2 by weight	Before the addition of H_2O_2	After the addition of H_2O_2		
		24 hours	48 hours	72 hours
(%)		— (count/ml.) —		
0.00	56,000	15,400,000	9,100,000	8,700,000
0.03	56,000	*	49	1,300
0.05	56,000	*	37	760
0.09	56,000	*	*	*
				1,220,000
				14,800
				9,200
				*

* Less than one per milliliter.

TABLE XIII
EFFECT OF DIFFERENT CONCENTRATIONS OF HYDROGEN PEROXIDE
ON BACILLUS MEGATERIUM IN STERILE MILK AT 37° C.

Concentration of H ₂ O ₂ by weight	Before the addition of H ₂ O ₂	After the addition of H ₂ O ₂		
		24 hours	48 hours	72 hours
(%)		(count/ml.)		
0.00	390,000	7,400,000	15,800,000	20,700,000
0.03	390,000	93	*	*
0.05	390,000	*	*	*
0.09	390,000	*	*	*

* Less than one per milliliter.

TABLE XIV
EFFECT OF DIFFERENT CONCENTRATIONS OF HYDROGEN PEROXIDE
ON BACILLUS POLYMYXA IN STERILE MILK AT 37° C.

Concentration of H ₂ O ₂ by weight	Before the addition of H ₂ O ₂	After the addition of H ₂ O ₂		
		24 hours	48 hours	72 hours
(%)		(count/ml.)		
0.00	430,000	272,000,000	> 300,000,000	6,300,000
0.03	430,000	*	*	*
0.05	430,000	*	*	*
0.09	430,000	*	*	*
				2,360,000

* Less than one per milliliter.

megaterium required a larger time than 24 hours for a more complete destruction with 0.03 per cent hydrogen peroxide. There was a complete destruction of this organism with 0.05 per cent hydrogen peroxide, which was in agreement with the report by Nambudripad, Laxminarayana, and Iya (74), who found 16 hours of exposure to 0.05 per cent hydrogen peroxide at 37° C. was necessary for full destruction of Bacillus megaterium in broth solutions. Nambudripad, Laxminarayana, and Iya (74) and Roushdy (99) have reported independently that aerobic spore-formers like Bacillus subtilis and Bacillus megaterium are more resistant to the action of hydrogen peroxide. But in this investigation it was found that Bacillus megaterium and Bacillus polymyxa were not so much resistant to peroxide treatment. The longer period of exposure, namely 24 hours, would have been the cause for this difference. Bacillus subtilis seemed to be more resistant than Bacillus polymyxa and Bacillus megaterium. The easy spore-forming ability of Bacillus subtilis might have been the reason for such resistance, as spores required higher concentration of peroxide and more time of exposure to bring about complete destruction.

The results of the investigation showed that 0.30 per cent hydrogen peroxide was the most effective concentration for the complete destruction of bacteria in raw milk. But the amount of hydrogen peroxide required to bring about a total destruction of the various organisms in pure culture seemed to be 0.05 per cent for all the bacteria except Bacillus subtilis, which required 0.09 per cent. There was a difference between the concentration required for the total destruction of bacteria in raw milk and that for the organisms of pure culture; a greater concentration of 0.30 per cent was required to bring about total destruction in raw milk whereas

0.09 per cent was effective for all organisms including aerobic spore-former like Bacillus subtilis. This difference may be explained on the basis of the fact that the influence of hydrogen peroxide was based more on the type and proportion of the various organisms, the catalase content of the milk, and the differences in the milk used namely raw milk and heat sterilized skim milk. Probably the raw milk contained initially more of catalase and a type of bacterial flora which was less susceptible to the hydrogen peroxide.

Hydrogen peroxide is unique among germicides. It can be used to destroy organisms in milk, and after it has done its work, it can be rapidly and completely broken down. Moreover, hydrogen peroxide as it is produced today is remarkably stable, and when used in conjunction with catalase, does not impart an off-flavor to the milk. For more than 50 years, hydrogen peroxide has been used from time to time in milk. The object of its use primarily was to extend the keeping quality of milk for a period of one or more days by preventing bacterial increase.

The hydrogen peroxide preservation of milk must be considered as an aid to farmers and the dairy industry in countries with tropical climates. Until economic conditions permit the necessary improvements in production conditions (cooling on the farm and during transport), the addition of hydrogen peroxide to milk can be recommended. The hydrogen peroxide is added to milk on the farms which are far from the receiving dairies and it has been effective in preventing souring during transport. The addition of hydrogen peroxide may be suggested as a method if anything unforeseen happens, such as delay in milk transport or any mechanical breakdown which would cause losses through souring of the milk. The

hydrogen peroxide treatment does not diminish the nutritive value of the milk to any great extent.

Peroxide processing of the milk, therefore, would appear to be a practical method of preserving large quantities from milk producing areas and require to be transported to long distances for consumption as raw milk or for manufacture of dairy products. Even though this method of preservation is useful to preserve good and fair quality milk for long periods, milk produced under unhygienic conditions is least suitable for preservation by this method. It has been reported by various workers that the pathogens are not completely destroyed by the normal concentrations being used for the preservation of good and fair quality milk. Even though higher concentrations of hydrogen peroxide and longer period of exposure may kill the pathogens and preserve poor quality milk, it is not practicable because of the larger volumes of hydrogen peroxide required and the development of pronounced oxidized flavor. Therefore for maximum preserving effect the milk has to be produced under hygienic conditions from animals free from any disease.

The results of the investigation showed that complete destruction of bacteria in raw milk can be produced by hydrogen peroxide treatment. This has a practical application in the cheese industry because the milk intended for cheese making can also be treated in a similar manner. This method of treatment with hydrogen peroxide has already been recommended in cheese industry by various workers (68, 98, 104). Therefore hydrogen peroxide treatment has been regarded as another approach to better quality and control in dairy products. In addition to the destruction of bacteria, the treatment offers an avenue to control partially selection

of types of organisms for specific uses. It gives another type of control of body and texture in cheese. Good quality Swiss and Domiatl cheeses have been successfully manufactured from hydrogen peroxide treated milk. The peroxide-catalase method of treating milk for cheese is easily applied and has good possibilities of improving cheese quality. But this treatment will not cure everything. Milk containing large number of undesirable organisms will definitely produce cheese of inferior quality. Therefore along with this treatment all efforts should be made to produce clean, good quality milk.

In the United States, permission from regulatory officials is required for the manufacture of cheese from hydrogen peroxide treated milk. When more and more evidences are collected in the harmlessness and effectiveness of the hydrogen peroxide treatment it may be approved by the Public Health Service, Food and Drug Administration and other health protection agencies, and find a place in the dairy industry.

CHAPTER V

SUMMARY AND CONCLUSIONS

The influence of 0.09, 0.15, and 0.30 per cent by weight of hydrogen peroxide on the total bacterial content of raw milk exposed to 37° C. for 24, 48, 72, and 96 hours was determined. The initial bacterial count of the raw milk before the addition of hydrogen peroxide varied from 28, 300 to 65,000 per milliliter. There was a total destruction of bacteria in 1 milliliter quantities in the milk containing 0.30 per cent hydrogen peroxide. The growth of the bacteria was greatly retarded in milk containing 0.15 and 0.09 per cent hydrogen peroxide. Bacteriological analyses were made to determine the effect of hydrogen peroxide on pure culture of representative species of organisms of common dairy importance. Concentrations of 0.03, 0.05, and 0.09 per cent hydrogen peroxide in sterile milk were used for this study. The coliform organisms, Escherichia coli and Aerobacter aerogenes were completely destroyed in 1 milliliter samples by 0.03 per cent hydrogen peroxide within 24 hours at 37° C. The organisms of the genus Streptococcus seemed to be more resistant than coliforms for peroxide treatment, thereby showing that the acid-producing, Gram-positive, aerobic bacteria required a higher concentration of 0.05 per cent of hydrogen peroxide for complete destruction in 1 milliliter samples at 37° C. The susceptibility of Lactobacillus group of organisms (anaerobic and microaerophilic in nature) to hydrogen peroxide treatment seemed to be great as in the case of coliforms. There was complete destruction within 24 hours in 1 milliliter samples with 0.03 per cent hydrogen peroxide at 37° C. Bacillus polymyxa and Bacillus megaterium

were completely destroyed in 24 hours at 37° C. by 0.03 and 0.05 per cent peroxide respectively in 1 milliliter samples. Bacillus subtilis required 0.09 per cent peroxide for complete destruction in 1 milliliter sample. The easy spore forming ability of Bacillus subtilis might have been the reason for the resistance to peroxide treatment. 0.03, 0.05, and 0.09 per cent concentrations of hydrogen peroxide in sterile milk brought about complete destruction of organisms in pure culture depending upon the type of the organism. At the same time 0.30 per cent hydrogen peroxide was the most effective concentration for the complete destruction of bacteria in raw milk. This difference in the effective concentrations required in either case was explained on the basis of the fact that the influence of hydrogen peroxide was based more on the type and proportion of the various organisms, the catalase content of the milk, and the differences in the milk used namely raw milk and heat sterilized milk.

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