To the Graduate Council:

I am submitting herewith a thesis written by Ong-Ard Praepanitchai entitled "The Influence of Pasteurization Temperatures, Homogenization Pressures and Storage Duration at 4°C on Activity of Milk Xanthine Oxidase." I recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Food Technology and Science.

B. J. Demott

B. J. Demott, Major Professor

We have read this thesis and recommend its acceptance:

Accepted for the Council:

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Vice Chancellor
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THE INFLUENCE OF PASTEURIZATION TEMPERATURES, HOMOGENIZATION PRESSURES AND STORAGE DURATION AT 4°C ON ACTIVITY OF MILK XANTHINE OXIDASE

A Thesis

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ABSTRACT

The influence of pasteurization temperatures, homogenization pressures and storage duration at 4°C on the activity of milk xanthine oxidase was studied.

The activity of xanthine oxidase in raw milk was increased after milk was stored at 4°C for 24 hours but decreased upon further storage. Raw milk contained 0.2275 ± 0.0051 units per ml.

The activity of xanthine oxidase was increased when raw milk was heated to 55°C for 5 minutes. Milk heated at 55°C for 5 minutes had an activity of 0.2356 ± 0.0206 per ml and 0.2373 ± 0.0146 units per ml after storage at 4°C for 2 and 24 hours, respectively.

The activity of xanthine oxidase in milk was increased gradually as the homogenization pressures were increased from 1000 to 4000 pounds per square inch (psi). Milk homogenized at 4000 psi had 0.2559 ± 0.0056 units per ml after being stored at 4°C for 2 hours. Milk homogenized at 4000 psi had 0.2661 ± 0.0051 units per ml after storage at 4°C for 24 hours and lesser amounts when stored for longer periods.

The xanthine oxidase activity was greater in whipping cream than in skimmilk, homogenized milk or half and half. The whipping cream contained 0.2766 units per ml when analyzed on the day of purchase but decreased upon further storage.

The skimmilk of different processors contained 0.0463 to 0.0680 units per ml when analyzed on the day of purchase but after 24 and 48 hours storage the activity decreased. The homogenized milk of different processors contained 0.0007 to 0.1202 units per ml when analyzed on the day of purchase but decreased upon further storage. Half and half contained 0.0094 units per ml when analyzed on the day of purchase and lesser amounts after 24 and 48 hours storage.

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CHAPTER I

INTRODUCTION

The biological function of the enzyme xanthine oxidase is to aid in the breakdown of purines and pyrimidine. Xanthine oxidase is involved in the metabolic pathway which has been responsible for development of gout in humans. The enzyme is stimulated by testosterone. but inhibited by progesterone, cortisone and corticosterone. which might explain the predominance of gout and higher average serum uric acid level in men as contrasted to women (43). About 60 years ago xanthine oxidase was found to be present in milk and various milk products.

Plasmalogens, a group of phospholipids, are present in large amounts in human heart and brain tissue. But in those areas of the body where xanthine oxidase is present in large amounts, the plasmalogens are absent. This was explained by Ross et al. (41) to be due to the fact that xanthine oxidase oxidized the aldehydes of acid-hydrolyzed plasmalogens. The authors further speculate that the stimulation of testosterone of xanthine oxidase activity might account for the higher frequency of atherosclerotic heart disease in men as compared to women.

Ross et al. (41) postulated that consumption of homogenized milk was a causative factor in cardiovascular

disease, based upon the assumption that xanthine oxidase oxidizes the "protective" plasmal present in the linings of human arterial walls and heart muscle, thus resulting in a loss of elasticity of the arterial wall.

Because xanthine oxidase has been implicated to be a factor related to human health, the activity of the enzyme in milk and milk products would be useful information.

CHAPTER II

LITERATURE REVIEW

I. HISTORICAL ASPECTS OF XANTHINE OXIDASE

In 1902 xanthine oxidase was known as the Schardinger's enzyme. This enzyme was first discovered in milk when formaldehyde and methylene blue were added and the latter was decolorized. This enzyme is sometimes known as hypoxanthine oxidase, aldehydrase or transhydrogenase (7,24).

An oxidase is an enzyme which catalyzes the addition of oxygen to a substance or the removal of hydrogen from it (48). Xanthine oxidase, xanthine:oxygen oxidoreductase, E.C.1.2.3.2., is an enzyme which is widely distributed in living organisms occurring in both bacteria and animals, especially in cow's milk and calf liver (8). Xanthine oxidase was not found in mare's or sow's milk but was found in some samples of human milk in maximum amounts on the third day postpartum (31,37,38). Xanthine oxidase activity was found in the β-globulin fraction of human milk and serum. It was measured spectrophotometrically and the fractionation was by dialysis and precipitation with ammonium sulfate and copper (48).

Nilson (36) found that milk samples from mastitis-infected quarters showed a markedly higher xanthine oxidase

activity than those from normal quarters. Although the xanthine oxidase activity of normal milk was positively related to the butter fat content of milk, no such relationship was found in mastitis milk. He suggested that a xanthine oxidase test might be suitable for the rapid detection of mastitis on the farm as well as in the dairy.

Greenbank et al. (17) reported that xanthine oxidase promotes the oxidation of numerous aldehydes. Through the combined action of this and other enzymes in milk, a person might postulate a series of reactions and explain in the formation of hydrogen peroxide as an end product, creating the possibility that this enzyme could be of great significance in the deterioration of milk and milk products.

II. THE STRUCTURE OF XANTHINE OXIDASE

The structure of the enzyme is fairly well known since Bergel and his colleagues (4,6) isolated its crystalline form from milk. Milk xanthine oxidase contains, per molecule, 2 molecules of flavin adenine dinucleotide (FAD), 8 atoms of nonheme iron, 2 atoms of molybdenum and 8 "labile sulfides" which are readily released as hydrogen sulfide from the protein by acidification or by boiling at pH 7.0. The metal ions and flavin are bound quite firmly to the protein and are not released by dialysis. Denaturation of the protein by heat or by treatment with acids releases the metal ions

and flavin but activity is irreversibly lost. The flavin can be removed by calcium chloride treatment; this hydrolyzes FAD to flavin mononucleotide (FMN) which, being much less tightly bound, dissociates from the protein (5,7,11,30,40,49).

The purified enzyme isolated from cow's milk is a reddish brown protein with a molecular weight of about 275,000 (1). The isoelectric point of the enzyme is 6.2 and the activity pH optimum is about 8.3 (7,49).

III. REACTIONS CATALYZED; SUBSTRATE SPECIFICITY

Xanthine oxidase is a rather nonspecific enzyme and a great variety of compounds have been found to serve as substrates (electron donors). The rate of oxidation varies quite markedly among the different types of substrates, with hypoxanthine and xanthine being the best substrates. The enzyme is about as nonspecific for the electron acceptor as it is for the electron donor.

Xanthine oxidase catalyzes the following reaction (12,24):

$$\text{Reduced Substrate} + \text{Hydrogen Acceptor} \xrightarrow[\text{Oxidase}]{\text{Xanthine}} \text{Oxidized Substrate} + \text{Reduced Form of Acceptor}$$

A variety of hydrogen acceptors are: (a) methylene blue, (b) molecular oxygen (O_2), (c) cytochrome C, (d) triphenyltetrazolium chloride (TTC), (e) 2,6 dichlorophenol indophenol, and (f) ferricyanide.

Among the substrate (hydrogen donators) are: (a) hypoxanthine, (b) xanthine, (c) aldehydes, (d) adenine, (3) guanine, and (f) vanillin.

IV. ASSAY OF ENZYME ACTIVITY

Among the methods for assaying xanthine oxidase activity is the methylene blue reduction technique (13,32), in which the methylene blue under anaerobic conditions replaces oxygen (O_2) as the terminal acceptor of electrons. The rate of reduction in blue color can then be followed continuously in a spectrophotometer. A second method for enzyme activity measurement is oxygen uptake (32). The rate of oxygen uptake can be measured conveniently in either a Warburg apparatus or with an O_2-sensitive electrode. A third method is based upon uric acid formation (7,25). The enzymatic oxidation of hypoxanthine or xanthine to uric acid results in a marked increase in the light absorption at 290 nanometers (nm). A fourth method of assay utilizes the reduction of cytochrome C (23) which can be reduced both aerobically and anaerobically by hypoxanthine in the presence of xanthine oxidase, the anaerobic reduction being the faster. Xanthine oxidase requires molybdenum for reaction with cytochrome C. A fifth method is based upon TTC reduction (51). Xanthine is the substrate and a dye, TTC, the hydrogen acceptor. It is red in color and can be extracted from the

reaction mixture with toluene for measurement of color intensity. The final method is based upon vanillin oxidation (28). Vanillin is the substrate for the enzyme to produce vanillic acid which then reacts with 2,6 dibromo-quinonechloroimide (BQC) to produce a blue color, the intensity of which is then measured on a spectrophotometer at 650 nm.

V. DISTRIBUTION OF XANTHINE OXIDASE IN NATURE

The xanthine oxidase concentration of milk is about 120-160 mg per liter (7,15), and has been shown to be associated with milk fat and especially with the fat globule membrane (21,33,34,39,52). Morton (34) demonstrated that xanthine oxidase is found in fresh milk in the form of lipid protein complexes occluded onto the surface of the fat globules. Kiermeier and Vogt (26) found that most of the xanthine oxidase remained in the cream during separation, and the enzyme activity was proportional to the fat content of cream. Rennet whey and rennet curds contained approximately similar amounts of the enzyme.

Xanthine oxidase activity was increased in fresh raw milk by storing at 4°C for 24 hours, heating at 70°C for 5 minutes and by homogenizing. Enzyme activity increased in both milk and cream, but not in skimmilk stored at 4°C for 24 hours (19).

Zittle et al. (52) showed that xanthine oxidase was more concentrated in cream than in skimmilk. Gudnason and Shipe (20) reported that there was remarkable consistency in the percentage of xanthine oxidase activity found in the skimmilk phase when stored at 4°C for 24 hours. It varied from 66 to 80%.

VI. EFFECT OF STORAGE UPON APPARENT XANTHINE OXIDASE ACTIVITY

Over the past 40 to 50 years, some investigators reported that storage of fresh raw milk at 0 to 5°C resulted in an apparent increase in xanthine oxidase activity, reaching a maximum after 2 to 4 days. They suggested that the oxygen in milk was used up by bacteria during storage, thus decreasing time needed for methylene blue discoloration. Such an effect would be interpreted as indicating an increase in enzyme activity (20).

Ball (7) reported that a large part of the xanthine oxidase was associated with the fat fraction. Since the portion in the skim fraction increased as the milk aged, he suggested that the enzyme was absorbed on the fat droplets, and could be forced into solution by causing the fat droplets to coalesce.

Gudnason and Shipe (20) showed that xanthine oxidase activity of milk reached a maximum level after storage at 4°C for 24 hours. They showed that the heat sensitivity of

the enzyme is increased by aging of the milk at 4°C for 24 hours and thought that this was due to a disintegration of the microsomes associated with the fat globule membrane.

VII. EFFECT OF HEATING ON XANTHINE OXIDASE ACTIVITY

Xanthine oxidase is more heat-stable than alkaline phosphatase. The activity of both enzymes in skimmilk is decreased at temperatures of 55 to 75°C (51). The xanthine oxidase is concentrated in the cream portion of milk and is considerably more resistant to the effect of heat in cream than when the enzyme is in skimmilk. After pasteurization of cream, only 2.3% of the xanthine oxidase was inactivated, whereas with whole milk it was 23.6% and in skimmilk, 27.9%. While low levels of heat tend to activate xanthine oxidase, the free and active enzyme is apparently more sensitive to heat deactivation (16).

Significant irregularities in the inactivation occurred in the region of 60 to 70°C, both in milk and cream. In some samples increases in the activity of the enzyme occurred when milk was heated to temperatures in this region. Temperatures of 70°C or above always decreased the activity, but in order to completely inactivate the enzyme, temperatures of over 85°C for 35 seconds were necessary (26). Inactivation of the vanillin and xanthine oxidizing activities of milk were caused by heating to 70°C (28).

VIII. EFFECT OF HOMOGENIZATION UPON XANTHINE OXIDASE ACTIVITY

The increases in apparent xanthine oxidase activity which occurred when fresh milk was subjected to homogenization and enzymatic activity indicate that some of the enzyme is not active in fresh uncooled milk. Homogenization produced about a two-fold increase in activity as determined by the manometric method. Increases in enzyme activity are always accompanied by great increases in activity of the skimmilk phase (20).

Greenbank and Pallansch (16) showed that concentrating heat-treated milk (195°F for 15 seconds) in a single effect pan to 50% solids content had little or no effect on xanthine oxidase activity. However, homogenizing the 50% concentrate at 4,500 psi was found to reactivate part of the xanthine oxidase. Since increasing the homogenization pressure over 4,500 psi did not increase the amount of activation over that homogenized at 4,500 psi, the workers (16) reasoned that two forms of xanthine oxidase are present in milk; one form of the enzyme being stabilized in an inactive form by a very close association with the fat globule. High pressure homogenization of high solids concentrates promotes a casein-fat interaction which may displace the enzyme from the fat surface, thereby activating it. Excessively high homogenization pressures, the workers reasoned, would not only free the

enzyme but rapidly denature its active and pressure-sensitive form. This deactivation was observed at homogenization pressures in excess of 4,500 psi.

IX. ENZYME INHIBITION

Hofstee's (22) data indicate that there must be two binding sites per molecule of xanthine oxidase, only one of which is enzymatically active, and binding of the substrate on the auxiliary site inhibits the enzymatic action at the active site.

Enzyme activity may be lost by the action of heavy metals, photooxidation or gross denaturation (9). The inhibition of xanthine oxidase can be induced by excess of hypoxanthine or xanthine (35). The optimal concentration of xanthine in the reaction mixture is 0.000088 to 0.00037 mole per liter. The influence of the excess of hypoxanthine or xanthine can be eliminated by the addition of histamine or by the removal of metallic ions from the reaction mixture. The addition of a minute amount of Cu^{++}, Pb^{++}, Ca^{++}, or Mg^{++} to the metal-free reaction system prepared by treatment with dithizone brought about the reappearance of the typical substrate inhibition. Therefore, the presence of a trace of some metallic ions in the reaction mixture may be responsible for the inhibition by the substrate.

Xanthine oxidase was inhibited in the presence of 0.016 M borate. Inhibition appeared to be due to the formation of a complex between the ribityl radical of riboflavin and borate; it was reversed completely by 0.04 M sorbitol and 0.1 M ribose in the presence of 0.032 M borate (42). The addition to milk of folic acid, a very effective competitive inhibitor for xanthine oxidase, failed to slow the rate of resazurin reduction significantly (10). The action of xanthine oxidase was found to be inhibited by the amino group reagents, 2,4 dinitrofluorobenzene; 2,4,6 trinitrobenzene sulphonic acid and benzaldehyde. The reactions were irreversible and occurred more rapidly at pH 10.8 than at pH 7.8 (18).

X. OXIDIZED FLAVOR AND XANTHINE OXIDASE

The development of spontaneous oxidation in milk has been attributed to its xanthine oxidase activity (2), copper content (27) and, more recently, to the copper and ascorbic acid content (44,45,46,47). The occurrence of a spontaneously oxidized flavor in milk was found to be dependent only upon a high level of xanthine oxidase activity. Milks that possessed xanthine oxidase activity between 120 and 140 units (measured by a manometric method, results expressed as microliters of oxygen absorbed per milliliter of milk per hour) showed rapid flavor development; milks possessing an activity of less than 100 units failed to develop spontaneous oxidized flavor; and

those with activity between 100 and 120 units were not consistent in the production of an oxidized flavor. Addition of xanthine oxidase to milks low in activity resulted in development of the flavor, as measured by the thiobarbituric acid method (2,3).

CHAPTER III

MATERIALS AND METHODS

I. SOURCE OF RAW MILK

The raw milk was obtained from the storage tank at the University of Tennessee Dairy Plant at approximately 8 AM on the day of the experiment. Such milk was a mixture of that which was taken from the cows on the University dairy farm the evening before and that which was taken from the cows on the morning of the experiment. The herd milk was handled in the conventional manner; that is, using a pipe line milker, cooling the milk in a bulk tank, mixing the morning's milk with the evening's milk, pumping the milk into a transport vehicle, moving the vehicle to the dairy processing plant, pumping the milk into a storage tank, from which the sample for experimental use was taken. When the milk was held for storage studies, glass containers protected from the light were used.

II. STANDARD CURVE

A unit of activity of xanthine oxidase, E.C.1.2.3.2, is that amount forming one micromole of uric acid per minute at 25°C (50). The enzyme, extracted from milk and suspended in

0.60 saturated ammonium sulfate, was purchased from ICN Pharmaceuticals, Inc., Cleveland, Ohio.

Each mg dry enzyme preparation contained 0.494 units xanthine oxidase. Each ml of the xanthine oxidase suspension contained 10 mg dry enzyme preparation. A standard curve was constructed by adding 5 ml of the xanthine oxidase suspension containing 24.7 units xanthine oxidase to 45 ml of distilled water to obtain a total volume of 50 ml. Each ml of the diluted xanthine oxidase suspension contained 0.494 units xanthine oxidase. Various volumes of this diluted xanthine oxidase suspension and various volumes of distilled water were mixed into 10 ml of milk which had been boiled for 30 minutes. The total volume of the mixture was 20 ml. The volumes of the xanthine oxidase suspension used for the construction of the standard curve were in 0.5 ml increments. The activity of xanthine oxidase preparations was determined by the method of Kuramoto et al. (28). Vanillin was used as a substrate for the enzyme to produce vanillic acid which then reacted with BQC to produce a blue color the absorbance of which was measured on a Coleman-Spectronic 20 spectrophotometer at 650 nm. The relationship between the units of xanthine oxidase activity (Y) and the absorbance values (X) as shown in Figure 1 was

$$Y = -0.0058 + 0.5431\ X$$

$$r = 0.9966$$

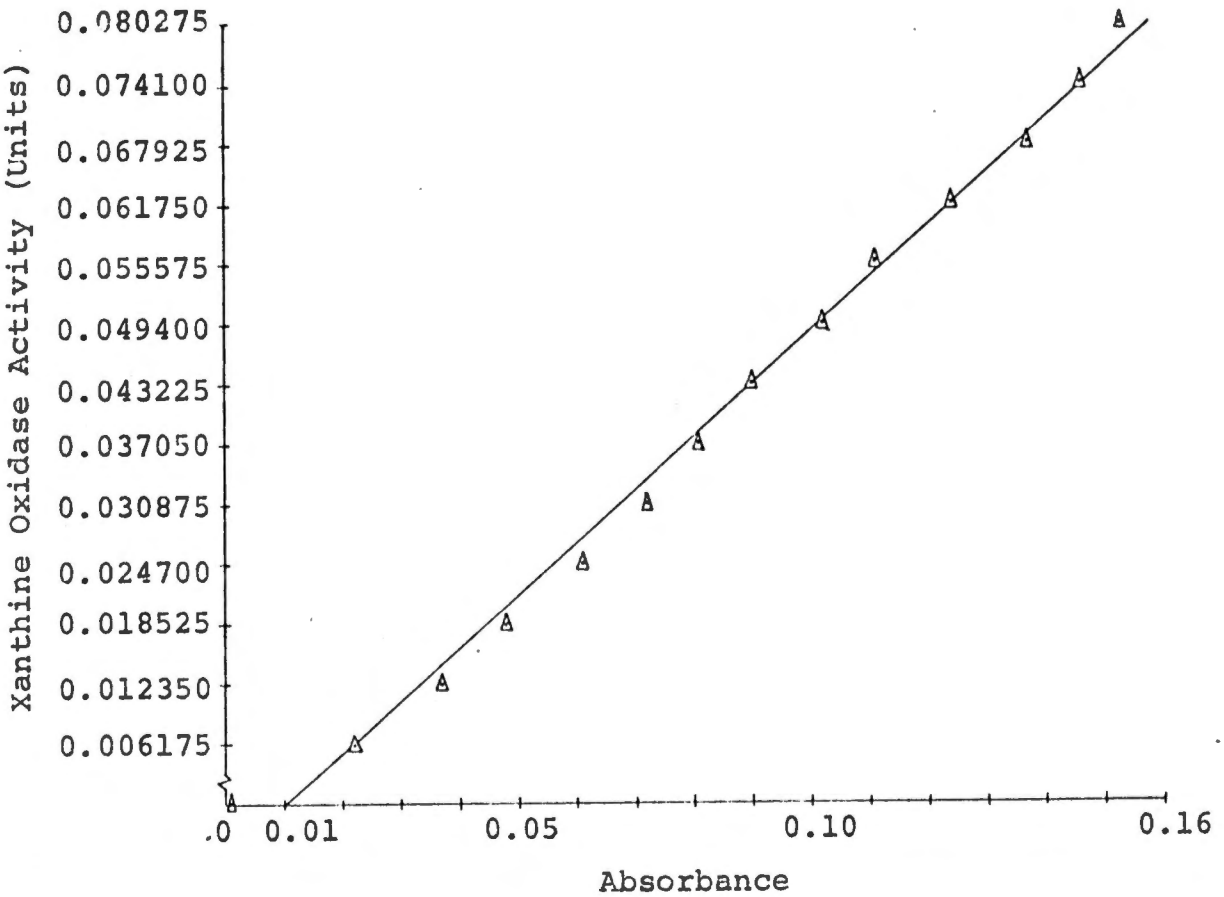

Figure 1. Relationship between xanthine oxidase activity and absorbance.

III. ANALYSIS OF SAMPLES

To 10 ml of each milk sample was added 10 ml of distilled water. The diluted milk was analyzed by the following procedure: To 0.5 ml of diluted milk in a 50 ml centrifuge tube, 1 ml of aqueous vanillin solution (1 mg per ml) was added. After incubating the mixture at 60°C for 5 minutes, 5 ml of borate buffer (pH 9.2) was added. After mixing, 0.025 ml of 0.4% ethanolic BQC solution was added. The mixture was shaken thoroughly and allowed to stand 15 minutes for color development. The resulting blue color was extracted with 20 ml of 1-butanol by first shaking virogously and then centrifuging. The absorbance of the butanol solution was measured at 650 nm by a spectrophotometer. Using the same preparation procedure a blank was prepared from a sample of milk which had been boiled for 30 minutes. The net absorbance values from the milk samples was inserted in the regression equation of the standard curve and the units of xanthine oxidase calculated. Inasmuch as the milk analysis used only 0.25 ml of milk, the observed xanthine oxidase activity was multiplied by four to express the activity on a per ml basis.

IV. EXPERIMENTAL TREATMENTS

To determine the influence of storage duration of raw milk on xanthine oxidase activity, the xanthine oxidase activity was determined within 2 hours after securing the

sample and again after 24, 48, 72 and 96 hours storage at 4°C. Eight trials were conducted.

To determine the influence of heat treatment and storage upon the xanthine oxidase activity, raw milk was heated at 55, 60, 65, 70 and 75°C for 5 minutes; cooled to 4°C, and the xanthine oxidase activity determined within 2 hours and again after 24 hours storage at 4°C. Eight trials were conducted.

To determine the influence of homogenization pressures on xanthine oxidase activity, raw milk was heated at 48°C for 5 minutes, homogenized in a single stage Gaulin homogenizer at 1000, 1500, 2000, 2500, 3000, 3500 and 4000 psi, cooled to 4°C and analyzed for xanthine oxidase activity within 2 hours. Six trials were conducted.

To determine the influence of homogenization pressures and storage duration on xanthine oxidase activity, raw milk heated at 48°C for 5 minutes was homogenized at pressures of 2500, 3000, 3500, and 4000 psi, cooled to 4°C and analyzed for xanthine oxidase activity within 2 hours and after storage for 24, 48, 72 and 96 hours. Six trials were conducted.

To gain some information relative to the xanthine oxidase concentration in fluid milk products offered for sale, samples of skimmilk, homogenized milk, half and half and whipping cream were purchased at a retail outlet and the xanthine oxidase activity determined on the day of

purchase, one day later and two days later. A total of eight different samples were analyzed.

V. STATISTICAL ANALYSIS

The xanthine oxidase activity in units per ml were used as data points and the data analyzed by an analysis of variance. Differences between means were determined by using Duncan's Multiple Range Test as illustrated by Larmond (29).

CHAPTER IV

RESULTS AND DISCUSSION

The activity of xanthine oxidase in raw milk was increased after milk was stored at 4°C for 24 hours but decreased upon further storage as shown in Figure 2. Xanthine oxidase activity was at its maximum level after the raw milk was stored at 4°C for 24 hours, containing 0.2275 ± 0.0051 units per ml. The xanthine oxidase activity decreased after the raw milk was stored at 4°C more than 24 hours. Duncan's Multiple Range Test showed a significant difference ($P < .05$) between samples stored for 2, 24, 48 and 96 hours, but the samples stored for 2 hours and 72 hours were not significantly different from each other (Table I). Analysis of variance of these data is presented in Table II. There was a significant difference ($P < .01$) in the xanthine oxidase activity in raw milk stored for different lengths of time. There was a significant difference ($P < .01$) in replications which might be due to differences in composition of the raw milk used. Regression analysis of xanthine oxidase activity over time yielded a quartic function (Figure 2) represented by the equation:

$$Y = 0.2085 + 0.0239X - 0.0092X^2 + 0.0012X^3 - 0.00005X^4.$$

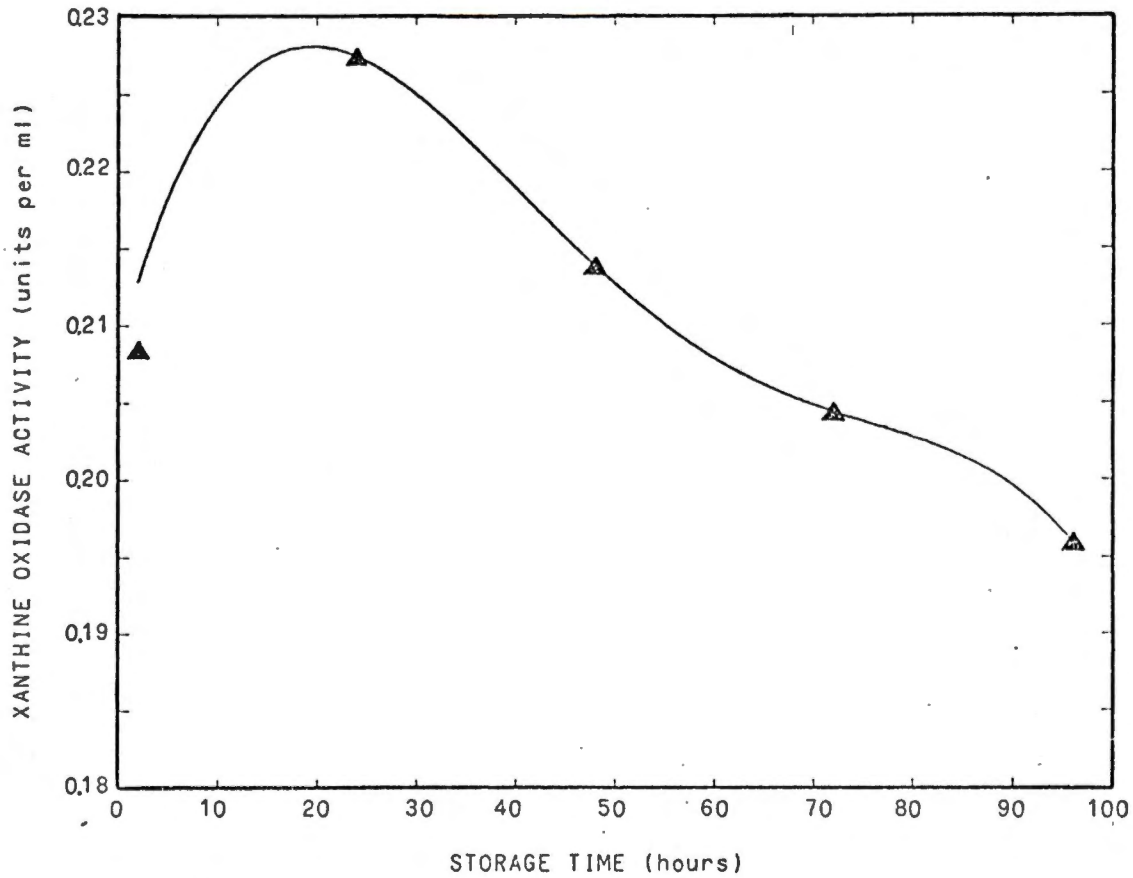

Figure 2. The influence of storage duration at 4°C on xanthine oxidase activity in raw milk. (Mean of eight trials.)

TABLE I

The Xanthine Oxidase Activity in Raw Milk as Influenced by Storage at 4°C

Storage (Hours)	Xanthine Oxidase Activity (Units per ml)									
	Replication								Mean	Std. Dev.
	1	2	3	4	5	6	7	8		
2	0.2116	0.2136	0.2136	0.2028	0.2072	0.2028	0.2072	0.2092	0.2085[c]	0.0043
24	0.2180	0.2288	0.2332	0.2268	0.2308	0.2352	0.2244	0.2224	0.2275[a]	0.0051
48	0.2072	0.2180	0.2136	0.2156	0.2180	0.2224	0.2092	0.2072	0.2139[b]	0.0056
72	0.1964	0.2008	0.2028	0.2092	0.2116	0.2116	0.2048	0.1984	0.2045[c]	0.0059
96	0.1896	0.1940	0.1984	0.2008	0.2008	0.2028	0.1920	0.1896	0.1960[d]	0.0053

Note: Means followed by the same letter are not significantly different at the 5% level.

Each data point is the mean of duplicate analyses.

TABLE II

Analysis of Variance of the Influence of Storage at 4°C for 2, 24, 48, 72 and 96 Hours on Activity of Milk Xanthine Oxidase

Source	Degrees of Freedom	Sum of Squares	Mean Squares	F Value
Storage duration	4	0.004389976	0.001097494	60.30**
Replication	7	0.000514672	0.000073525	4.04**
Residual error	28	0.000509608	0.000018200	
Total	39	0.005414256	0.000138827	

**P < .01.

Figure 3 shows the influence upon xanthine oxidase activity in raw milk given heat treatment of 55, 60, 65, 70 and 75°C for 5 minutes and subsequently stored at 4°C for 2 and 24 hours. The xanthine oxidase was activated when raw milk was heated to 55°C for 5 minutes. The Student's t test showed this difference to be significant at the 0.01 level of probability. Raw milk given no heat treatment had xanthine oxidase activity of 0.2085 ± 0.0043 and 0.2275 ± 0.0051 units per ml after storage at 4°C for 2 and 24 hours, respectively (Figure 2, page 21) whereas this same milk heated at 55°C for 5 minutes had an activity of 0.2356 ± 0.0206 and 0.2373 ± 0.0146 units per ml after storage at 4°C for 2 and 24 hours, respectively. Xanthine oxidase is partially inactivated when exposed to temperatures of 60, 65 and 70°C for 5 minutes and is almost completely inactivated when heated at 75°C for 5 minutes. Duncan's Multiple Range Test showed that samples heated at 55 or 60°C were not significantly different ($P > .05$), but these samples were significantly different ($P < .05$) from milk heated at 65, 70 or 75°C. The latter three samples were also significantly different ($P < .05$) from each other (Table III). The xanthine oxidase activity of samples heated at 55, 60 and 65°C were not significantly different ($P > .05$), but these three samples had different values from those samples heated at 70 or 75°C, and the latter two samples were also significantly different

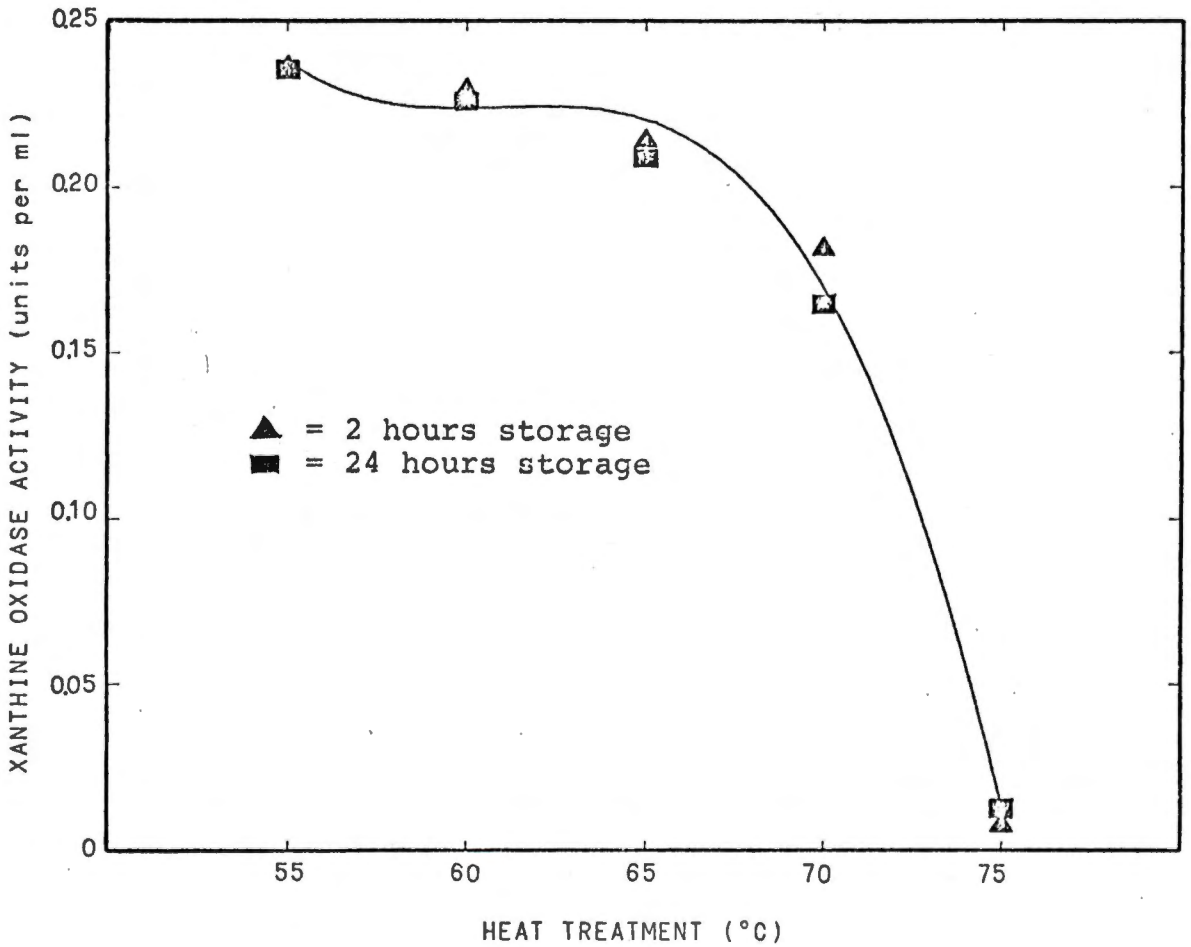

Figure 3. The activity of xanthine oxidase of milk as influenced by heat treatment and storage at 4°C. (Mean of eight trials.)

TABLE III

The Activity of Xanthine Oxidase in Milk as Influenced by Heat Treatment and Storage at 4°C for 2 Hours

Heat Treatment °C	Xanthine Oxidase Activity (Units per ml)									
	Replication								Mean	Std. Dev.
	1	2	3	4	5	6	7	8		
55	0.2332	0.2352	0.2680	0.2484	0.2352	0.2376	0.1940	0.2332	0.2356[a]	0.0206
60	0.2288	0.2308	0.2572	0.2352	0.2244	0.2244	0.1788	0.2288	0.2261[a]	0.0218
65	0.2224	0.2180	0.2460	0.2200	0.2072	0.2092	0.1464	0.2028	0.2090[b]	0.0286
70	0.1616	0.1744	0.2200	0.2008	0.1180	0.1616	0.1160	0.1656	0.1648[c]	0.0359
75	0.0008	0.0204	0.0008	0.0204	0.0116	0.0224	0.0008	0.0244	0.0127[d]	0.0105

Note: Means followed by the same letter are not significantly different at the 5% level.

Each data point is the mean of duplicate analyses.

($P < .05$) from each other (Table IV). Analysis of variance of the influence of heat treatment of milk at these temperatures, presented in Table V, shows a significant difference ($P < .01$) in the xanthine oxidase activity in raw milk given the various heat treatments. The xanthine oxidase activity was decreased when the raw milk was heated to temperatures greater than 55°C for 5 minutes. There was no significant difference ($P > .05$) between samples stored for 2 hours and those stored for 24 hours. There was a significant difference ($P < .01$) in replications which might be due to differences in composition of the raw milks used. There was no significant ($P > .05$) interaction between temperature and storage time. Regression analysis of xanthine oxidase activity over heat treatment yielded a cubic function (Figure 3, page 25) represented by the equation:

$$Y = 17.7139 - 0.8619X + 0.0141X^2 - 0.00008X^3$$

The activity of xanthine oxidase in milk gradually increased as the homogenization pressures increased from 1000 to 4000 psi. Figure 4 shows the influence of homogenization pressures of 1000, 1500, 2000, 2500, 3000, 3500 and 4000 psi on xanthine oxidase activity in milk. Milk homogenized at 1000 psi contained 0.2201 ± 0.0081 units per ml after being stored at 4°C for 2 hours, and milk homogenized at 4000 psi contained 0.2559 ± 0.0056 units per ml. Duncan's

TABLE IV

The Activity of Xanthine Oxidase in Milk as Influenced by Heat Treatment and Storage at 4°C for 24 Hours

Heat Treatment °C	Xanthine Oxidase Activity (Units per ml)									
	Replication								Mean	Std. Dev.
	1	2	3	4	5	6	7	8		
55	0.2396	0.2420	0.2572	0.2484	0.2308	0.2376	0.2072	0.2352	0.2373[a]	0.0146
60	0.2308	0.2376	0.2572	0.2396	0.2224	0.2308	0.1960	0.2288	0.2304[a]	0.0173
65	0.2268	0.2268	0.2572	0.2244	0.2048	0.2180	0.1528	0.2092	0.2150[a]	0.0297
70	0.1832	0.2004	0.2308	0.2072	0.1224	0.1940	0.1420	0.1768	0.1821[b]	0.0352
75	0.0008	0.0180	0.0008	0.0116	0.0008	0.0160	0.0008	0.0204	0.0087[c]	0.0087

Note: Means followed by the same letter are not significantly different at the 5% level.

Each data point is the mean of duplicate analyses.

TABLE V

Analysis of Variance of the Influence of the Heat Treatment at 55, 60, 65, 70 and 75°C and Subsequent Storage at 4°C for 2 and 24 Hours on Activity of Milk Xanthine Oxidase

Source	Degrees of Freedom	Sum of Squares	Mean Squares	F Value
Temperature	4	0.55903208	0.13975802	643.09**
Storage duration	1	0.00051207	0.00051207	2.36
Temperature x Storage	4	0.00098821	0.00024705	1.14
Replication	7	0.02709484	0.00387069	17.81**
Residual	63	0.01369132	0.00021732	
Total	79	0.60131852	0.00761163	

**P < .01.

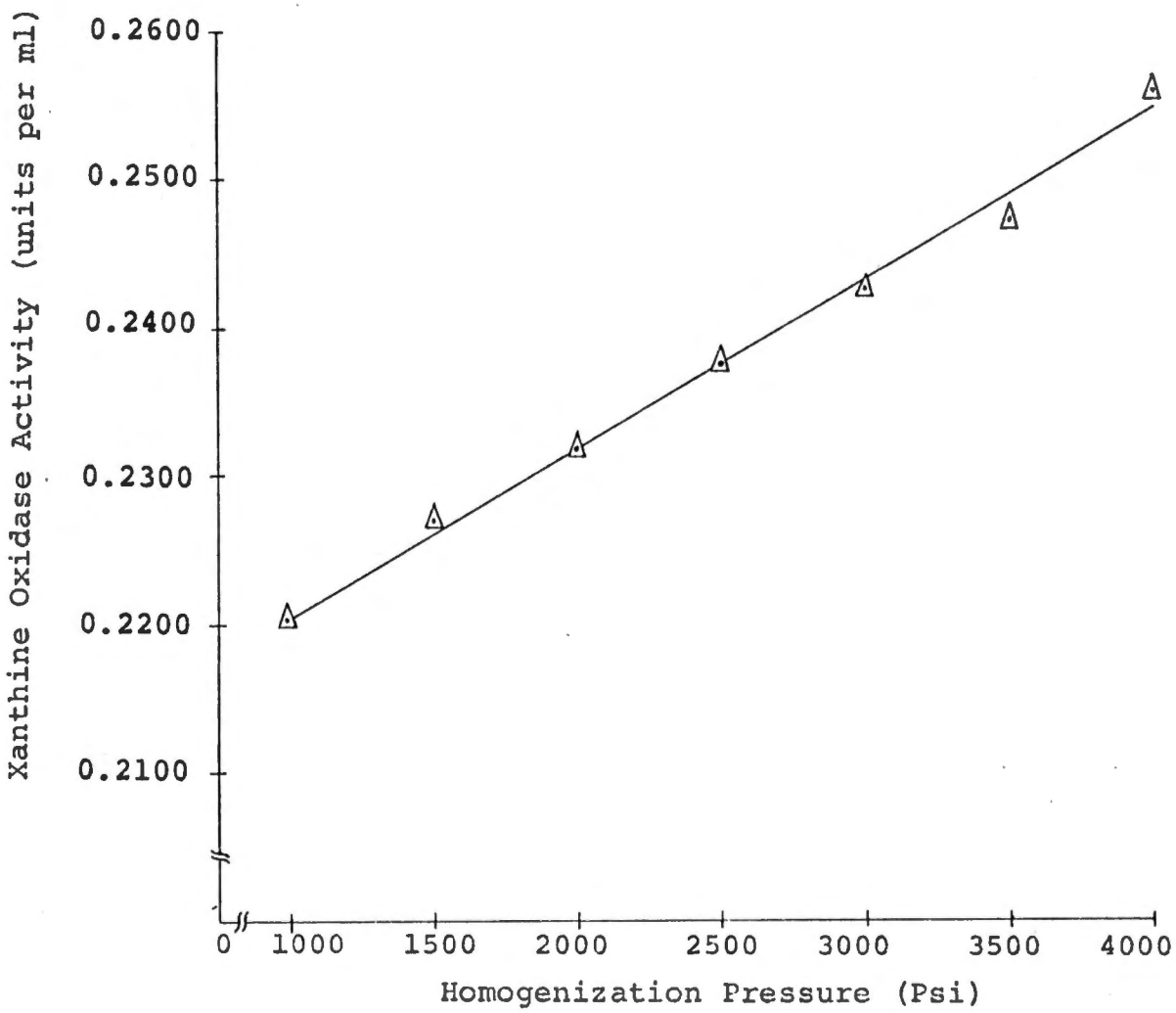

Figure 4. The activity of xanthine oxidase of milk as influenced by homogenization pressures. (Mean of six trials.)

Multiple Range Test showed a significant difference ($P < .05$) between samples homogenized at the various pressures and stored at 4°C for 2 hours, with the exception that the samples homogenized at 3000 psi and 3500 psi were not significantly different from each other (Table VI). The analysis of variance of influence of homogenization pressures of milk on xanthine oxidase activity (Table VII) shows a significant difference ($P < .01$) in the xanthine oxidase activity in milk homogenized at different pressures. There was also a significant difference ($P < .01$) in replications which might be due to the differences in composition of the raw milks used. Regression analysis of xanthine oxidase activity over different pressures yielded a linear function (Figure 4) represented by the equation:

$$Y = 0.20905 + 0.0000113X$$

$$r = 0.9962$$

Figure 5 shows the influence of milk homogenized at 2500, 3000, 3500 and 4000 psi and subsequent storage at 4°C for 2, 24, 48, 72 and 96 hours upon the xanthine oxidase activity. The activity increased after homogenized milk was stored at 4°C for 24 hours, but decreased upon further storage. The milk homogenized at 4000 psi contained 0.2661 ± 0.0051 units per ml after storage at 4°C for 24 hours and

TABLE VI

The Activity of Xanthine Oxidase in Milk as Influenced by Homogenization Pressures

Homogenization Pressures psi	Xanthine Oxidase Activity (Units per ml)							
	Replication						Mean	Std. Dev.
	1	2	3	4	5	6		
1000	0.2180	0.2224	0.2136	0.2308	0.2268	0.2092	0.2201[f]	0.0081
1500	0.2288	0.2244	0.2180	0.2352	0.2332	0.2224	0.2270[e]	0.0066
2000	0.2308	0.2288	0.2224	0.2396	0.2376	0.2308	0.2317[d]	0.0062
2500	0.2396	0.2352	0.2332	0.2396	0.2440	0.2308	0.2371[c]	0.0049
3000	0.2460	0.2396	0.2376	0.2420	0.2504	0.2396	0.2425[b]	0.0048
3500	0.2548	0.2504	0.2376	0.2440	0.2304	0.2440	0.2469[b]	0.0062
4000	0.2612	0.2572	0.2528	0.2504	0.2636	0.2504	0.2559[a]	0.0056

Note: Means followed by the same letter are not significantly different at the 5% level.

Each data point is the mean of duplicate analyses.

TABLE VII

Analysis of Variance of the Influence of Homogenization Pressures of 1000, 1500, 2000, 2500, 3000, 3500 and 4000 psi on Xanthine Oxidase Activity in Milk after Storage at 4°C for 2 Hours

Source	Degrees of Freedom	Sum of Squares	Mean Squares	F Value
Pressure	6	0.0053920914	0.000898682	58.09**
Replication	5	0.0008613486	0.000172269	11.14**
Residual error	30	0.0004640914	0.00001547	
Total	41	0.0067175314	0.000163842	

**P < .01.

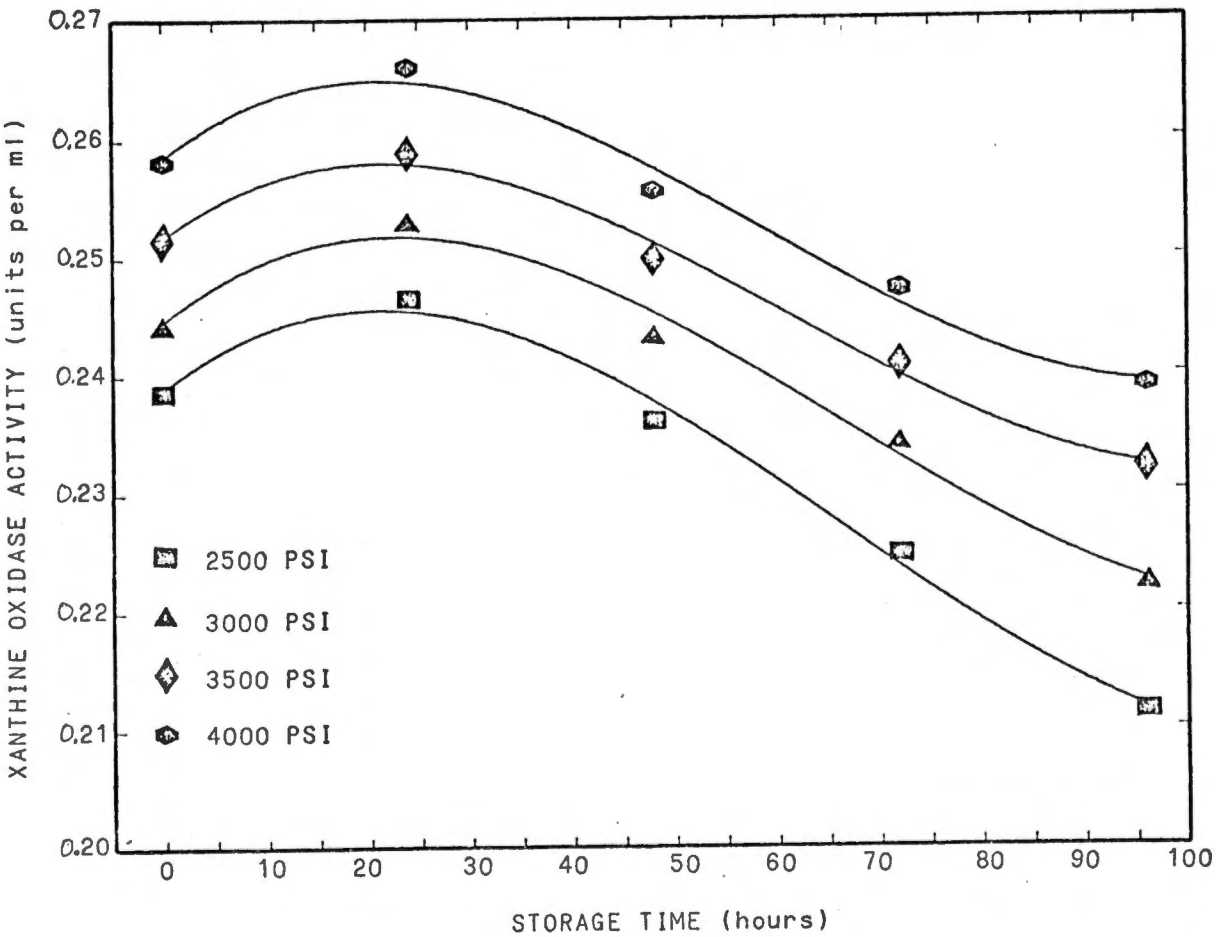

Figure 5. The activity of xanthine oxidase of milk as influenced by homogenization pressures and storage time. (Mean of six trials.)

lesser amounts when stored for longer periods. Duncan's Multiple Range Test showed that, regardless of the pressures used, homogenized milk stored for 2 or 48 hours had xanthine oxidase activities which were not significantly different ($P > .05$) from each other. But these two samples were different from those samples stored for 24, 72 or 96 hours ($P < .05$), and the latter three samples were different from each other (Tables VIII to XI). The analysis of variance of these data, presented in Table XII, shows a significant difference ($P < .01$) in the xanthine oxidase activity in milk homogenized at different pressures and a significant difference ($P < .01$) when the homogenized milk was stored for different lengths of time. There was also a significant difference ($P < .01$) in replications which might be due to the differences in composition of the raw milks used. Regression analysis of xanthine oxidase activity at different pressures over time yielded cubic functions (Figure 5) represented by the equations:

$$2500 \text{ psi}, \quad Y = 0.2389 + 0.0068X - 0.0019X^2 + 0.0001X^3$$

$$3000 \text{ psi}, \quad Y = 0.2446 + 0.0069X - 0.0019X^2 + 0.00009X^3$$

$$3500 \text{ psi}, \quad Y = 0.2518 + 0.0064X - 0.0018X^2 + 0.0001X^3$$

$$4000 \text{ psi}, \quad Y = 0.2585 + 0.0067X - 0.0020X^2 + 0.0001X^3$$

The xanthine oxidase activity in commercial milk products is presented in Table XIII. The whipping cream of

TABLE VIII

The Activity of Xanthine Oxidase in Milk as Influenced by Homogenization at 2500 psi and Storage at 4°C

Storage (Hours)	Xanthine Oxidase Activity (Units per ml)							
	Replication						Mean	Std. Dev.
	1	2	3	4	5	6		
2	0.2352	0.2396	0.2420	0.2332	0.2440	0.2376	0.2386[b]	0.0041
24	0.2440	0.2460	0.2484	0.2420	0.2484	0.2504	0.2465[a]	0.0031
48	0.2332	0.2376	0.2396	0.2332	0.2376	0.2352	0.2361[b]	0.0026
72	0.2288	0.2332	0.2244	0.2224	0.2200	0.2200	0.2248[c]	0.0052
96	0.2156	0.2180	0.2112	0.2112	0.2048	0.2072	0.2113[d]	0.0049

Note: Means followed by the same letter are not significantly different at the 5% level.

Each data point is the mean of duplicate analyses.

TABLE IX

The Activity of Xanthine Oxidase in Milk as Influenced by Homogenization at 3000 psi and Storage at 4°C

Storage (Hours)	Xanthine Oxidase Activity (Units per ml)							
	Replication						Mean	Std. Dev.
	1	2	3	4	5	6		
2	0.2420	0.2460	0.2504	0.2396	0.2460	0.2420	0.2443[b]	0.0039
24	0.2548	0.2572	0.2548	0.2484	0.2528	0.2504	0.2531[a]	0.0032
48	0.2460	0.2420	0.2440	0.2420	0.2420	0.2440	0.2433[b]	0.0016
72	0.2352	0.2332	0.2332	0.2352	0.2352	0.2332	0.2342[c]	0.0011
96	0.2268	0.2224	0.2200	0.2200	0.2200	0.2244	0.2223[d]	0.0028

Note: Means followed by the same letter are not significantly different at the 5% level.

Each data point is the mean of duplicate analyses.

TABLE X

The Activity of Xanthine Oxidase in Milk as Influenced by Homogenization at 3500 psi and Storage at 4°C

Storage (Hours)	Xanthine Oxidase Activity (Units per ml)							
	Replication						Mean	Std. Dev.
	1	2	3	4	5	6		
2	0.2504	0.2528	0.2548	0.2460	0.2504	0.2548	0.2515[b]	0.0034
24	0.2592	0.2612	0.2592	0.2528	0.2572	0.2636	0.2589[a]	0.0037
48	0.2484	0.2504	0.2528	0.2460	0.2484	0.2528	0.2498[b]	0.0027
72	0.2396	0.2420	0.2440	0.2352	0.2420	0.2420	0.2408[c]	0.0031
96	0.2308	0.2352	0.2268	0.2332	0.2308	0.2352	0.2320[d]	0.0032

Note: Means followed by the same letter are not significantly different at the 5% level.

Each data point is the mean of duplicate analyses.

TABLE XI

The Activity of Xanthine Oxidase in Milk as Influenced by Homogenization at 4000 psi and Storage at 4°C

Storage (Hours)	Xanthine Oxidase Activity (Units per ml)							
	Replication						Mean	Std. Dev.
	1	2	3	4	5	6		
2	0.2572	0.2504	0.2612	0.2572	0.2528	0.2636	0.2582[b]	0.0049
24	0.2612	0.2656	0.2680	0.2700	0.2592	0.2724	0.2661[a]	0.0051
48	0.2572	0.2572	0.2548	0.2548	0.2504	0.2592	0.2556[b]	0.0031
72	0.2460	0.2484	0.2460	0.2460	0.2420	0.2548	0.2472[c]	0.0043
96	0.2396	0.2376	0.2396	0.2352	0.2332	0.2484	0.2389[d]	0.0053

Note: Means followed by the same letter are not significantly different at the 5% level.

Each data point is the mean of duplicate analyses.

TABLE XII

Analysis of Variance of Xanthine Oxidase Activity of Milk Homogenized at 2500, 3000, 3500 and 4000 psi and Subsequently Stored at 4°C for 2, 24, 48, 72 and 96 Hours

Source	Degrees of Freedom	Sum of Squares	Mean Squares	F Value
Pressure	3	0.007868196	0.002622732	243.36**
Storage duration	4	0.012778635	0.003194659	296.42**
Pressure x Storage	12	0.000203637	0.000016970	1.57
Replication	5	0.000328183	0.000065636	6.09**
Residual error	95	0.001023844	0.000010777	
Total	119	0.022202495	0.000186576	

**P < .01.

TABLE XIII

The Xanthine Oxidase Activity of Commercial Milk Products

Product	Processor	Product Analyzed: On the Day Day Purchase	Product Analyzed: 24 Hours after Purchase	Product Analyzed: 48 Hours after Purchase
		Units per ml		
Skimmilk	A	0.0680	0.0572	0.0507
	B	0.0463	0.0376	0.0333
	C	0.0593	0.0550	0.0507
Homogenized milk	A	0.0963	0.0854	0.0680
	B	0.1202	0.1093	0.1006
	C	0.0007	-	-
Whipping cream	C	0.2766	0.2701	0.2614
Half and half	C	0.0094	0.0072	0.0007

processor C contained 0.2766 units per ml when analyzed on the day of purchase and 0.2701 and 0.2614 units per ml respectively when analyzed 24 and 48 hours later. The skimmilk of different processors contained 0.0463 to 0.0680 units per ml when analyzed on the day of purchase but after 24 and 48 hours storage the activity was decreased. The homogenized milk of different processors contained 0.0007 to 0.1202 units per ml when analyzed on the day of purchase but the activity decreased after 24 and 48 hours storage. Half and half contained 0.0094 units per ml when analyzed on the day of purchase and lesser amounts after 24 and 48 hours storage. Thus the xanthine oxidase activity was greatest in whipping cream, a product containing the greatest fat concentration of those tested. The heat treatment given the commercial products was not available.

The results of these experiments show that the xanthine oxidase activity was at its maximum level after the raw milk was stored at 4°C for 24 hours but decreased upon further storage which is in agreement with the work of Gudnason and Shipe (20). The xanthine oxidase activity was increased when raw milk was heated to 55°C for 5 minutes and stored at 4°C for 24 hours but decreased upon further storage. The xanthine oxidase was partially inactivated when the milk was exposed to temperatures of 60, 65 and 70°C for 5 minutes and is almost completely inactivated when heated at 75°C for 5

minutes which is in agreement with the work of Kiermeier and Vogt (26). The activity of xanthine oxidase in milk gradually increased as the homogenization pressures increased from 1000 to 4000 psi which is in agreement with earlier work (17,20).

The xanthine oxidase activity was greater in whipping cream than in skimmilk, homogenized milk or half and half which agrees with the finding of Zittle et al. (52).

CHAPTER V

SUMMARY AND CONCLUSIONS

Raw milk obtained from the storage tank at the University of Tennessee Dairy Plant was studied for the influence of pasteurization temperatures, homogenization pressures and storage duration at 4°C on activity of xanthine oxidase.

General conclusions in this study may be summarized in the following statements:

The xanthine oxidase activity in raw milk was increased by storing milk at 4°C for 24 hours but decreased upon further storage. Raw milk stored for 24 hours contained 0.2275 ± 0.0051 units per ml.

The xanthine oxidase was activated when raw milk was heated at 55°C for 5 minutes. Milk heated at 55°C for 5 minutes had an activity of 0.2356 ± 0.0206 and 0.2373 ± 0.0146 units per ml after storage at 4°C for 2 and 24 hours, respectively. Xanthine oxidase was partially inactivated when exposed to temperatures of 60, 65 and 70°C for 5 minutes and was almost completely inactivated when heated at 75°C for 5 minutes.

The activity of xanthine oxidase in milk gradually increased as the homogenization pressures were increased from 1000 to 4000 psi. Milk homogenized at 4000 psi contained 0.2559 ± 0.0056 units per ml after being stored at 4°C for

2 hours, 0.2661 ± 0.0051 units per ml after storage at 4°C for 24 hours and lesser amounts when stored for longer periods.

The whipping cream contained 0.2766 units per ml when analyzed on the day of purchase but decreased upon further storage. The skimmilk of different processors contained 0.0463 to 0.0680 units per ml when analyzed on the day of purchase but after 24 and 48 hours storage the activity was decreased. The homogenized milk of different processors contained from 0.0007 to 0.1202 units per ml when analyzed on the day of purchase but decreased upon further storage. Half and half contained 0.0094 units per ml when analyzed on the day of purchase and lesser amounts of activity after 24 and 48 hours storage. The xanthine oxidase activity was greater in whipping cream than in skimmilk, homogenized milk or half and half.

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