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ARTICLE

Hydrogen Peroxide-Dependent Conversion of Nitrite to Nitrate as a Crucial Feature of Bovine Milk Catalase

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The enzyme catalase is well-known to catalyze the disintegration of hydrogen peroxide to water and oxygen; however, this study shows that its main function in bovine milk is oxidation of nitrite to nitrate. This process depends on hydrogen peroxide, of which the main source appears to be hydrogen peroxide formation that is coupled to the conversion of purines—xanthine in the present study—to uric acid by milk xanthine oxidase. However, additional secondary sources of hydrogen peroxide appear to be important during the relatively long storage of milk in the gland cistern. This paper demonstrates that the oxidation of nitrite to nitrate is necessary to prevent accumulation of free radicals and oxidative products during storage of milk in the gland and during the unavoidable delay between milking and pasteurization in dairy plants. Recommendations for minimizing the deterioration in milk quality during commercial storage are presented.

KEYWORDS: NO cycling; peroxidation; preformed radical products

INTRODUCTION

Catalases (CAT; e.g., human erythrocyte CAT, EC 1.11.1.6) are tetramers of four polypeptide chains, each over 500 amino acids long, that contain four porphyrin heme (iron) groups. Catalases are found in all kinds of living organisms and are best known for catalyzing the decomposition of hydrogen peroxide to water and oxygen (*1*), with turnover rates among the highest of all enzymes. One molecule of CAT can convert millions of molecules of H_2O_2 to water and oxygen each second because its catalytic efficiency is equal to the diffusion rate of H_2O_2 (*1*). The lopsided degradation of H_2O_2 by CAT depends on a heme cofactor with a bound iron atom, which is cycled between oxidation states (*2*). Many CATs have been shown also to be peroxidases and can oxidize short-chain alcohols, including ethanol, and other substrates in a two-step reaction that is H_2O_2 dependent (*3*−*6*). In the peroxidative mode, a CAT−H_2O_2 intermediate is formed, and the overall H_2O_2 decomposition reaction proceeds more slowly than when H_2O_2 is decomposed directly. These basic peroxidative functions of CAT were studied mostly more than five decades ago (*3*−*6*). Later it was reported that CAT is involved in the metabolism of ethanol and methanol in the liver (*7*, *8*) and in the central nervous system (*9*). However, the biological significance of many other peroxidative reactions of CAT (e.g., oxidation of formaldehyde, formalin, and nitrite) remained elusive.

Lactation is a metabolically highly demanding process of synthesis and transport of carbohydrates, proteins, and lipids, and it is crucial for the development of the newborn (*10*−*12*). In cows, every 10 L increase in milk production is associated with roughly doubling the maintenance requirements (*13*), and glucose turnover (*14*) and milk yield may vary between 30 and 70 L day^{-1} in modern dairy farms in many countries (*15*). The net uptake of glucose by the mammary glands of a lactating cow accounts for 60−70% of the whole body glucose turnover, and ∼70% of this is used for lactose synthesis (*14*). High glucose uptake by cells can result in increased oxidative stress because of excessive production of oxygen radicals during the autoxidation of glucose or glycosylated proteins or stimulation of cytochrome P450-like activity by the excessive NADPH produced by glucose metabolism (*16*, *17*). It appears that lactation in modern dairy cows is associated with considerable enhancement of glucose oxidation and, consequently, most probably with unavoidable imposition of oxidative stress.

Milk is a very complicated biochemical and biophysical substance (*18*). We have shown that bovine milk is a live bioreactor, into which H_2O_2 and NO are being constantly surged from the surrounding epithelial cells and milk leukocytes by the activities of enzymes such as xanthine oxidase (XO), superoxide dismutase, and nitric oxide synthase (*19*). Nitric oxide (NO) is constantly cycling in milk, through its autoxidation to nitrite and the conversion of nitrite to nitric dioxide by H_2O_2-dependent lactoperoxidase activity. In turn, the interaction of NO with cysteine-bearing groups on proteins forms nitrosothiols, which serve as a pool that constantly delivers NO into the system (*19*). Twice or thrice daily milkings are commonly practiced in modern dairy farming, which means that milk produced immediately after a milking is stored in the udder for as long as 8−12 h, during which the accumulated activity of free radicals may impair the milk quality.

Despite the fact that nitrite is the first product of NO autoxidation, in milk, as in blood, the NO-derived species accumulate

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mainly in the form of nitrate (*18*, *19*), which is much less active than nitrite (*20*). In blood, residual amounts of NO react with water to form nitrite, which, in the presence of heme groups in proteins such as myoglobin or hemoglobin, rapidly oxidizes to nitrate and the corresponding met-heme protein (*20*). However, milk does not contain hemoglobin or myoglobin; therefore, it is obvious that the equivalent mechanism in milk is different; in light of the fact that the pro-oxidative reaction repertoire of CAT includes the capability of oxidizing nitrite to nitrate (*6*), we hypothesized that the candidate for this function in milk is the bovine milk CAT (*19*). Saturation of the CAT capacity to convert nitrite to nitrate shifts the NO cycle to produce more nitric dioxide. This response would boost the protection against invasion of the mammary gland lumen by pathogens, albeit with reduction of the nutritive quality of milk, and increase susceptibility of the gland tissue to oxidative scarring (*18*, *19*, *21*).

The first aim of the present study was to provide direct evidence that CAT is the enzyme responsible for conversion of nitrite to nitrate in bovine milk. Second, we knocked down CAT activity with a specific inhibitor in order to assess the importance of CAT in maintaining the oxidative stability of milk during its storage in the gland for 8−12 h and during the 2−5 days that elapse between milking and pasteurization in the dairy plant.

MATERIALS AND METHODS

Spiking Experiments. In experiment 1, 50 mL samples of milk were obtained from the udders of each of six Israeli-Holstein cows. The sample from each cow was taken from a mixed yield of the udder. Preliminary analysis of every gland confirmed that the milk was taken from bacteria-free udders (*22*). The somatic cell count (SCC) in these samples was ~70000 mL^{-1}, which is typical of milk from bacteria-free udders (*22*). The milk samples were immediately stored in crushed ice and were transported to a nearby laboratory, where they arrived at a temperature of 6−10 °C and were analyzed within ~60 min. A duplicated 5 mL aliquot of each sample was transferred to tubes held in a rotating water bath that was thermostatically controlled at 37 °C. The samples were spiked with 40 μM nitrite and with 0, 10, 50, or 150 μM xanthine (Xa). Subsamples, each of 200 μL, were taken every 5 min for 60 min, for nitrite and nitrate determination. The rate of nitrite disappearance was calculated from the linear regression of log nitrite concentration with time. The effect of Xa on nitrite disappearance was further analyzed by plotting Xa concentration against the calculated rate constant during the first 30 min of the experiment.

In experiment 2, milk from six cows' udders was sampled and handled as in experiment 1. All samples were spiked with 40 μM nitrite and 150 μM Xa and with 0, 5, 10, or 30 mM 3-amino-1,2,4-triazole (3-AT), an inhibitor of CAT. Subsamples, each of 200 μL, were taken every 5 min for 30 min, for nitrite and nitrate determination. The rate constant was calculated as described above, and the effect of 3-AT on enzyme activity was analyzed by means of a Dixon plot, that is, from the regression of 3-AT concentration against the reciprocal of the nitrite disappearance rate constant.

In experiment 3, milk from six cows' udders was sampled and handled as in experiment 1. The rate of oxidation of 5-thio-2-nitrobenzoic acid (TNB) to 5,5′-dithiobis(2-nitrobenzoate) (DTNB) was measured as described previously (*19*) in intact milk and in milk that had been spiked with 30 mM 3-AT, 50 μM Xa, 10 μM nitrite, or a mixture of 10 μM nitrite and 30 mM 3-AT.

In experiment 4, a short-term storage test, milk samples were treated as in experiment 3 and were then held for 6 h in the dark at 4 °C. Lipid peroxidation was measured at the start and at the end of incubation.

Long-Term Storage Experiment. Six cows in which one gland was infected with coagulase negative staphylococci (CNS) and one or more of the other glands were free of bacteria were preidentified as described previously (*22*). CNS are the most prevalent bacteria in the infected mammary gland in Israel and in many other countries (*22*); therefore, we thought it interesting and appropriate to examine if infection with CNS increases the nitrosative stress in milk. About 50 mL of milk was obtained from each of the infected and noninfected glands of each of the cows and treated as in experiment 1. At the laboratory, subsamples from each gland were stored for 4 days in the dark at 4 °C, with and without addition of 30 mM 3-AT. The concentrations of nitrotyrosine, lipid peroxidation products, and carbonyls were determined at the start of incubation and at 1 day intervals.

Analytical Procedures. The XO (*19*) and CAT (*23*) activities were routinely determined in each of the milk samples with the aid of fluorescent substrates. Nitrate in raw udder-milk samples and nitrite and nitrate in spiked samples were determined with Griess reagent as described previously (*19*). Nitrotyrosine and carbonyls were determined in whey proteins (*24*, *25*). As the last stage in both determinations was colorimetric, the concentration was determined after clarification of the milk to a crystal-like solution following skimming and casein precipitation by ultracentrifugation, according to the method of Silanikove and Shapiro (*26*). Nitrotyrosine was determined by ELISA with monoclonal antibodies against nitrotyrosine (*24*), and carbonyls were determined by color reaction (*25*); both were related to the protein content in the whey, which was determined colorimetrically (*26*). Lipid−peroxides were determined in the separated milk fat samples (*26*) as described by Ostdal et al. (*27*). The TNB in milk samples was converted to DTNB according to the method of Li et al. (*28*), with slight modifications essentially as described previously (*19*).

Statistical Analysis. The short-term spiking experiments were analyzed with Graphpad prism 5 software (Graphpad, Chicago, IL). The nitrite oxidation constant was calculated from the linear regression of log nitrite concentration against time. The extent of inhibition of nitrite conversion to nitrate by 3-AT and the value of K_i, that is, the concentration of 3-AT that totally inhibited nitrite oxidation, were evaluated from the intercept of the Dixon plot, under conditions described in the text. The dependency of the nitrite conversion constant on XO activity was analyzed with the Michelis−Menten equation, and the parameters V_{max} and K_m were derived from the Lineweaver−Burk plot.

The data sets of the long-term storage experiment were analyzed by using repeated-measures analysis to model correlated residuals within cow, as described previously (*29*). The analysis concentrated on the effects of bacterial infection status (+ or −), day of storage (0−4), and (bacterial status) × (day of storage) interactions. The effects of bacterial infection status, (bacterial status) × (day of storage), parity, and number of days in milk were not significant ($P > 0.25$) and therefore are not included in the analyses presented here.

RESULTS

Spiking Experiments Confirmed That Milk Catalase Converts Nitrite to Nitrate. Milk samples in these experiments typically contained ~30 mU mL^{-1} of XO and 1.9 U mL^{-1} of CAT, which is consistent with previously reported data (*19*).

The data in **Figure 1A** show that in the absence of added Xa, a putative milk enzyme converts nitrite to nitrate at a rate of ~1 μM h^{-1}. Previously, we have shown that milk XO converted an added dose of 30 μM Xa to urate within 30 min ($t_{1/2}$ of ~10 min), in association with apparent formation of H_2O_2, with an H_2O_2 to uric acid molar ratio of 2:1 (*21*). Thus, the nitrite to nitrate conversion in the absence of added Xa should represent the basal conversion capacity of this enzyme in the gland cistern. Spiking milk with Xa increased the conversion of nitrite to nitrate in a dose-dependent manner, indicating that the activity of this enzyme is rate-limited by H_2O_2 availability (**Figure 1**). The rate constant of nitrite disappearance (nitrate formation) per minute for each level of Xa addition during the first 30 min was used to derive the disappearance rate constant (**Figure 1B**). Plotting the nitrite disappearance rate constant against Xa concentration shows classical Michelis−Menten kinetics (**Figure 1C**), indicating that the nitrite to nitrate conversion is indeed an enzymatic process. However, it is not possible to evaluate the actual dependency between the H_2O_2 concentration and the enzyme activity, because milk contains lactic peroxidase (LPO), which is the main consumer of H_2O_2 in milk (*19*).

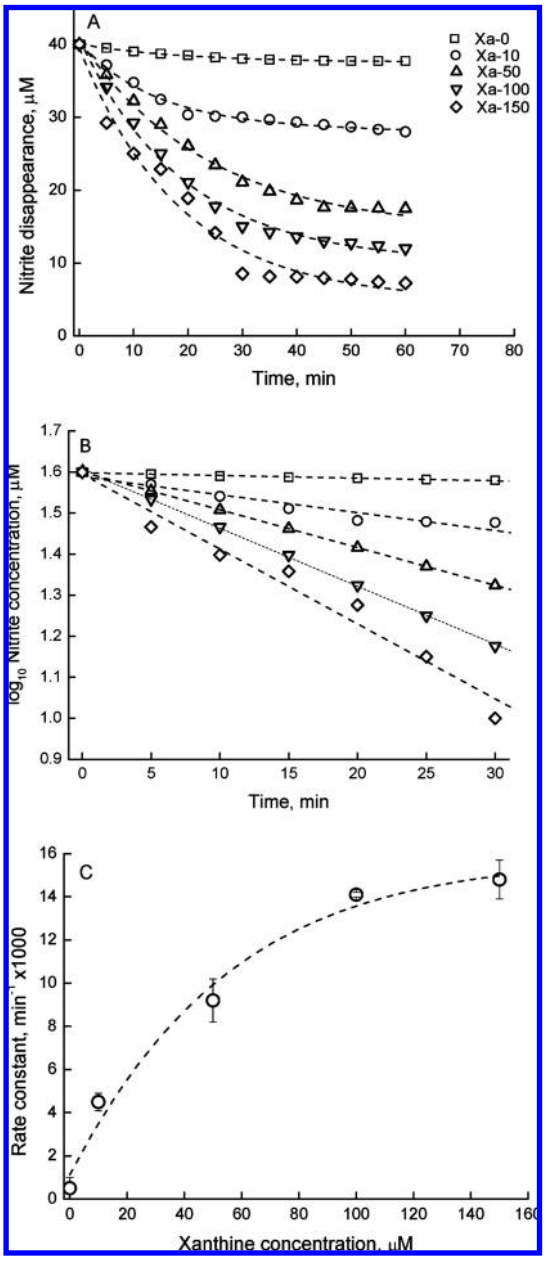

Figure 1. Effects of spiking milk with 40 μM nitrite in the presence of various levels of xanthine on (**A**) nitrite disappearance, (**B**) disappearance rate constant, and (**C**) dependency of the rate constant on xanthine level. It was verified that nitrite was converted to nitrate in a 1:1 molar ratio. The experiments were carried out in a rotating bath at 37 °C. The antilog of the intercepts did not differ from 40 μM, and R^2 of the regression lines = 0.97−0.99. Xa-0−Xa-150 = xanthine concentration in μM.

When the rate constant of nitrite disappearance was analyzed as a function of Xa concentration for the samplings between 30 and 60 min, the difference between treatments faded or disappeared. This result is in accordance with our previous finding that, after 30 min of incubation, virtually all of the Xa in milk was converted by XO to urate (*21*).

It is well-known that 3-AT is a noncompetitive inhibitor of CAT (*30*). A Dixon plot of 3-AT concentration against the reciprocal of the maximal conversion rate of nitrite to nitrate (**Figure 2**) clearly shows that 3-AT reduced the conversion of nitrite spiked into milk to nitrate in a dose-dependent manner. Thus, the data strongly suggest that the indigenous milk enzyme that is responsible for the conversion of nitrite to nitrate is CAT.

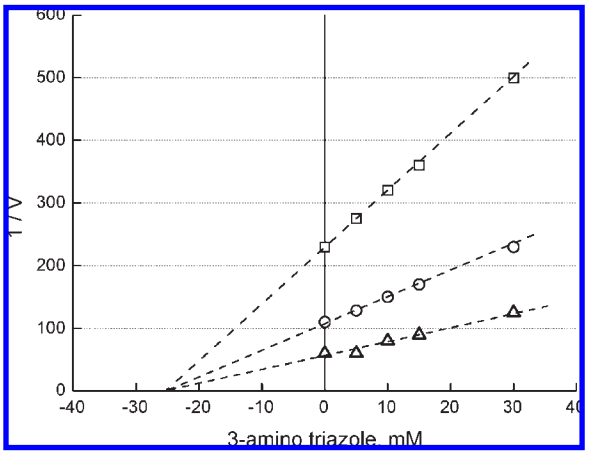

Figure 2. Dixon plot of the relationship between various levels of milk spiked with 3-AT and the reciprocal of the nitrite disappearance rate constant under three levels of xanthine spiking. The experiments were carried out in a rotating bath at 37 °C. Xanthine spiking levels were 50, 100, and 150 μM.

Table 1. Effects of Various Treatments of Milk Samples on the Immediate Oxidation of TNB to DTNB at Room Temperature and on the Accumulation of Lipid Peroxidation Products of Milk Samples Stored for 6 h under Cold and Dark Conditions

treatment	increase in lipid peroxidation, %	rate of DTNB formation, μM s^{-1}
no addition	8 ± 0.02	2 ± 0.02
+ 30 mM 3-AT	25 ± 0.2	4.7 ± 0.09
+ 50 mM Xa	8 ± 0.03	2 ± 0.08
+ 10 μM nitrite	12 ± 0.04	2.9 ± 0.04
+ 10 μM nitrite + 30 mM 3-AT	150 ± 5.2	20 ± 1.3

According to **Figure 2**, a concentration of ~25 mM 3-AT would result in complete inhibition (K_i) of CAT activity, which is consistent with the results of Margoliash and Schejter (*30*).

Physiological Range of Catalase Activity Protects Milk from Oxidation. Figure 1C enables derivation of the operative V_{max} and K_m of the peroxidative conversion of nitrite to nitrate by CAT in bacteria-free raw milk. Calculating V_{max} and K_m according to the Lineweaver−Burk plot (i.e., 1/xanthine concentration vs $1/V$) yielded a straight line with $R^2 = 0.98$ from which V_{max} of 17.4 min^{-1} × 1000 and K_m of 31.6 μM were derived. The physiological range of hypoxanthine (hXa) and/or Xa in alveolar milk was shown to be ~40 μM (*21*), indicating that milk CAT operates in the alveolus at ~25% above its operative K_m. According to **Figure 1B**, the activity of CAT under such conditions is ~1 μM min^{-1}. In the gland cistern, where no Xa was present (*21*), CAT oxidized nitrite to nitrate at a rate of ~1 μM h^{-1}, which is ~1.7% of its activity in the alveolus.

The TNB spiked into fresh raw milk was immediately converted into DTNB at a rate of ~1.5 μM s^{-1} (**Table 1**), in line with previous results (*19*). Inhibition of CAT with 30 mM 3-AT significantly increased the rate of DTNB formation 3-fold ($P < 0.01$). Spiking the milk with 50 μM Xa did not increase the rate of TNB oxidation, suggesting that the increased H_2O_2 formation under such conditions did not lead to increased NO_2 radical formation (*19*). Spiking the milk with 10 μM nitrite significantly increased the rate of DTNB formation by 50% ($P < 0.01$). The combination of spiking the milk with 10 μM nitrite and inhibiting CAT with 30 μM 3-AT increased the rate of DTNB formation 13-fold, to ~20 μM s^{-1} (**Table 1**).

Catalase Activity Is Essential for Maintaining Milk Quality during Short- and Long-Term Storage. Incubation of raw milk for

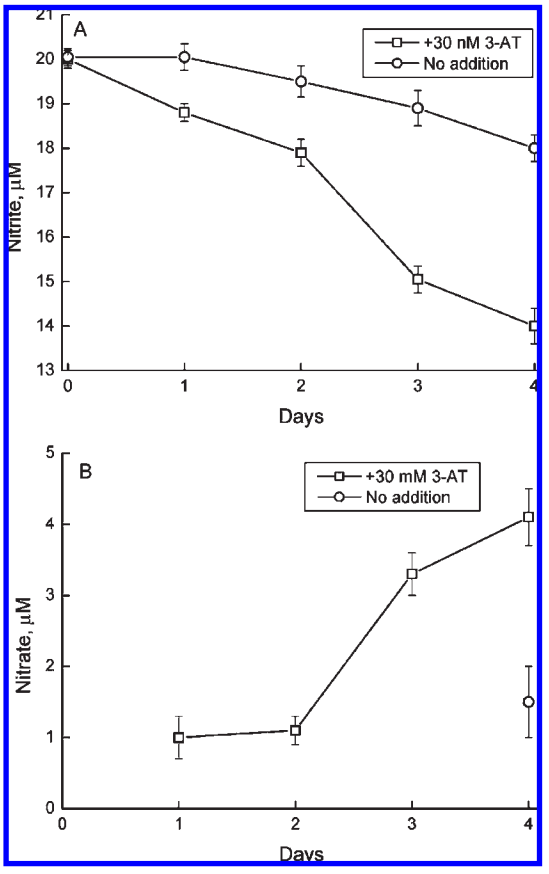

Figure 3. Effects of cold storage of milk samples for 4 days in the dark, with and without 30 mM 3-AT, on concentrations of nitrate and nitrite. Milk samples from six bacteria-free cows and six cows infected with CNS were used. As no differences between noninfected and infected cows were found (see Materials and Methods), the results were pooled. 3-AT, 3-aminotriazole.

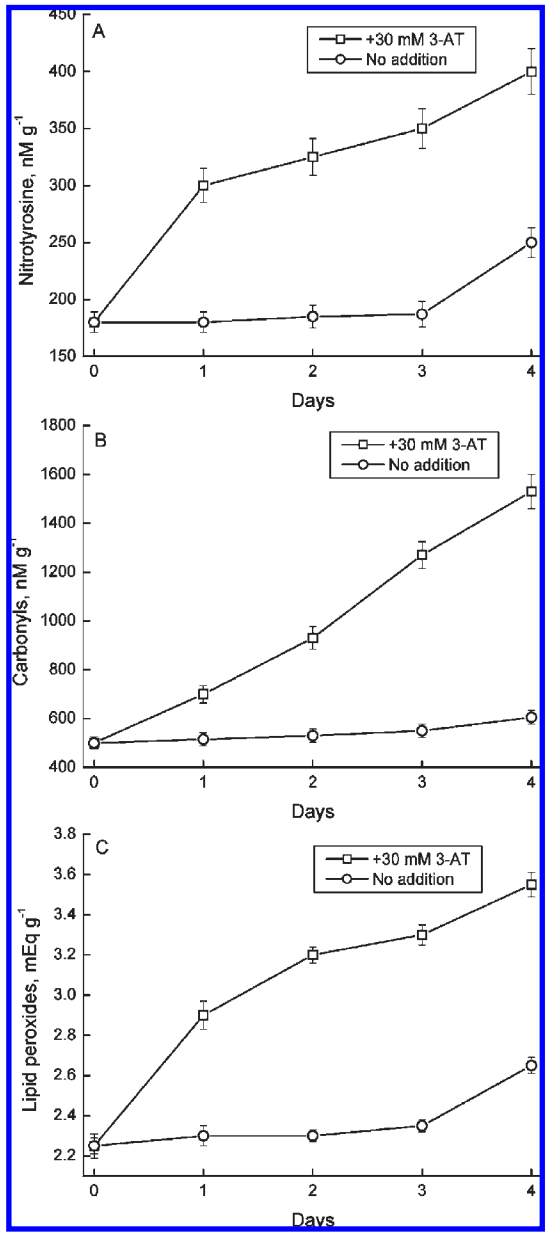

Figure 4. Effects of cold storage of milk samples for 4 days in the dark, with and without 30 mM 3-AT on concentrations of nitrotyrosine, carbonyls, and lipid peroxidation products. Milk samples from six bacteria-free cows and six cows infected with CNS were used. As no differences between noninfected and infected cows were found (see Material and Methods), the results were pooled. 3-AT, 3-aminotriazole.

6 h in the dark at 4 °C was associated with a ~5% increase in lipid peroxide (Lpx) products in milk fat (**Table 1**), consistent with the continuous production of free radicals in milk (*19*). Inhibiting CAT with 30 mM 3-AT increased the content of Lpx by ~20%, equivalent to a 4-fold increase over that in intact milk ($P < 0.01$). Spiking the milk with 50 μM Xa did not increase the Lpx content. This result shows that XO-derived H_2O_2, which is essential for the activity of CAT, does not induce an oxidation response in milk. Spiking the milk with 10 μM nitrite increased Lpx content by 10%, doubling the increase compared with that in intact milk ($P < 0.01$). The combination of spiking the milk with 10 μM nitrite and inhibiting CAT with 30 mM 3-AT increased Lpx concentration by ~150%, a significant ($P < 0.01$) 30-fold increase over that in intact milk (**Table 1**).

Milk samples for the long-term (4-day) storage experiment were taken individually from glands of 12 cows. Six of the samples were identified as infected with CNS and six were from bacteria-free glands (see Materials and Methods). These samples were stored for 4 days in the dark, with or without 30 mM 3-AT, and the results are depicted in **Figures 3** and **4**. Statistical comparison to determine the effect of infection showed that the results obtained from the CNS-infected glands did not differ from those of the bacteria-free glands; therefore, the data from bacteria-free glands and CNS-infected glands were pooled.

Fresh samples of milk contained ~20 μM nitrate, in line with previous findings with Holstein cows from the same herd (*19*). The nitrite concentration in these samples was below the nitrite detection level with Griess reagents (~1 μM), which is consistent with the finding of nitrite at about 200 nM in bacteria-free fresh milk (*19*). Nitrate concentrations in the 3-AT-treated samples dropped at a rate of 1−3 μM day^{-1}, to ~14 μM on the fourth day of storage; the largest decrease (from 18 to 15 μM) occurred between days 2 and 3. In parallel, nitrite concentration increased every day, to a level of 4.1 μM on day 4, with the largest increase (from 1.1 to 3.3 μM) occurring between days 2 and 3 (**Figure 3**). In milk samples with intact CAT activity, changes in nitrite/nitrate concentration were observed only during the fourth day of storage, when there was a drop in nitrate concentration to ~18 μM and an increase of nitrite to ~1.5 μM.

Fresh milk samples contained ~180 nmol g^{-1} of nitrotyrosine (NTyr) and ~490 nmol g^{-1} of carbonyls in the whey proteins and ~2.25 mequiv g^{-1} of Lpx in the milk fat, indicating that these

substances were formed either during milk storage in the gland or during its synthesis in the epithelial cells (**Figure 4**).

Inhibition of CAT with 3-AT resulted in a 70% increase in NTyr concentration (to ~300 nmol g^{-1}) in whey proteins on day 1, after which the NTyr concentration increased steadily on each additional day of storage, so that on day 4 it reached ~400 nmol g^{-1}, that is, a 2.2-fold increase above the basal level. In the intact samples, a significant increase in NTyr concentration to ~250 nmol g^{-1}, that is, a 20% increase over the basal level, was observed on day 4 (**Figure 4**).

Inhibition of CAT with 3-AT resulted in a 50% increase in carbonyls concentration, to ~750 nmol g^{-1} in whey proteins on day 1, and a steady increase on each additional day of storage so that on day 4 the concentration reached ~1500 nmol g^{-1}, that is, a 3.1-fold increase over the basal level. In the intact samples, a significant increase in carbonyls concentration to ~600 nmol g^{-1}, that is, a 20% increase over the basal level, was observed on days 1, 2, and 3. A further increase to ~1200 nmol g^{-1}, that is, a 2.2-fold increase over the basal level, was measured during the fourth day (**Figure 4**).

Inhibition of CAT with 3-AT resulted in a 30% increase in Lpx concentration (to ~2.9 mequiv g^{-1}) in milk fat on day 1 and a further steady increase on each additional day of storage, so that on day 4 the concentration reached ~3.5 mequiv g^{-1}, that is, a 60% increase over the basal level. In the intact samples, a significant increase in Lpx concentration to ~2.6 mequiv g^{-1}, that is, a 20% increase over the basal level, was observed only on day 4 (**Figure 4**).

DISCUSSION

The Fundamental Role of CAT in Milk Is the Conversion of Nitrite to Nitrate. Thurlov (cited in ref *31*) showed in 1925 that nitrite could be oxidized by milk peroxidase in the presence of the xanthine oxidase system. Heppel and Porterfield (*31*) showed in 1949 that the rate of nitrite oxidation by homogenates of rat liver and kidney depended on the content of CAT, that it could be increased by addition of crystalline CAT, and that it was coupled to peroxide-forming systems such as XO and D-amino acid oxidase. Thus, in the present study (**Figure 1**), we reconfirmed the previous conclusion (*31*), that a milk peroxidase, most probably CAT, exhibits its peroxidase function by utilizing H_2O_2 for the coupled oxidation of nitrite. Chance (*6*) showed in 1950, by applying his newly invented in vitro stop-flow technique, that pure CAT oxidized nitrite to nitrate following the formation of a CAT−H_2O_2 intermediate. Margoliash and Schejter (*30*) were among the first to show that 3-AT is a specific inhibitor of the formation of the CAT−H_2O_2 intermediate. Thus, the inhibition of nitrite oxidation in 3-AT-spiked milk, as shown in **Figure 2**, further supports the conclusion that CAT is the enzyme responsible for the oxidation of nitrite in milk.

One unit of CAT forms O_2 (or, conversely, degrades H_2O_2) at a rate of 1 μM min^{-1} (*1*), whereas its typical activity in bovine milk is ~2 U mL^{-1}. It is obvious that if CAT reacts in milk according to the classical picture, it would reduce the H_2O_2 content of the milk rather rapidly. However, relatively high levels of H_2O_2 at the micromolar range in various mammals indicate that in these cases CAT does not function in the milk according to the classical picture (*19*, *32*−*34*). It was also noted by Heppel and Porterfield (*31*) that CAT concentration required for nitrite oxidation in homogenates of rat liver and kidney was 1000 times that necessary for the ordinary catalytic decomposition of H_2O_2. Thus, one important mystery that remained to be solved lies in the mechanism responsible for shifting CAT activity from H_2O_2 disintegration reaction mode to the pro-oxidative mode.

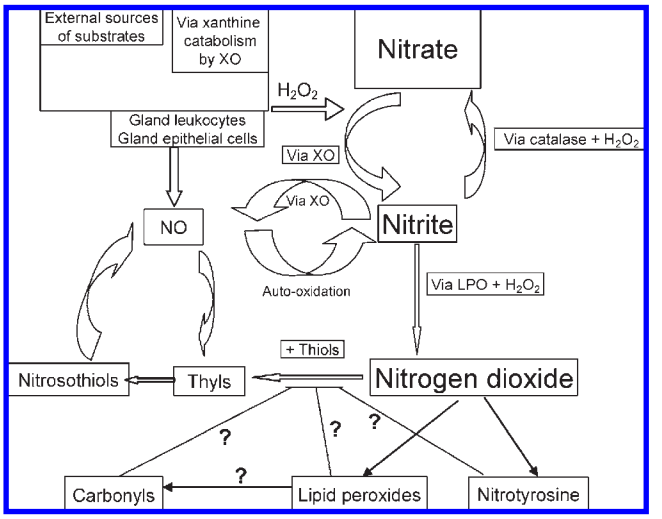

Figure 5. Scenario of NO cycling and the formation of nitric dioxide oxidation products in bovine milk. See ref *19* for details and justification of the basic NO cycle. The present data have shown that inactivation of catalase enhanced the formation of nitrotyrosine, lipid peroxidation products, and carbonyls. Question marks represent unknown pathways. XO, xanthine oxidase; NO, nitric oxide; LPO, lactic peroxidase.

High CAT activity was found in various exocrine secretions, such as saliva (*35*, *36*), nasal cavity fluid (*37*), lung fluid (*38*), seminal fluid (*39*, *40*), and tears (*41*). It is also noteworthy that tears in various mammals contain a relatively high concentration—in the range of 30−80 μM—of H_2O_2 (*41*, *42*). It is, therefore, tempting to speculate that one of the main roles of CAT in these exocrine fluids, as in milk, is the detoxification of nitrite and of foreign substances such as alcohols, formaldehyde, and formic acid. In general, in nature (*35*−*41*) the mucosal surfaces of exocrine secretions contain a mixture of XO (a superoxide and an H_2O_2-forming enzyme), peroxidative enzymes such as LPO (e.g., ref *43*), and antioxidants such as superoxide dismutase and CAT and have a physiological significance in antimicrobial defense. Thus, the NO cycle observed in bovine milk (*19*) (**Figure 5**) may also function in other exocrine secretions.

Cellularly Preformed Radical Products Are Secreted into Milk. The presence of NTyr and carbonyls on proteins and Lpx products in the fat in cow's milk at milking indicates that these substances were formed either in the epithelial cells during milk synthesis, during the storage of milk inside the glands, or both. In light of their high basal level at milking and of the absence or slow evolution rates of NTyr, carbonyls, and Lpx during storage in CAT-intact milk (**Figures 3** and **4**), it is most probable that these substances are produced in the epithelial cells and are then secreted into the milk. This conclusion is consistent with new evidence and reinterpretation of existing knowledge that have led to the hypothesis that oxidative damage to the mammary gland epithelial cells is among the causes of the declining phase of lactation in mammals (*44*). In mice, deletion of the PPAR-γ gene produced milk with elevated levels of oxidized lipids and caused inflammation, alopecia, and growth retardation in the nursing neonates (*45*).

In the present study and elsewhere it has been shown that cow's milk contains NTyr (present study and ref *46*), carbonyls (present study) on proteins, and carbonyls (*47*) and Lpx on fat (present study and refs *47*−*49*, for example). Nitrite and nitrates—which are precursors of free radicals—are constituents of cow's milk (*19*), and their content in foods is under legislative constraints in many countries. Bovine milk is a major raw material for manufacturing infant formula and, in light of the present

results, it should come as no surprise that such formulas contain Lpx products (*50*) and, most likely, components such as NTyr and carbonyls. The presence of free radical products and their precursors in the food chain may contribute to the total reactive oxidative load that infants have to deal with, and they are considered to be factors in the etiology of common infants' and preterm infants' pathogenesis, such as necrotizing enterocolitis (*51*, *52*), bronchopulmonary dysplasia (*52*), and type I diabetes (*53*). Epidemiological studies have shown that bovine milk is a safe food that contributes positively to preventing obesity and metabolic syndromes (*54*, *55*) in addition to being an almost irreplaceable source of dietary calcium, particularly for adolescents (*56*) and postmenopausal women (*57*). Nevertheless, there also is epidemiological evidence that consumption of cow's milk during the first year of life predisposed infants to type I diabetes, although the basis for that remained elusive (*53*). Currently, the National Health and Medical Research Council of Australia (*58*) and the American Academy of Pediatrics (*59*) recommend that cow's milk should not be used by infants aged less than 12 months, other than in small amounts in food.

The combination of the present results, which show that bovine milk contains free radicals, their precursors, and oxidative modified products, on the one hand, and the concept that type I diabetes is possibly due to the selective death of β-cells as a result of a nonspecific inflammatory attack by radicals (*53*, *60*), on the other hand, opens a new way to account for the link between consumption of cow's milk by infants under the age of 12 months and susceptibility to type I diabetes. Because of infants' lower body mass and higher surface-to-mass ratio than those of adults, consumption of food with free radicals exposes them to relatively higher burdens of these substances. Thus, we would suggest that there is an urgent need to develop meticulous safety criteria and safety standards that would limit the contents of radical precursors and radical-preformed oxidized substances in dairy products intended for use in infants' formulas, in a manner that could be knowingly applied by the food industry.

CAT Protects Milk Quality during Storage of Milk in the Udder. In the present study we found that the CAT-dependent conversion rate of nitrite to nitrate in the absence of Xa in the medium was low compared with that in the presence of Xa. However, the former process is still of physiological significance because the normal nitrite content in milk from bacteria-free glands is ~0.2 μM. CAT activity at a rate close to the enzyme K_m for nitrite oxidation in the alveolus and its residual activity in the gland cistern is the most likely reason for the low nitrite level in milk. The cows sampled in the present experiment produced ~40 L milk per day with 20 μM nitrate, indicating that at least 0.8 mM nitrite had to be oxidized to nitrate. We have shown that with a typical substrate (Xa or hXa) concentration at 50 μM, milk CAT can convert ~1.4 mM nitrite to nitrate, which explains the low level of nitrite in milk. Furthermore, despite the upsurge in NO formation during bacterial infection (*21*) and the inflammation response in the mammary gland (*19*), the nitrite concentration remains relatively low (up to 10 μM) compared with the increase in nitrate (up to 1200 μM) concentration, which most likely relates to CAT activity and is consistent with increase in CAT activity during mastitis (*18*).

The occurrence of CAT activity in the absence of Xa suggests that secondary sources of substrate for CAT are present in milk and accounts for the formation of H_2O_2 in fresh milk at a rate in the low micromole per hour range. Although these sources were not identified in the present study, the presence of glucose−glucose oxidase, free L-alanine−L-alanine oxidase, or gluthathion−gluthathion oxidase in bovine milk and milk of other mammals (*18*) suggests that these systems are the source of low-level H_2O_2 formation. The ability of glucose−glucose oxidase (*61*) and L-alanine−L-alanine oxidase (*32*) to serve as effective sources of H_2O_2 in milk was demonstrated. Thus, CAT appears to play an essential role in maintaining milk quality during storage in the gland, which identifies it as a component of the anti-inflammatory restraining arm of the mammary gland innate immune system.

CAT Is Essential To Protect Milk Quality during Commercial Storage. In dairy farming in Israel, the milk is stored for 1−3 days in the farm tank, so that milk in the dairy silo may be up to 3−5 days old before its pasteurization. It is commonly recognized that milk quality deteriorates with time and that holding milk for a long time impairs its quality with respect to bacteriological standards as well as with regard to suitability for cheese production (*61*, *62*). It is also well documented that poor animal health impairs milk quality through the activation of the innate immune system, which increases the release of deteriorative enzymes into the milk, the quality of which is subsequently affected by their action (*22*, *61*, *62*). In practice, bulk milk with high SCCs results in diminished cheese yield. Thus, in light of the above findings, it was considered interesting to examine the role of CAT under conditions that simulate its action during commercial milk storage.

The present results show unequivocally that the presence of intact CAT in milk is essential for preventing accumulation of basal NTyr, Lpx, and, to a lesser extent, carbonyls, under conditions simulating commercial milk storage. The accumulation of these products in milk samples in which CAT was inactivated is consistent with the concept that free radicals, which are derived mainly from NO metabolism, are constantly cycled in milk and that CAT plays an essential role in restraining this cycle by oxidizing nitrite to nitrate (see **Figure 5** for further explanation). The lack of effects of CNS infection on indices reflecting nitrosative stress is consistent with a recent finding (*21*). Thus, in cows the damage caused by CNS infection is mostly proteolytic (*18*, *22*), but not oxidative.

The elevation in NTyr, Lpx, and carbonyls contents in the unspoiled samples that were stored for more than 3 days suggests that CAT became ineffective, most likely because the secondary sources (glucose and free amino acids) for H_2O_2 formation had been exhausted by then. Whether nitrites, nitrates, and *N*-nitroso compounds in foods and drinking water affect human health is a controversial issue (*63*). Available epidemiological information regarding the effects of consumption of carcinogenic *N*-nitroso compounds relates mainly to ingestion of the foreign substance *N*-nitrosodimethylamine (*63*). Increases in NTyr, Lpx, and carbonyls levels are a common outcome of systemic oxidative stress, and there is a general agreement that increased concentrations of these substances predispose humans to diseases such as cancer and atherosclerosis. However, in the literature, we could not find data on the significance of these metabolites in foods. Because newborn infants are particularly susceptible to the detrimental effects of nitrite, the regulatory upper limits applied to the production of milk-based infant formulas are generally low: 40 mg kg^{-1} for nitrate and 0.5 mg kg^{-1} for nitrite (*64*). The milk samples used in the present study contained nitrate and nitrite at 12 and 0.1 mg kg^{-1}, respectively, that is, well within these limits. These contents were calculated on the basis of typical dry matter content in milk of 100 g kg^{-1}; the value for nitrite was calculated according to the method given in ref *19*. However, by the fourth day of storage the nitrite level in intact milk rose to 0.7 mg kg^{-1}, which is above the permitted level. Thus, our data suggest that these levels should be evaluated under farming conditions, and if the present results are confirmed, the issue of allowable storage duration of unprocessed milk, particularly for baby food formulation, should be evaluated by the appropriate authorities.

The present results as summarized in **Figure 5** are consistent with the essential function of CAT in restraining this cycle by oxidizing nitrite to nitrate. The potential interactions between the formation of NTyr and lipid peroxidation products, on the one hand, and the formation of carbonyls, on the other hand, as well as the competition of formation of these substances with that of nitrosothiols are theoretically possible (*65*, *66*). Blocking the formation of nitrosothiols would further divert nitric dioxide toward the formation of oxidized products. Some of the present results indirectly imply that carbonyls are formed as second-wave products of preformed nitrotyrosine and lipid peroxides: (i) catalase attenuated only the accumulation of carbonyls and (ii) the contents of carbonyls, NTyr, and lipid peroxidation products increased sharply between days 2 and 3 of storage of milk treated with 3-AT.

In humans, CAT gene polymorphism is a familiar problem, associated with a range of stress-related oxidative diseases such as atherosclerosis, diabetes, dyslipidemia, and neurodegenerative disease (*67*). We could not find equivalent information regarding CAT polymorphism in bovines. However, in light of its essential contribution to milk-quality maintenance and because of the importance of cow's milk in human nutrition, we emphasize the importance of ensuring that this enzyme is fully functional in each cow acquired by a commercial dairy farm.

In conclusion, this study highlights the presence of oxidized substances in the form of nitrotyrosine, lipid peroxidation products, and carbonyls, which are most likely related to oxidative stress reactions in the secretory epithelial cells. We show that prevention of further accumulation of these substances during the unavoidable storage of milk in the udder, and also between milking and pasteurization in the dairy, depends to a large extent on the presence of intact catalase in the milk. We show that this attribute of catalase relates to H_2O_2-dependent oxidation of nitrite to nitrate. As bovine milk is a major raw material for infant food formulations, we suggest that further research into the role of this enzymatic system in milk is needed and that this could lead to establishment of new regulations to ensure safety in this sector.

ABBREVIATIONS USED

CAT, catalase; 3-AT, 3-amino-1,2,4-triazole; CNS, coagulase negative staphylococci; DTNB, 5,5′-dithiobis(2-nitrobenzoate); hXa, hypoxanthine; LPO, lactic peroxidase; Lpx, lipid peroxide; NTyr, nitrotyrosine; TNB, 5-thio-2-nitrobenzoic acid; Xa, xanthine; XO, xanthine oxidase.

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