PREVENTION OF THE SULFIDE FLAVOR IN HEATED MILK BY OXIDATION WITH HYDROGEN PEROXIDE

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ABSTRACT

PREVENTION OF THE SULFIDE FLAVOR IN HEATED MILK

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By

Richard Marc Perlmutter

The sulfide flavor of heated milk results from the release of volatile sulfides from the heat unstable sulfhydryl groups located mainly in the fat globule membrane proteins and β-lactoglobulin. Hydrogen peroxide was used to oxidize these -SH groups to a heat stable form which prevented the volatilization of the sulfur.

To quantify the effect of the hydrogen peroxide treatment, a model system was used consisting of 150 mg β-lactoglobulin AB and 1.00 g lactose dissolved in 20 ml of simulated milk ultrafiltrate. Hydrogen peroxide concentrations were varied from 0.01 to 0.10% (w/v). Heat-treated samples were processed at 85 C for 12 min; other samples were maintained at room temperature. In all cases the peroxide was destroyed by the addition of an excess of catalase following the heat treatment.

When peroxide was present in the system during heating, all the -SH groups of the protein were destroyed, but none of the disulfides were destroyed. Amino acid analyses revealed that all the cysteine residues were oxidized to cysteic acid residues in the 0.10% peroxide-containing samples. But only about 30% was converted in the samples containing 0.01% peroxide. Through the whole range of peroxide

concentrations used, no -SH or -SS- destruction was found in the absence of the heat treatment. Methionine destruction increased from 7 to 45% through the range of peroxide concentrations in the heat-treated samples; and there was no methionine destruction in the samples not receiving a heat treatment. Amino acid analyses of the hydrogen peroxide-containing, heat-treated samples failed to detect the presence of either methionine sulfoxide or methionine sulfone, both possible oxidation products of methionine.

Changes in solution opacity and polyacrylamide gel electrophoretic patterns indicated that the peroxide treatment decreased protein polymerization in the heated samples. Also, nonprotein nitrogen determinations indicated that none of the treatments caused a significant amount of protein breakdown.

Whole milk containing 0.01% (w/v) hydrogen peroxide was heat treated at 120 C for 4 sec and fed to young rats to measure possible nutritional loss due to processing. The rats gained the same amount of weight consuming this milk as they did consuming a pasteurized whole milk control. Thus, the treatment did not adversely affect the nutritional value of the milk.

Another lot of whole milk was treated with 0.01% (w/v) hydrogen peroxide, heat sterilized at 143 C for 2 sec, and aseptically canned. In taste panels conducted 3 and 9 weeks following processing, panelists exhibited a marked preference for a commercially pasteurized whole milk control over the peroxide-treated, heat-sterilized milk. Though the peroxide treatment prevented sulfide formation, it failed to inhibit the development of the caramelized flavors associated with heat-sterilized milk.

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INTRODUCTION

Heat-sterilized milk has never enjoyed wide consumer acceptance in the United States because of its cooked flavor. One component of this flavor is caused by the volatilization of sulfur from the sulfhydryl groups present in the milk proteins. When milk is heated above a critical temperature of around 70 C, sulfhydryl groups are activated and sulfur is volatilized, resulting in a sulfide flavor.

In this research a means was sought to prevent the volatilization of sulfur and the concomitant appearance of a sulfide flavor. Oxidation of the sulfhydryl groups was carried out using hydrogen peroxide which was later destroyed with catalase. The rationale was to convert cysteine sulfur to a heat-stable form, such as to disulfides or cysteic acid residues. Hydrogen peroxide was chosen as the oxidizing agent because it is one of the very few chemicals that can legally be added to milk, though presently only as a preservative in the manufacturing of cheese. Also, it can be decomposed in the milk to water and oxygen by the addition of the enzyme catalase.

Quantitative experiments were performed using a model system consisting of β-lactoglobulin AB dissolved in simulated milk ultrafiltrate containing lactose. Sulfhydryl groups, disulfides, and methionine were measured before and after treatment with hydrogen peroxide and/or heat. In selected samples all the amino acids were assayed to detect changes of nutritional importance. Physical changes in the protein were investigated using polyacrylamide gel electrophoresis. Determinations of

solution opacity and nonprotein nitrogen were also made.

Following tests conducted with the model system solutions, a batch of hydrogen peroxide-, heat-treated milk was prepared and fed to young rats to determine if this treatment reduced the nutritional value of the milk. Also, milk processed in a similar way was aseptically canned and stored at room temperature for up to nine weeks. This milk was judged organoleptically and compared to commercially pasteurized milk to evaluate its acceptance by consumers.

REVIEW OF LITERATURE

In keeping with the emphasis of this research, the literature review will relate mainly to β-lactoglobulin and how it is affected by treatment with heat and hydrogen peroxide.

β-Lactoglobulin: Its Discovery

β-Lactoglobulin, the most abundant protein in milk serum, was first prepared in pure form by Palmer (1934). It accounts for 7-12% of the total milk protein and about half of the whey protein (Jenness *et al.*, 1956). Pedersen (1936) subjected Palmer's protein to ultracentrifugal and electrophoretic analysis and concluded that the protein was homogeneous. It was given the name β-lactoglobulin by Cannan *et al.* (1942) based upon its location in the sedimentation pattern of milk serum.

Later electrophoretic studies by Li (1946) and Polis *et al.* (1950) indicated that β-lactoglobulin is not homogeneous. The latter workers reported that it was probably a mixture of two similar proteins which they designated β_1-lactoglobulin and β_2-lactoglobulin. Aschaffenburg and Drewry (1955) purified β-lactoglobulin from individual cows and found the cows had one, or the other, or both electrophoretically discernible types described by Polis *et al.* (1950). Further work by Aschaffenburg and Drewry (1957a) showed that the biosynthesis of the two types of β-lactoglobulin was genetically controlled. In keeping with the nomenclature of genetic variants, they renamed the types

β-lactoglobulin A and β-lactoglobulin B, the A variant having the faster electrophoretic mobility at pH 8.6.

More recently Bell (1962) discovered a third variant, β-lactoglobulin C. It migrates more slowly than variants A and B in zonal electrophoresis performed at alkaline pH. A D variant also has been reported (Grosclaude *et al.*, 1966). And there are variants associated with Australian Droughtmaster cattle (Bell *et al.*, 1966) which are believed to be alleles of the A and B variants. These have been designated as variants A_{Dr} and B_{Dr}.

Only one or two variants are present in the milk of an individual cow. The A and B variants occur in the milk of most common dairy breeds, the B variant occurring more frequently than the A variant. Variant C has been found almost exclusively in the milk of the Jersey breed; and variant D is present in the milk of Montbéliarde, Simmental, and Danish Jersey cows (Aschaffenburg, 1968).

β-Lactoglobulin: Its Physico-chemical Properties

β-Lactoglobulin is a member of the lactalbumin fraction of milk proteins because it is soluble in a one-half saturated solution of ammonium sulfate at neutral pH or in a saturated magnesium sulfate solution (Jenness *et al.*, 1956). But it behaves like a globulin by virtue of its insolubility in water and its solubility in dilute salt solutions near its isoelectric point of pH 5.2 (Tilley, 1960).

From studies done by Green and Aschaffenburg (1959), crystalline β-lactoglobulin has been shown to be composed of two identical subunits related by a twofold axis. The molecular weight of the dimer is 36,000 Daltons and it has a sedimentation coefficient of 2.7 S. (Townend *et al.*, 1964). The dimer, however, is not the major configuration under many

conditions. Temperature, protein concentration in solution, nature of the solvent, pH, and type of genetic variant present influence the association properties of the monomer. For a review of the effects of these variables see McKenzie (1967).

Titration experiments by Cannan *et al.* (1942) indicated that the dimer unit had 58 carboxyl, 34 amino, 6 imidazole, and 6 guanidino groups. Later determinations made using column chromatography techniques showed that some of these values were incorrect. Tanford and Taggert (1961), also using titration curve data, showed that the conformational transition in β-lactoglobulin near pH 7.5 is due to the exposure of two buried carboxyl groups. Below pH 7.5 these groups are unavailable for titration. This transition is accompanied by a change in the sedimentation constant and optical rotation properties of the protein.

Also of importance are the tendencies for the A variant to undergo a dimer-octamer association reaction, especially in the cold (Timasheff and Townend, 1961), and to be more resistant to heat denaturation than are the other variants (Gough and Jenness, 1962; Sawyer, 1968).

The amino acid compositions of the A and B variants have been reported by Gordon *et al.* (1961) and Piez *et al.* (1961). Kalan *et al.* (1964) determined the amino acid composition of the C variant and Brignon *et al.* (1969) reported the composition of the D variant. There are 324 amino acid residues in the dimer unit of all the variants studied. Amino acid differences among the variants of known composition are:

Variant:	A	B	C	D
	Asp	Gly		
	Val	Ala		
		Gln	His	
		Glu		Gln

With respect to the other milk proteins synthesized in the mammary gland, β-lactoglobulin is unique because it is the only one known to contain free -SH groups. The protein contains two cysteine and four cystine residues in the 36,000 Dalton dimer unit.

The Effect of Hydrogen Peroxide Treatment

Hydrogen peroxide has been used as a milk preservative since before the turn of the century (Lück, 1956), but only in recent years has its effect on milk constituents, especially the proteins, been investigated.

Milk is treated with hydrogen peroxide to destroy bacteria and hence preserve the milk. It is used mainly in hot climates where dairy farms lack refrigeration. Before consumption the milk containing the peroxide is treated with catalase, an enzyme which breaks down hydrogen peroxide to water and oxygen. Following such treatment, the milk is free of the undesirable metallic flavor imparted by the peroxide.

Regulations regarding the use of hydrogen peroxide in milk in the United States are given in the Code of Federal Regulations, Title 21, Part 19.5, which allows the use of hydrogen peroxide only in milk used for the manufacture of Cheddar, Swiss, washed curd, colby, and granular cheese. Milk can be treated with hydrogen peroxide at a concentration of no more than 0.05% by weight. The peroxide must be destroyed with catalase added to the milk at a concentration of not more than 20 ppm.

Hydrogen peroxide, especially when used with high temperature storage, destroys most of the vitamin C in milk (Lück, 1956). However, because milk is not a major source of this vitamin, the loss is not considered to significantly decrease the nutritional value of the milk. Gregory *et al.* (1961) used a microbiological assay technique to determine the effect of hydrogen peroxide treatment on the vitamins in milk other

than vitamin C. The concentration of peroxide used was 0.05% (w/v); and the milk was stored for 8 hr at 24 C, and then treated with catalase to destroy the peroxide. They reported no significant loss of biotin, nicotinic acid, calcium pantothenate, riboflavin, thiamin hydrochloride, vitamin B_{12}, vitamin A, carotene, or α-tocopherol in the hydrogen peroxide-treated milk.

In a similar study Tepley *et al.* (1958) employed a microbiological assay procedure to measure vitamin losses in cheese made from hydrogen peroxide-treated milk. They used hydrogen peroxide concentrations ranging from 0.1 to 0.5% (w/v); and the milk samples were held at 120 F (49 C) for 10 min or pasteurized at 160 F (71 C) for 20 min prior to the addition of peroxide. In all the samples tested they found no significant loss of niacin, thiamin, riboflavin, vitamin B_6, pantothenic acid, folic acid, vitamin B_{12}, or β-carotene.

Experiments investigating changes in amino acid concentration following hydrogen peroxide treatment indicated a loss in cystine, methionine, lysine, and tyrosine. Tepley *et al.* (1958) found decreases in lysine and methionine in the range of 10 to 25%. They called these losses induced losses because they were found only in the cheese, and not in the milk used, or the whey produced, in the cheese manufacture. Gregory *et al.* (1961) measured only methionine and found a 15 and 7% drop in 'available' and total methionine, respectively. Both groups of workers used microbiological assays to measure the methionine lost.

Schmidt *et al.* (1969) relied on a chemical test to measure the methionine content of isoelectric casein. They found a 19% loss following treatment with 1% hydrogen peroxide at 54 C for 15 min. They postulated that this methionine loss may be a cause of the softness sometimes found in rennet cheese made from hydrogen peroxide-treated milk. The

hydrogen peroxide oxidizes methionine which is the N-terminal end of the glycomacropeptide split off by the rennet. The oxidized form is not split off, thus blocking the primary phase of rennet action.

Zweig and Block (1953) studied the sulfhydryl content of skimmilk treated with 0.03% hydrogen peroxide and held 20 hr at 25 C. Using an amperometric titration apparatus, they found no decrease in -SH content following the peroxide treatment.

The nutritive value of milk proteins is only slightly affected by hydrogen peroxide treatment. Tepley *et al.* (1958) reported no change in protein quality on the basis of rat feeding studies; and Gregory *et al.* (1961) found that the total nutritive value (biological value x true digestibility x 100) was 6% lower for the hydrogen peroxide-treated milk than for the untreated controls. They attributed this loss to the partial destruction of methionine.

The effect of hydrogen peroxide on the electrophoretic gel patterns of milk proteins has been studied by Grindrod and Nickerson (1967). They treated selected proteins in solution with 1% hydrogen peroxide which was destroyed after 30 min with catalase. For the first 10 min the test solutions were agitated at 49.9 C, and for the next 20 min they were held at 26 C in a quiescent state. No generalizations could be made about the results. The mobility of the α_s-casein was decreased by hydrogen peroxide treatment. β-Lactoglobulin mobility was also decreased and the initial two bands corresponding to the A and B variants were replaced by one band. The mobility of β-casein increased and its electrophoretic band became more diffuse. Bovine serum albumin also increased in mobility. However k-casein and α-lactalbumin exhibited no change due to the peroxide treatment. The two bands of β-lactoglobulin

AB gradually disappeared as exposure to 1% hydrogen peroxide was increased from 10 min to 24 hr. These workers also reported a slight decrease in whey protein nitrogen accompanied by a slight increase in nonprotein nitrogen as skimmilk was exposed to 0.5% hydrogen peroxide for increasing time periods.

The Effect of Heat Treatment

Heating causes many changes in milk, including such diverse phenomena as enzyme inactivation, changes in ionic equilibria, and protein-protein interactions. However, only those changes germaine to this study are discussed here.

The most immediately noticeable characteristic of heat-treated milk is the development of a sulfide flavor caused by raising the milk above a critical temperature of about 70 C. According to Blankenagel and Humbert (1963), this phenomenon is actually the last of a series of changes that milk undergoes when heated. First the proteins in the milk are denatured; then the sulfhydryl groups are activated (made more accessible for reaction); and finally a cooked flavor appears. They also found that the -SH titer (thiamine disulfide reducing compounds) and the cooked flavor intensity both reached a maximum when the skimmilk used was heated to 265 F (129 C) for 3.5 sec. Sulfur in unheated milk does not react with thiamine disulfide (i.e., TDS). But in milk heated above the critical temperature, the sulfur causes a positive TDS reaction.

In a somewhat similar study, Boyd and Gould (1957) made simultaneous determinations of volatile -SH (measured as hydrogen sulfide by the methylene blue test (Fogo and Popowsky, 1949)) and nonvolatile activated -SH (TDS test) in heated milk. Thiamine disulfide values were highest

at the time the maximum heating temperature of 90 C was first reached. With holding, hydrogen sulfide was liberated and the thiamine disulfide values dropped 20-22%. From these findings they concluded that the formation of -SH volatiles included a step where the sulfur was reactive to thiamine disulfide. Zweig and Block (1953) also measured the sulfhydryl content of milk during heating. They noted that at the critical temperature the -SH content began a sharp decrease and that a sulfide flavor appeared. They concurred with the findings of Larson and Jenness (1952) that the loss of -SH followed first order reaction kinetics. An excellent review which discusses the activity of -SH groups in heated milk was published by Jenness (1954).

The source of the -SH groups responsible for sulfide flavor development has been investigated by several workers. Townley and Gould (1943a) discovered that the easily volatilized sulfur was associated with the milk serum and the fat globule membrane. They found more sulfur in the serum than in the membrane. But the sulfur in the membrane had a lower critical temperature and was more easily volatilized than the sulfur in the milk serum. Boyd and Gould (1957) found that the amount of hydrogen sulfide liberated and the TDS values decreased for the following products in the order listed: buttermilk, cream, milk, and whey. This sequence is in agreement with the findings of Townley and Gould (1943a).

Hutton and Patton (1952), after screening the various fractions of milk, reported that the -SH in β-lactoglobulin accounted for practically all of the -SH in milk. To confirm the role of the -SH groups of β-lactoglobulin, they doubled the content of the protein in a sample of milk and heated it. The number of activated -SH groups and the intensity of the sulfide flavor were both noticeably greater than in a sample of normal milk receiving the same heat treatment. They suggested that the

conversion of protein-SH to hydrogen sulfide may be the primary cause of cooked flavor.

According to Boyd and Gould (1957), low temperature preheating (66 C for 30 min) prior to high temperature treatment (90 C for 30 min) was responsible for a significant drop in both thiamine disulfide values and the evolution of hydrogen sulfide. However, Townley and Gould (1943a) had previously observed that similar preheating caused almost no decrease in hydrogen sulfide liberation when the milk was later heated to 95 C. Zweig and Block (1953) added the oxidizing agents hydrogen peroxide and potassium bromate to unheated milk and found no decrease in -SH groups after a holding period of 20 hr at 25 C. Boyd and Gould (1957) added cupric ions ($CuSO_4$) to milk both before and after heating. In both cases there was a marked reduction in hydrogen sulfide production and a smaller but still significant decrease in thiamine disulfide values.

Townley and Gould (1943b), in separate trials, added cupric, mercuric, and silver salts to milk and found them to be equally effective in decreasing sulfide liberation. Furthermore, the anion associated with the cation had no bearing on the extent of the inhibition. The addition of cysteine hydrochloride stimulated hydrogen sulfide formation, but the addition of cystine inhibited it. The addition of as little as 0.25 g cystine per liter of milk almost entirely prevented sulfide liberation. Townley and Gould (1943b) also investigated the effect of pH on the evolution of hydrogen sulfide. Below pH 4 and above pH 10 the amount of sulfide liberated was small. Between these extremes of pH values the amount of sulfides liberated increased to a maximum at about pH 9. The normal pH of milk is about 6.6.

Townley and Gould (1943b) concluded that the addition of reducing agents such as cysteine hydrochloride increased hydrogen sulfide production, and that oxidizing catalysts such as metallic salts decreased sulfide production. They suggested that the enhancing action of reducing substances was due to their ability to favor the formation of sulfhydryl groups. Likewise, oxidizing substances destroyed sulfhydryl groups by converting them to disulfides or other oxidation products. The addition of various sugars at a 5% level also decreased hydrogen sulfide production. Sucrose, a nonreducing sugar, resulted in a 13% decrease, while glucose and lactose, both reducing sugars, caused a 30 and 39% decrease, respectively.

Besides liberating sulfur, high heat treatment of milk proteins causes extensive denaturation and aggregation. Zittle and DellaMonica (1957) proposed that the opacity of heated β-lactoglobulin solutions was due to disulfide interchange polymerization. The addition of large amounts of mercaptoethanol before heating prevented the solutions from becoming opaque, presumably because disulfide linkages were unable to form. These results agreed with those of Hospelhorn and Jensen (1954), who performed analagous experiments with bovine serum albumin. They noted that SH-containing albumin aggregated more rapidly when heated than did SH-free albumin. During thermal denaturation, sulfhydryl groups promote the lateral association of the protein molecules through a chain reaction with disulfides. This action is largely responsible for the increased turbidity.

Thermal denaturation and aggregation of β-lactoglobulin has been described by Sawyer (1968) as a two-step process. The first stage is characterized by intermolecular disulfide bond formation; but the second stage does not involve disulfide linkages. In the second stage larger

polymers are formed than in the first stage. Also primary stage aggregation is favored by heating at 97.5 C, and secondary stage aggregation at 75 C. By measuring the turbidity developed by heating before and after the addition of a -SH blocking agent, Sawyer was able to approximate the extent of each type of aggregation.

Kresheck *et al.* (1964) found that the weight average molecular weight of β-lactoglobulin increased from 40,000 to 3,020,000 after 9 hr of heating at 90 C. The turbidity of the β-lactoglobulin solutions also rose dramatically during the heat treatment. Damicz (1966) determined that β-lactoglobulin was denatured completely following heat treatments of 80 C for longer than 10 min or 90 C for longer than 30 sec. He also noted a correlation between denaturation and cooked flavor.

Another important result of heat treating milk is the interaction between β-lactoglobulin and k-casein. On the basis of electrophoretic evidence, McGugan *et al.* (1954) predicted that casein and β-lactoglobulin associate upon heating. Later Zittle *et al.* (1962) presented additional proof that the interaction does take place and that specifically it is an interaction between β-lactoglobulin and k-casein. An article reviewing research concerning this interaction was recently published (Sawyer, 1969).

The importance of sulfhydryl groups in the interaction was illustrated by the work of Sawyer *et al.* (1963). If the -SH blocking agent N-ethylmaleimide (i.e., NEM) was added before heating, no interaction took place between the k-casein and β-lactoglobulin. Also electrophoretic patterns of the two proteins, when heated separately and subsequently reduced with mercaptoethanol, were similar to the pattern of the reduced complex. These results led Sawyer *et al.* (1969) to propose that the

formation of an intermolecular disulfide bond between the two proteins is the mechanism of interaction.

The Effect of Combined Heat and Hydrogen Peroxide Treatments

Research on the combined effects of hydrogen peroxide and high heat treatment has been mainly directed toward the β-lactoglobulin--k-casein complex. Grindrod and Nickerson (1968) observed from electrophoretic patterns that the interaction did not occur in the presence of hydrogen peroxide. Using the same technique, Fish and Mickelson (1967b) found that hydrogen peroxide partially retarded the heat-induced interaction. Electrophoretic gels from the latter study showed a smear for the hydrogen peroxide-, heat-treated β-lactoglobulin with some residue appearing in the large pore spacer gel.

Fish and Mickelson (1967a) also monitored the change in whey protein nitrogen as a measure of whey protein denaturation in heat-treated skimmilk to which increasing levels of hydrogen peroxide were added. They measured whey protein nitrogen after removal of denatured proteins by the Harland-Ashworth procedure (Harland and Ashworth, 1947). The concentrations of hydrogen peroxide added ranged from about 0 to 0.30%. After the heat treatment the peroxide was destroyed with catalase. There was 65% protein denaturation in the control sample (no peroxide added). At levels of peroxide ranging from 0 to 0.07%, the amount of denaturation decreased from 65 to 41%. There was no further decrease when greater concentrations of peroxide were used. These workers suggested three reasons for the observed results:

(a) The β-lactoglobulin--k-casein interaction may be inhibited;

(b) β-Lactoglobulin is changed by the peroxide treatment;

(c) A casein protein may be affected.

These explanations were based on gel electrophoretic patterns of the protein solutions used in their study.

Lück and Schillinger (1958) measured the effect of heat and hydrogen peroxide on the sulfhydryl content of milk. In milk containing hydrogen peroxide that was heated to 51 C for 30 min, there was only a slight loss of -SH groups. Higher temperatures and/or longer holding times were necessary to significantly lower the sulfhydryl group content.

The work of Townley and Gould (1943b) comes closest to anticipating the results presented in this research project. They added small amounts of hydrogen peroxide to milk before heating or after heating milk (but before cooling) momentarily to 90 C and measured the volatile sulfides liberated. The following table reports their findings.

	Sulfur liberated as volatile sulfides	
Hydrogen peroxide 30%	Added before heating	Added after heating
0.00 ml/l	0.236 mg/l	0.236 mg/l
0.25	0.089	0.047
0.50	0.064	0.023

The nitroprusside test (for activated -SH groups) was positive in all cases except for those samples receiving 0.50 ml of peroxide per liter of milk after heating. For these samples the test was either slightly positive or negative. Also the expected cooked flavor was altered, being more accurately described as a smoky flavor. To completely stop the evolution of sulfides, Townley and Gould (1943b) found it necessary to add a minimum of 2.5 ml of 30% peroxide per liter of

milk before heating, or 0.75 ml after heating. These corresponded to concentrations of 0.075 and 0.0225% hydrogen peroxide, respectively.

In a recent publication Koops and Westerbeek (1970) improved the heat stability of concentrated milk by adding 0.05% hydrogen peroxide before preheating the milk. The addition of hydrogen peroxide also shifted the pH of maximum heat stability in the alkaline direction. Oxidation of -SH and -SS- groups and the subsequent inhibition of intermolecular disulfide bond formation was offered as an explanation for the improved heat stability.

Heat-Sterilized Milk: Sterilization Procedures

Traditionally milk to be sterilized is given a pre-sterilization heat treatment, filled in a can, and sealed. Then it is given a more severe heat treatment known to produce sterility. This final heat treatment often does a significant amount of damage to the product. In recent years the trend has been toward ultra-high temperature processing (i.e., UHT) followed by aseptic packaging (i.e., AP). Product and container are sterilized separately. This procedure requires a higher temperature but a much shorter holding time than the post-packaging sterilization procedure. Consequently less heat damage is produced in the product (Mitten, 1968).

Before the widespread use of UHT-AP equipment, at least one manufacturer tried using hydrogen peroxide to reduce the severity of in-can heating necessary to sterilize milk (Anonymous, 1953). Hydrogen peroxide was added to 100 F (38 C) milk to a final concentration of 0.2 to 0.4%. After a 30 min incubation period the peroxide was destroyed by the addition of catalase. The peroxide-free milk was then run through a vacuumizer and homogenizer. It was canned, placed in an end-over-end sterilizer, and quickly heated to 265 F (129 C). The cans were immediately water-

cooled to 90 F (32 C) and removed from the sterilizer.

According to Rippen (1970), in the UHT-AP processing procedure, sterility is usually achieved by heating the product from 140.6 to 148.9 C (285 to 300 F) for 2.5 to 8.0 sec. In the United States the sterile product is usually packaged in sterile cans utilizing the Dole system. In Europe the laminated paper container, Tetra Pak, is the most popular.

Heat-Sterilized Milk: Changes in the Product

The body and flavor of UHT-AP milk deteriorates over time, especially if stored at or above room temperature. At the time of packaging the most objectionable flavor is the sulfide flavor arising from the release of volatile sulfur compounds. This off-flavor disappears with time due to the oxidation of -SH groups by the small amount of oxygen present in the milk (Clark, 1967). This is the same explanation offered by Jenness (1954) to account for the loss with time of -SH groups in heated β-lactoglobulin.

During storage UHT-AP milk loses its freshness and develops a stale flavor. The compounds responsible for this off-flavor have been studied by Kirk *et al.* (1968). By means of gas chromatographic techniques, they found an increase in the more volatile flavor compounds and a complete loss of the less volatile compounds as the freshly processed milk became stale. Compounds present in the stale milk, not present in the fresh milk, were butanal, hexanal, and 2-heptanone. The rate of stale flavor development was faster in milk stored at higher temperatures.

Another flavor change is caused by the reaction between the proteins and reducing sugars (Jordan, 1968). This is most often described

as a slight cooked or caramel-like flavor. Also, the formation of δ-lactones causes a coconut-like flavor.

Regarding body, UHT processing can destabilize the fat emulsion causing fat agglomeration. The defect is prevented by homogenization downstream from the UHT heating stage (Clark, 1967). Destabilization of the other milk solids can also occur, causing the formation of sediment at the bottom of the milk container. This, too, can be prevented by downstream homogenization. Age thickening, which is really incipient gelation, is another common defect that develops in sterile milk during storage.

With all of these potential deteriorative changes, it is little wonder that Kosikowski (1969) reported only marginal consumer acceptance of sterilized whole milk. He stated that research is urgently needed in the production of sterile milk products having uniform flavor and texture stability.

Besides these external changes, UHT-AP affects milk in other ways. Melachouris and Tuckey (1966) heated milk for 2.08 sec at temperatures ranging from 93.3 to 143.3 C. With increasing temperature they observed increasing whey protein denaturation as measured by the Harland-Ashworth test (Harland and Ashworth, 1947). At the highest temperature 57% of the whey protein was denatured. β-Lactoglobulin was the most sensitive component, undergoing 70% denaturation at 143.3 C. Denatured whey protein in milk increases the reflectance of light, making UHT milk appear whiter than unheated milk.

Heat-sterilized milk behaves differently than does raw milk in the presence of rennin. During the primary phase of rennin action, the release of glycomacropeptides proceeds at the same rate for both raw and heat-sterilized milk. Yet, coagulation is slower for the

heat-sterilized milk (Hindle and Wheelock, 1969). Pyne (1945) found that an increase in the amount of Ca^{+2} adsorbed on the caseinate complex as calcium phosphate slows the secondary phase of rennin action on milk. Because the heating of milk forces the free-Ca^{+2} $\rightleftharpoons$ Ca^{+2}-adsorbed on the caseinate complex equilibrium toward the adsorbed state, the secondary phase coagulation takes more time in heated milk than in unheated milk. However when the heated milk is cooled, it slowly reestablishes its original Ca^{+2} equilibrium position.

High temperature processing also lowers the quality of milk proteins, mainly by reducing the availability of lysine (Porter, 1964). Ford (1962) heated thin films of freeze-dried skimmilk at 121 C for various time intervals. He used a microbiological assay procedure to measure the loss of 'available' amino acids and an acid hydrolysis procedure to measure the loss of total amino acids in the heated films. After 30 min of heating only 14% of the original available lysine remained; and only 42% of the original available arginine and histidine remained. The fraction of the loss recovered by acid hydrolysis was one-third, one-third, and three-fourths, respectively. For the other amino acids tested there was a slow loss of availability with lengthening time of heating. But this loss was recovered with acid hydrolysis in every case. In a similar experiment, Pol and Groot (1960) monitored amino acid changes in milk sterilized by holding it before bottling at 120 C for 20 sec and after bottling at 118 C for 14 min. Following sterilization they measured a 6 and 13% loss of lysine and cystine, respectively. Ford (1962) did not measure cystine in his work.

Ford *et al.* (1969) and Pol and Groot (1960) also assayed for vitamin losses in heat-sterilized milk. Pol and Groot (1960), working with milk sterilized in the manner described above, reported no destruction

of vitamin A, carotene, riboflavin, niacin, and pantothenic acid. However, sterilization destroyed 24% of the thiamin and 17% of the vitamin B_6. Vitamin C destruction was about 60%. Ford *et al.* (1969) did their work on milk sterilized and cooled by various commercial methods. The temperature and time of heating ranged from 138 to 145 C for from 2 to 4 sec. Upon processing and during subsequent storage for 90 days there was no loss of vitamin A, carotene, vitamin E, thiamin, riboflavin, pantothenic acid, biotin, or nicotinic acid. There was little or no loss of vitamins B_6 or B_{12} on processing, but losses during storage were up to 50%.

All the dehydroascorbic acid and 20% of the ascorbic acid (vitamin C) were lost on processing. When there was no residual oxygen in the milk there was no ascorbic acid destruction during storage. But if there was greater than 1 ppm residual oxygen, all the remaining ascorbic acid was lost within 14 days. Processing caused a 20% loss of folic acid, and if there was no residual oxygen present, there was no further loss during storage. Ford *et al.* (1969) emphasized the desirability of there being no residual oxygen from a nutritional standpoint, but acknowledged that oxygen is needed to rapidly disperse the sulfur flavor of the freshly sterilized product.

EXPERIMENTAL

Apparatus and Equipment

Laboratory balances used in this research project were a Mettler Type K7T, Mettler Type H16, and a Sartorius Series 2400 balance. They were accurate to the nearest hundredth of a gram, tenth of a gram, and hundredth of a milligram, respectively.

The centrifuging required in the preparation procedure for β-lactoglobulin was done in a Sorvall RC2-B refrigerated centrifuge equipped with a type GSA rotor. All pH measurements were made on either a Corning Model 12 pH meter or an Instrument Laboratories, Inc. Model 245 Delta-Matic pH meter.

Spectrophotometric measurements were made with a Beckman DK-2A ratio recording spectrophotometer equipped with visible and ultraviolet light sources. Samples for analysis were placed in 1-cm path length silica glass cuvettes.

Ion exchange chromatography was performed with an ion exchange chromatographic column purchased from Pharmacia Fine Chemicals, Inc. The column height was 30 cm and the internal diameter was 0.85 cm. Protein eluted from the column was detected with an Instrumentation Specialties Co., Inc. Model UA-2 ultraviolet analyzer.

Flatbed gel electrophoresis was performed in a laboratory-constructed apparatus assembled from Plexiglass. Vertical gels were run on an EC470 Vertical Electrophoresis Apparatus manufactured by the E-C

Apparatus Corp. Direct current was supplied by a Heathkit Variable Voltage Regulated Power Supply Model PS-3. Gel destaining was done in a laboratory-constructed electrolytic destainer.

All freeze drying was done on a laboratory-constructed lyophilizer which was connected to a Cenco Hyvac 28 vacuum pump.

Amino acid analyses were performed with a Beckman 120C Amino Acid Analyzer. Protein samples were hydrolyzed for analysis in a Stabil-Therm Gravity Oven constructed by the Blue M Electric Co.

β-Lactoglobulin model system solutions were heated in a laboratory-constructed water bath apparatus equipped with a constant temperature circulator supplied by the Brownwill Scientific Division of the Will Corp.

Milk processing for rat feeding studies was done at the Michigan State University dairy pilot plant in E. Lansing. Ultra-high temperature processing was carried out in a No-Bac Spiratherm manufactured by the Cherry-Burrell Corp.; Cedar Rapids, Iowa. The milk was homogenized in a Manton-Gaulin two-stage homogenizer.

Milk processing for organoleptic testing was done at the University of Illinois dairy pilot plant in Urbana. The milk was heat-sterilized in a Mallory small-tube heater and homogenized in a two-stage homogenizer. Both were installed by the Illinois Creamery Supply Company of Chicago, Ill. This equipment was described in detail by Herreid and Tobias (1959). After processing, the milk was canned in a Martin Aseptic Canning System using a licensed process of the James Dole Engineering Company; San Francisco, Calif. The canning equipment was on loan from the Continental Can Company.

Chemicals and Materials

The principal chemicals used in this study are listed below. All chemicals were reagent grade unless otherwise stated. The water used was deionized and distilled.

Chemicals used in the β-lactoglobulin preparation procedure: The ammonium sulfate and the sodium sulfate used in the salting-out fractionations were supplied by the Fischer Scientific Company; Fair Lawn, N.J., and Merck & Company, Inc.; Rahway, N.J., respectively. Hyflo Super-Cel, filter aid, was purchased from the Celite Division, Johns-Manville Corp.; New York, N.Y.

Chemicals used to prepare and process β-lactoglobulin model system test solutions: Hydrogen peroxide, 35% (food grade), was donated by the Shell Chemical Company, division of Shell Oil Company; New York, N.Y. Food grade fungal catalase (i.e., Fermcolase) was donated by Fermco Laboratories, a division of G. D. Searle; Chicago, Ill. According to the manufacturer 1 ml of the enzyme preparation was rated to decompose 264 g hydrogen peroxide. Lactose was purchased from Eimer and Amend; New York, N.Y.

Chemicals used in nitrogen and nonprotein nitrogen (micro-Kjeldahl) determinations: Tris(hydroxymethyl) aminomethane, primary standard (i.e., Sigma 7-9), for standardization of hydrochloric acid solution was supplied by Sigma Chemical Co.; St. Louis, Mo. The dyes bromocresol green and methyl red WS used in the indicator solution were purchased from Matheson Coleman & Bell; Norwood, Ohio, and Nutritional Biochemicals Corp.; Cleveland, Ohio, respectively. Selenium dioxide was supplied by K & K Laboratories; Plainview, N.Y. The trichloroacetic acid used in the nonprotein nitrogen determinations and the 30% hydrogen peroxide were purchased from Mallinckrodt Chemical Works; St. Louis, Mo.

Chemicals used in the sulfhydryl groups determinations: Ellman's reagent, 5,5'-dithiobis-(2-nitrobenzoic acid), also known as DTNB, was purchased from the Sigma Chemical Company as was the disodium salt of ethylenediamine tetraacetic acid, di-Na EDTA. Sodium lauryl sulfate was bought from Fischer Scientific Company.

Chemicals used in the sulfhydryl groups plus disulfides determinations: The DTNB and di-Na EDTA came from the same sources as above. Ultra-pure urea was purchased from Schwarz Bioresearch, Inc.; Orangeburg, N.Y., and the sodium borohydride was manufactured by the Metal Hydrides Division of Ventron; Beverly, Mass. L-cysteic acid monohydrate was purchased from Nutritional Biochemicals Corp.

Chemicals used in the methionine determinations: DL-methionine was obtained from Matheson Coleman & Bell and sodium nitroprusside from the J. T. Baker Chemical Co.; Phillipsburg, N.J. L-methionine DL-sulfoxide and L-methionine sulfone were both purchased from Sigma Chemical Co.

Chemicals used in the tryptophan determinations: DL-tryptophan was supplied by the Nutritional Biochemicals Corp. and Pronase, B grade, by Calbiochem; Los Angeles, Calif. The p-dimethylaminobenzaldehyde came from Eastman Organic Chemicals, division of Eastman Kodak Co.; Rochester, N.Y.

Chemicals used in the determination of amino acids: Chemicals needed to run the amino acid analyzer included sodium citrate, thiodiglycol, and the detergent, BRIJ-35. All were supplied by Bio-Rad Laboratories; Richmond, Calif. These chemicals were especially prepared for use in amino acid analyzers. The preservative, pentachlorophenol, was purchased from Eastman Organic Chemicals. The standard amino acid calibration mixture was bought from Bio-Rad and norleucine

from Nutritional Biochemicals Corp. The suppliers of L-cysteic acid monohydrate, L-methionine DL-sulfoxide, and L-methionine sulfone are listed above.

Chemicals used in the hydrogen peroxide determinations: The sodium oxalate was purchased from the J. T. Baker Co., and the potassium permanganate from Mallinckrodt Chemical Works.

Chemicals used in the nitroprusside determinations of activated sulfhydryl groups: The suppliers of the sodium nitroprusside and ammonium sulfate used in this test are listed above.

Chemicals used in the electrophoretic studies: Tris(hydroxymethyl) aminomethane, reagent grade (i.e., Trizma Base), was supplied by Sigma Chemical Co. Cyanogum 41, a prepared mixture of acrylamide and N,N'-methylenebisacrylamide, was supplied by the E-C Apparatus Co.; Philadelphia, Pa. Ammonium persulfate was purchased from the J. T. Baker Chemical Company. The dye brom phenol blue, sodium salt, was obtained from the National Aniline Division of the Allied Chemical Corp.; New York, N.Y. Buffalo Black, NBR, which was used in the amido black staining solution, was supplied by the Industrial Chemicals Division of the Allied Chemical Corp. N,N,N',N'-tetramethylethylenediamine (i.e., TEMED) was manufactured by Eastman Organic Chemicals.

DEAE-Sephadex A-50, used to separate the genetic variants of β-lactoglobulin, was obtained from Pharmacia Fine Chemicals, Inc.; Piscataway, N.J.

Preparative Procedures

Preparation of β-Lactoglobulin AB

β-Lactoglobulin AB was prepared from mixed milk of Holstein cows that were a part of the Michigan State University dairy herd. The

fractionation procedure used was essentially that of Aschaffenburg and Drewry (1957b) and the reprecipitation purification procedure used was that of Larson and Jenness (1955). The protein was fractionated from four 10-liter batches of milk.

Whole milk, not allowed to cool following milking, was warmed to about 40 C, and while stirring, sodium sulfate was added slowly to a final concentration of 20 g per 100 ml of milk. After the salt had dissolved and the temperature had dropped to about 24 C, the mixture was filtered through E & D No. 515 filter paper. In this step the casein and fat were removed. Concentrated HCl was added to the filtrate to lower the pH to 2 (about 1 ml acid per 100 ml filtrate). All the remaining proteins except β-lactoglobulin were precipitated at this time. To separate the filtrate from the precipitate, the mixture was centrifuged at 16,300 x g for 20 min and the supernatant was filtered through Whatman No. 4 filter paper.

The pH of the filtrate was raised to about 6 with the addition of concentrated ammonium hydroxide (about 0.6 ml per 100 ml filtrate). Ammonium sulfate was added in the ratio of 20 g per 100 ml of filtrate to precipitate the β-lactoglobulin. When the protein had flocculated it was filtered by suction through a thin pad of the filter aid Hyflo Super-Cel deposited on Whatman No. 1 filter paper which was placed in a Büchner funnel. The filtrate was discarded and the filter cake was slurried with a little water to produce a paste fluid enough to transfer into a dialysis sac.

The dialysis sac (i.e., Visking cellulose) containing the paste was dialyzed overnight at 4 C with stirring against water. A little toluene had been added to the water to prevent microbial growth. The sac contents were filtered through Whatman No. 1 filter paper to remove the

filter aid. The pH of the clear filtrate was adjusted to 5.8 and dialysis was continued for 24 hr. The dialysate was adjusted to pH 5.2, the isoelectric point of β-lactoglobulin, and the dialysis continued until the protein had completed precipitation as an oily mass.

The precipitate was separated from the supernatant. A little water plus enough sodium chloride was added to the precipitate to resuspend the protein. The pH was adjusted to 5.2 and dialysis was continued as before until there was again complete precipitation. The reprecipitation procedure was repeated two more times to insure the elimination of contaminating proteins. Finally the β-lactoglobulin AB was freeze-dried, further dried in a vacuum desiccator over P_2O_5 for 24 hr, and stored in a desiccator at 4 C.

Preparation of β-Lactoglobulin Model System Solutions

A model system consisting of β-lactoglobulin AB and lactose dissolved in simulated milk ultrafiltrate was used extensively in this research project. The reason was that protein changes due to heat and/or hydrogen peroxide treatment were more easily determined in the model system than in whole milk or skimmilk.

Model system solutions were prepared by dissolving 150 mg β-lactoglobulin AB and 1 g lactose in 20 ml of the simulated milk ultrafiltrate described by Jenness and Koops (1962). The β-lactoglobulin concentration was 0.75%; and the lactose concentration was 5.0% in the model system solutions. For whole milk these values are 0.41 and 4.9%, respectively (Webb and Johnson, 1965).

Below are listed the salts and the amounts of each needed to prepare 2 liters of the simulated milk ultrafiltrate.

KH_2PO_4	3.16 g
K_3 citrate·H_2O	1.02
Na_3 citrate·$2H_2O$	3.58
Mg_3 citrate·H_2O	1.01
K_2SO_4	0.36
K_2CO_3	0.60
KCl	2.15
$CaCl_2 \cdot 2H_2O$	2.65

All these, but the last, were combined and put in solution with about 1800 ml of water. To this was added a separate solution of the 2.65 g $CaCl_2 \cdot 2H_2O$ in about 100 ml water. The pH was adjusted to 6.6 with 1.0 N KOH and the solution was brought to a final volume of 2 liters with water. If the calcium chloride was combined with the other salts before they were dissolved, some of the salts began to precipitate several days after the solution was prepared.

Model system solutions (20 ml volume) were subjected to various treatments with heat and/or hydrogen peroxide. They were placed in unstoppered, thick-walled Pyrex tubes with a height and internal diameter of 20 and 2.0 cm, respectively. Heating was carried out in a water bath maintained at 85 $\pm$ 0.5 C.

Model system samples to be heated were immersed in the hot water bath for 12 min and cooled by swirling in an ice water bath for 2 min. While in the hot water bath the samples required about 2 min to reach the temperature necessary (i.e., about 70 C) to activate the sulfhydryl groups. Thus the samples were at or above activation temperature for approximately 10 min. Figure 1 illustrates the temperature profile of the samples undergoing the hot water bath, ice water bath treatment.

When hydrogen peroxide was used, it was added undiluted as a 40% (w/v) solution with a microsyringe 10 min prior to immersion in the hot water bath. Catalase, to destroy the peroxide, was added as a 5% (v/v) solution with a microsyringe 36 min after removal from the ice water bath. Therefore the total contact time between the protein and

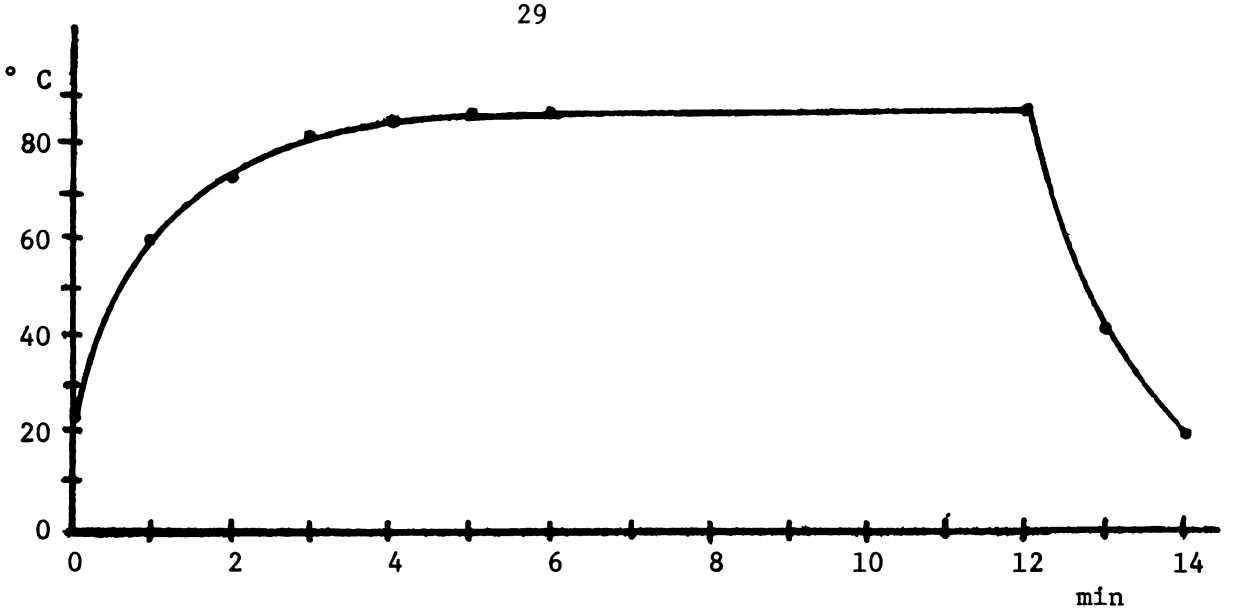

FIGURE 1. Time-temperature profile of the heat treatment of model system solutions.

the hydrogen peroxide was 1 hr. The amount of catalase added was sufficient to destroy the hydrogen peroxide within 30 min.

A few drops of a freshly prepared 25% (w/v) solution of potassium iodide added to a few drops of a solution containing hydrogen peroxide forms a yellow color resulting from the oxidation of the iodide to free iodine. Enough catalase was added to prevent the formation of a yellow color between the potassium iodide solution and the model system solutions after 30 min.

Following treatment, solution opacity was measured spectrophotometrically as per cent transmittancy (i.e., %T) at 720 nm. The model system solutions were then dialyzed against water (changed several times) at 4 C for 3 days to remove lactose and salts. The remaining protein from each solution was freeze-dried, further dried in a vacuum desiccator over P_2O_5 for 24 hr, and stored in a desiccator at 4 C.

In Table 1 are listed the names of the individual model system samples and the details regarding their treatment. A cursory inspection should reveal the logic behind the code name designations.

Preparation of Milk Samples for Rat Feeding Trials

Milk used in the rat feeding study was mixed milk obtained from the Holstein dairy herd of Michigan State University. Three types of processing treatments were used: a pasteurized control run; a UHT control run; and a UHT-, hydrogen peroxide-treated run. Following processing the milk samples were assayed for per cent fat and methionine content.

Pasteurized control sample. Approximately 30 lb of raw milk contained in a 5-gal milk can were heated to 66 C (150 F) and maintained at that temperature for 30 min. The heating was done in a large, steam-jacketed kettle containing hot water. After pasteurization the milk was cooled to about 38 C (100 F) and homogenized in a Manton-Gaulin two-stage homogenizer at 2500-500 psi. It was then placed in a 5-gal milk can and cooled in a 0 C (32 F) cold room.

Two lots of pasteurized milk were processed; the first on the day before the feeding study began, and the second 6 days into the feeding study. It was felt that one lot of milk would go sour if kept for the entire 12 day duration of the study.

UHT control sample. Approximately 40 lb of raw milk were heated in a Spiratherm tubular heat exchanger to 120 $\pm$ 1 C (245 F) and held at that temperature for 4 sec. The milk was immediately cooled to 38 C (100 F) and then homogenized and refrigerated in the same manner as above.

TABLE 1. Nomenclature and details of treatment of model system solutions

Code name	µl 40%* H_2O_2 added	Final %* H_2O_2 conc.	Heat treatment C/min	µl 5%** catalase added	Excess catalase***
St	0	0.00	none	0.00	-
1H	0	0.00	85/12	0.00	-
2.01	5	0.01	none	19.0	125
2.03	15	0.03	none	19.0	42
2.05	25	0.05	none	19.0	25
2.07	35	0.07	none	26.5	25
2.10	50	0.10	none	38.0	25
3H.01	5	0.01	85/12	19.0	125
3H.03	15	0.03	85/12	19.0	42
3H.05	25	0.05	85/12	19.0	25
3H.07	35	0.07	85/12	26.5	25
3H.10	50	0.10	85/12	38.0	25

*(w/v). **(v/v). ***1 = minimum calculated amount needed to destroy H_2O_2.

Review of time sequence:

Min	Manipulation
0	Add H_2O_2 to model system solutions.
10	Immerse in hot water bath.
22	Remove from hot water bath and immerse in ice water bath.
24	Remove from ice water bath.
60	Add catalase.
90	No remaining H_2O_2 as shown by negative KI test reaction.

UHT treated sample. To 41 lb of raw milk were added 4.1 ml of 40% (w/v) hydrogen peroxide. The final peroxide concentration was 0.01 (w/v). The milk was manually stirred for about 10 min and then heated and homogenized as described above. Following homogenization the lot of milk weighed 35 lb due to some loss during processing. To this was added 3.5 ml of a 10% (v/v) catalase solution. According to the manufacturer this was 67 times the minimum amount needed to decompose the hydrogen peroxide. The milk was stirred manually for about 5 min and then placed in a cold room (0 C) where it remained for 72 hr. The milk was then reheated to 120 ± 1 C (245 F) for 4 sec and recooled in a cold room to 0 C. The milk was heat-treated a second time to inactivate the remaining catalase.

Preparation of UHT-AP Milk Samples for Organoleptic Evaluation

Milk for ultra-high temperature processing was obtained from the mixed breed University of Illinois dairy herd, Urbana. Two separate sample runs were made; a UHT control run and a UHT-, hydrogen peroxide-treated run.

UHT control sample. About 80 lb of raw milk were heated to 143 C (290 F) for 2 sec in a Mallory small-tube heater, cooled to about 38 C (100 F), homogenized at 2500-500 psi in a two-stage homogenizer, further cooled to about 4.5 C (40 F), and aseptically canned. Canning was done in a Martin Aseptic Canning System and 49 cans, holding about 175 ml of milk apiece, were filled. The entire system was previously sterilized by circulating 149 C (300 F) water through the processing equipment.

UHT treated sample. To 81 3/4 lb of raw milk were added 8.2 ml of 40% (w/v) hydrogen peroxide. The final peroxide concentration was 0.01% (w/v). The milk was stirred manually for about 10 min and then heated and homogenized as described previously. After homogenizing, the milk

was further cooled to 20.5 C (69 F) and collected in a 10-gal milk can. Sixty-nine lb of milk were collected. Six and nine-tenths ml of a 10% (v/v) catalase solution were added to this. According to the manufacturer this was a 67 times excess.

The milk was manually stirred for 30 min to insure complete mixing of the catalase. The milk was then placed in a cold room (40 F) where it remained for 70 hr. The milk was reheated to 143 C (290 F) for 2 sec, cooled to 4.5 C (40 F), and aseptically canned in the same manner as the UHT control. Forty-two cans of treated milk were filled. As in the case of the milk processed for the rat feeding trials, this milk was heated a second time to inactivate the remaining catalase.

Chemical Methods

Nitrogen

A micro-Kjeldahl apparatus was used for the nitrogen determinations. The digestion mixture was composed of 5.0 g $CuSO_4 \cdot 5H_2O$ and 5.0 g SeO_2 in 500 ml concentrated sulfuric acid. Approximately 10 mg dry protein was digested with 4 ml of the digestion mixture over a gas flame for 1 hr. Each digestion was done in a micro-Kjeldahl digestion flask. After cooling, 1 ml of a 30% hydrogen peroxide solution was added to each flask and digestion was continued for another hour. Each flask was cooled and rinsed with a little water.

The digested sample mixture was neutralized with about 25 ml of a 40% (w/v) NaOH solution. The released ammonia was steam distilled into a receiving flask containing 15 ml of a 4% boric acid solution and two drops of indicator. The indicator consisted of 400 mg bromocresol green and 40 mg methyl red WS in 100 ml of 95% ethanol. This indicator is blue in alkaline media and yellow in acid media. The distillation was

continued until 70 ml of solution was collected in the receiving flask. The ammonia-borate complex was titrated with 0.02 N HCl which had been standardized prior to titration with a solution of 0.1000 N tris(hydroxymethyl) aminomethane. Along with the protein samples a blank was run plus a tryptophan standard to determine average per cent recovery of nitrogen.

Nonprotein Nitrogen

Ten ml of a 45% (w/v) trichloroacetic acid (i.e., TCA) solution was added to 20 ml samples of a 0.75% protein solution (the model system samples). The final TCA concentration was 15% (w/v). After shaking well and waiting about 5 min, the resulting mixture was filtered through Whatman No. 43 filter paper. The per cent nitrogen was determined using 10 ml aliquots of the filtrate.

The nitrogen determinations were done as above except that 8 ml (not 4 ml) of the sulfuric acid digestion mixture and 2 ml (not 1 ml) of the 30% hydrogen peroxide solution were added during digestion. Boiling chips were found to be helpful in preventing excessive bubbling during digestion.

Sulfhydryl Groups

Sulfhydryl groups were determined by a modification of the procedure developed by Ellman (1959). To 5 mg dry protein were added 5 ml of a 0.01 M sodium phosphate buffer, pH 8.0, containing 1.0% sodium lauryl sulfate and 0.04% di-Na EDTA. This solution was allowed to stand for 30 min and then 0.02 ml of a 5,5'-dithiobis-(2-nitrobenzoic acid) solution, DTNB, was added. The DTNB solution was prepared by dissolving 40 mg DTNB in 10 ml 0.1 M sodium phosphate buffer, pH 7.0. The color was allowed 45 min to develop, and absorbancy at 412 nm was read in a

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spectrophotometer. Concentration of the sulfhydryl groups was determined using an extinction coefficient of 12,000 as reported by Flavin (1962). A blank was run along with the protein samples.

Sulfhydryl Groups Plus Disulfides

In this test disulfides were first reduced with sodium borohydride to form sulfhydryl groups. The reducing agent was destroyed with acid and acetone and the concentration of sulfhydryl groups was determined. The method is a modification of the one developed by Cavallini *et al.* (1966) and used by Manning *et al.* (1969).

About 0.8 mg dry protein was placed in 1 ml 0.05 M sodium phosphate buffer, pH 7.4, containing 10 ml 0.02 M di-Na EDTA per 200 ml of buffer. To this solution was added 2 µl octyl alcohol, an antifoaming agent. One ml of a urea-sodium borohydride solution was added. The solution contained 10 g ultra-pure urea, 0.25 g sodium borohydride, and 10 ml of water. The protein, urea, borohydride mixture was shaken and incubated in a 40 C water bath for 30 min. After cooling to room temperature, 0.5 ml of a low pH buffer was added. It consisted of 13.6 g KH_2PO_4 plus 1.66 ml concentrated HCl brought to a final volume of 100 ml with water. This solution was introduced dropwise to prevent excessive foaming. After the addition was completed, care was taken to see that the walls of the reaction vessel were completely wetted by the low pH buffer.

Five minutes reaction time was allowed and 1 ml acetone was added to complete the borohydride destruction. Again the mixture was shaken to wet the walls of the reaction container. Finally 0.02 ml of the same DTNB solution that was used in the sulfhydryl group determination was added and absorbancy at 412 nm was measured after 45 min. As previously stated an extinction coefficient of 12,000 was used. It was found

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unnecessary to purge the test solution with nitrogen gas before adding DTNB as recommended in the references cited. There was no color development when the determination was run on a L-cysteic acid standard.

This test was conveniently performed in glass-stoppered, 5-ml volumetric flasks. A blank determination was run in parallel to the test solutions to check for complete destruction of the sodium borohydride. Undestroyed borohydride would reduce the DTNB causing the blank to become yellow and absorb light at 412 nm.

Methionine

This determination was adapted from the modification by Block and Bolling (1951) of the procedure developed by McCarthy and Sullivan (1941).

Twenty mg dry protein were added to 2 ml of once-distilled 6 N HCl in a 16 x 250 mm glass-stoppered Pyrex test tube. The stoppered tube was placed in an oil bath and the contents hydrolyzed at 110 $\pm$ 0.1 C for 2 hr. After the tube had cooled to room temperature, the following three reagents were added in the order listed: a) 2 ml 7.5 N NaOH; b) 0.5 ml of a 1% glycine solution; and c) 0.1 ml of a 10% (w/v) sodium nitroprusside solution. The tube was shaken after each addition. The sodium nitroprusside solution was stored in the dark at 4 C previous to use.

The tube and its contents were warmed in a water bath to 40 C and held at that temperature for 15 min. The temperature was then lowered to about 10 C by immersing the tube in an ice water bath. One ml of 6 N HCl was added to the tube, and then the tube was shaken. After allowing 15 min for color development, absorbancy at 510 nm was read. Two blank determinations were run in parallel to the sample: one without sodium nitroprusside to measure the absorbancy contribution of the partially

hydrolyzed protein, and the other without protein to measure the absorbancy contribution of the chemicals used in the test. Also, there was no color development when the determination was run on solutions of L-methionine DL-sulfoxide and L-methionine sulfone.

A standard curve was made by dissolving from 0 to 1.04 mg dry DL-methionine in 2 ml of once-distilled 6 N HCl. Color was developed in the same way it was developed for the protein.

Tryptophan

Tryptophan was measured according to Procedure W of Spies (1967). Five mg dry protein were directly weighed into a 2-ml glass vial with screw cap. To this was added 0.2 ml of a freshly prepared solution containing 10 mg Pronase, B grade, per 2 ml 0.1 N sodium phosphate buffer, pH 7.5. The cap was screwed on and the contents were incubated at 40 C for 24 hr in a water bath. When the vial had cooled to room temperature, 1 ml of the phosphate buffer was added to the vial. After making sure that the vial was clean and dry on the outside, it was placed in a 50 ml Erlenmeyer flask containing a freshly prepared solution of 10.0 ml 21.2 N sulfuric acid plus 30 mg p-dimethylaminobenzaldehyde.

The unscrewed vial was tipped over and the contents quickly mixed by gentle swirling. The flask was stoppered and placed in the dark for from 6 to 8 hr. Next, 0.1 ml of a 0.045% sodium nitrite solution was added. The solution was shaken, the color allowed to develop for 30 min, and absorbancy at 590 nm was measured. Two blanks were run along with this determination. One contained no protein to find the absorbancy contribution of the tryptophan in the Pronase solution; and the other contained no sodium nitrite to measure the absorbancy contribution of the other chemicals used in the test.

A standard curve was prepared by performing the determination on weighed portions (0 to 2.03 mg) of tryptophan dissolved in 10 ml of 19 N sulfuric acid containing 30 mg p-dimethylaminobenzaldehyde as described in Procedure E of Spies and Chambers (1948).

Amino Acids

Amino acid analyses were performed on 22 and 72 hr acid hydrolyzates of protein samples. The amino acids were separated by column chromatography and quantitatively determined by automatically recording the intensity of the color produced by their reaction with ninhydrin (Moore and Stein, 1954; Moore *et al.*, 1958; Spackman *et al.*, 1958).

Four mg portions of dry protein were weighed directly into 10-ml glass ampules and then 5 ml of once-distilled 6 N HCl were added. The contents of each ampule were frozen in a dry ice-ethanol bath, evacuated with a high-vacuum pump, and allowed to melt slowly under vacuum to remove dissolved gases. The contents were again frozen and the ampules sealed with an air-propane flame. The sealed ampules were placed in an oil bath set in a 110 $\pm$ 0.1 C hydrolysis oven. After hydrolysis (22 or 72 hr) the ampules were removed and allowed to cool.

The ampules were broken open on top and 1 ml of a 2.5 μM per ml norleucine solution was added to each as an internal standard to measure transfer losses. The contents of each ampule were quantitatively transferred to a pear-shaped evaporating flask. The flask was connected to a rotary evaporator, slowly immersed in a 55 C water bath, and rotated under vacuum until the acid was removed. A small amount of water was added to resuspend the protein hydrolyzate and the rotary evaporation was repeated. This was done three times in succession to completely remove the acid. The acid-free hydrolyzate of each sample was dissolved

in 0.067 M sodium citrate-HCl buffer, pH 2.2. This buffer contained the methionine antioxidant, thiodiglycol; the detergent, BRIJ-35; and the preservative, pentachlorophenol. Each solution was transferred to a 5-ml volumetric flask and brought to volume with additional buffer. An 0.20 ml portion was removed from each sample and applied to the analyzer for analysis.

The resulting chromatograms were compared to those of standard amino acid calibration mixtures. Standards containing cysteic acid, methionine sulfone, and methionine sulfoxide were also run. The ratio of areas under the curve for the samples and the standards were compared and converted to give the amino acid composition of the sample.

Hydrogen Peroxide

The determination of hydrogen peroxide was taken from a procedure by Bonnichsen *et al.* (1947) reported by Maehly and Chance (1954).

Ten ml of an approximately 35% (w/v) hydrogen peroxide solution were diluted to 200 ml total volume with water. Two ml of the once-diluted solution were added to 25 ml 0.01 M sodium phosphate buffer, pH 7.0. Three ml of this solution were added to 5 ml of a 2% sulfuric acid solution. This solution was titrated with 0.01 M potassium permanganate which had been standardized by titration with an acidified sodium oxalate solution of known concentration. In both titrations the appearance of the purple potassium permanganate signified the equivalence point. The hydrogen peroxide concentration was then calculated stoichiometrically from the equation of the titration reaction: $5H_2O_2 + 2KMnO_4 + 3H_2SO_4 \longrightarrow K_2SO_4 + 2MnSO_4 + 8H_2O + 5O_2$ (Parkes, 1967).

Nitroprusside Test

The nitroprusside test is a fast method for determining the presence of activated sulfhydryl groups. The procedure was described by Josephson and Doan (1939) and later used by Patton and Josephson (1949). Five ml of sample cooled to below 20 C were saturated with ammonium sulfate. To this were added 5 drops of a 4.5% (w/v) sodium nitroprusside solution and 5 drops of concentrated ammonium hydroxide. The solution was shaken after each addition. The intensity of the pink color formed was roughly proportional to the extent of formation of activated -SH groups in the sample.

Physical Methods

Flatbed Gel Electrophoresis

The procedure used for this electrophoretic technique was described by Melachouris (1969). It utilizes a discontinuous buffer system and a spacer and running gel of different gel concentrations. The running gel solution (9% concentration) was prepared by dissolving 45 g of Cyanogum 41 in 0.38 M tris-HCl buffer, pH 8.9, and making it up to a total volume of 500 ml with the buffer. One-half ml of N,N,N',N'-tetramethylethylenediamine (i.e., TEMED) was added to this solution. Cyanogum 41 at a 5% concentration in 0.062 M tris-HCl buffer, pH 6.7, was prepared as the spacer gel solution. The catalyst TEMED was added in the ratio of 0.1 ml per 100 ml of spacer gel solution.

The running and spacer gel solutions were poured into a Plexiglass gel bed (26 x 12 x 0.4 cm). A Plexiglass strip was placed 6.3 cm from and parallel to one end of the gel bed to divide the two gel solutions. It was lightly coated with silicone grease to prevent seepage. The large area of the gel bed was filled with 190 ml of the running gel solution

to which 1.9 ml of a freshly prepared 10% (w/v) ammonium persulfate solution had been added. The gel was given time to polymerize (about 20 min) under an atmosphere of nitrogen. The spacer strip was removed and the smaller area was filled with 90 ml of the spacer gel solution containing 1 ml of the persulfate solution.

A slot former containing 6 slots (1 x 0.1 x 0.3 cm) was inserted into the still liquid spacer gel parallel to the spacer-running gel interface and 1.2 cm from it (measured on a perpendicular from the slots to the interface). After about 30 min under an atmosphere of nitrogen the spacer gel had polymerized and the slot former was carefully removed.

The protein samples were dissolved at a 2% concentration in the spacer gel buffer. A drop of brom phenol blue dye solution was added to each sample as a marker. From 4 to 40 µl of each protein solution were applied to a slot with a microsyringe. The slots were covered with mineral oil and the entire gel bed was covered with Saran Wrap to reduce evaporation. Cool water was circulated through the gel bed to prevent overheating. Buffer tanks were placed at both ends of the gel bed and each was filled with 1600 ml of a 0.046 M tris-glycine buffer, pH 8.3. Platinum electrodes were inserted into each buffer tank with the negative electrode placed nearest the spacer gel. The electrophoretic run was then carried out at a constant voltage of 190 to 200 volts. After 14 hr the current was stopped. The buffer front had migrated 18 cm from the sample slots.

The gel was removed from the bed and stained for 10 min in an amido black dye staining solution. The solution contained 4 g Buffalo Black, NBR, 500 ml methanol, 100 ml glacial acetic acid, and 500 ml water. The excess dye in the gel was removed electrolytically in an electric destaining cell containing 7% acetic acid.

Vertical Gel Electrophoresis

An EC470 Vertical Electrophoresis Apparatus was used with a 7.5% gel and a discontinuous buffer system. The gel solution was prepared by dissolving 15 g Cyanogum 41 in 0.32 M tris-HCl buffer, pH 8.9, and bringing it up with additional buffer to 200 ml total volume. To this was added 0.2 ml of the catalyst TEMED. Immediately before use, 2 ml of a freshly prepared 10% (w/v) ammonium persulfate solution were added. About 140 ml of the gel solution were carefully poured into the gel bed which was in a horizontal position. The bed had been precooled by running cool water through it. Before polymerization a Plexiglass slot former containing 8 slots (1 x 0.1 x 1 cm) was inserted into the gel.

After allowing 30 min for polymerization, the gel around the slot former was removed. The apparatus was set upright and about 1200 ml of 0.05 M tris-glycine buffer, pH 8.3, were placed in each of the two buffer compartments. After checking that the buffer solution was above the level of the gel, the slot former was removed.

Protein samples were dissolved in 0.32 M tris-HCl buffer, pH 8.9, at a 1 or 2% concentration. Enough sucrose was then added to saturate the sample solutions. The sucrose was necessary to raise the density of the sample solutions so they would layer into the sample slots. From 3 to 50 μl of the sucrose-containing samples were applied with a microsyringe to each slot. The power supply was connected and the electrophoresis was begun with a voltage of about 150 volts. At 15 min intervals for the next 30 min the voltage was increased by 15 volt increments. In three hr the buffer front had migrated 11.5 cm.

The power supply was disconnected. The gel was removed from the gel bed, stained, and destained in the same manner as was the flatbed gel.

Ion Exchange Chromatography

Diethylaminoethyl- (i.e., DEAE-) Sephadex A-50 was used to separate the genetic variants of the β-lactoglobulin AB preparation. The Sephadex was prepared for the separation by first allowing it to hydrate overnight in 0.5 N acetic acid. It was then equilibrated with the starting buffer; 0.05 N sodium acetate, pH 6.0; of the pH gradient elution system used. This was done by suspending the ion exchange resin in the buffer, letting the resin settle, and pouring off the buffer. More buffer was added and the process was repeated. This was done six times. Finally the Sephadex was allowed to sit overnight in the buffer. The equilibrated resin was poured into a 30 cm high, 0.85 cm internal diameter, plastic chromatographic column to a height of 25 cm. The Sephadex was gently stirred to dislodge all air bubbles and then allowed to evenly settle in the column. The layer of buffer which formed above the resin as it settled was periodically removed with a syringe.

The pH gradient employed to elute the protein began with 0.05 N sodium acetate buffer, pH 6.0, and ended with 0.05 N sodium acetate buffer, pH 4.0. Seventy-five ml of each buffer were poured into separate 125-ml Erlenmeyer flasks. These flasks were connected by plastic tubing; and tubing was also connected from the flask containing the pH 6.0 buffer to the top of the chromatography column. As buffer from the pH 6.0 buffer flask dripped onto the column, it was replaced by buffer from the pH 4.0 buffer flask.

Two ml of a 1% β-lactoglobulin AB in starting buffer solution were applied with a syringe to the top of the column. The pH gradient system was attached and the ion exchange elution was begun. The solution leaving the column passed through an ultraviolet monitor that measured absorbance at 254 nm and recorded it on a strip chart. When the

absorbance reading rose above the baseline, indicating the elution of a protein constituent, the eluant was collected. This eluant was later analyzed by polyacrylamide gel electrophoresis to identify the eluted proteins.

The procedure was taken from a paper published by Kiddy *et al.* (1965). They used DEAE-cellulose (not DEAE-Sephadex) and a pH gradient of 0.02 N sodium acetate buffer from pH 5.2 to pH 4.5.

Methods of Evaluation of Treated Milk

Rat Feeding Trials Procedure

A rat feeding study was conducted to compare the nutritive value of UHT-, hydrogen peroxide-treated milk with the nutritive value of pasteurized and UHT milk not receiving a peroxide treatment.

Thirty, 21 day old, male, Sprague-Dawley strain rats obtained from Spartan Research Animals, Inc.; Haslett, Mich., were used in the study. The rats were divided into 3 groups of 10, each group of rats having nearly the same average weight and weight distribution. Group A rats were given pasteurized milk, the control. Group B rats were given the UHT milk not receiving a peroxide treatment. And Group C rats were given the UHT-, hydrogen peroxide-treated milk.

The rats were caged singly in a room provided with a thermostatically regulated temperature control and a 12 hr, 12 hr light-dark daily illumination cycle. Each rat was given a known quantity of milk twice daily in a 100 ml capacity feed cup. These feedings were done at about 8:30 a.m. and 7:30 p.m. If feeding was done only once each day the milk turned sour within the 24 hr. At each feeding the volume of milk consumed since the previous feeding was determined.

Because milk is not a complete ration in itself, a mineral supplement was also given. Its composition was as follows:

KI	0.10 g
$FeSO_4 \cdot 7H_2O$	2.50
$MnSO_4 \cdot H_2O$	0.45
$CuSO_4 \cdot 5H_2O$	0.28
sucrose	200.00

For each rat, approximately 1 g of mineral supplement was given per 50 ml of milk consumed. The supplement was provided daily at the morning feeding only. It, too, was served in a 100 ml capacity feed cup.

The feeding trials lasted 12 days, and the rats were weighed every other day during the test. The supplement was first given on the third day of the feeding trials, after the rats had become accustomed to the milk diet.

Organoleptic Evaluation Procedure

The cans of UHT-AP milk processed at the University of Illinois were stored at room temperature and subjected to organoleptic evaluation at 3 and 9 weeks following processing.

Untrained judges were asked to compare the flavor of 3 samples of milk. One was the UHT-, hydrogen peroxide-treated sample; one the UHT control receiving no peroxide; and one a sample of commercially processed, homogenized, pasteurized milk. The evaluations were made in a taste panel room equipped with individual flavor judging carrels. The milk samples were served at about 10 C (50 F) in clear plastic cups, each holding a 0.5 fl oz portion. A glass of water was served along with the samples.

Each participant was asked to judge the samples on a 1 to 5 basis, depending on how well he liked each sample. If he did not like a particular sample he was asked to write down his criticism. Figure 2 is a copy of the juding form used.

November 4, 1970

Milk Samples

Please score the milk samples on a 1 to 5 basis, where:

1 = very good

2 = good

3 = mediocre or fair

4 = bad

5 = very bad

If you score a sample 3, 4, or 5; please write down your criticism.

Sample code	Score	Reason
3X	_____	_____
7A	_____	_____
2K	_____	_____

FIGURE 2. Milk flavor judging form.

RESULTS AND DISCUSSION

Preparative Procedures

Preparation of β-Lactoglobulin AB

About 12 g of dry β-lactoglobulin AB were isolated from four 10-liter batches of raw cows' milk. This was a rather low yield and was due, at least in part, to a loss in the filtrate when the salted-out protein was deposited on the filter aid. When suction was first applied the filtrate was clear, but as the filtering progressed the filtrate became slightly cloudy. The presence of protein may have accounted for this cloudiness.

After three reprecipitations, the protein was viscous, oily, and dull tan in appearance. No attempt was made to crystallize it, because in the crystalline form β-lactoglobulin is sticky and difficult to work with. The protein was evaluated in three ways to determine its purity and genetic variant distribution.

Kjeldahl nitrogen. The purity of the freeze-dried β-lactoglobulin preparation was determined by measuring its nitrogen content. The determination was done in triplicate and an average value of 15.3% nitrogen was obtained. The accuracy of the determination was checked by measuring the per cent nitrogen in tryptophan. Duplicate samples were run and an average 97.6% of the nitrogen, based on the molecular formula, was recovered. Correcting for the per cent recovery of tryptophan, the nitrogen content of β-lactoglobulin was calculated to be 15.7%. This

compares favorably with a value of 15.60% cited by Larson and Jenness (1955).

Gel electrophoresis. Flatbed gel electrophoresis was used to check for extraneous protein contamination in the β-lactoglobulin preparation. Whey (minus milk salts and lactose) and individual β-lactoglobulin polymorphic variants were run as standards of comparison. The individual variants were supplied by Dr. Marvin Thompson of the U.S.D.A. Eastern Regional Research Laboratory in Philadelphia, Pa. The gel patterns (Figure 3) indicated that the β-lactoglobulin preparation was electrophoretically pure and was composed entirely of the A and B variants, which are the only variants present in Holstein milk.

Ion exchange chromatography. The prepared β-lactoglobulin AB was chromatographed on a column of DEAE-Sephadex to separate and quantify the amount of each variant present. Two ml of a 1% protein solution were applied to the column. Two protein components were eluted from the column as determined by monitoring the eluant at 254 nm. The first peak eluted at about pH 4.65 and the second at pH 4.45. The peak areas were almost identical, indicating that the two components were present in approximately equal quantities (see Kiddy *et al.*, 1965).

The aliquots of eluant containing the protein constituents were identified by vertical polyacrylamide gel electrophoresis. The gel patterns illustrated that the first peak contained the B variant and the second peak contained the A variant. The A and B variants used as standards of comparison were those supplied by Dr. Thompson.

Figures 4 and 5 represent the ion exchange elution profile and the gel patterns, respectively. The peaks from the elution profile were very broad because the elution rate was slow; i.e., about 10 ml per hr.

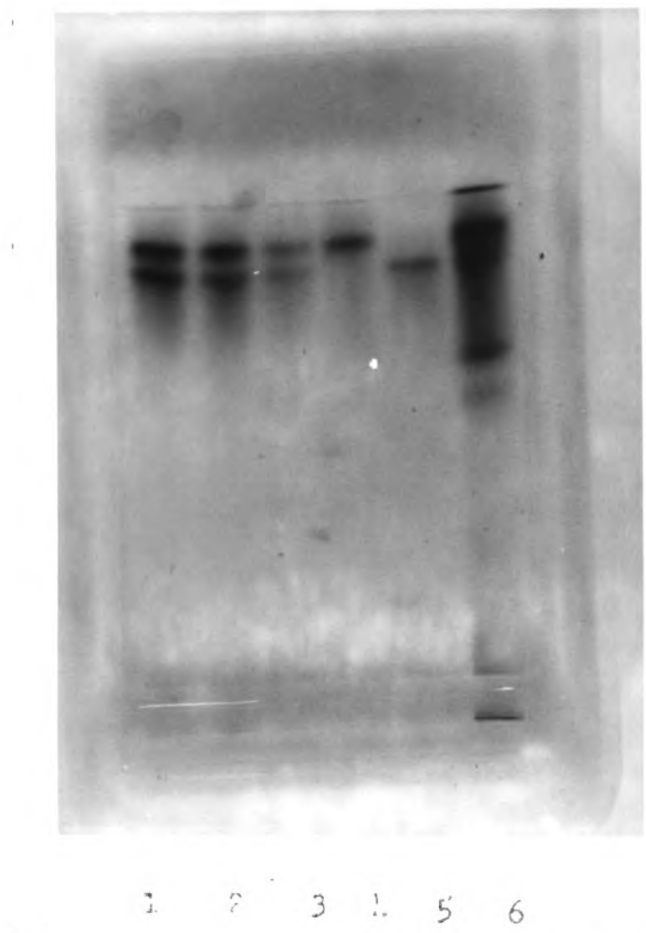

FIGURE 3. Polyacrylamide gel electrophoretic patterns of whey, β-lactoglobulin A, β-lactoglobulin B, and prepared β-lactoglobulin AB. Slot no. 1, 2, & 3, prepared β-lactoglobulin AB; slot no. 4 & 5, β-lactoglobulin A and B, respectively; slot no. 6, whey proteins.

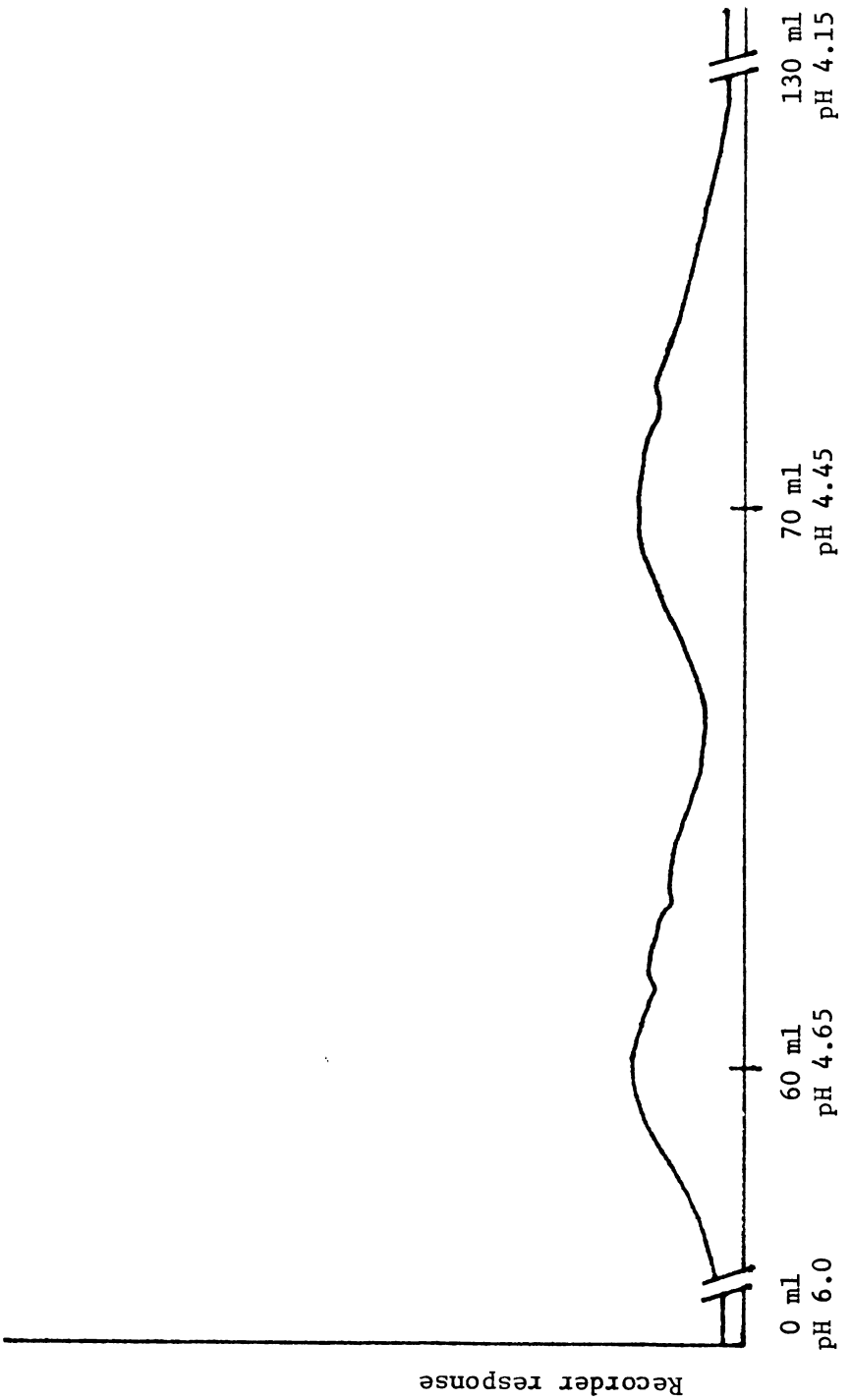

FIGURE 4. Ion exchange elution profile of prepared β-lactoglobulin AB.

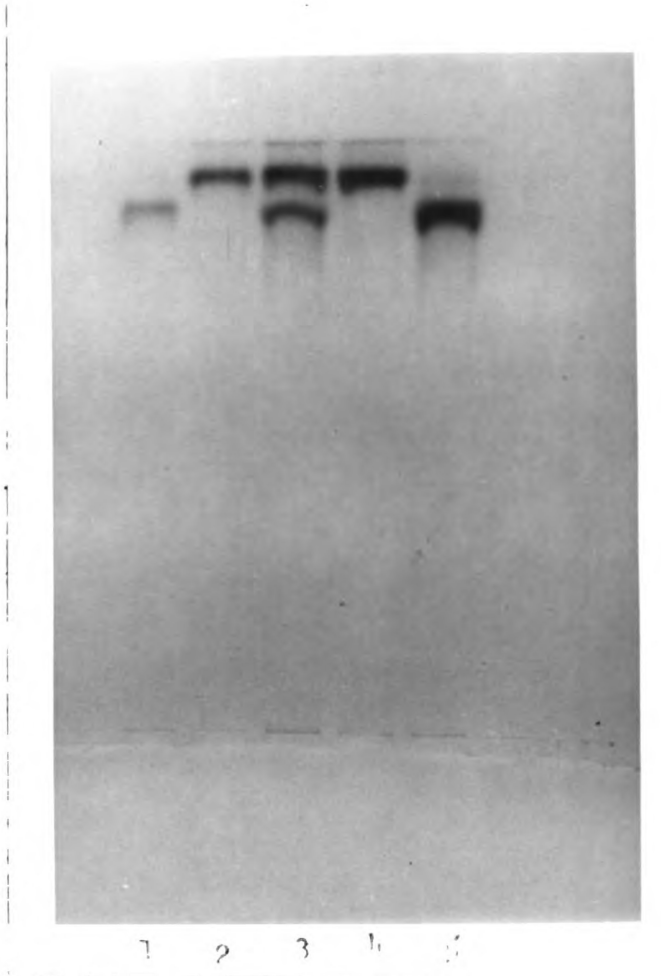

FIGURE 5. Polyacrylamide gel electrophoretic patterns of β-lactoglobulin A (contained in peak no. 2) and β-lactoglobulin B (contained in peak no. 1) eluted from the ion exchange column and corresponding protein standards of comparison. Slot no. 1, solution eluted as peak no. 1 (β-lactoglobulin B); slot no. 2, solution eluted as peak no. 2 (β-lactoglobulin A); slot no. 3, prepared β-lactoglobulin AB; slot no. 4 & 5, β-lactoglobulin A and B, respectively.

Complete separation was not achieved as seen by the failure of the profile to return to baseline between peaks. When 1 ml (not 2 ml) of the 1% β-lactoglobulin solution was applied to the column, there was complete separation as indicated by a return to baseline between peaks.

Preparation of β-Lactoglobulin Model System Solutions

The hydrogen peroxide concentration in the preparation supplied by Shell Chemical Company was determined to be 40% (w/v). According to the manufacturer's label the hydrogen peroxide concentration was 35%. Close to the completion of this research project the hydrogen peroxide concentration was measured again to check for possible decomposition. It measured 40% (w/v) the second time, also.

The only difficulty encountered with the model system samples became noticeable after the proteins were freeze-dried prior to analysis. In the freeze-dried state, most of the samples were similar in appearance; i.e., white and fluffy. However, the protein from the 1H (heated) sample was brittle, tan in color, and was the only sample that would not disperse in a weak salt solution.

Chemical Analyses of β-Lactoglobulin

Sulfhydryl Groups, Disulfides, and Methionine

Various constituents were measured in the protein isolated from the treated model system solutions. Sulfhydryl groups, disulfides, and methionine were determined in all the samples and nonprotein nitrogen was measured in selected samples. The number of disulfides was calculated to be one-half of the difference in -SH groups before and after reduction of the protein with sodium borohydride. The results, which are the average of duplicate determinations, are summarized in Table 2. The

TABLE 2. Number of -SH, -SS-, and methionine residues* and the per cent nonprotein nitrogen in β-lactoglobulin AB from model system solutions

Code name	Residues -SH (unreduced protein)	Residues -SH (reduced protein)	Residues -SS-**	Residues methionine	% nonprotein nitrogen
Lit.***	2.00	10.00	4.00	8.00	0.00
St	2.07	9.94	3.93	8.33	1.27
1H	-****	8.39	-	8.30	1.00
2.01	2.04	9.03	3.50	8.64	1.39
2.03	2.03	9.84	3.91	9.15	-
2.05	2.00	9.74	3.87	8.90	-
2.07	2.03	10.32	4.15	8.60	-
2.10	1.94	9.94	4.00	9.03	1.04
3H.01	0.00	8.70	4.35	7.80	1.28
3H.03	0.00	7.79	3.90	6.95	-
3H.05	0.00	8.00	4.00	6.70	-
3H.07	0.00	7.79	3.90	5.88	-
3H.10	0.00	7.61	3.81	4.43	1.50

* No. of residues/36,000 Daltons.

** Residues -SS- = 1/2 [residues -SH (reduced protein) - residues -SH (unreduced protein)].

*** Literature values: Piez *et al.*, 1961; Gordon *et al.*, 1961.

**** Insoluble.

amino acids are expressed as number of residues per 36,000 Daltons. The number of -SH groups (reduced protein) and -SH groups (unreduced protein) are presented graphically in Figure 6. The number of methionine residues is presented graphically in Figure 7. The lines of the graphs are connected to the average values of duplicate determinations. The values of the individual determinations are shown above and below the lines.

The data show a small decrease in the number of -SH groups (reduced protein) in the heat-treated (1H) β-lactoglobulin sample. The loss was attributed to volatilization of some of the cysteine sulfur (Zweig and Block, 1953). The -SH and -SS- groups of the protein were unaffected by the peroxide treatment in the absence of heat. This confirms the findings of Zweig and Block (1953), who reported no -SH loss in unheated milk containing peroxide. β-Lactoglobulin samples treated with both hydrogen peroxide and heat lost all of their -SH groups, but none of their -SS- groups.

The -SH groups not determined as cysteine were assumed to be the reduced form of the cystine disulfides. This assumption may be criticized as too simplistic. Perhaps some of the -SH groups were oxidized to the -SS- groups in the hydrogen peroxide-, heat-treated protein. However, the nearly constant difference of 8 -SH groups between the reduced and unreduced protein samples is most logically explained as having originated from the four cystine residues of the protein. This interpretation will be more fully discussed with the amino acid analyzer data.

The methionine content of β-lactoglobulin was unaffected by treatment with heat or hydrogen peroxide alone. However, combined hydrogen

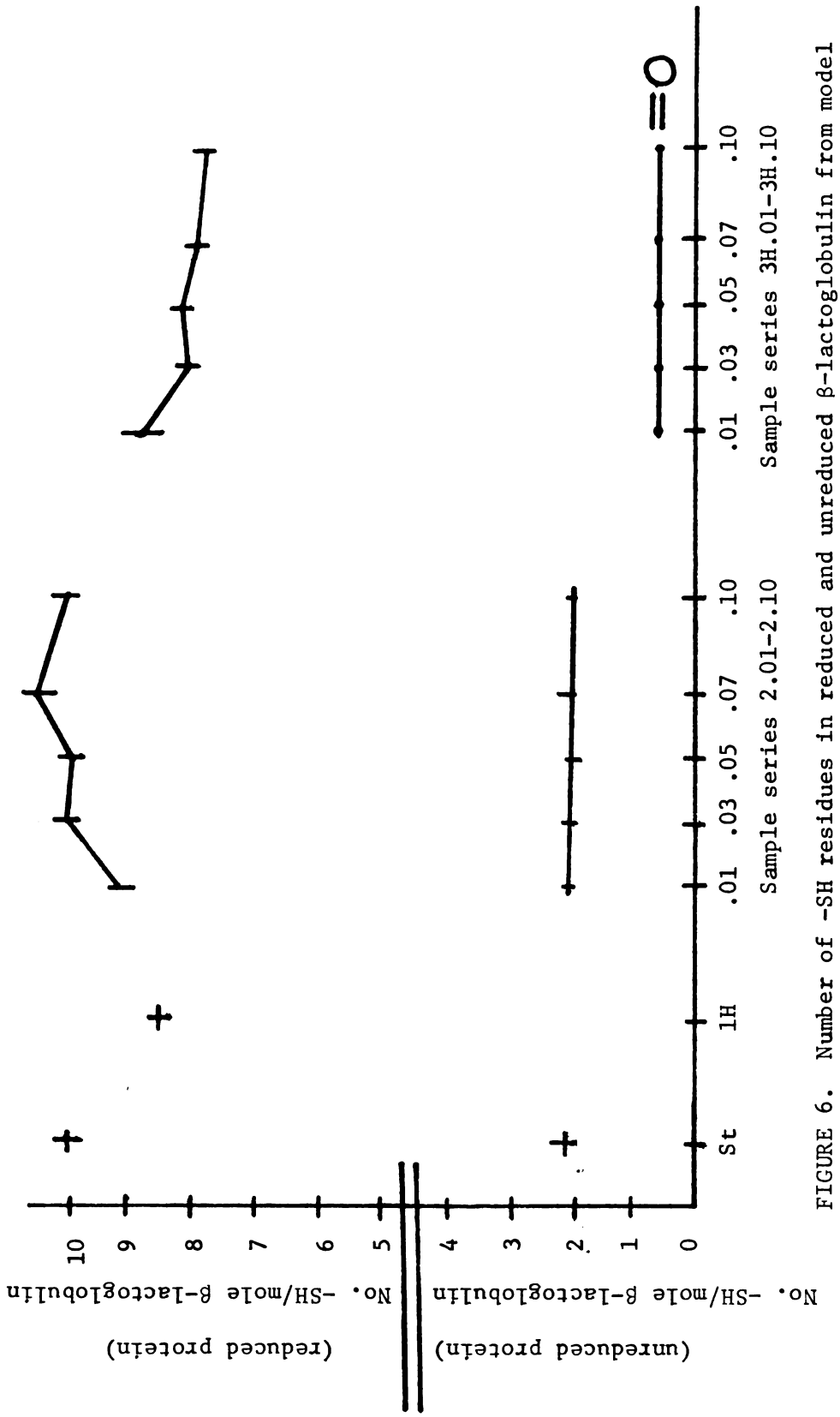

FIGURE 6. Number of -SH residues in reduced and unreduced β-lactoglobulin from model system solutions.

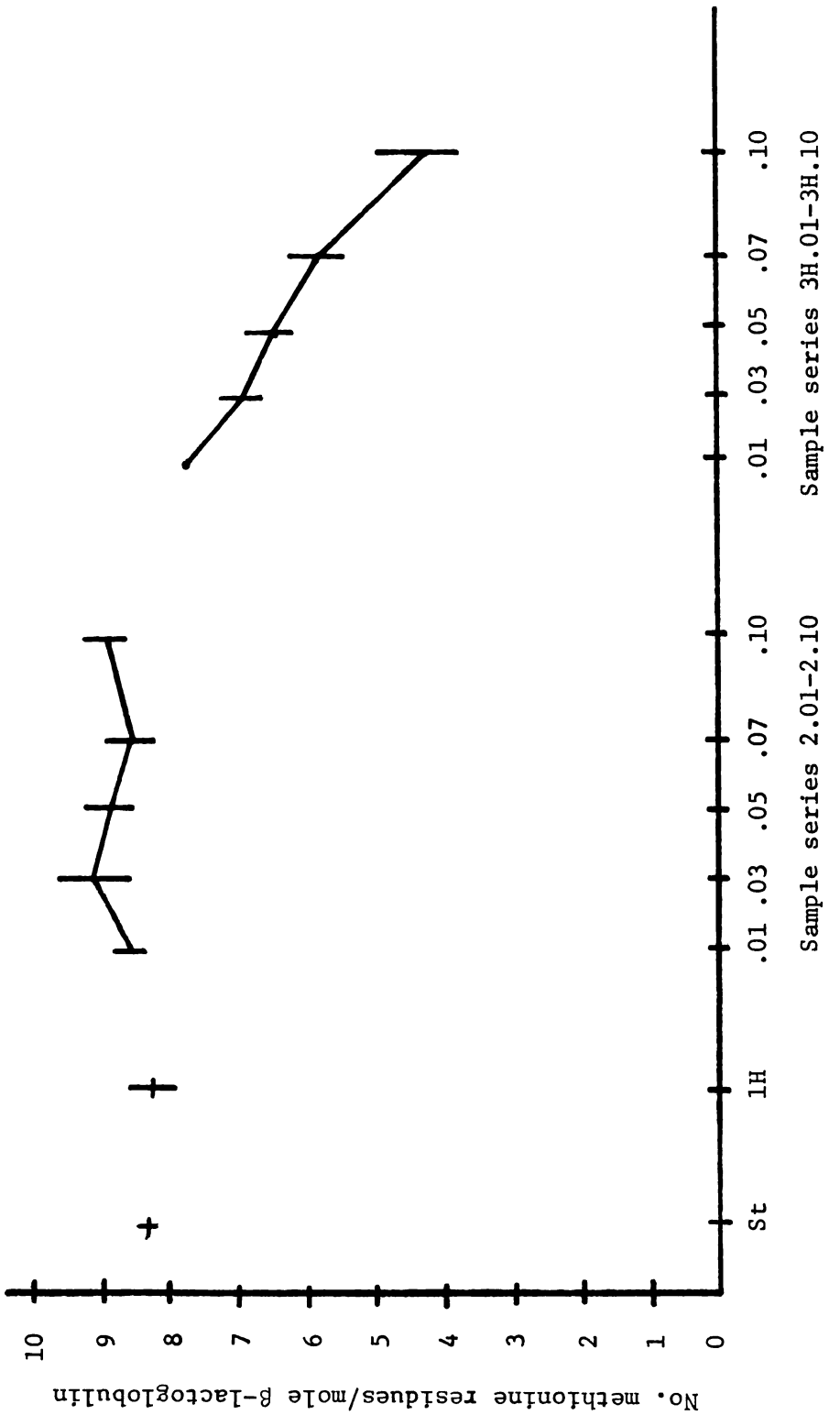

FIGURE 7. Number of methionine resfdues fn β-lactoglobulfn from model system solutions.

peroxide and heat treatments destroyed some methionine; the amount depending upon the concentration of hydrogen peroxide in the model system solution. At the 0.01% (w/v) level about 7% of the methionine was destroyed; and at the 0.10% (w/v) level about 45% was destroyed. Other investigators (Tepley *et al.*, 1958; Gregory *et al.*, 1961; Schmidt *et al.*, 1969) reported methionine losses in milk treated with hydrogen peroxide. However, none of their samples were heated to as high a temperature as was done in the present study.

Amino Acids

The amino acid analyzer data are presented in Table 3. The data are tabulated as the number of residues per 324 residues, which is the number of residues in the 36,000 Dalton dimer unit of β-lactoglobulin. A standard, a heated sample, and hydrogen peroxide-treated samples, both heated and unheated, were analyzed. Only the samples from the latter two groups containing 0.01% (w/v) and 0.10% (w/v) peroxide were analyzed. Corrections for losses of threonine, serine, cystine, and tyrosine during acid hydrolysis were made using the equation given by Hirs *et al.* (1954):

$$\log A_o = \frac{t_2}{t_2 - t_1} (\log A_1) - \frac{t_1}{t_2 - t_1} (\log A_2)$$

where A_1, A_2, and A_o are the quantities of amino acids after t_1, t_2, and t_o hours of hydrolysis, respectively. Times t_1 and t_2 correspond to 22 and 72 hr of hydrolysis, respectively. The other amino acids were determined as the simple average of the 22 and 72 hr results. All calculations were done by a computer program except for the 3H.01 and 3H.10 calculations which were done by hand because the computer program was not written to accommodate cysteic acid. Tryptophan is destroyed by acid hydrolysis and was measured independently by the method of Spies (1967).

TABLE 3. Amino acid analyses* of β-lactoglobulin AB from selected model system solutions

Amino acid	Lit.** A	Lit.** B	Code name St	1H	2.01	2.10	3H.01	3H.10
lys	30		29.49	29.77	29.44	29.47	29.81	30.32
his	4		4.68	4.62	4.86	4.12	4.01	4.08
arg	6		6.19	5.74	6.02	5.59	6.05	5.87
asp	32	30	32.15	33.36	32.71	33.99	32.41	33.14
thr	16		16.90	17.32	16.61	16.00	16.71	16.73
ser	14		16.36	15.76	16.16	12.16	16.34	15.60
glu	50		47.79	48.34	48.24	49.95	49.18	48.75
pro	16		16.22	15.03	15.22	18.08	16.77	16.09
gly	6	8	7.32	7.57	7.58	8.08	7.61	7.59
ala	28	30	28.70	28.98	29.27	30.41	28.62	29.01
1/2-cys	10		10.50	8.96	9.27	8.81	8.33	7.99
cysteic	0		0.00	0.00	0.00	0.00	0.62	2.07
val	20	18	21.93	22.17	22.27	22.24	20.96	21.62
met***	8		3.75	3.68	4.08	4.32	4.72	3.64
isoleu	20		19.15	18.57	19.39	19.14	18.21	19.09
leu	44		43.61	43.75	43.87	42.42	44.04	43.72
tyr	8		7.77	8.00	7.70	8.29	7.55	7.47
phe	8		7.98	8.71	8.10	7.43	8.44	8.22
try	4		3.52	3.66	3.22	3.48	3.62	3.00

* No. of residues/324 residues.

** Literature values: Piez *et al.*, 1961; Gordon *et al.*, 1961.

*** No methionine sulfoxide or methionine sulfone was found in any of the samples analyzed.

Cysteine and cystine are both assayed as cystine in the analyzer, but reported as 1/2-cystine (i.e., 1/2-cys) because one mole of cystine combines with two moles of ninhydrin. An air oxidation step is sometimes used (Moore and Stein, 1963) to convert the cysteine to cystine after acid hydrolysis. It was unnecessary to use the procedure in the present work because the sulfhydryl groups were oxidized to disulfides during the normal preparation of samples for analysis.

Inspection of the tabulated results reveals that selected treatments affected the 1/2-cystine values. The methionine values failed to support any trend and all the other amino acids were unaffected by the treatments.

Before discussing the significance of these data, mention should be made of the somewhat 'out-of-line' residue values for the 2.10 sample. The serine and 1/2-cystine contents were both low, and the proline content was high when compared to other analyses. Some of the other values were also not in complete agreement.

A low 1/2-cystine value is often accompanied by a high proline value because any cysteine not oxidized to cystine appears in the position of proline on the amino acid chromatogram. Proline is measured at 440 nm and cysteine is measured at 570 nm; so it is possible to distinguish between the two on the chromatogram (Moore and Stein, 1963). In the case of the 2.10 sample the chromatogram of both the 22 and 72 hr hydrolyzates failed to show cysteine in the proline position. Therefore, unoxidized cysteine is not the reason for the anomalous 1/2-cystine and proline values.

Because the chemical tests indicated that cysteine and methionine residues were sometimes destroyed, the amino acid analyzer was used to identify and quantitatively determine possible destruction products. A

known amount of cysteic acid was chromatographed on the analyzer, it being a likely oxidation product of cysteine. Methionine sulfoxide and methionine sulfone were also assayed, both being possible oxidation products of methionine. The cysteic acid and methionine sulfone standards were stable to 22 hr acid hydrolysis at 110 C--a necessary prerequisite for detection by amino acid analysis. However a methionine sulfoxide standard was unstable and about half of it reverted to methionine during a 22 hr period of acid hydrolysis. A much smaller amount appeared as minor unidentified peaks at other places in the amino acid chromatogram.

The analyzer results compare well with those found by chemical methods. The heated (1H) sample lost some of its 1/2-cys (-SH) which may be explained by sulfide liberation. The unheated hydrogen peroxide-treated samples lost no 1/2-cys, nor did they gain any cysteic acid. In the heated samples containing peroxide, there was a loss of about two 1/2-cys (-SH) groups which was accompanied by the appearance of cysteic acid. At the 0.10% peroxide level the change was quantitative; all the 1/2-cys lost could be accounted by the cysteic acid gained. But at the 0.01% peroxide level, the 1/2-cys decrease was not matched by an equivalent gain in cysteic acid.

This difference in the amounts of cysteic acid formed may be due to a difference in oxidizing strengths of the 0.01 and 0.10% peroxide concentrations used. The peroxide was active enough at the 0.10% level to completely oxidize the cysteine residues to cysteic acid residues; but at the 0.01% level it was not active enough to effect a complete conversion. Therefore, at a lower concentration some of the cysteine residues may have been converted to intermediate oxidation products which were destroyed during acid hydrolysis. A similar explanation was offered by Thompson and O'Donnell (1959). From amino acid analyzer data they

found that peracetic acid oxidized wool incompletely and that the more active performic acid oxidized it completely--even cleaving and converting cystine residues to cysteic acid residues. In the case of the incomplete oxidation the two workers hypothesized the formation of intermediate oxidation products such as -SOH and $-SO_2H$ which may have been destroyed by acid hydrolysis prior to amino acid analysis.

The chemical and analyzer determinations of -SH residues in the hydrogen peroxide-, heat-treated samples complement each other. There were about 8 -SH groups in the reduced protein which were not initially present as cysteine nor labile to oxidation with hydrogen peroxide when heated. By the coincidence of numbers, the 8 groups correspond to the 4 reduced disulfides of cystine in the β-lactoglobulin dimer. Also in support of the same conclusion, the -SH groups of β-lactoglobulin are known to be quite stable (Larson and Jenness, 1952; Townend *et al.*, 1969). They do not form disulfide bridges readily as is true of other SH-containing proteins. If this stability is due to steric hindrance, the same explanation would apply to the absence of disulfide bond formation following oxidation with hydrogen peroxide (Cecil, 1963). Steric hindrance would force the oxidation of the sulfhydryl groups beyond the disulfide stage (Chinard and Hellerman, 1954).

Further support is indicated by the work of Larson and Jenness (1952). Using a Warburg apparatus they measured oxygen uptake by the heat-activated -SH groups of β-lactoglobulin. They determined that about 0.7 moles of O_2 was taken up per mole of activated cysteine (i.e., -SH). Because only 0.25 moles is needed per mole of cysteine to form disulfides, they proposed that oxidation beyond the disulfide stage was taking place.

The analyzer results for methionine are quite disappointing. They varied in a random fashion around 4 methionine residues per 324 residues.

Except for the 3H.01 and 3H.10 samples, values of 8 were expected. Not only was the recovery low, but there was no trace of the probable oxidation products. This may be because: 1) acid hydrolysis destroyed some of the methionine prior to analysis; and 2) the oxidation products, whatever they were, were completely destroyed by acid hydrolysis. A similar explanation is suggested by the work of Brunfeldt and Thomsen (1966). They found that methionine and its oxidation product methionine sulfoxide were both destroyed during the acid hydrolysis of casein. These findings demonstrate the importance of conducting an independent assay for methionine when determining a protein's amino acid composition after acid hydrolysis.

Nitroprusside Test

The rather inelegant nitroprusside test contributed supporting evidence to many of the above conclusions. A distinctly positive nitroprusside reaction, indicating activated -SH groups, was achieved only with the 1H sample. The St and 2.01 samples yielded negative results and the reaction for the 3H.01 sample was only slightly positive. This was probably due to trace amounts of nitroprusside reactive material (other than -SH) that were activated by the heat treatment.

Nonprotein Nitrogen

There was almost no increase in the nonprotein nitrogen of the model system solutions following the various heat and/or hydrogen peroxide treatments (see Table 2). This observation suggests that the treatments did not rupture peptide bonds, breaking up the protein. To the contrary, the treated samples were not characterized by protein breakdown, but by protein aggregation, as will be seen in the following section.

Physical Analyses of β-Lactoglobulin

Opacity of Model System Solutions

Opacity measurements (see Table 4) were made on the model system solutions after treatment. Measurements were made three times on separate samples of the 3H.01 through 3H.10 sample series. These measurements were made at 720 nm, a transmittancy maximum for the solutions. All the samples that received a heat treatment were opaque, indicating, in most instances, some protein aggregation (Zittle and DellaMonica, 1957). The heated (1H) sample was by far the most turbid. It was necessary to make a 1:19 dilution with water to bring the turbidity into a readable range. The turbidity was indicative of an extremely high degree of disulfide linking. This polymer formation could also account for the insolubility of the protein after freeze-drying. It is interesting to note that when this protein sample was reduced with sodium borohydride in the disulfide assay procedure, it regained its solubility in a dilute salt solution. Presumably the reducing agent cleaved the disulfides, breaking up the aggregates.

Because no heat was applied, the St and 2.01 through 2.10 samples were not at all turbid. However, there was significant turbidity in the peroxide-containing samples receiving a heat treatment. This turbidity decreased (i.e., per cent transmittancy increased) in the heated samples as the concentration of hydrogen peroxide was increased. Assuming that the turbidity was due to the reflection of light off large protein aggregates formed by intermolecular disulfide linkages, one can suggest an explanation for the effect of peroxide on the turbidity measurements. With no peroxide present the activated -SH groups of the heated protein may be either liberated as sulfides or joined by disulfide linkages to

form aggregates. In the presence of peroxide there is a third alternative--the activated -SH groups of the cysteine residues may undergo oxidation to cysteic acid, or some intermediate oxidation product. This latter alternative, if favored, greatly reduces the formation of protein aggregates and the liberation of sulfides. With increasing peroxide concentration, the oxidation becomes the preferred pathway followed.

Fish and Mickelson (1967a) found that there was a gradual decrease in whey protein denaturation of heated skimmilk as the hydrogen peroxide concentration was increased from 0 to 0.07%. Above 0.07% there was no further decrease in denaturation. The decrease in opacity between model system solutions containing 0.07 and 0.10% peroxide is not as great as for the samples containing a lower peroxide concentration. Thus the opacity data tend to agree with the whey protein denaturation data. However, there is not enough information available to draw any reliable conclusions.

Finally it can be seen that the blank, the simulated milk ultrafiltrate plus 5% lactose (no protein), was quite opaque when heated. In one series of samples it was as opaque as the protein solutions containing 0.07 and 0.10% peroxide. According to Jenness and Koops (1962), heat treatment forces calcium phosphate to precipitate from the simulated milk ultrafiltrate. The precipitate tends to redissolve on cooling, but requires many days to completely resolubilize.

Gel Electrophoresis

Vertical gel electrophoresis patterns further illustrate the effect of the different treatments (see Figure 7). The patterns of the St and 2.01 through 2.10 samples appeared to be similar--showing no alteration from the expected pattern of β-lactoglobulin AB. Most of the protein from

TABLE 4. Opacity (per cent transmittancy) of model system solutions

Code name	Series number 1	2	3	Average*
St	100.0%	100.0%	100.0%	100.0%
2.01	100.0	100.0	-	100.0
2.03	100.0	-	-	100.0
2.05	100.0	-	-	100.0
2.07	100.0	-	-	100.0
2.10	100.0	100.0	-	100.0
1H**	47.0	67.5	49.0	54.5
3H.01	17.0	23.0	8.0	16.0
3H.03	18.5	35.0	27.5	27.0
3H.05	33.0	43.0	23.5	33.0
3H.07	49.5	47.0	30.5	42.0
3H.10	47.5	55.0	31.5	45.0
Blank	49.0	77.5	-	63.0

* Average rounded to nearest 0.5%. ** 1:19 dilution with water.

the 1H sample remained in the slot, indicating that it was too aggregated to enter the gel. All the 3H samples produced rather diffuse smears, though maintaining two major zones which decreased in electrophoretic mobility with increasing peroxide concentration. Also, they were all partially aggregated, as the protein remaining in the sample slots illustrates.

Evaluation of Treated Milk

Nutritional Evaluation: Rat Feeding Trials

The results of the rat feeding study are given in Table 5. The weight gain is the average weight gain per rat over the 12 day feeding period plus the sample standard deviation for each group of 10 rats. The average milk consumed was calculated by summing the volume of milk consumed by all the rats of each group over the 12 day feeding period.

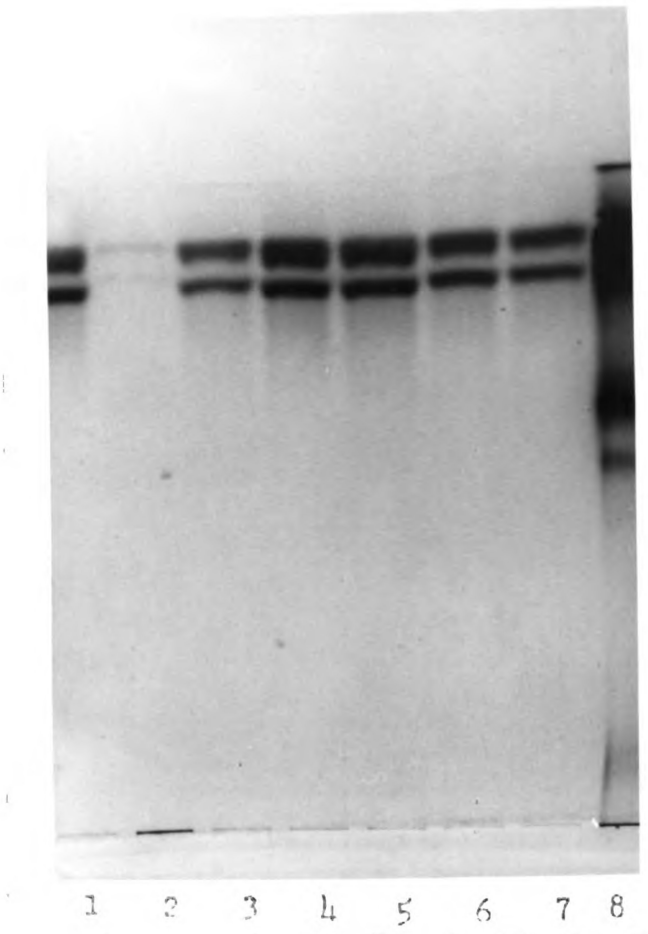

FIGURE 8. Polyacrylamide gel electrophoretic patterns of β-lactoglobulin AB from model system solutions. Slot no. 1, St; slot no. 2, 1H; slot no. 3, 2.01; slot no. 4, 2.03; slot no. 5, 2.05; slot no. 6, 2.07; slot no. 7, 2.10; slot no. 8, whey proteins.

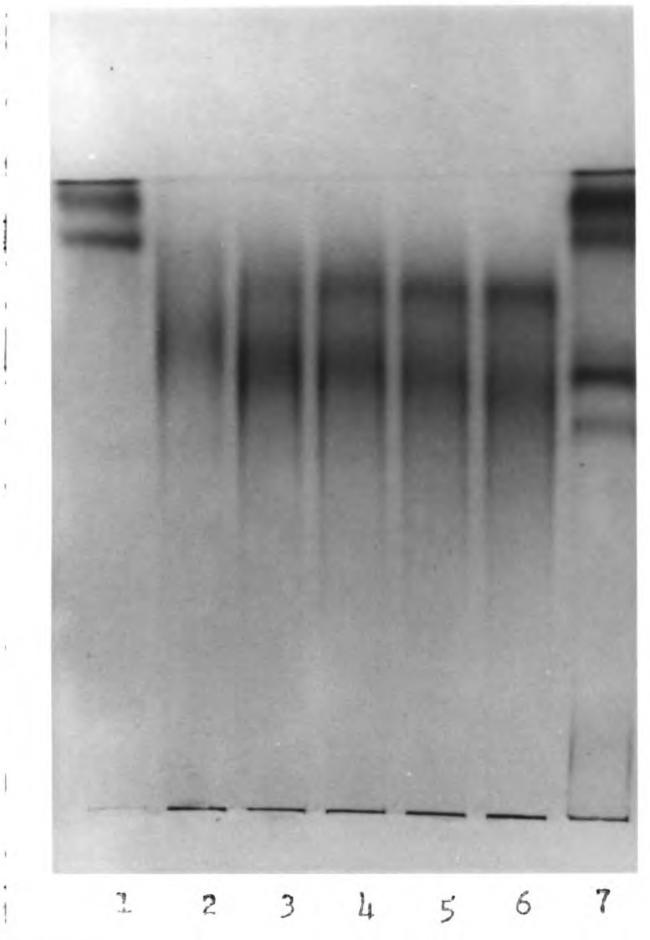

FIGURE 8. (Continued) Slot no. 1, St; slot no. 2, 3H.01; slot no. 3, 3H.03; slot no. 4, 3H.05; slot no. 5, 3H.07; slot no. 6, 3H.10; slot no. 7, whey proteins.

This result was divided by 10 to yield the average total milk consumption for a rat of each group. Per cent fat in the milk samples was measured by the Mojonnier modification of the Roese Gottlieb fat determination (Milk Industry Foundation, 1959) using a Mojonnier Milk Tester manufactured by the Mojonnier Bros. Co.; Chicago, Illinois.

TABLE 5. Results of the rat feeding trials

Group*	Average rat wt_i (g)	Average rat wt_f (g)	Weight gain (g)	Av. vol. milk consumed (ml)	% fat
A	52.9	120.8	67.9 $\pm$ 10.36	659	3.58
B	52.4	118.6	66.2 $\pm$ 8.74	672	3.56
C	51.6	116.6	65.0 $\pm$ 8.14	674	3.50

*Group A: pasteurized control; B: UHT control; C: UHT treated.

None of the differences in weight gain among the three groups was statistically significant. Nor were there any large differences in the amount of, or fat content of, the milk samples consumed. Therefore the three differently processed milk samples appear to have the same nutritional value.

An attempt was made to measure methionine in the milk samples. The aqueous fraction from the Roese Gottlieb test was used as the protein source. The McCarthy and Sullivan (1941) methionine assay of this fraction indicated that all of the amino acid was destroyed. Probably the fat extraction was responsible for the methionine destruction.

The assay was done on 2 lots of skimmilk which had been processed in a fashion similar to the milk given rat Groups B and C. Before

analysis the skimmilk was exhaustively dialyzed against water to remove salts and lactose, and freeze-dried. Results of the assay were that the UHT control and UHT treated skimmilk samples contained 0.030 and 0.0295 g methionine per g of protein, respectively. The insignificant loss of methionine in the treated sample was expected considering the low (0.01% (w/v)) concentration of hydrogen peroxide used. The difference in methionine content between the 1H and 3H.01 β-lactoglobulin samples was also small. These samples received treatments analagous to the treatments given to the milk samples that were fed rat Groups B and C.

Organoleptic Evaluation: Taste Testing Panels

Scores from the taste panels were compiled and their differences were tested for statistical significance. The average score for each milk sample and its sample standard deviation are presented in Table 6. A 5-point rating system was used to score the samples ranging from 1 = very good to 5 = very bad. The commercial sample was commercially homogenized, pasteurized whole milk.

TABLE 6. Results of organoleptic evaluation of UHT-AP milk samples

Milk sample	Time in storage at room temperature	Number of taste panel judges	Average score
Commercial	-	24	1.83 ± 0.47
UHT control	3 weeks	24	2.96 ± 1.17
UHT treated	3	24	3.17 ± 1.14
Commercial	-	25	1.80 ± 0.63
UHT control	9	25	2.88 ± 0.88
UHT treated	9	25	3.20 ± 1.06

The differences in score between the UHT control and UHT treated milks were not significantly different for either 3 or 9 week storage period evaluations. Nor were the differences significant between the same milk samples judged at 3 and 9 weeks following processing. However, in both the 3 and 9 week tests the commercial milk sample was judged significantly better ($\alpha = 0.01$, one-tailed test) when compared to the control or treated sample.

As indicated by the rather large sample standard deviations for both the UHT control and UHT treated samples, the taste panelists' scoring were not uniform. However, most did express a preference for the commercial sample. And they criticized the control and treated samples for having a cooked flavor after 3 weeks of storage, and for having a cooked and/or caramel flavor after 9 weeks of storage. Furthermore, between the third and ninth week of storage the control and treated samples both became slightly brown in color. At no time, though, was there evidence of fat separation or destabilization of other milk solids.

On the day the UHT milk samples were processed, the control sample had a strong sulfide flavor that was absent in the treated sample. However, by the time the first taste panel was conducted, the sulfide flavor in the control sample had dissipated.

CONCLUSION

The goals of this research project were twofold. The first was to explain why the addition of hydrogen peroxide prevents sulfide flavor development in high temperature processed milk. The second was to use the peroxide treatment with heat sterilization to produce a sterilized milk acceptable to consumers.

The first goal was accomplished. It was shown that hydrogen peroxide oxidizes the -SH of the cysteine residues in milk proteins, preventing the evolution of sulfides. The second goal was not realized. The peroxide treatment did nothing to decrease the caramelized flavors normally associated with heat-sterilized milk. These flavors make the milk unacceptable to consumers. Furthermore, the sulfide flavor, which was specifically prevented by the addition of peroxide, dissipates over time in normally processed UHT milk. Thus the peroxide treatment is of value only if the heat-sterilized milk is to be consumed shortly after processing.

The other interesting finding, which is actually an extension and confirmation of work done by others, is that hydrogen peroxide destroys methionine. The extent of destruction decreases with decreasing peroxide concentration. At a low peroxide concentration (0.01% (w/v)), it is possible to prevent sulfide flavor development in UHT milk, yet not destroy enough methionine to measurably decrease the milk's nutritional quality.

Judging from the data obtained on β-lactoglobulin samples, at higher peroxide concentrations (up to 0.10% (w/v)) none of the amino acids other than cysteine and methionine is affected by a hydrogen peroxide, high temperature treatment.

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