THE OCCURRENCE AND POSSIBLE PRECURSOR OF METHYL SULFIDE IN MILK FROM COWS FED ALFALFA FORAGES

by

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B. S., Kansas State University, 1959

A MASTER'S THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

Department of Dairy and Poultry Science

KANSAS STATE UNIVERSITY

Manhattan, Kansas

1967

Approved by:

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INTRODUCTION

The importance of the flavor of milk cannot be over emphasized. No other characteristic of milk is as important as flavor for determining acceptability to the consumer. Although off-flavors in milk can cause great economic loss to the dairyman, considerable milk produced has flavor defects. The most common of these off-flavors are caused by feed.

During the past forty years, much research has been directed toward better understanding of the feed flavor problem in milk. Until recently, the progress of researchers has been restricted since the compounds responsible for feed flavors were not definitely known. With the identification of methyl sulfide (Me_2S) as an important compound associated with the alfalfa and silage feed flavor defects, advances probably will be made in controlling feed flavors in milk.

Although the chemical nature of the flavoring compound responsible for alfalfa feed flavors in milk is known, the precursor of Me_2S has not been established. Previous research indicated an apparent decreased availability of the Me_2S precursor when alfalfa hay was fed as compared to fresh alfalfa. Since low levels of Me_2S have been shown to be a desirable component of dairy products, a means of controlling and stablizing the level of Me_2S in milk would seem appropriate. Therefore, the need for a better understanding of the precursors of Me_2S is apparent.

The research presented here in was designed to:

(a) determine the effect of drying alfalfa on the availability of the precursor for the production of Me_2S.

(b) determine the effect of storing alfalfa hay on the availability of the precursor for the production of Me_2S.

(c) investigate the possibility of methionine, methionine sulfoxide, and

methionine sulfone as precursors of Me_2S in vitro.

(d) determine the effectiveness of methionine and sulfur as precursors of Me_2S in vivo.

REVIEW OF LITERATURE

Importance of Flavor in Milk

The flavor of milk is one of the most important characteristics governing its market value. Milk having an unpleasant flavor is one of the main reasons for rejection by both the processor and the consumer. Therefore, a uniform palatable product must be produced from day to day in order to maintain the consumption of fluid milk.

Lucas (1929) classified flavor defects in milk as those that were present at the time of milking and those that developed with age. Flavor defects present at the time of milking were believed caused by feed or the condition of the cow. These defects do not intensify with age and cannot be transferred from one lot of milk to another. Flavor defects that develop after storing were usually believed due to bacterial fermentation and are not related to feed flavors. Bacterial flavor defects can be transferred from one lot of milk to another.

One of the first flavor defects caused by feed was reported by Strobel et al., (1953) to have occurred in England in 1757 and was associated with feeding turnips. Surveys have shown that flavors caused by feed are the most prevalent flavor defects observed in milk (Weaver et al., 1935A; Dahlberg et al., 1953; and Jensen, 1960). As a result, methods for controlling feed flavors in milk have attracted the attention of many researchers during the last four decades. Yet, recently Ship (1964) reported a survey in which 39% of the samples tasted had feed flavor defects.

The continued existence of feed flavors in milk does not reflect the progress made toward understanding the problems. Instead, dairymen have been reluctant to adopt recommendations for controlling feed flavors in milk since

certain feeds usually must be avoided or limited, and management problems are created. Therefore, further studies are needed so that practical rations can be utilized and milk free of flavor defects can be produced.

One of the most important forages for dairy cattle is alfalfa. Although alfalfa is usually criticized for causing bloat in cattle, it also produces an undesirable milk flavor. With the use of a bloat preventing compound now available (poloxalene) and probable increased utilization of alfalfa pasturage, the importance of alfalfa feed flavor undoubtedly will be accentuated.

Characteristics of Milk Flavoring Compounds

Experienced judges of dairy products are proficient in evaluating the flavor of milk, but the chemical nature of the flavors has not been completely characterized. Before a particular compound can be said to contribute significantly to the flavor of milk, the concentration of the compound in milk must be measured and the organoleptic threshold of the compound must be determined. Patton and Josephson (1957) stated that a compound will contribute to flavor if it is present in greater than threshold concentrations.

Davis (1950) attributed the flavor of freshly drawn milk to a complex mixture of lower fatty acids and their condensation or oxidation products, acetone bodies, carbon dioxide, and other volatile products occurring normally in small quantities in tissue fluids. Wynn et al. (1960) believed that the flavor of milk was influenced by acetone, acetaldehyde, and methyl sulfide (Me_2S). However, Potts and Kessler (1957) found no relationship between the concentration of acetone bodies in individual cow's milk and the flavor. Acetone and 2-butanone were identified in milk by Loney et al. (1963), but the off-flavor noted was not related to the concentration of either compound.

Methyl sulfide was isolated from the volatile constituents of normal milk

by Patton et al. (1956). The odor of Me_2S, the volatiles from raw milk, and a cow's breath were reportedly similar, therefore, it was suggested that Me_2S contributed to the flavor of raw milk. The flavor threshold of Me_2S in distilled water was 12 ppb. Concentrations slightly above threshold produced a milk-like flavor, while at concentrations well above threshold a cowy or malty flavor was observed. This is the characteristic description of the flavor defect in milk from cows fed fresh alfalfa. The presence and importance of Me_2S in the flavor of fresh milk and cream was confirmed by Wong and Patton (1962).

Shipe et al. (1962) concluded that feed flavors in milk were caused by compounds other than carbonyls. When carbonyl-free neutral fractions from steam distillates of grass and corn silage were introduced into the lungs or rumens of cows, normal feed flavors were produced. The potency of Me_2S as a flavoring compound was indicated, since 1 to 5 ml placed in the rumen produced a strong feed flavor in the milk. Day et al. (1964) observed that the concentration of Me_2S in butter was highest during the spring and was related to feed flavors. This report noted that below threshold concentrations Me_2S tends to mask the harsh flavors of diacetyl and acids associated with culture flavors.

A positive correlation between the flavor defect produced by fresh alfalfa and the concentration of Me_2S was observed by Reddy (1966). In this study the organoleptic threshold of Me_2S in pasteurized homogenized milk was 19 ppb. Methyl sulfide in milk from cows receiving freshly chopped alfalfa ranged from 27 to 35 ppb as compared to less than 10 ppb from cows eating rye or brome grass pasture.

Factors Responsible for Alfalfa Flavors in Milk

The literature contains numerous reports of alfalfa causing feed flavors in milk. Although the chemical nature of the flavoring compound believed responsible was not known until recently, the typical description of alfalfa-produced flavors was noted.

It is generally accepted that alfalfa flavors are more prevalent during periods when alfalfa is fed as a succulent feed. Many milk flavor studies use green alfalfa or alfalfa silage in the experimental ration and alfalfa hay in the control ration. Lucas (1929) noted off-flavors in milk were more noticeable during the spring and autumn months when succulent pastures were being utilized. According to Roadhouse et al. (1926), alfalfa fed as hay, pasturage, or freshly cut produced a distinct feed flavor in milk two hours later. However, the flavor produced by pasturage or freshly cut alfalfa was more intense than the flavor produced by hay. This report concluded that flavors observed in milk varied due to individual cows, and whether or not the alfalfa had been cured.

The flavor provoking ability of alfalfa silage was demonstrated by Gamble and Kelly (1922). A distinct feed flavor was produced one hour after feeding 5.9 to 7.9 kg alfalfa silage and a noticeable flavor when these amounts were fed after milking. Wide variation among individual cows in the flavor and odor of the milk produced was also noted.

Other factors that apparently affect the intensity of the flavor defect produced by alfalfa include: amount consumed; time between feeding and milking; and stage of maturity of the alfalfa. A pronounced feed flavor in milk was observed by Weaver et al. (1933B) when alfalfa hay was fed less than five hours before milking. The feed flavor was not as noticeable four hours after

four hours after feeding and was practically eliminated five hours after feeding. The effects of different quantities of alfalfa on the flavor intensity were studied by Roadhouse and Henderson (1935). A feed flavor was observed two hours after feeding 5.9 kg alfalfa hay or 13.4 to 15.7 kg green alfalfa. A more intense flavor was produced, however, by green alfalfa. It was found that as little as 3.9 kg green alfalfa or 2.0 kg hay would impart an undesirable flavor to milk. Reddy (1966) found that the flavor produced in milk by green alfalfa was less intense as the alfalfa matured. The improved flavor was correlated with a lower Me_2S level in the milk.

Methods of Controlling Alfalfa Flavors in Milk

Research conducted by Weaver et al. (1935B) and Roadhouse and Henderson (1937) indicated that the flavor defect caused by alfalfa could be prevented by withholding the forage five hours before milking. This practice has not been readily accepted by dairymen due to the management problems created. As a result, research has been directed toward methods for removing the feed flavor from milk.

Methods for removing the defect due to feeding alfalfa are based upon the volatility of the flavoring compound. Gamble and Kelly (1922) and Babcock (1938) recommended aeration of freshly drawn milk as a means of correcting the flavor defect due to alfalfa. Later MacCurdy and Trout (1940) reduced the intensity of feed flavors by pasteurization.

More recently, volatile flavoring compounds have been removed successfully with reduced pressure at or near pasteurization temperature. Wynn et al. (1960) reported removing Me_2S from milk by distillation at 50 C under 2.5 cm mercury vacuum. Reddy (1966) subjected milk from cows receiving green alfalfa to two-stage vacuum pasteurization. Gas chromatographic analysis

indicated that the Me_2S concentration was lowered below threshold.

The research conducted by Reddy (1966) also included a study of the effects of feeding a nonionic surface active agent as a means of controlling the alfalfa flavor defect. His hypothesis held that a lower rumen fluid surface tension would allow the release of volatile flavor compounds rapidly, thereby avoiding the feed flavor defect. Apparently lowering the rumen fluid surface tension had no effect on milk flavor.

Possible Precursors of Methyl Sulfide

Several possibilities have been suggested as precursors of Me_2S, yet none has been isolated from alfalfa except methionine and sulfate. Day et al. (1964) suggested that the Me_2S normally found in milk was a metabolic product of methionine. They also postulated that the concentration of Me_2S found in milk with a feed flavor defect was due to superimposing Me_2S resulting from the metabolism of certain feeds upon the normal concentration. Kiribuchi and Yamanishi (1963) apparently produced Me_2S by simple hydrolysis of methyl-methionine-sulphonium salt found in green tea leaves. After heating in water, the sulphonium salt yielded Me_2S and homoserine. Challenger (1959) identified dimethyl-beta-propiothetin in algae and methyl-methionine-sulphonium salt in asparagus as precursors of Me_2S. The Me_2S was liberated by treating with cold sodium hydroxide and boiling alkali, respectively.

Although specific pathways for the production of Me_2S in the rumen have not been elucidated, there is one unique pathway which may explain this phenomenon. White et al. (1964) reported that mutants of Aerobacter aerogenes synthesize methionine by the direct transfer of a thiomethyl group (transmethiolation) from thiomethyladenosine to alpha-amino-butyric acid. Thus, methylation of the thiomethyl groups would yield Me_2S. An observation

of Toan et al. (1965) tends to support this phenomenon. While studying milk flavor defects due to bacterial fermentations, strains of Aerobacter aerogenes in milk produced quantities of Me_2S well above the taste threshold.

According to Samuelsson (1962) Me_2S can be formed in the body tissues. After administering S^{35} labeled methionine through the left pudic artery of a lactating cow, S^{35} was found in the Me_2S fraction of the volatiles from milk. However, this theory does not eliminate the possibility of the labeled methionine being recycled to the rumen in saliva. White et al. (1964) reported that the reverse of the transmethiolation reaction occurred in the liver of the rat, which may account for Samuelsson's observation.

Because Me_2S is found in milk soon after feeding, it seems likely that Me_2S from microbial fermentations in the rumen would be more significant than the Me_2S produced in the tissues. This agrees with the results of a study by Pereira et al. (1964) in which sulfur appears to be a precursor of Me_2S. When S^{35} labeled sulfate was placed in the rumen, labeled hydrogen sulfide, mercaptans, sulfides, disulfides, thionals, and thiones were collected from the volatiles of the milk. The quantities of these labeled compounds were greater than those produced after infusing S^{35} labeled methionine into the blood stream.

Although little is known about the precursor of Me_2S in alfalfa, there is indication that it is not volatile. Morgan and Pereira (1963) were unable to detect Me_2S in the volatile compounds collected from green alfalfa. The main constituent believed responsible for the grassy aroma of alfalfa was trans-2-hexenal. Previously Morgan and Pereia (1962) isolated Me_2S from the volatile compounds in grass and corn silage which indicates that microorganisms probably alter the precursor of Me_2S in forages during fermentation.

Research reported by Roadhouse and Henderson (1932) indicated that the

precursor of Me_2S was a soluble constituent of alfalfa plants. After administering alfalfa juice as a drench to dairy cows, a feed flavor defect was observed in milk twenty to thirty minutes later.

Physiological Transmission of Flavors

Feed flavors observed in milk are believed exogenous with respect to the udder. There is no evidence that the synthesizing mechanism of the udder produces any flavors similar to feed flavors. Hence, the flavoring compound must be in the blood and the membranes of the udder must be permeable to it. The concept of blood transporting off-flavors to the udder is supported by numerous reports of feed flavors developing in milk soon after feeding and then decreasing in intensity after the feed is withheld. The dynamic state of flavoring compounds in milk indicates the existence of an equilibrium between blood and milk. Petersen and Brereton (1942) pointed out that the possible avenues through which flavoring compounds may gain entrance to the blood are: through the walls of the alimentary tract from ingested materials; through the lungs from inhaled substances and eructated substances from the rumen; and through the skin from contacted substances.

It is generally known that the consumption of flavor provoking feeds or the inhalation of certain odors produce flavors in milk. However, the physiological mechanism involved in transmitting flavors had not been studied until recently. Dougherty and Ghipe (1960) developed a technique for studying the various pathways of flavor transmission. Two cows with ruminal and trachael fistulas were fitted with cannulas so that test substances could be placed in the rumen or lungs. When substances were tested by placing the material in the rumen, fresh air could be supplied directly to the lungs via an endotrachael catheter, thus eructated gases from the rumen were avoided.

Results of this study led to the speculation that eructated gases could be an important factor in the production of off-flavors in milk.

Later, Dougherty et al. (1962a) found that an onion slurry had to be incubated about thirty minutes in rumen ingesta before a flavor defect could be imparted to milk. Apparently the main route for absorption of the flavoring compound was via the lungs. When an onion slurry was introduced through the ruminal cannulas, while the cows were breathing fresh air via endothrachael catheters, only a slight off-flavor appeared in the milk. The intensity increased after the endothrachael catheters were removed and the cows were breathing normally. An intense off-flavor also developed when air was bubbled through rumen ingesta containing onions and was passed directly to the lungs. These results indicated that flavoring compounds enter the blood stream more readily from the lungs than from the rumen. These workers agreed with Petersen and Brereton (1942) that onion odors failed to impart a typical onion flavor to milk.

While studying eructation, Dougherty et al. (1962b) found that the glottis remained open during the active phase of eructation and that a significant amount of eructated gas was forced into the lungs. This indicates the importance of the lungs as a route for transmitting flavoring compounds to the blood. In another study, Dougherty et al. (1962c) reported that eructated ruminal gas was absorbed as evidenced by the concentration of carbon dioxide, methane, hydrogen sulfide, and oxygen in the pulmonary blood.

The occurrence, level, and disappearance of volatile compounds in biological fluids were studied by Bassette et al. (1966). Blood, milk, and urine samples were collected before feeding alfalfa hay and grain, three hours later, and every hour thereafter through at least the first twelve

hours. Methyl sulfide usually reached maximum concentration about four and one half hours after feeding, and returned to approximately the same concentration observed before feeding after about eight hours. The concentration of Me_2S in blood, urine and milk followed similar patterns which indicates an apparent equilibrium amoun these fluids. Consistently higher concentrations of Me_2S were observed in blood than in urine or milk.

Oxidation of Methionine

Alfalfa hay fed two to three hours before milking often produces a flavor defect in milk, however, the flavor defect produced by green alfalfa is apparently more intense. According to Lucas (1929), the flavor of milk from cows fed alfalfa hay is not criticized by many people. As noted earlier (Roadhouse et al., 1926), feed flavors in milk varied among cows and between cured alfalfa and green alfalfa. Roadhouse and Henderson (1935) concluded that as little as 2 kg alfalfa hay or 3.9 kg green alfalfa produced a detectable feed flavor in milk. When the amount of dry matter from these two sources is considered, it would appear that the flavor produced per unit of dry matter is not as strong in the case of hay.

The fact that numerous researchers use alfalfa hay in the control ration in milk flavor studies implies that the flavor produced by hay is not as serious as when succulent feeds are fed. Reddy (1966) used alfalfa hay as the control in a study of the effects of feeding a surface active agent for controlling feed flavors. Compared to the milk produced by cows fed green-chopped alfalfa, the milk produced by those fed the control ration had a more desirable flavor and a lower concentration of Me_2S. The results reported by Bassette et al. (1966) were similar. Low concentrations of Me_2S in blood, milk, and urine were observed after feeding alfalfa hay adlibitum

during a one hour period.

Since the flavor in milk produced by alfalfa hay is apparently less intense, a relationship between curing or storing and the amount of available Me_2S is suggested. Because Morgan and Pereira (1962) did not detect Me_2S in the volatile compounds collected from fresh alfalfa, Me_2S apparently is not evaporated from the plant while curing. Yet, the precursor of Me_2S may be volatilized or chemically changed during curing or storage, which would result in a lower production of Me_2S.

When the possible precursors of Me_2S are considered, it is difficult to explain a chemical change that will account for a lower availability of the Me_2S precursor, except for methionine. Evidence indicating that methionine can be destroyed in fish meal was reported by Miller (1956). While investigating the destruction of lysine in fish meal due to the Maillard browning reaction, the protein quality was improved more by adding methionine than by adding lysine. Likewise, Njaa (1961) found that the protein quality of herring meal was improved for rats by supplementing with methionine more than would be expected when the methionine content of the meal was considered. Lea et al. (1958) believed that methionine sulfoxide ($CH_3SOCH_2CH_2CHNH_2COOH$) may be formed in fish meal in the presence of peroxidizing fat. However, Njaa (1962) found that methionine sulfoxide was utilized equally as well as methionine, and that the sulfone derivative of methionine ($CH_3SOOCH_2CH_2CH\ NH_2COOH$) was not utilized by rats. It was therefore postulated that the decreased utilizable methionine in fish meal was due to oxidation of methionine to the sulfone stage. This oxidation was accounted for by the presence of peroxides formed during autoxidation of unsaturated fats with molybdenum as a catalyst.

Although the literature does not mention the oxidation of methionine in alfalfa, there are certain facts that support this hypothesis. Black

(1963) has isolated methionine sulfoxide from plants. Hence, in the presence of the predominantly unsaturated fats and molybdenum in alfalfa, methionine sulfoxide conceivably could be oxidized to the sulfone stage.

The isolation of dimethyl sulfone (CH_3SOOCH_3) from cattle blood by Ruzicka et al. (1940) indicated that the sulfone derivative of methionine may be present in the feed consumed by cattle. Apparently dimethyl sulfone is absorbed rather than being reduced to methyl sulfide. Therefore, a reduced amount of methyl sulfide would be produced.

The rate at which methionine sulfone is reduced in the rumen may be another factor which makes it less available for Me_2S production. The rate of reduction of sulfate and sulfide in the rumen of sheep was studied by Anderson (1956). When sulfate was fed to sheep, reduction was slower than when sulfide was fed. Rate of reduction was determined by the concentration of sulfide remaining in the rumen after certain intervals. The greatest concentration of sulfide was reached between two and one half hours and five hours after sulfate feeding. When sulfide was given, more than half disappeared from the rumen within the first thirty minutes and very little remained after two and one half hours. Lewis (1954) reported similar results and noted that the hydrogen donors for sulfate reduction could be the following: glucose, formate, fructose, lactate, pyruvate, succinate, ethanol, citrate, and malate. Therefore, if dimethyl sulfone is reduced at a slower rate, the amount of Me_2S available for absorption at any one time should be less than with methionine.

EXPERIMENTAL PROCEDURE

Experiment I. The effect of drying alfalfa. A pair of identical twin cows in late lactation was confined in stanchions in a room maintained at a constant temperature and humidity. Each cow received 1.8 kg concentrate containing 16% protein twice daily. Each member received either oven-dried or fresh alfalfa ad libitum prior to the experimental period. The alfalfa was fourth cutting and had about thirteen days regrowth at the start and was nearing full bloom at the end of the experiment.

During the experimental periods, one twin received 11.4 kg fresh alfalfa twice daily. The same amount was dried in a hot air oven at 45 C to about 30% of the original weight (about three hours) and was fed to the other at the succeeding feeding. Alfalfa was cut each morning and evening to avoid deterioration due to storage of the green material. Feed was limited to facilitate rapid consumption. Using this procedure, the cows consumed the forage offered in one and one half hours. Grain was fed after the samples had been collected to avoid measuring MegS produced from the concentrate mixture.

Blood and milk samples were collected during two trials, each three days in length with a seven day preliminary period and a ten day change over between periods. During the first period, the cows were milked by machine at 6 a.m. At 10 a.m. (zero hour) milk samples were taken and the forage was fed. Milk samples were collected each half hour during the next four hours. Approximately 20 ml milk was collected at each sampling by hand-milking each quarter. The milk samples were refrigerated and analyzed the same day by gas chromatography (GLC) according to the procedure of Toan et al. (1965). Blood

samples were taken by jugular venipuncture before feeding and two and one half hours after feeding. Coagulation was prevented by adding 0.1 ml 40% sodium citrate per 10 ml blood. Plasma was removed by centrifugation and was refrigerated until GLC analysis could be performed.

During the second period, the treatments were reversed. The same routine was followed as in the first period, except the first milk sample after feeding was omitted, and blood was sampled each time milk samples were taken.

Peak heights (percent full-scale deflection X attenuation factor) of Me_2S in milk were converted to ppb to relate the concentration of Me_2S to the organoleptic threshold. The standard curves used for converting peak heights to ppb were those established by Reddy (1966).

Rumen fluid samples were collected on two days following the second period. The cows were assigned to the same treatment, with respect to the type of forage received, as in the second period. A 25 ml syringe fitted with a 25 cm, 14 gauge hypodermic needle was used to withdraw 15 ml rumen fluid. The rumen was punctured on the left side in the center of the paralumbar fossa. The samples were neutralized with 1.7 ml 0.1 N sodium hydroxide to avoid contaminating the GLC columns with volatile fatty acids. The rumen fluid was diluted to avoid contaminating the laboratory with volatile compounds by adding 1 ml neutralized rumen fluid to 9 ml cold distilled water. This procedure also retarded fermentation. The diluted samples were refrigerated until analyzed by GLC. After the samples had been collected on the first day, each cow received a 20 ml intramuscular injection containing 2 g terramycin to guard against infection. Rumen fluid samples were not taken again until three days later.

Experiment II. The effect of storing alfalfa hay. Two trials were conducted. During the first trial, the same identical twin Jersey cows were used

as in Experiment I. The cows were housed, fed and managed as previously described. Each member was fed 3.2 kg first cutting, sun-cured alfalfa hay which had been stored in bales for approximately three and one half months. Milk and blood samples were collected before feeding, and one, two, two and one half, three, three and one half and four hours later. Samples were collected on three consecutive days following a seven day adjustment period. Methyl sulfide concentrations were determined by GLC.

During the second trial, fourth cutting, sun-cured alfalfa hay was compared with oven-dried alfalfa hay from the same field. The oven-dried hay was cut during a three day period due to the limited capacity of the drying oven. Both hays were stored loose for three months (Sept. 20 to Dec. 20). A pair of identical twin Guernsey cows in late lactation were used. The cows were housed in a stanchion barn and were fed the same kind and quantity of concentrate as described in previous experiments. During the three day test period, preceded by a seven day adjustment period, one cow was fed 3.2 kg oven-dried and the other 3.2 kg sun-cured hay twice daily. Milk and blood samples were collected and analyzed by the procedure outlined for the first trial.

Experiment III. Comparison of methionine, methionine sulfoxide, and methionine sulfone as precursors of MeSH in vitro.

Methionine sulfoxide was synthesized from dl-methionine by the method of Waelsch et al. (1946). Methionine sulfone was synthesized by the method of Toennies and Kolb (1941) as modified by Njaa (1962).

The compounds were tested qualitatively to ascertain that different derivatives of methionine had been synthesized. Descending paper chromatography was used to separate the three compounds. The solvent used was N-butanol, formic acid, and water (100:30:25). The chromatogram was run for

twenty six hours. The solvent front ran off the paper so the R_f values could not be determined. This procedure was used since methionine sulfoxide and methionine sulfone migrate at nearly the same rate. However, this technique gave good separation of methionine, methionine sulfone, and methionine sulfoxide, named in the order of decreasing distance from the starting line. Ninhydrin (0.3% (W/V) in methanol) was used as the developing reagent. The samples tested were chromatographically pure with the exception that the methionine sulfoxide contained a trace of methionine. When a mixture of the amino acids was spotted and treated with 30% hydrogen peroxide before subjection to the solvent, the resulting spot appeared identical to the methionine sulfone spot. This observation agrees with that of Dent (1948). A typical chromatogram is shown in Figure 1.

The odor and solubility characteristics were also used for detecting differences in the three forms of methionine. The sulfoxide derivative had the typical odor of methionine and was about twelve times as soluble in water as was methionine, as reported by Toennies and Kolb (1939). The sulfone derivative was about seven times as soluble in water as was methionine, and the odor of methionine was entirely absent. These characteristics were also observed by Toennies and Kolb (1941).

Due to the expensive procedure for producing the derivatives of methionine in quantities large enough for animal feeding trials, a technique requiring small amounts of the amino acids was needed to determine whether methionine, methionine sulfoxide, and methionine sulfone are sources of Me_2S when acted upon by the rumen microorganisms. Hence, a modified artificial rumen was selected. The use of the artificial rumen also was convenient for studying the rate of Me_2S production from these sources.

Fig. 1. A typical chromatogram showing the separation of methionine and its two oxidized derivatives. Note the trace of methionine above the methionine sulfoxide spot.

A modification of the invitro fermentation technique of Baumgardt et al. (1962) was used. The fermentation was not stopped by adding 4 N sulfuric acid because the pH of the artificial rumen fluid samples had to be near neutrality to avoid contaminating the chromatographic columns with volatile fatty acids. Instead, the fermentations were retarded by diluting the 1 ml samples of the artificial rumen fluid with 9 ml cold distilled water.

Substrates used were: 1 g ground alfalfa hay (control); 1 g ground alfalfa hay plus 0.015 g dl-methionine; 1 g ground alfalfa hay plus 0.0165 g

dl-methionine sulfoxide; and 1 g ground alfalfa hay plus 0.018 g dl-methionine sulfone. These amounts of amino acids represent approximately 0.1 milliequivalent. No allowance was made for the methionine contained in methionine sulfoxide.

Rumen fluid used for inoculation for the in vitro fermentation was collected from a cow fitted with a permanent rumen cannula. The cow was being fed alfalfa hay. Inoculum was obtained at least twelve hours after the last feeding to avoid Me_2S or possibly a precursor in the inoculum.

The artificial rumen consisted of a 125 ml Erlenmeyer flask and a rubber stopper equipped with a Bunsen valve and a glass tube. The glass tubing extended into the contents of the flask so that samples could be taken during the fermentation period. To maintain anaerobic conditions within the flask, a short piece of teflon tubing, which could be sealed with a pinch clamp, was attached to the exposed end of the glass tube. One ml samples were removed from the flask by attaching a 1 ml pipette to the teflon tubing. The flasks were incubated in a shaking water bath at 39 C.

Samples were collected after two, three, four, five and six hours of incubation. To determine the amount of Me_2S present in the flask at the start of the incubation period, a 1 ml sample of the inoculum and buffer solution, mixed in proper proportion and diluted in 9 ml cold distilled water, was analyzed by GLC.

Experiment IV. The effects of feeding methionine or sulfur. Six cows, three Holsteins and three Guernseys, were used in two 3 X 3 Latin squares. The cows were confined during a six hour period each morning in a stanchion barn. The remainder of the time was spent in a dry lot where the cows had access to water, salt, a mineral mixture, and 3.2 kg alfalfa hay per animal daily. This hay was fed early in the afternoon so that the animals would have

an appetite for the test meal each morning. The cows were fed 1.8 kg of a concentrate mixture containing 14% protein twice daily. A seven day adjustment period was followed by three one day experimental periods. During a preliminary study, it was determined that the treatment caused no carry over effect from day to day, therefore, no change over period was used.

At eight a.m. (zero hour) each cow was given a gelatin capsule containing either 13 g 90% feed grade methionine, 2.5 g sulfur or an empty capsule (control), depending on the treatment designated for each day. This amount of methionine represented approximately the amount of methionine contained in 3.2 kg alfalfa hay, while 2.5 g sulfur is approximately the amount of sulfur contained in the methionine treatment. Each cow was fed 3.2 kg first cutting alfalfa hay at eight a.m. Concentrate was fed after the samples had been collected. About 20 ml milk were collected by hand-milking each quarter at the time of feeding hay, and at one hour intervals for the next four hours. The milk samples were analyzed by GLC the same day as collected.

RESULTS AND DISCUSSION

Experiment I. Results of feeding oven-dried and fresh alfalfa on the production of Me_2S in milk are presented in Figure 2 and Table 1. When these concentrations are related to the taste threshold established by Reddy (1966), either form of alfalfa was obviously a good source of Me_2S.

Fresh alfalfa produced higher ($P<0.25$) concentrations of Me_2S than oven-dried alfalfa in the three and four hour samples. However, these differences are probably of minor importance, since small variations in rate of eating would accentuate these differences. Concentrations of Me_2S during Period 1 were greater one, two, two and one half, three, three and one half, and four hours after feeding when compared to the more mature alfalfa fed during Period 2. These results agree with those reported by Reddy (1966), in which a trend toward lower levels of Me_2S was noted as the alfalfa matured. However, the concentration of Me_2S in milk produced during Period 2 was high when related to the organoleptic threshold.

Although the plot of the average concentration of Me_2S revealed a rather large difference between oven-dried and fresh alfalfa forages from two and one half to four hours after feeding, these differences were not highly significant since the variability among days was high. During Period 1 a difference ($P<0.25$) among days occurred two and on half, three, and four hours after feeding. Differences among days during Period 2 were not significant. The rather consistent trend toward lower levels of Me_2S produced by oven-dried alfalfa was probably due to a loss of leaves during drying and handling.

The differences between periods occurring two and one half and three hours after feeding are probably the most important in illustrating the effect of maturity on the production of Me_2S. Apparently, enough more Me_2S precursor was available in the immature alfalfa so that greater concentrations of Me_2S

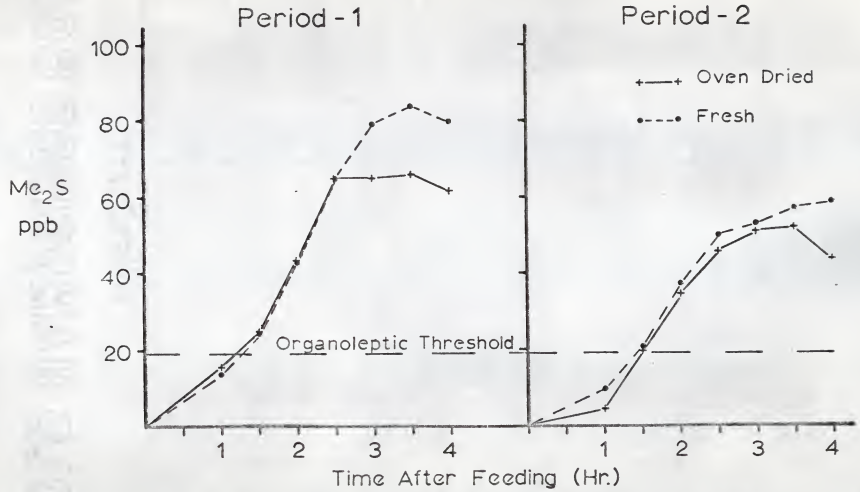

Fig. 2. Average concentrations of Me_2S in milk produced by fresh and oven-dried alfalfa at two stages of maturity.

were produced in milk than when more mature alfalfa was fed. This may be due to lower solubility of the precursor with increasing lignification.

Concentrations of Me_2S in blood plasma are presented as peak heights in Figure 3 and Table 2. A standard curve was not available for converting peak heights to actual concentrations. However, only relative concentrations were pertinent. The high Me_2S concentration observed two and one half hours after feeding during Period 1 illustrates the importance of blood as a Me_2S transporting medium. When blood samples were collected with each milk sample during Period 2, the concentration of Me_2S in blood plasma could be related to that in milk. The concentration of Me_2S in blood plasma usually reached a maximum two and one half hours after feeding. Plots of the Me_2S peak heights are similar to those for milk. Unusually large Me_2S peaks found in milk were usually present in blood plasma at the corresponding time. This indicated an apparent equilibrium between blood and milk.

Maximum concentrations of Me_2S in milk usually occurred about three hours after feeding, while the concentrations of Me_2S in plasma usually reached maxima one half hour earlier. The Me_2S concentration in milk usually decreased four hours after feeding. These results agree with those of Weaver et al. (1935b) and Roadhouse and Henderson (1937) who reported that flavor defects due to feeding alfalfa could be prevented by withholding the forage five hours before milking.

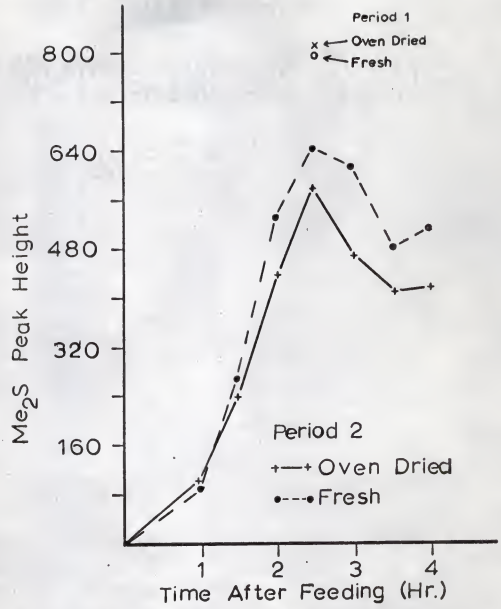

Fig. 3. Average peak heights of Me_2S in blood plasma produced by fresh and oven-dried alfalfa.

Concentrations of Me_2S in rumen fluid are presented as peak heights in Figure 4 and Table 3. Peak heights were not corrected for the dilution factor, since the addition of water as a diluent probably made Me_2S less soluble. Hence, the actual peak heights probably were distorted. The large Me_2S peaks produced by rumen fluid samples point out the importance of microbial fermentations for Me_2S release.

Experiment II. The effects of feeding alfalfa hay that had been stored on the production of Me_2S in milk and blood plasma are shown in Figures 5 and 6, and Tables 4 and 5. Peak heights were converted to ppb Me_2S in milk. The time after feeding when Me_2S reached maximum concentrations was similar to the results of Experiment I.

When first cutting alfalfa was fed after three and one half months storage, the average concentrations of Me_2S were always below the taste threshold. Average Me_2S concentrations produced by oven-dried and sun-cured alfalfa after three months storage were only slightly above the taste threshold. However, during both trials the concentrations of Me_2S occasionally exceeded the taste threshold on certain days (Table 4). During Trial I, there was considerable variation among days, possibly due to variation in hay.

Since a different pair of twin cows was used for Trial 1 than for Trial 2, the two trials could not be compared statistically due to the variability between pairs of cows. However, Trial 1 of Experiment II can be compared with Periods 1 and 2 of Experiment I, since the same pair of twins was used. The concentration of Me_2S in milk and blood plasma produced by oven-dried or fresh alfalfa was highly significantly ($P<0.001$) greater than that produced by first cutting hay three hours after feeding.

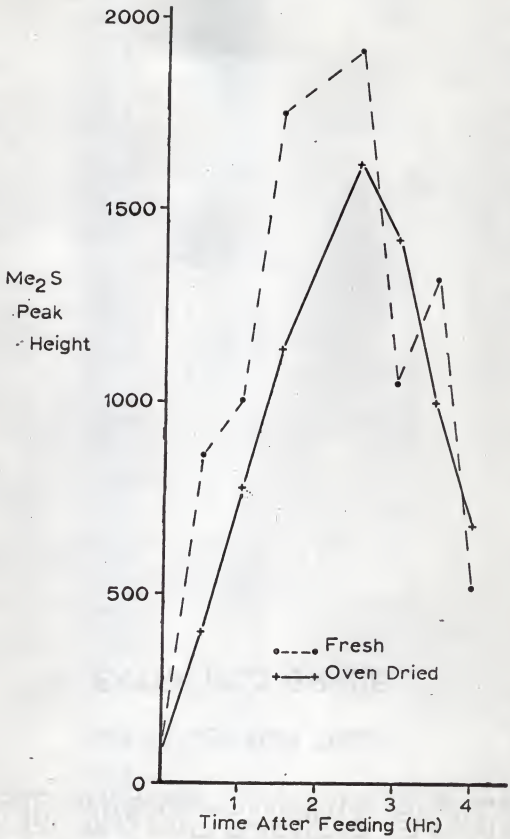

Fig. 4. Average peak heights of Me_2S in rumen fluid.

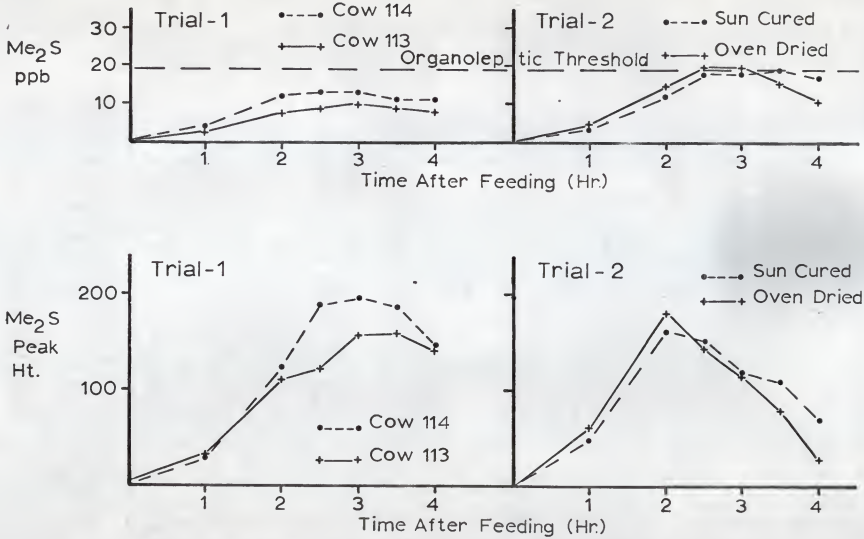

Fig. 6. Average peak height of Me_2S in blood plasma produced by stored first cutting (Trial 1) and stored fourth cutting sun-cured and oven-dried alfalfa (Trial 2).

Experiment III. The artificial rumen proved to be a valuable tool for studying the rate of Me_2S production by rumen microorganisms. The in vitro technique has the advantage over in vivo studies in that results are not affected by rate of feed consumption, regurgitation, or differences among animals. Variability among days, which is probably due to the inoculum, can be eliminated statistically. Preliminary work indicated the necessity for using inoculum from a donor cow which had been fasted for twelve hours to avoid Me_2S in the inoculum.

Chromatographic peak heights of Me_2S in artificial rumen fluid are presented as averages in Figure 7 and Table 6. Variability between duplicates was usually less than 10%. Analysis of variance indicated that the three forms of methionine produced highly significantly ($P<0.01$) more Me_2S at each sampling period than did the control. Methionine and methionine sulfoxide produced highly significantly ($P<0.01$) more Me_2S than that produced by methionine sulfone until the six hour sample. At this time, methionine sulfoxide was significantly higher ($P<0.05$ and $P<0.01$) than methionine and methionine sulfone, respectively, while methionine was higher ($P<0.05$) than methionine sulfone. The difference in concentrations between methionine and methionine sulfoxide was probably due to the trace of methionine contained in methionine sulfoxide.

The slower rate of Me_2S production from methionine sulfone is the interesting part of this experiment. To interpret the plots of concentrations in Figure 7, it must be remembered that Me_2S was escaping from the flasks due to the evolution of gases which escape through the Bunsen valves. Therefore, when the production of Me_2S was greater than the amount lost, the concentration increased within the flask. After three hours incubation the concentration of Me_2S in the control flask declined steadily (Figure 7). The

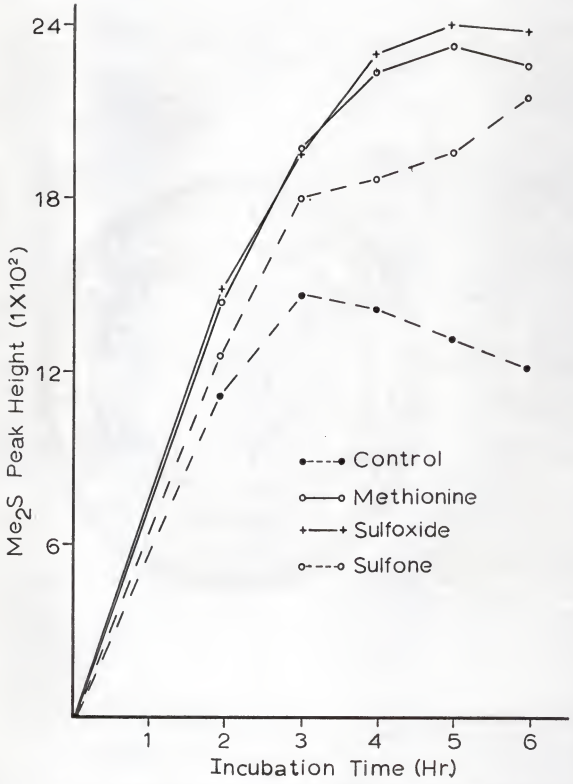

Fig. 7. Average peak heights of Me_2S in artificial rumen fluid.

concentration of Me_2S produced from methionine or methionine sulfoxide did not decrease until six hours after incubation. The amount of Me_2S produced from methionine sulfone was still increasing six hours after incubation. However, the rate of production was apparently greater from the fifth to the sixth hour than from the third to the fifth hour. Hence, methionine sulfone reduction is apparently slow enough that Me_2S is not produced as rapidly as when methionine or methionine sulfoxide is fermented. The apparent decreased rate of Me_2S production by methionine sulfone after three hours incubation also corresponds with the time when the control produces less Me_2S than escapes.

Experiment IV. The concentrations of Me_2S produced by methionine, sulfur, and the control during the first three hours after feeding were about equal, as shown in Figure 8 and Table 7. The concentrations of Me_2S produced by the sulfur treatment and the control had begun decreasing four hours after feeding, while the methionine treatment produced a greater average concentration than was observed in the three hour sample. However, these differences in the four hour samples were not significant.

The cause for methionine not producing greater concentrations of Me_2S in milk than the control, which would be expected from the results of Experiment III, is not understood. It may be related to the order of reaction. If the rate of Me_2S formation depends on the concentration of Me_2S precursor, then the reaction would be a first order reaction and high concentrations would be observed soon after feeding. However, if large amounts of Me_2S are released rapidly, then large amounts of Me_2S probably would escape absorption. Hence, the effects of administering methionine would be masked by high

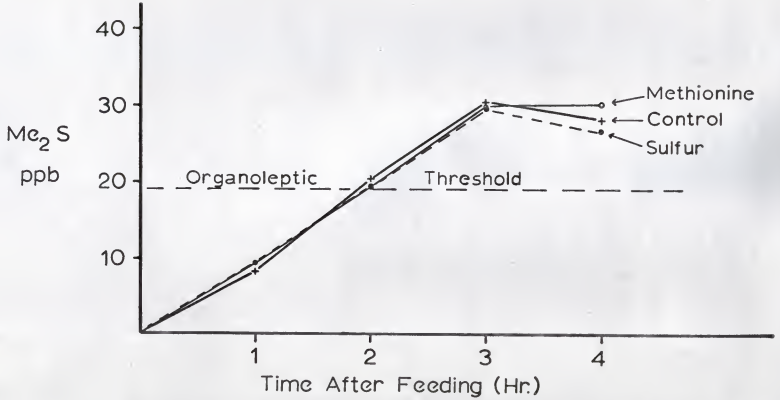

Fig. 8. Average concentrations of Me_2S in milk produced by cows fed control, sulfur and methionine rations.

substrate concentrations. The high concentration of Me_2S produced by the control ration indicates that the concentration of substrate in the hay was high. This would be expected since the hay had been stored only about two weeks.

If the concentration of substrate was high enough for the rate of reaction to be zero order, then higher concentrations of Me_2S produced by methionine would not be observed until after the precursor in the hay had been utilized. To determine this, a longer sampling period would be needed. Since the amount of Me_2S produced in the in vitro study appeared relatively constant during the first four hours of incubation, or while the substrate concentration was probably high, a zero order reaction is suggested.

In this experiment differences between the ability of individual cows to produce Me_2S was highly significant ($P<0.01$) at two and four hours after feeding, and significant ($P<0.05$) at one and three hours after feeding. Roadhouse et al. (1926) also reported that flavors in milk varied due to individual cows.

From the results of these experiments a definite precursor of Me_2S cannot be identified. Apparently, Me_2S or the precursor are not volatile constituents of alfalfa forage, since oven-dried and fresh alfalfa produced similar concentrations of Me_2S in milk. The most noticeable effect during Experiment I was that lower concentrations of Me_2S occurred with increased maturity. Yet, the mature alfalfa produced concentrations of Me_2S well above the organoleptic threshold. Hence, the generally accepted theory that alfalfa hay produces a better flavored milk than does fresh alfalfa cannot be attributed to the stage of maturity alone. The greatest effect for lowering the concentration of Me_2S in milk was that of storage.

The results of the in vitro study indicated that methionine and methio-

nine sulfoxide were readily available sources of Me_2S, and that methionine sulfone produced Me_2S at a slower rate. From these results it appeared that the lower concentration of Me_2S produced by stored alfalfa hay could be due to the oxidation of methionine to methionine sulfone during storage. However, under the conditions of Experiment IV it could not be established that methionine was a precursor of Me_2S. The problem needs further study under conditions where there is not as much Me_2S precursor available as there apparently was in this study.

SUMMARY

In an attempt to identify the precursor of methyl sulfide (Me_2S) in alfalfa forages, some possible precursors of Me_2S in alfalfa and the fate of those precursors was studied. Since cows fed alfalfa hay apparently produced a better flavored milk than cows fed fresh alfalfa, there is an indication that Me_2S or the precursor is evaporated during drying, or that the precursor is lost during storage. Identical twin cows were fed fresh, oven-dried, oven-dried and stored, or sun-cured and stored alfalfa forages to determine the effects of drying, storage, and maturity of the production of Me_2S in milk and blood plasma. The difference between fresh and oven-dried alfalfa for the production of Me_2S was not significant, indicating that Me_2S or the precursor is not a volatile constituent of alfalfa. Cows fed immature alfalfa during the first part of the experiment produced highly significantly ($P<0.005$) greater quantities of Me_2S in milk than when fed more mature alfalfa later. However, observed concentrations of Me_2S associated with feeding mature alfalfa were above the organoleptic threshold. Storing alfalfa had the most depressing effect on concentrations of Me_2S in milk, since concentrations near or below the organoleptic threshold were observed when stored alfalfa was fed. The concentration of Me_2S in blood plasma showed similar patterns to that of the milk, except maximum concentrations were usually reached about one half hour earlier. The importance of the rumen microorganisms for releasing Me_2S was indicated by gas chromatography (GLC) analysis of rumen fluid.

To account for the lower concentration of Me_2S produced by stored alfalfa, an in vitro study was conducted to investigate the possibility that methionine is a precursor of Me_2S and that methionine is oxidized during hay

storage. Two oxidized derivatives of methionine, methionine sulfoxide and methionine sulfone, were synthesized from dl-methionine for comparison of rates of Me_2S formation. Artificial rumens which had been modified to allow the removal of samples withoutdisturbing the anaerobic conditions were used. Substrates used were: alfalfa (control) and alfalfa plus equimolar quantities of dl-methionine sulfoxide or dl-methionine sulfone. The three forms of methionine produced highly significantly ($P<0.01$) more Me_2S in the artificial rumen than the control at intervals during a six hour incubation. Methionine and methionine sulfoxide produced highly significantly ($P<0.01$) more Me_2S than methionine sulfone during the first five hours of incubation. At the sixth hour the concentrations of Me_2S were more nearly alike from the three treatments. Hence, a slower rate of Me_2S production by methionine sulfone was indicated.

Methionine and slufur were tested as precursors of Me_2S in vivo. Six cows in two 3 X 3 Latin squares were fed alfalfa hay (control), alfalfa hay plus 13 g 90% methionine and alfalfa hay plus 2.5 g sulfur daily. No significant difference in Me_2S concentration in milk was observed between treatments. The difference between concentrations of Me_2S in individual cow's milk was highly significant ($P<0.01$).

Conclusions

From the results of this study it was concluded that:

1. The Me_2S content of milk from cows fed alfalfa decreased with advancing maturity of the alfalfa, was the same for green and freshly dried alfalfa and decreased with alfalfa hay storage.
2. The concentration of Me_2S in milk varied among cows.
3. Methyl Sulfide was produced in artificial rumens from methionine, methionine sulfoxide and methionine sulfone, with methionine sulfone having the slowest rate of production.

ACKNOWLEDGMENTS

The author wishes to express his grateful appreciation to Dr. G. M. Ward, Department of Dairy and Poultry Science, for his valuable advice during this investigation and for his guidance in the preparation of this thesis.

Grateful acknowledgment is extended to Dr. Richard Bassette, Department of Dairy and Poultry Science, for his advice and help during the investigation.

Acknowledgment is due Dr. C. L. Norton, Head, Department of Dairy and Poultry Science, Dr. E. E. Bartley, Department of Dairy and Poultry Science, and Dr. K. Burkhard, Department of Biochemistry, for their critical review of this manuscript.

Sincere appreciation is expressed to M. C. Reddy, graduate student, Department of Food Science and Technology, Oregon State University, Corvallis, for his help in the collection and analysis of milk, blood, and rumen fluid samples.

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APPENDIX

Table-1. Average concentrations of Me_2S in milk produced by cows fed fresh and oven-dried alfalfa cut at two stages of maturity.

Time after feeding	Period-1 Me_2S		Period-2 Me_2S		Probability of significance between	
	Dry	Fresh	Dry	Fresh	Treatments	Periods
(hr.)	(ppb)				P	P
Before	0	0	0	0		
½	7	4				
1	16	14	5	10		0.01
1½	25	24	20	21		
2	44	43	35	37		0.25
2½	65	65	46	50		0.05
3	65	79	51	53	0.25	0.005
3½	66	84	52	57		0.25
4	62	80	44	59	0.25	0.25

Table-2. Average GLC peak heights of Me_2S in blood plasma of cows fed fresh and oven-dried alfalfa.

Time after feeding (hr.)	Period-1		Period-2	
	Dry	Fresh	Dry	Fresh
Before	0	0	3	3
1			108	91
1½			255	279
2			439	539
2½	806	786	571	642
3			468	604
3½			405	479
4			412	507

Table-3. Average GLC peak heights of Me_2S in rumen fluid of cows fed fresh and oven-dried alfalfa.

Time after feeding (hr.)	Dry	Fresh
Before	76	58
½	396	862
1	780	1005
1½	1136	1754
2½	1619	1914
3	1422	1048
3½	1003	1314
4	681	524

Table-4. Average Me_2S concentrations in milk produced by cows fed stored first cutting (Trial-1) and stored fourth cutting sun-cured and oven-dried alfalfa hays (Trial-2).

Time after feeding (hr.)	Trial-1 Cow 113	Trial-1 Cow 114	Trial-2 Sun-cured	Trial-2 Oven-dried
	(ppb)			
Before	0	0	0	0
1	3	4	3	5
2	8	12	12	15
2½	9	13	18	20
3	10	13	18	19
3½	9	11	19	16
4	8	11	17	11

Table-5. Average GLC peak heights of Me_2S in blood plasma produced by cows fed stored first cutting (Trial-1) and stored fourth cutting sun-cured and oven-dried alfalfa hays (Trial-2).

Time after feeding (hr.)	Trial-1 Cow 113	Trial-1 Cow 114	Trial-2 Sun-cured	Trial-2 Oven-dried
Before	6	3	2	0
1	34	29	48	62
2	113	114	162	182
2½	123	190	152	145
3	159	196	119	117
3½	161	187	110	79
4	143	148	71	29

Table-6. Average GLC peak heights of Me_2S in artificial rumen fluid produced by alfalfa (control), alfalfa plus methionine, alfalfa plus methionine sulfoxide, and alfalfa plus methionine sulfone.

Incubation time (hr.)	Control	Methionine	Methionine sulfoxide	Methionine sulfone
Before*	0	0	0	0
2	1109	1443	1498	1258
3	1469	1953	1947	1787
4	1419	2235	2319	1862
5	1319	2315	2408	1951
6	1212	2263	2388	2147

* The sample taken before incubation was a mixture of innoculum and buffer.

The means lying above the same horizontal line are not significantly different; those over different lines ($P<0.01$),

	2 hr incubation		
Treatment	Sulfoxide	Methionine	Sulfone	Control
Mean	1498	1443	1258	1109

	3 hr incubation		
Treatment	Methionine	Sulfoxide	Sulfone	Control
Mean	1953	1947	1787	1469

	4 hr incubation		
Treatment	Sulfoxide	Methionine	Sulfone	Control
Mean	2319	2235	1862	1419

	5 hr incubation		
Treatment	Sulfoxide	Methionine	Sulfone	Control
Mean	2408	2315	1951	1319

	6 hr incubation		
Treatment	Sulfoxide	Methionine	Sulfone	Control
Mean	2388	2263	2147	1212

Table-7. Average concentrations of Me_2S in milk produced by cows fed alfalfa (control) alfalfa plus sulfur, and alfalfa and methionine.

Time after feeding (hr.)	Control	Sulfur	Methionine
---	---	---(ppb)---	
Before	0.0	0.0	0.0
1	8.5	9.5	9.5
2	20.5	19.5	19.5
3	30.5	29.5	30.0
4	28.0	26.5	30.5

Table-8. Concentrations of Me_2S in individual cows milk produced by control, sulfur, and methionine rations.

Time after feeding (hr.)	Cow 59C			Cow A148			Cow B10		
	Control	Sulfur	Methionine	Control	Sulfur	Methionine	Control	Sulfur	Methionine
	(ppb)								
Before	0	0	0	0	0	0	0	0	0
1	10	10	9	5	6	8	5	8	7
2	21	25	23	16	15	14	10	8	14
3	33	38	37	27	28	21	14	15	18
4	34	32	45	18	26	29	13	15	19

Time after feeding (hr.)	Cow B101			Cow 408C			Cow 406C		
	Control	Sulfur	Methionine	Control	Sulfur	Methionine	Control	Sulfur	Methionine
	(ppb)								
Before	0	0	0	0	0	0	0	0	0
1	9	9	12	11	10	9	11	13	11
2	30	23	27	24	24	19	22	23	19
3	45	33	49	30	31	23	32	32	32
4	47	27	36	25	34	21	30	26	34

Table-8 (cont.). The means lying above the same horizontal line are not significantly different; those over different lines (P<0.01).

1 hr after feeding

Cow	406C	H101	59C	408C	H103	A148
Mean	11.67	10.00	9.67	9.67	6.67	6.33

2 hr after feeding

Cow	H101	59C	408C	406C	A148	H103
Mean	26.67	23.00	22.33	21.33	15.00	10.67

3 hr after feeding

Cow	H101	59C	406C	408C	A148	H103
Mean	43.00	36.00	32.00	28.00	25.33	15.67

4 hr after feeding

Cow	59C	H101	406C	408C	A148	H103
Mean	37.00	36.67	30.00	26.67	24.33	15.67

THE OCCURRENCE AND POSSIBLE PRECURSOR OF METHYL SULFIDE IN MILK FROM COWS FED ALFALFA FORAGES

by

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B. S., Kansas State University, 1959

AN ABSTRACT OF A MASTER'S THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

Department of Dairy and Poultry Science

KANSAS STATE UNIVERSITY

Manhattan, Kansas

1967

In an attempt to identify the precursor of methyl sulfide (Me_2S) in alfalfa forages, some possible precursors of Me_2S in alfalfa and the fate of those precursors was studied. Since cows fed alfalfa hay apparently produced a better flavored milk than cows fed fresh alfalfa, there is an indication that Me_2S or the precursor is evaporated during drying, or that the precursor is lost during storage. Identical twin cows were fed fresh, oven-dried, oven-dried and stored, orsun-cured and stored alfalfa forages to determine the effects of drying, storage, and maturity on the production of Me_2S in milk and blood plasma. The difference between fresh and oven-dried alfalfa for the production of Me_2S was not significant, indicating that Me_2S or the precursor is not a volatile constituent of alfalfa. Cows fed immature alfalfa during the first part of the experiment produced highly significantly ($P<0.005$) greater quantities of Me_2S in milk than when fed more mature alfalfa later. However, observed concentrations of Me_2S associated with feeding mature alfalfa were above the organoleptic threshold. Storing alfalfa had the most depressing effect on concentrations of Me_2S in milk, since concentrations near or below the organoleptic threshold were observed when stored alfalfa was fed. The concentration of Me_2S in blood plasma showed similar patterns to that of the milk, except maximum concentrations were usually reached about one-half hour earlier. The importance of the rumen microorganisms for releasing Me_2S was indicated by gas chromatography (GLC) analysis of rumen fluid.

To account for the lower concentration of Me_2S produced by stored alfalfa, an in vitro study was conducted to investigate the possibility that methionine is a precursor of Me_2S and that methionine is oxidized during hay storage. Two oxidized derivatives of methionine, methionine sulfoxide and methionine sulfone, were synthesized from dl-methionine for

comparison of rates of Me_2S formation. Artificial rumens which had been modified to allow the removal of samples without disturbing the anaerobic conditions were used. Substrates used were alfalfa (control) and alfalfa plus equimolar quantities of dl-methionine, dl-methionine sulfoxide, or dl-methionine sulfone. The three forms of methionine produced highly significantly ($P<0.01$) more Me_2S in the artificial rumen than the control at intervals during a six hour incubation. Methionine and methionine sulfoxide produced highly significantly ($P<0.01$) more Me_2S than methionine sulfone during the first five hours of incubation. At the sixth hour the concentrations of Me_2S were more nearly alike from the three treatments. Hence, a slower rate of Me_2S production by methionine sulfone was indicated.

Methionine and sulfur were tested as precursors of Me_2S in vivo. Six cows in two 3 X 3 Latin squares were fed alfalfa hay (control), alfalfa hay plus 13 g 90% methionine and alfalfa hay plus 2.5 g sulfur daily. No significant difference in Me_2S concentration in milk was observed between treatments. The difference between concentrations of Me_2S in individual cow's milk was highly significant ($P<0.01$).