Table II. Identity of Methyl Esters of Free Acids in Figure 8

Peak	Identity
1	Ethyl acetate, artifact from extraction
2	Methyl butyrate
3	Methyl pentanoate
4	Methyl hexanoate
5	Methyl heptanoate
6	Methyl octanoate
7	Methyl nonanoate

methyl esters of butyric, hexanoic, octanoic acids, and phenyl acetonitrile. Nitriles previously have been found associated with mustard oils (Challenger, 1959). The same acids, especially butyric, were extracted in the free form from the puree and were identified as their methyl esters (Figure 8; Table II) after treatment with diazomethane. Formation of the free acids probably is the major cause of off-odor in the puree product. In addition, there is evidence of traces of pentanoic, heptanoic, and nonanoic acids.

Puree prepared by the improved processing method differs from the fresh fruit puree in that it contains hexanal, heptanal, benzaldehyde, and traces of α-terpineol. These may be present in low concentration in the commercial puree and in fresh fruit, but have not yet been detected. The nonacidic volatiles found in puree prepared by commercial methods, but not found in the puree prepared by improved processing method, are the methyl esters of butyric, hexanoic, and octanoic acids.

SUMMARY

Off-flavor development in papaya puree can be of either microbial or enzymatic origin. Acidification of papaya puree to pH 3.5 was beneficial in retaining quality by reducing microbial growth. Acidification is recommended as part of an improved processing method. Data from the gc–ms study of papaya flavor components show that the unpleasant odorous compounds, butyric, hexanoic, and decanoic acids and their methyl esters, are present in purees with off-odors and off-flavors. They are not present in purees prepared by the improved processing method.

LITERATURE CITED

American Public Health Association, "Standard Methods for the Examination of Dairy Products," 12th ed., 1967, pp 129, 141.
Angara, F. S., Jr., Wang, J.-K., Agena, U., *Trans. Amer. Soc. Agr. Eng.* **12**, 745 (1969).
Association of Official Agricultural Chemists, "Methods of Analysis," 9th ed., 1960.
Brekke, J. E., Chan, H. T., Cavaletto, C. G., *Food Prod. Develop.* 6(6), 36 (1972a).
Brekke, J. E., Chan, H, T., Cavaletto, C. G., Research Bulletin No. 170, Hawaii Agricultural Experiment Station, University of Hawaii, 1972b.
Challenger, F., "Aspects of the Organic Chemistry of Sulfur," Butterworths, London, 1959, pp 120–132.
Ettlinger, M. G., Hodgkins, J. E., *J. Org. Chem.* **21**, 204 (1956).
Flath, R. A., Forrey, R. R., unpublished data, 1972.
Forss, D. A., Jacobsen, V. M., Ramshaw, E. H., *J. Agr. Food Chem.* **15**, 1104 (1967).
Katague, D. B., Kirch, E. R., *J. Pharm. Sci.* **54**, 891 (1965).
Tang, C.-S., *Phytochemistry* **10**, 117 (1971).

Received for review January 15, 1973. Accepted March 15, 1973. Reference to a company or product name does not imply approval or recommendation of the product by the U. S. Department of Agriculture or the Hawaii Agricultural Experiment Station to the exclusion of others that may be suitable. Presented at Symposium on Biogenesis of Flavor Components, 164th National Meeting of the American Chemical Society, New York, N. Y., Aug–Sept, 1972.

Lipoxygenase-Mediated Pentane Production: Characterization of the System

Elizabeth B. Johns, Harold E. Pattee,* and John A. Singleton

Pentane production was studied in a model system consisting of soybean lipoxygenase and linoleic acid. After an initial lag period of 3.5 min, there was a linear increase in pentane concentration up to 20 min. Pentane production increased linearly with the amount of enzyme between 35 and 75 μg of protein. The optimum linoleic acid concentration for pentane production was 2.1 m*M*, while higher levels were inhibitory. Lipoxygenase has pH optima at 7.1 and 9, while pentane production has a broad alkaline optimum with the maximum at 9. Purification of the enzyme on Sephadex G-150 and DEAE-Sephadex yields two lipoxygenase fractions with pH optima at 7 and 9, respectively. Only the pH 9 fraction produced pentane.

Pentane has only recently been identified as a constituent of plant products. Buttery *et al.* (1961) found pentane in dehydrated potatoes and Pattee *et al.* (1969) showed that pentane was a normal volatile constituent of raw peanuts. Horvat *et al.* (1964) first demonstrated pentane production in a model system by headspace analysis of the autoxidation products of methyl linoleate. Evans *et al.* (1967) formed pentane by the thermal decomposition of 13-hydroperoxylinoleic acid, which is the primary product of the reaction of crystalline soybean lipoxygenase (EC 1.13.1.13) with linoleic acid. Pattee *et al.* (1970) found a rough correlation between pentane content and lipoxygenase activity during the maturation of peanut seeds and postulated that lipoxygenase might be involved in pentane production. Meanwhile, Garssen *et al.* (1971) demonstrated that 13-oxotridecadienoic acid and pentane would be formed in an anaerobic system containing soybean lipoxygenase, 13-hydroperoxy linoleic acid, and linoleic acid.

We report here the partial characterization of a pentane-producing system containing linoleic acid as substrate and soybean lipoxygenase as catalyst.

Mid-Atlantic Area, Market Quality Research Division, Agricultural Research Service, United States Department of Agriculture, and Department of Botany, North Carolina State University, Raleigh, North Carolina 27607.

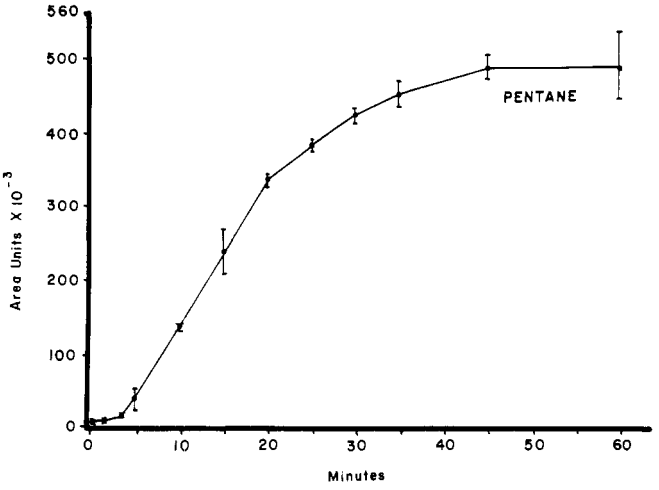

Figure 1. Pentane production as a function of time. The reaction was started by adding 50 μg of lipoxygenase to a reaction mixture (3 ml) containing 2.1 m*M* of linoleic acid in 0.1 *M* borate buffer at pH 9.

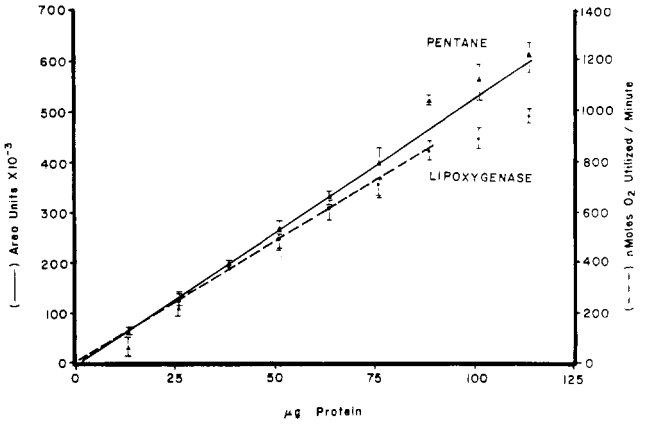

Figure 2. Effect of catalyst concentration on pentane production and lipoxygenase activity. The reaction was started by adding lipoxygenase to a reaction mixture (3 ml) containing 2.1 m*M* of linoleic acid in 0.1 *M* borate buffer at pH 9. Pentane was sampled at 15 min.

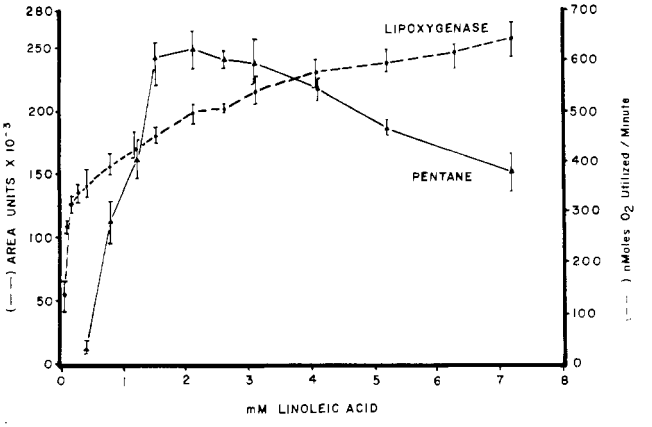

Figure 3. Effect of substrate concentration on pentane production and lipoxygenase activity. The reaction was started by adding 50 μg of lipoxygenase to a reaction mixture (3 ml) containing various concentrations of linoleic acid in 0.1 *M* borate buffer at pH 9. Pentane was sampled at 15 min.

EXPERIMENTAL SECTION

Soybean lipoxygenase was obtained from Nutritional Biochemicals Corporation, high-purity grade linoleic acid came from the Hormel Institute, and Sephadex G-150 and DEAE-Sephadex were from Pharmacia Fine Chemicals.

Lipoxygenase activity (measured by O_2 consumption) and pentane production were measured simultaneously at room temperature (25 ± 2°) in a glass vessel (22 ml volume) fitted with a Clark oxygen electrode and a serum stopper. The vessel contained 2.1 m*M* of linoleic acid and 0.07% Tween 20 in 0.1 *M* borate buffer pH 9.0, and enzyme (0.25 mg/ml in 0.01 *M* phosphate buffer, pH 7.0) in a total volume of 3 ml. Values for O_2 concentration (Hodgeman, 1954) were assumed to be 260 nmol/ml and were not corrected for the effects of ionic solutes. Lipoxygenase activities were calculated from initial reaction rates. At a given time interval a 5-ml gas volume was withdrawn with an air-tight syringe and injected into a Model 1840 Varian Aerograph gas chromatograph. A Chromosorb 102 column operated isothermally at 140° was used to determine pentane. Peak areas were integrated using an Infotronics CRS-100 digital readout system and pentane data are presented as integrator area units. Protein content was determined spectrophotometrically as described by Layne (1957). Peroxidase activity was assayed as described by Shannon *et al.* (1966) by following the increase in absorbance at 460 nm due to *o*-dianisidine oxidation in the presence of H_2O_2 and enzyme. Reaction mixtures were incubated at 25° and contained 0.5% *o*-dianisidine, 0.1 ml of 0.1 *M* H_2O_2, 0.1 ml of enzyme, and 2.75 ml of 0.05 *M* phosphate buffer, pH 6.5.

Each data point on the graphs represents the mean of at least three determinations (generally 4–6), while the bars depict absolute deviations from the mean.

RESULTS AND DISCUSSIONS

Time Course. When pentane production was measured over time (Figure 1), there was an initial lag period of about 3.5 min, a subsequent linear increase to 20 min, and then a decline in the rate of production. In this system it required approximately 1.5 min for the available O_2 in the reaction mixture to be depleted; thus pentane was produced only after the system became anaerobic. This agrees with the results of Garssen *et al.* (1971) who showed that 13-oxotridecadienoic acid, which should be formed concomitantly with pentane, was not produced until all available O_2 was depleted.

Linearity with Catalyst Concentration. A comparison of lipoxygenase activity and pentane production as a function of enzyme concentration showed that the rate of O_2 consumption was linear between 12 and 87 μg, while the linear range for pentane production was between 35 and 75 μg of protein (Figure 2). In this linear range for pentane production, 1.2 to 2.5 min was required to reach the zero O_2 level, while at the lower protein concentrations 5–11 minutes was required. Since pentane was sampled at 15 min regardless of the time required to reach low O_2 levels, the lower protein concentrations gave pentane values lower than expected.

A decrease in the lipoxygenase reaction velocity with time was also observed at the lower protein levels (12 and 25 μg), while at levels below 12 μg of protein the reaction stopped before the O_2 was depleted. These observations are in agreement with those of Smith and Lands (1972), who suggested that lipoxygenase may catalyze its own destruction during the oxygenation reaction.

Effect of Substrate Concentration on Lipoxygenase Activity and Pentane Production. The optimum linoleic acid concentration for pentane production was 2.1 m*M*; higher levels were inhibitory (Figure 3). However, lipoxygenase activity continued to increase, even at the highest substrate concentration used (7.2 m*M*). At low substrate concentrations (0.4 m*M*) conditions were encountered such that the O_2 was not depleted and hence there was no pentane production.

Our substrate curve for lipoxygenase (Figure 3) was similar to that of Tappel *et al.* (1953) in that after the

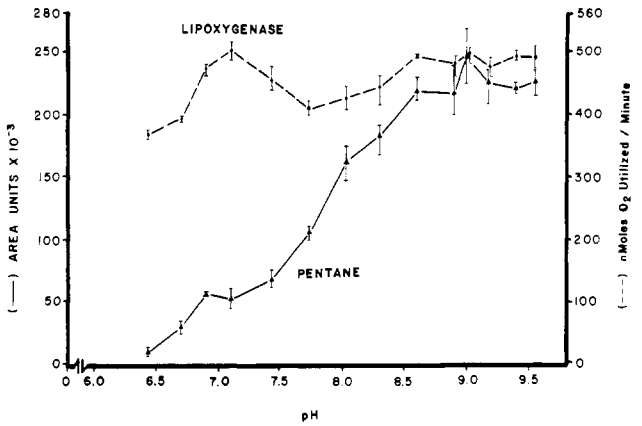

Figure 4. Effect of pH on pentane production and lipoxygenase activity. The reaction was started by adding 50 μg of lipoxygenase to a reaction mixture (3 ml) containing 2.1 m*M* of linoleic acid in 0.1 *M* borate buffer at the indicated pH's. Pentane was sampled at 15 min.

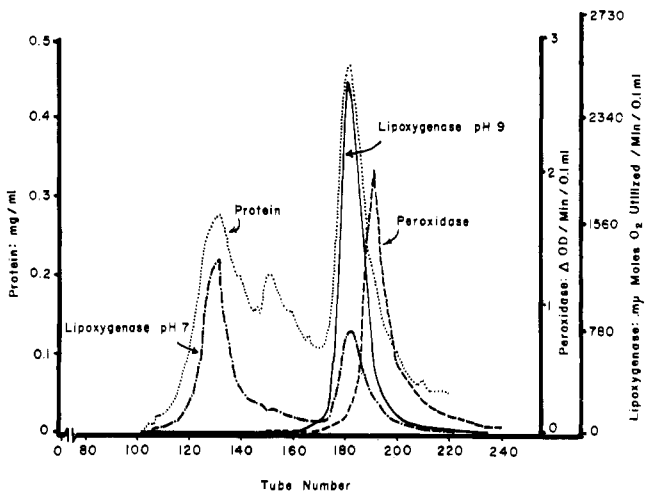

Figure 5. DEAE-Sephadex chromatography of soybean lipoxygenase after partial purification on Sephadex G-150. The column (2.5 × 90 cm) was equilibrated with 0.1 *M* Tris buffer, pH 8.5. The enzyme solution (120 mg) was applied in the same buffer and elution was performed (40 ml/hr) with a linear gradient of NaCl (0–0.4 *M*) in 0.1 *M* Tris buffer, pH 8.5, at 6°. Fractions of 5 ml were collected and protein content and enzyme activities were measured as previously described.

rapid rise in initial velocity at low substrate concentrations there was a subsequent slower increase in reaction velocity at the higher substrate concentrations. However, Tappel *et al.* (1953) did report a decrease at their highest substrate level (about 7 m*M*).

The decrease in pentane production at the higher substrate levels could be explained by the kinetic formulation for lipoxygenase action given by Smith and Lands (1972). They assumed that binding sites existed for both product (P, the hydroperoxide) and substrate (S), and that S could bind to the P site but P could not bind to the S site. At high substrate levels these conditions could lead to a decrease in availability of the P binding site for P. If under anaerobic conditions the P binding site were the site of hydroperoxide cleavage, the high substrate conditions would lead to a decrease in pentane production as indicated in the present results.

Effect of pH on Lipoxygenase Activity and Pentane Production. Lipoxygenase has pH optima at 7.1 and 9, although activity is high from 8.6 to 9.6 (Figure 4). This double optimum with soybean enzyme can also be seen in the profiles of Ames and King (1966), although the activity maxima were different (7.7 and 10 in one case) than found here. This may be explained in part by the observation of Ben-Aziz *et al.* (1970) that at high linoleate concentrations there was a shift in optimal pH toward 7. Pentane production has a broad alkaline optimum with the maximum at 9, with only small amounts produced at pH 7 (Figure 4).

Table I. Lipoxygenase Activity and Pentane Production by the pH 7 and 9 Isoenzymes

	Lipoxygenase nmol of O_2 utilized/min/mg assay at		Pentane area units × 10^3/min/mg assay at	
Isoenzyme	pH 7	pH 9	pH 7	pH 9
pH 9	8656	32,500	186	863
pH 7[a]	17,463	1365	24	5

[a] Assays run in the presence of 6.7×10^{-4} *M* $CaCl_2$.

Purification of the soybean enzyme by chromatography on Sephadex G-150 and subsequently on DEAE-Sephadex yielded two fractions with lipoxygenase activity (Figure 5). Since the fractions from the first protein peak have maximal lipoxygenase activity at pH 7 and those from the third protein peak have maximal activity at pH 9, these fractions probably correspond to the lipoxygenase 2 and 1 fractions, respectively, which were demonstrated by Christopher *et al.* (1970). Pentane production, however, was limited almost exclusively to the pH 9 fraction (Table I). When the reaction is assayed at pH 7, the pH 7 isoenzyme is about twice as active as the pH 9 isoenzyme, yet the latter catalyzes the production of almost eight times more pentane. When comparing the pH 7 isoenzyme (assayed at pH 7) and the pH 9 isoenzyme (assayed at pH 9), there is a twofold difference in lipoxygenase activity but a 36-fold difference in pentane production.

Christopher and Axelrod (1971) have also shown that lipoxygenase 1 (pH 9 enzyme) produces the 13-hydroperoxide almost exclusively, while lipoxygenase 2 produces approximately equal amounts of the 9- and 13-hydroperoxides. Since pentane should be produced only from the 13-hydroperoxide, we would expect lipoxygenase 1 to mediate pentane production. However, if lipoxygenase 2 (pH 7 enzyme) produces equal amounts of the two hydroperoxide isomers, this enzyme might also be expected to mediate pentane production. We find almost no pentane production with the lipoxygenase 2. It is, of course, possible that lipoxygenase 2 is quite different from lipoxygenase 1 and is incapable of participating in pentane production even though the 13 isomer is present and other conditions are optimum.

Further work on the isolation of the reaction products from the two lipoxygenase fractions and further characterization of the pentane-producing system are in progress.

LITERATURE CITED

Ames, G. R., King, T. A., *J. Sci. Food Agr.* **17,** 301 (1966).
Ben-Aziz, A., Grossman, S., Ascarelli, I., Budowski, P., *Anal. Biochem* **34,** 88 (1970).
Buttery, R. G., Hendel, C. E., Boggs, M. M., *J. Agr. Food Chem.* **9,** 245 (1961).
Christopher, J., Axelrod, B., *Biochem. Biophys. Res. Commun.* **44,** 731 (1971).
Christopher, J., Pistorius, E., Axelrod, B., *Biochem. Biophys. Acta* **108,** 12 (1970).
Evans, C. D., List, G. R., Dolev, A., McConnell, D. G., Hoffman R. L., *Lipids* **2,** 432 (1967).
Garssen, G. J., Vliegenthart, J. F. G., Boldingh, J., *Biochem. J.* **122,** 327 (1971).
Hodgeman, C. D., "Handbook of Chemistry and Physics," 36th ed., Chemical Rubber Co., Cleveland, Ohio, 1954–1955, p 1610.
Horvat, R. J., Lane, W. G., Ng, H., Shepard, A. D., *Nature (London)* **203,** 523 (1964).
Layne, E., *Methods Enzymol.* **3,** 451 (1957).

Pattee, H. E., Singleton, J. A., Cobb, W. Y., *J. Food Sci.* **34**, 625 (1969).
Pattee, H. E., Singleton, J. A., Johns, E. B., Mullin, B. C., *J. Agr. Food Chem.* **18**, 253 (1970).
Shannon, L. M., Kay, E., Lew, J. Y., *J. Biol. Chem.* **241**, 2166 (1966).
Smith, W. H., Lands, W. E. M., *J. Biol. Chem.* **247**, 1038 (1972).
Tappel, A. L., Boyer, P. D., Lundberg, W. O., *J. Biol. Chem.* **199**, 267 (1953).

Received for review November 1, 1972. Accepted February 20, 1973. Presented at symposium on Biogenesis of Flavor Components, 164th National Meeting of the American Chemical Society, New York, N. Y., Aug-Sept 1972. Paper Number 3770 of the Journal Series of the North Carolina State University Agricultural Experiment Station, Raleigh, N. C. Use of trade names of specific materials does not constitute a recommendation by the U. S. Department of Agriculture to the exclusion of others which also may be available.

Biogenesis of Cheese Flavor

Thorvald Kristoffersen

Cheese flavor results from the action of microorganisms and enzymes on the carbohydrates, fat, and proteins of the milk and curd. A multitude of breakdown products contribute to the flavor, such as acids, particularly short-chain fatty acids and acetic and lactic acid, alcohols, aldehydes, ketones, esters, ammonia, amines, sulfides, and mercaptans. Characteristic flavor of cheese varieties is related to the concentrations and balance of relatively few key components. Control of flavor development is exercised through selection of microorganisms and enzymes, milk treatment, and manufacturing and curing procedures. However, ultimately the development of characteristic cheese flavor appears to be determined by the ability of protein-based sulfur groups to accept hydrogen resulting from oxidative ripening processes. Results leading to this conclusion are reviewed.

Over 800 different cheeses are recognized in the world and these possess a wide spectrum of flavors. Most cheese is produced from cow's milk but cheese is also made from the milk of other domesticated animals and from nonanimal sources, such as soybeans. The flavor of cheese is determined in part by the source of the raw material.

Collectively, over 100 volatile and nonvolatile potential flavor compounds have been identified in ripening cheese. With respect to their contribution to flavor, suppositions have centered primarily on acetic acid and other short-chain fatty acids, amino acids, alcohols, aldehydes, ketones, esters, ammonia, amines, sulfides, and mercaptans.

FLAVOR DEVELOPMENT AND BALANCE

Control of desirable cheese flavor most generally is presumed to begin with the manufacturing process during which the internal environmental conditions for subsequent biological activities are established. Agreement exists with regard to the essential role of acidification by bacterial action of the milk and curd. This involves the selection of proper types and strains of lactic acid bacteria and the formation of lactic acid at the proper rate and in the proper concentration according to the cheese variety. Agreement also exists that the degree of curd dehydration and the salt content affect cheese flavor development, and the addition of specific flavor-producing microorganisms or enzymes aids in the formation of characteristic flavor in such cheese varieties as Swiss, Blue, and Romano, but not necessarily in all varieties.

During the curing process, the external environmental conditions are controlled to allow for proper flavor development. These conditions vary widely according to the kind of cheese. Basically, cheese flavor results from the action of microorganisms and enzymes on the lactose, fat, and protein of the cheese. The breakdown of these components to lactic acid, fatty acids, and amino acids, respectively, has been termed the primary step of cheese ripening (Harper and Kristoffersen, 1956). With the exception of short-chain fatty acids, the primary ripening compounds contribute little to characteristic cheese flavor. Such flavor results from subsequent action of microorganisms and enzymes on the primary ripening compounds.

Attempts to relate the characteristic flavor and flavor quality of a given variety of cheese to a single or a group of closely related compounds have generally proved futile. More success has been achieved when the relative concentrations of products resulting from the breakdown of protein and fat (Harper and Long, 1956: Kristoffersen and Gould, 1960; Long and Harper, 1956) or protein and carbohydrate (Kristoffersen and Slatter, 1959) have been related to flavor. For example, for Cheddar cheese there appears to be a definite relationship between the relative concentrations of free fatty acids and hydrogen sulfide and flavor quality (Table I).

BIOLOGICAL FUNCTION OF SULFUR GROUPS IN CHEESE RIPENING

Despite the mass of knowledge which has been accumulated on cheese manufacturing and curing procedures, the microorganisms and enzymes involved during the ripening process, and the flavor compounds produced, many instances occur when cheese fails to develop uniform, full, high quality flavor, even under apparently ideal conditions. For the past several years our research has sought an explanation for this incongruity, with emphasis on the sulfur groups of milk and cheese protein. Evidence accumulated indicates that the sulfur groups of these proteins are intimately involved in determining the quality characteristics of cheese and that they exert control over cheese ripening in concert with other agents.

The state of sulfur groups in milk may be subject to time–temperature dependent storage changes as revealed by studies of H_2S from milk (Kristoffersen *et al.*, 1962). The amount of H_2S released by heat from milk stored at 37° varied with time of storage of the milk (Figure 1). Similar but delayed changes were shown for milk stored at 4 and 15°. Assuming that the amount of H_2S released from milk is a function of the state of the sulfur groups, and relatively high concentrations of H_2S are released from the -SH state and relatively low concentrations are

Department of Food Science and Nutrition, The Ohio State University, Columbus, Ohio 43210.