

CHAPTER 5

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The Lipids of Milk:
Deterioration

Daniel P. Schwartz

Part I. Lipolysis and Rancidity

Market milk and some products manufactured from milk at times possess a flavor described as rancid. This term as used in the dairy industry denoted implicitly the flavor due to the accumulation of the proper concentration and types of free fatty acids hydrolytically cleaved from milkfat under the catalytic influence of the lipases normally present in milk.

The development of rancid flavor in milk and some other fluid products is usually undesirable and detracts from their market value. In contrast, the popularity of certain dairy products, notably some cheese varieties, and also some confectionery items containing milk as an ingredient, is thought to be partially due to the proper intensity of the rancid flavor. Hence, knowledge of the factors involved in the development of rancidity is of great practical importance to several industries.

The literature on the subject is quite large. The present review has been limited to milk lipases, but good reviews on this, other dairy products, milk esterases, and on microorganisms are available.^{11,92,145,147,162,163,195,223,313,318}

GENERAL

A lipase has been defined as an enzyme hydrolyzing the esters from emulsified glycerides at an oil/water interface.⁶¹ Adherence to this definition has been maintained in this review; as a consequence, investigations which involved water-soluble substrates or substrates containing an alcoholic moiety other than glycerol have not been included.

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The term enzyme system has been proposed to include multiple enzymes.¹⁵⁴ It has been suggested that the term "lipase system" be used in dairy literature to express the lipase multiplicity in milk.¹⁰⁸ This expression will be adopted here and will be used synonymously with lipases and lipolytic enzymes.

It should be mentioned at the outset that a number of variables affect lipase activity. Unlike most enzymic reactions, lipolysis takes place at an oil/water interface. This rather unique situation gives rise to variables not ordinarily encountered in enzyme reactions. Factors such as the amount of surface area available, the permeability of the emulsion, the type of glyceride employed, the physical state of the substrate (complete solid, complete liquid or liquid-solid), and the degree of agitation of the reaction medium must be taken into account for the results to be meaningful. Other variables common to all enzymic reactions—such as pH, temperature, presence of inhibitors and activators, concentration of enzyme and substrate, light, and the duration of the incubation period—will affect activity and the subsequent interpretation of the results. Many of these variables have not been standardized in milk lipase studies, and it is difficult to assess results and draw concrete conclusions in a number of instances.

Enzymes are produced and elaborated by living cells—a fact that has prompted some investigations into the origin of milk lipases. It is only recently that synthesis of glycerides by milk lipases has been demonstrated.^{205,229,231,232} Using tripalmitin isotopically labeled in both the glycerol and fatty-acid moieties, Koskinen *et al.*²⁰⁵ demonstrated that glyceride synthesis occurs in freshly drawn milk, and that synthesis and hydrolysis occur simultaneously.²²⁹ Luhtala *et al.*²³¹ showed that intracellular enzymes isolated from homogenized somatic cells of milk were capable of synthesis and lipolysis of milk triglycerides. However, there has been no evidence that any of the lipases of milk are identical with the lipase(s) involved in milkfat synthesis *in vivo*. It is of interest in this line of investigation to note that Morton²⁴⁵⁻²⁴⁷ has shown that milk phosphatase is derived from mammary gland microsomes released into the milk during the normal secretory process.

Bovine blood serum is lipolytically active, but cows producing milk which goes rancid quickly do not have sera more lipolytically active than those producing normal milk.²⁸⁴ Leucocytes, which are present in large numbers in milk, are especially high in mastitic milk; they are the source of milk catalase, but are apparently not the source of milk lipases.²⁵²

The lipases of milk are apparently inactive in the udder and at the time of milking.¹⁴⁵ Milk always contains relatively large proportions of unesterified fatty acids,³⁶⁰ but these may be left over from the

metabolic pool. The milk lipases are unusually slow-acting unless some physical or thermal treatment is applied to the milk. This may account for the inactivity in the udder, but no experiments have been conducted to substantiate this.

FARM FACTORS AND LIPOLYSIS

Spontaneous Rancidity

Studies have been undertaken to determine how widespread rancidity really is. Hemingway *et al.*¹⁴¹ examined 12 herds and reported that about 50% of the herd samples showed some initial rancidity and 21% of samples from 15 cows were rancid. Differences in degree of rancidity were marked. Another report contends that from 2 to 22% of cows in a herd produce milk which goes rancid quickly.¹⁵⁰ Milk which inherently possesses the quality of high susceptibility toward rancidity has been variously termed "naturally rancid milk"⁶³, "bitter milk of advanced lactation"²⁵⁸, "naturally active" or "naturally lipolytically active",^{214,343,345,348} "normally active",³⁶⁰ and "spontaneous".^{345,348} The latter term has been more or less adopted in recent years. These various designations were introduced in an effort to distinguish such milk from "non-spontaneous" (normal) milk.

Lipolysis in freshly drawn milk normally proceeds at a very slow rate even upon prolonged incubation, unless proper thermal or mechanical treatment is applied to the milk. This, of course, always occurs in practice as raw, warm milk is never consumed on the market. It is through these necessary practices that lipolysis in normal milk is accelerated. As a consequence, milk may be made rancid either deliberately or accidentally. The so-called spontaneous type of milk needs no treatment other than cooling to 15 to 20°C when drawn or shortly afterward to hasten lipolysis.³⁵⁴ Once the milk has been cooled, lipolysis is not materially affected whether the milk is aged in the cold or rewarmed to 20, 30, or 37°C and aged at these temperatures. Lipolysis in normal milk is not accelerated to the same degree by cooling and aging.

The reason that rancid milk is not more prevalent in market milk is due to the fortuitous fact that spontaneous rancidity can be prevented or reduced by mixing such milk within 1 hr after milking with 4 to 5 times its volume of normal milk.³⁴⁸ Since usually only about 1 out of 5 cows in a herd produces spontaneous milk, this defect is thus almost automatically eliminated or reduced. It is clear, however, that farmers with only a few cows are likely to encounter spontaneously rancid milk during the lactation period.

The dilution of normal milk which has been activated by thermal or mechanical treatment does not diminish activity of the lipases.^{121,220,244}

Feed.—The cow's feed has been shown to be an important practical factor in influencing the susceptibility of the milk to rancidity. Feeding experiments and practical observations have demonstrated that green pasture decreases and dry feed increases the incidence of rancidity.⁴⁴ Poor quality ration fed at reduced energy levels can significantly increase the incidence of rancidity¹¹⁵, as can the feeding of a high-carbohydrate diet.¹⁹⁷

Lactation.—Individual cows maintained under identical conditions seem to vary markedly in the susceptibility of their milk to rancidity.^{176,206} An increased incidence of rancidity has also been associated with advanced lactation, particularly during long lactation periods.^{8,16,48,70,83,102,150,199,258} There are reports, however, which fail to show a correlation between rancidity and advanced lactation.^{149,268}

Mastitis.—Mastitis has been implicated in rancidity^{16,127,338,358}; according to Guthrie and Herrington¹²⁷ and Tarassuk *et al.*³⁵⁸, it may be more important than late lactation. Luhtala and Antila²³⁰, however, found lower lipolytic activity in mastitic milks. They also reported that lipase activity was higher in foremilk than in strippings.

Estrous

The effect of the estrous period on rancidity has also been investigated. According to Wells *et al.*³⁷⁰, who studied lipase activity in the milk and blood of cows throughout their lactation periods, the peak blood plasma lipase values occur about 24 hr before the onset of observed estrous. Changes in the blood lipase activity were reflected and magnified in the milk, although it was noted that the increase in the lipase level in milk occurred 9 to 15 hr after it was observed in the blood. Bachmann¹⁶ also has indicated that hormonal disturbances are linked to rancidity. He differentiates between rancidity produced by cows in late lactation and rancidity due to hormonal disturbances on the basis of an increase in lipase concentration in the latter.

Pipeline Milkers

The increased use of pipeline milkers and farm tanks on dairy farms has coincided with a noticeable increase in rancidity.^{114,146,167,178,257,327,359} About 6 times as much rancid milk has been reported from pipeline milkers as compared to nonpipeline systems.¹⁶⁸ The trouble has been traced to risers in the pipelines, that is, vertical sections connecting one pipeline to another at a higher level.

Air leaking excessively into the milk lines primarily at the claw, teat cups, milk hose and loose line joints causes considerable foaming of the warm, raw milk lifted in the risers under reduced pressure.⁴⁴ The formation of foam due to air agitation was found to be an important feature of the mechanism involved in the acceleration of lipolysis and the resultant appearance of rancid flavor in milk from pipeline milkers. Optimal conditions for activation by air agitation appear to be foaming with the continuous mixing of foam and milk at temperatures that keep the milkfat liquid.³⁴⁶

Remedial measures that suppress foaming and agitation in pipeline milkers have been recommended. The use of a pipeline located below the cow was reported to virtually eliminate rancidity or to significantly reduce the acid degree value which is defined as ml N KOH required to neutralize the free fatty acids in 100 gm fat.¹¹⁴ Shortening of the main pipeline and minimizing the number of risers, joints, and sharp bends will also reduce foam formation and subsequent rancidity.^{160,243}

DISTRIBUTION AND PURIFICATION OF MILK LIPASES

Sufficient evidence now exists to support the view that a number of lipases exist in milk. This was first proposed by Herrington and Krukovsky¹⁴⁹ on the basis of the effect of formaldehyde on lipase activity. Schwartz *et al.*³⁰⁹ presented kinetic data and conducted more detailed studies on formaldehyde inhibition,³¹⁰ the results of which were interpreted in favor of the existence of two or three lipases in skim milk. Albrecht and Jaynes⁴ and Nelson²⁵¹ obtained multiple pH optima on tributyrin hydrolysis with their enzyme source. The relative specificity of the milk lipase system toward a variety of substrates was shown to be different for individual milks; and this observation, together with data obtained using a number of selective inhibitors, also led to the conclusion that more than one lipase is normal to milk.^{106-108,347}

There seems to be little doubt that the majority of the cell-free lipases of milk are bound in some manner to the casein complex,^{65,66,67,68,111,139,295,318} although it has not been firmly established that the lipase activity is associated with any particular casein species. Lipase activity has been reported to be in the α -casein fraction,³²⁰ the β -casein fraction,²⁹⁶ and the κ -casein fraction.^{104,380} The association of lipases with the casein fraction of milk can be rationalized from the fact that lipases in general show a strong affinity for interfaces. Baskys *et al.*²¹ took advantage of this fact and used adsorption of the enzyme at an ether/water interface as their initial purification step. A recent report²⁷⁵ has shown that lipoprotein lipase as well as pancre-

atic lipase will attack triolein impregnated onto "Celite", demonstrating that creation of this type of interface will attract the enzyme. This experiment also may indicate that emulsification of the substrate is not essential. It may offer a means for simple standardization of lipolytic substrates.

Downey and Andrews⁶⁶ showed that, upon addition of pancreatic lipase to milk, a decrease in activity is manifested due to binding of the enzyme to both micellar and soluble casein complexes. They demonstrated that the binding of the lipase to casein was not dependent upon the presence of colloidal phosphate; hence, complete micellar structure is not essential for association of lipases with casein. Downey and Murphy⁶⁸ intimated that lipases in milk appear to be involved in the equilibrium between micellar and soluble casein in that activity is apparently influenced by this equilibrium.

The binding of milk lipases to casein micelles apparently imparts some stability to the enzymes, for as purification progresses the milk lipases become less stable, and more so as the concentration of casein decreases.⁶⁶

Lipases associated with the casein micelles in skimmilk are not fully active, but both dilution and the addition of sodium chloride stimulate or restore activity, presumably by dissociating the micelle-lipase complex. Sodium chloride is an inhibitor of lipolysis (see p. 232), but the proper dilution and addition of this salt can elicit maximal activity.⁶⁶

Other lipases are present in milk but are associated with cells or cell debris. Somatic cells isolated from separator slime were found to contain lipase activity^{110,231} and lipases have been isolated and purified to a high degree from separator slime.^{10,285} The cell-bound lipases are ostensibly distinct entities and differ from the lipases normally associated with casein.

Milk also contains a lipoprotein lipase similar to that found in mammalian adipose and heart tissue.^{204a} The enzyme is highly specific for lipoproteins and probably is not involved in rancidity development to any great extent.

In order to explain the phenomenon of spontaneous rancidity, Tarassuk and Frankel³⁴⁷ presented evidence to show that when freshly drawn milk is cooled, irreversible adsorption of a lipase on the membrane surrounding the fat globules occurs. This enzyme, termed "membrane lipase", was thought to occur in very low concentration in normal milk and in relatively high concentration in spontaneous milk. Thus, the accelerated lipolysis in spontaneous milk would be due, according to Tarassuk and Frankel³⁴⁷, to the higher concentration of "membrane lipase". These workers acknowledged that at least one other lipase is present in milk; it is associated with casein, and is not active to

any extent in spontaneous lipolysis, but can be activated by proper physical means (see below).

The appearance of more elegant techniques for purification of proteins has resulted in an increase in the number of attempts to purify the lipases of milk. Purification of cell-free lipases usually begins with the casein complex. At least 5 distinct lipases all capable of catalyzing the hydrolysis of tributyrin are associated with casein.⁶⁷ These enzymes can be dissociated from casein with 0.75M sodium chloride and subsequently separated to some degree by gel filtration.⁶⁵⁻⁶⁷ All the lipases separated in this manner show the characteristic property of lipases, namely, that they hydrolyze emulsified triglycerides at a greater rate than they hydrolyze the dissolved triglyceride, triacetin. Molecular weights of the lipases were estimated as between 35,000 and 180,000.

Curd from rennet casein was used by Gaffney *et al.*¹¹¹ as a starting point for fractionating the lipases on DEAE cellulose and on Sephadex G-35. Their results indicated that 11 of the fractions showed lipase activity.

Fox and Tarassuk,¹⁰³ also starting from rennet curd, isolated a lipase with a specific activity 500 times that of the original skim milk. The molecular weight of their preparation was 210,000. The lipase had a pH optimum at 9.2 on milkfat and a temperature optimum at 37°C.

Other lipases have been isolated from the separator slime deposited from milk. Chandan and Shahani⁴¹ purified a lipase which had a molecular weight of about 7,000, a pH optimum of 9.0 to 9.2, and a temperature optimum at 37°C. It hydrolyzed both milkfat and tributyrin and was inhibited by sulfhydryl reagents. The lipase showed little activity toward simple esters.

Foissy,⁹² working with separator slime, showed that three proteins isolated by gel electrophoresis exhibited lipolytic activity.

A recent report by Richter and Randolph²⁸⁵ described the isolation of a lipase from separator slime with a molecular weight of 8,500 which contained carbohydrate, had a pH optimum at 9.2, and also had a temperature optimum at 37°C. It hydrolyzed simple short-chain fatty-acid triglycerides more rapidly than long-chain glycerides, but had little specificity for natural oil emulsions. Whether this is the same lipase reported by Chandan and Shahani was not unequivocally established.

ACTIVATION OF LIPASES

Homogenization and Agitation

All methods of agitation of milk appear to increase the rate of lipolysis. The increased incidence of rancidity in pipeline milkers as

opposed to conventional milking procedures due to foaming and agitation has already been discussed. Homogenization (a more violent form of agitation) of raw milk when conducted at temperatures between 37.7°C and 54.4°C will render milk rancid within a very short time in some cases in only a few minutes.^{145,366} The length of time of homogenization as well as the homogenization pressure²⁵³ will influence subsequent lipase activity, lipolysis increasing within limits, as the magnitude of these variables increases.^{230,253}

Other forms of agitation, including shaking raw milk containing liquid fat,^{51,60,213,319} the churning of raw milk or cream, and pumping³⁷² will accelerate lipolysis. The severity of agitation and the temperature at which it is conducted are of prime importance.

Foaming due to agitation also promotes lipolysis, but the increased activity in foam is probably independent of the accelerated lipolysis due to agitation. According to Tarassuk and Frankel,³⁴⁶ foaming promotes lipolysis by providing (a) greatly increased surface area, (b) selective concentration of enzyme at the air-liquid interface, (c) "activation" of the substrate by surface denaturation of the membrane materials around the fat globules, and (d) intimate contact of the lipases and the "activated" substrate.

All forms of agitation, with the exception of churning, increase the surface area of the substrate, and this is the foremost reason for the increase in lipase activity. However, agitation produces other effects which are conducive to lipase action. The process of diffusion, which has been shown to be very important,²³⁹ is speeded up. Diffusion permits the lipases to migrate more readily to the oil/water interface while simultaneously allowing the fatty acids produced in lipolysis to leave the interface.

Thermal Manipulation

Unlike spontaneous milk, normal (nonspontaneous) milk requires additional thermal "shocking" beyond the first cooling to activate the milk lipase system. Krukovsky and Herrington²¹² were the first to demonstrate that lipolysis in normal milk could be hastened by warming cold milk to 29.4°C, and then recooling beyond the solidifying point of the fat. Most samples of milk subjected to this treatment will become rancid within 24 hr.¹⁴⁵ The temperature of approximately 30°C is critical, and heating below or appreciably beyond that point diminishes the degree of activation that can be obtained. This type of activation is of great practical importance because it can happen accidentally. For example, if warm morning milk is added to a can of milk refrigerated from the night before and the whole cooled again, the milk may be rancid by the time it is ready for processing.

Milk containing fat globules with a natural fat globule membrane can be activated, deactivated, and reactivated by proper changes in temperature. The phenomenon of temperature activation is found only when the fat globules have their natural layer of adsorbed materials. Neither homogenized milk, nor emulsions of tributyrin, nor of butter oil emulsified in skim milk can be activated in this manner.

Several hypotheses have been advanced to explain the peculiar phenomenon of temperature activation. These include the attainment of a favorable liquid-to-solid glyceride ratio^{144,215}, an increase in the permeability of the fat globule membrane to the lipases,³⁵³ and reorientation of glycerides more susceptible to lipolysis toward the fat/water interface.²⁸³ However, the first and latter hypotheses would seem to be inconsistent with the fact that homogenized milk cannot be temperature-activated.

The freezing of raw milk followed by thawing to 4°C causes an increase in lipolysis compared to unfrozen controls stored at 4°C, but the increase in activity varies considerably. Repeated freezing and thawing also causes a notable increase in lipolytic activity. The temperature of freezing has a marked effect, the increase in lipolysis being most pronounced when the temperature is lowered from -10 to -20°C; little further increase in activity occurs between -20 and -33°C. Slow freezing causes greater lipolysis than does rapid freezing.³⁷¹

Chemical Activation

Downey and Andrews' experiments⁶⁶ have indicated that there is a bivalent cation requirement for full milk lipase activity. Dunkley and Smith⁸¹ had previously stated that small amounts of CaCl₂ accelerate lipolysis. These observations are in keeping with those made on lipases from other sources where Ca⁺⁺ was found to stimulate activity.³⁷⁶

Pitocin, a hormone, was reported to increase lipolysis,^{179,180} and another hormone, diethylstilbestrol, is said to increase lipase activity toward tributyrin but not toward milkfat.⁸⁴ Shahani and Chandan³¹⁴ found that purified lipase from separator slime can be activated by the proper concentration of pseudoglobulin, euglobulin, lactalbumin, blood serum albumin, and bovine plasma albumin.

The milk lipase system is reported to be activated by mercuric chloride. Raw milk preserved with corrosive sublimate contains, in some instances, a much larger concentration of free fatty acids than unpreserved samples. Pasteurized milk preserved in a similar fashion does not show an increase in free fatty acids.²³⁵

INHIBITION OF LIPASES

Thermal Inhibition

Heat-treatment of milk is the most important practical means for inactivation of its lipases. The temperature-time relationship necessary for partial or complete inactivation has been extensively studied, but a number of discrepancies have been apparent. These are probably due to several factors, among which might be mentioned the sensitivity of the assay procedure, length of the incubation period following heating, the presence and concentration of fat and solids-not-fat in the milk at the time of heating, and the type and condition of the substrate. In view of these variables, references to a number of early studies on heat inactivation have been omitted.

The data of Nilsson and Willart²⁵⁴ indicate that heating at 80°C for 20 sec is sufficient to destroy all lipases in normal milk. Their studies included assays after 48 hr incubation following the heat-treatment. At lower temperatures for 20 sec, some lipolysis was detected after the 48 hr incubation period after heating. Thus, 10% residual activity remained at 73°C. Below a temperature of 68°C the amount of residual activity was enough to render the milk rancid in 3 hr, and temperatures below 60°C had no appreciable effect on lipolysis. With holding times of 30 min, 40°C produced only slight inactivation, and at 55°C, 80% inactivation was reported. The effect of other temperature-time relationships was studied; the results are given in Table 5.1.

Table 5.1

INFLUENCE OF HEATING TIME ON THE
HEAT-INACTIVATION OF MILK LIPASE(S)^a

Temperature °C	Heating Time Sec	% Inactivation Determined after Incubation for	
		3 Hr	24 Hr
65	5	23	20
	22	52	50
	36	70	57
	74	100	81
72	5	85	30
	22	96	91
	36	100	96
	72	100	100

^a Data of Nilsson and Willart.²⁵⁴

The data of Harper and Gould¹³⁸ are essentially in agreement with those of Nilsson and Willart. They also detected no inactivation until a temperature of 60°C for 17.6 sec was reached. At 87.7°C (17.6 sec) some lipase still survived.

Schwartz³⁰⁸ investigated the effect of various heat treatments on the pH-activity curve of the lipase system in raw skimmilk powder. For this purpose the powder was reconstituted and the milk exposed to the temperature and times listed in Table 5.2.

Fat apparently protects the lipases to some extent from heat inactivation, 1° to 2°C higher temperatures being necessary for whole milk than for skimmilk.^{109,137,138,254,297} The influence of the fat content of milk on heat inactivation of the milk lipase system is given in Table 5.3.

Harper and Gould¹³⁸ indicate that besides the protective effect of fat on lipase inactivation, the solids-not-fat content is also a factor. Higher solids-not-fat concentration, within limits, afforded some protection.

Inhibition by Light and Ionizing Irradiation

The milk lipase system shows a remarkable sensitivity to light. Kay¹⁷⁵ exposed fresh milk in glass vessels to bright summer sunshine for 10 min and found that 40% of the lipolytic activity was destroyed. Exposure for 30 min resulted in a loss of 80%, and exposure to an 800-watt quartz mercury-vapor lamp at a distance of 15 cm destroyed 75% of the activity. He noted, however, that if oxygen was first removed from the system before exposure to sunlight, the effect of the light was greatly diminished. Kannan and Basu¹⁷² observed that in some cases exposure to ultraviolet light destroyed the lipase system and diffused daylight brought about a partial inactivation.

Table 5.2

EFFECT OF VARIOUS HEATING PROCEDURES ON LIPOLYSIS^a

Temp. °C	Time Sec ^b	pH Levels						
		6.2	6.6	7.0	7.5	7.9	8.5	9.5
		Percent Inactivation						
60.0	17.4	73.5	67.4	55.0	45.8	49.7	41.3	53.5
66.8	14.2	65.1	61.4	65.7	65.4	47.8	48.4	90.7
72.0	14.4	91.0	90.1	84.4	83.5	82.1	83.8	91.3

^a Data of Schwartz.³⁰⁸

^b Milk attained temperature at or within these times.

Table 5.3

INFLUENCE OF FAT CONTENT ON THE HEAT-INACTIVATION
OF MILK LIPASES^a

Fat	% Inactivation after Heating at 55°C	
	5 min	15 min
0.1	50	70
5	40	63
10	35	60
20	29	57

^a Data of Nilsson and Willart.³⁵⁴

Frankel and Tarassuk¹⁰⁹ exposed a layer of raw skim milk 1 cm thick to direct sunlight at room temperature and noted a loss in lipase activity of 84% in 5 min and 96% after 10 min. In diffuse daylight inactivation was less, but 71% was lost in 1 hr. The loss of activity by light was independent of the temperature of the milk, equal losses being observed at 0°C and at 37°C. The enzymes are markedly protected against light inactivation by the presence of fat.

Stadhouders and Mulder³²⁹ confirmed Kay's observation that the shorter wavelengths (about 4300 Å) of the spectrum are most destructive to milk lipases. The destructive effect of light could be repressed by addition of reducing agents such as metol, hydroquinone, and especially by hydrogen sulfide. Ascorbic acid and methionine had no effect, but cysteine afforded a significant protection. Lipases which had been inactivated by light were not reactivated by treating milk with hydrogen sulfide.

Irradiation by ionizing radiation and its effect on milk lipase activity has also been studied.³⁶⁷ Irradiation doses of 6.6×10^4 rads destroyed 70% of the activity. The udders of lactating cows when exposed to cobalt-60 gamma rays gave milk with decreased lipase and esterase activity.²³³

Chemical Inhibition

A large variety of chemical compounds have been added to milk or purified lipase preparations in order to determine their effect on lipase activity. The conditions under which the inhibitor is studied are very important. Factors such as pH, temperature, time of addition of the chemical, sequence of addition of reactants and the presence or absence of substrate are undoubtedly involved. The presence of substrate appears to offer some degree of protection to the enzymes. Consequently, in lipase studies the surface area of the emulsified substrate is probably also important.

Heavy metals usually affect enzymes adversely, and milk lipases are no exception. Copper, cobalt, nickel, iron, chromium, manganese, and silver are inhibitors.⁵⁷ Raw skimmilk treated with 5 to 20 ppm Cu^{++} for 15 min at room temperature caused 7 to 17% loss of lipolytic activity, whereas 5 ppm at 37°C for 1 hr resulted in a 69% loss. There was less inhibition in the presence of substrate.¹⁰⁹ Earlier, however, Krukovsky and Sharp²¹⁶ showed that Cu^{++} was ineffective as a lipase inhibitor in nonhomogenized milk if oxygen was absent. At the same time they also found that oxygen alone is an active inhibitor, its effect being magnified by the presence of low percentages of copper.

A number of salts inhibit lipolysis, the most effective being sodium chloride.^{39,117,269,373} Lipolysis in cream was found to be insignificant in the presence of 4% sodium chloride and in homogenized milk containing 5 to 8% of this salt.¹¹⁷ It should be noted that Downey and Andrews⁶³ used 0.75M sodium chloride (about 4.3%) to dissociate the lipases from the casein micelles as the first step in their fractionation procedure, but still went on to isolate 5 active lipases. It is not known whether loss of part of the activity of their lipases was due directly to the salt treatment, or whether activity was restored by subsequent purification.

Phosphate buffer (0.6M) slightly inhibited lipolysis, but the same concentration of borate and barbiturate buffers was without effect.²⁶⁶

Zinc chloride, potassium cyanide, manganese sulfate, cysteine, and magnesium chloride retarded milk lipase activity to various degrees. All these compounds were tested at pH 8.5 with tributyrin as substrate during a 30-min incubation period.²⁶⁶

N-Ethyl maleimide inhibits lipase activity in milk activated by shaking, temperature fluctuations and homogenization, 0.02M being completely inhibitory.³⁵⁷ An equimolar concentration of glutathione markedly reduces inhibition by *N*-ethyl maleimide. This reagent can also completely inhibit lipolysis in spontaneous milk.³⁵⁷ It was concluded on the basis of these experiments that sulfhydryl groups are essential sites of activity on milk lipases. This is supported by the ability of reducing agents such as glutathione, hydroquinone, and potassium thiocyanate to offer stability to the milk lipase system during storage.¹⁰⁹ It was also concluded that purified lipase from separator slime contains 1 free and 1 masked sulfhydryl group, which are essential for activity. Sulfhydryl reagents such as *p*-chloromercuribenzoate, iodoacetic acid, formamidine disulfide, and *N*-ethyl maleimide are potent inhibitors of slime lipase.⁴³

Other chemicals which inhibit the milk lipase system include hydrogen peroxide, animal cephalin,³⁷ sodium arsenite, diisopropyl fluorophosphate, 2,4-dinitro-1-fluorobenzene, *p*-hydroxymercuri-

benzoate,²⁹⁰ potassium dichromate,³⁷⁵ lauryl dimethyl benzyl ammonium chloride,²⁹⁶ aureomycin, penicillin, streptomycin, and terramycin.⁴⁰

The most studied chemical inhibitor of lipolysis has been formaldehyde, for the reason that formaldehyde had been widely used as a preservative in milk lipase studies without knowledge of its effect. After the existence of a lipase in milk had been established, Herrington and Krukovsky¹⁴⁸ postulated that there was more than one lipase in milk, since experiments had suggested that there was a formaldehyde-sensitive and a formaldehyde-tolerant lipase in milk. Other investigators^{226,289} published data which essentially substantiated this, but there was also information obtained under different experimental conditions in which formaldehyde was shown to be noninhibitory.^{117,344}

An extensive study of the effects of formaldehyde in milk lipase inhibition showed that formaldehyde acts as a competitive inhibitor and also, under the proper conditions, selectively inhibits the lipases of raw skimmilk.³¹⁰ It was shown in this study that the inhibitory effect of formaldehyde was dependent on such factors as pH, time of addition of inhibitor, length of incubation period, concentration and availability of substrate, and concentration of inhibitor. Many of the conflicting results encountered with formaldehyde can be explained on the basis of dependence on one or more of these factors.

PROPERTIES OF MILK LIPASES

Specificity

A review of the specificity of a purified slime lipase from milk and also of pancreatic lipase has been published by Jensen,¹⁶³ who has been one of the foremost investigators in this field. Pancreatic lipase and the purified slime lipase exhibit similar characteristics in their specificities, and although slime lipase represents only one of a number of lipases in milk, it is probable that other lipases in milk will show similar specificities as they are isolated and purified.

A study of lipase specificity requires that the enzyme and substrates be virtually pure. Contamination of the lipase preparation with esterases will give rise to misleading results. Pure, synthetic substrates of known configuration are essential, and the same available surface area should be present after emulsification for meaningful data to be obtained. Since most of the earlier workers have disregarded one or more of these variables, their data are useless for all practical purposes, and will not be included here.

There are four types of specificity which may be considered: (1) a

specificity between glycerides, i.e., intermolecular specificity; (2) a specificity between different fatty acids in the same position of a glyceride, that is, the 1- and 3-positions, also called intramolecular specificity; (3) positional specificity, i.e., a specificity for the 2-position over the 1- and 3-positions, or vice versa; and (4) stereospecific specificity, i.e., whether lipases preferentially differentiate between the 1- and 3-positions when they are occupied by the same fatty acid.

Purified slime lipase shows an apparent specificity for short-chain fatty acid-containing triglycerides relative to longer chain-containing glycerides including milkfat. This was shown by Jensen *et al.*¹⁶⁵ It also shows a specificity for triolein over saturated triglycerides such as trilaurin or trimyristin, but this may have been because the latter two are solids and consequently are more difficult to emulsify to the same degree as liquid glycerides.

Purified slime and pancreatic lipases, among others, have been shown to have no intramolecular specificity, hydrolyzing both short- and long-chain acids at the same rate when the acids occupy the 1- and 3-positions in the same molecule.¹⁶³ This observation refuted earlier work, which had indicated that pancreatic lipase shows a specificity for shorter-chain acids in a similar situation.

Positional specificity is exhibited by slime and pancreatic lipases. Fatty acids in the 1- and 3-positions are hydrolyzed at a greatly accelerated rate compared to fatty acids in the 2-position. In fact, there is some question whether the 2-position is attacked at all, since acyl migration from the 2-position to the 1-position occurs to some extent.¹⁶³

Stereospecificity has not been studied on milk lipases, although it has been demonstrated to be absent for pancreatic lipase.¹⁷⁴

pH Optimum

Enzymes usually exert their catalytic influence over a somewhat restricted pH range. Within this range the activity passes through a maximum, commonly called the pH optimum, and then falls off again. Although the pH optimum and the pH range are generally characteristic of a given enzyme, they may in some instances be altered by such factors as type and strength of buffer, ionic strength, temperature, type of substrate employed, and, in the case of lipases, the condition of the interface where lipolysis must proceed.

Lipases are sensitive to extremes in pH, and even in the vicinity of the pH optimum, where enzymes are supposedly more stable, marked inhibition may occur.¹⁰⁸ Thus, it must be borne in mind also that the length of the incubation period and the prior history of the preparation can influence the range and perhaps the shape of the pH-activity curve.

The incubation of raw skimmilk at pH 6.0 and at pH 8.9 for 1 hr at 37°C in the absence of substrate was subsequently shown to cause a 47% and 40% decrease, respectively, in lipase activity when the milk was later incubated with milkfat. When tributyrin was the substrate the inhibition was even more marked. Although some of the inactivation was due to temperature, the majority of it is attributable to pH exposure. Stadhouders and Mulder³³⁰ have also demonstrated that milk lipase subjected to incubation at pH 5.0 is almost completely destroyed.

The point on the acid side of the pH curve where milk lipase activity ceases is of considerable practical importance, but there is still controversy regarding it. Bosco³³ found that milk lipase is active down to pH 4.7, whereas Schwartz *et al.*³⁰⁹ could detect no activity at pH 5.2 on butterfat. Although Peterson *et al.*²⁶⁷ found no milk lipase activity on tributyrin at pH 7.0, activity has been reported on this substrate at pH 5.0 and even at pH 4.7 when 24-hr incubation periods were used.³³⁰ Willart and Sjostrom³⁷⁴ have also observed activity in the range pH 4.1 to 5.7.

It has been reported that there are lipases and esterases operating in the range of pH 5.0 to 6.6 on tributyrin and simple esters. These lipases were stated to have pH optima at 5.4 and 6.3 and to be inhibited by formaldehyde.⁴ For two enzymes to operate in such a restricted range is unusual, and may indicate that nonenzymatic entities are present in milk which can promote hydrolysis of esters.

The pH activity curves for the milk lipase system were shown by Schwartz *et al.*³⁰⁹ to vary with the concentration of milkfat used as substrate. They observed activity between pH 5.2 and 9.8. At high substrate concentrations (16.5% milkfat), optima were observed at pH 8.5, 6.5 to 7.0, and 7.9; at a very low concentration of substrate (0.92% milkfat), a broad optimum between pH 7.0 and 8.0 was noted.

In general, when the substrate concentration is not limiting, the pH optimum most frequently observed for the milk lipase system lies between pH 8.5 and 9.0. The lipase isolated and purified from separator slime of milk shows an optimum at 9.0 to 9.2.⁴²

Apparent Temperature Optimum

A rise in temperature has a dual effect upon an enzyme-catalyzed reaction: it increases the rate of the reaction, but it also increases the rate of thermal inactivation of the enzyme itself. Like the pH optimum, the temperature optimum may in certain instances be altered by environmental conditions, e.g., pH, type and strength of buffer, etc. The term "temperature optimum," therefore, is useless unless the incubation time and other conditions are specified. A more enlightening

nomenclature is "apparent temperature optimum," which indicates that the optimum has been obtained under a certain set of conditions, and may or may not hold when these conditions are changed.

The apparent temperature optimum for the milk lipase system is reported to be around 37°C both on milkfat and on tributyrin.^{106,289} This temperature has been recorded both at pH 8.9 and pH 6.6 for milkfat^{106,289} and at pH 8.0 and pH 6.6 on tributyrin.¹⁰⁶

Stadhouders and Mulder,³²⁸ however, using tributyrin and milkfat together as substrates, found that at pH 9.1 under their conditions poorer activity was apparent at 37°C compared to 25°C or 15°C after 1, 4, and 24 hr of incubation. In fact, 90% less activity occurred at 37°C at 24 hr of incubation than at 15°C. The activity at 15°C was quite constant, indicating complete stability of the lipase system under the conditions used. Downey and Andrews⁶⁶ have stated in this regard that the lipases which they isolated are unstable even at 37°C, but are more active at this temperature than at 25°C. Their results suggest that it is unwise to assay at 37°C.

Stability

Some discussion regarding stability of milk lipases was presented in the preceding section. Other workers have also studied the stability of the milk lipase system under different circumstances. Schwartz³⁰⁸ found that raw skimmilk showed no loss of activity at pH 6.2, 6.6, 7.0, 7.5, and 8.5 after the milk had been stored at pH 6.6 for 6 hr in the dark at 4°C prior to assay. Frankel and Tarassuk¹⁰⁹ permitted raw skimmilk to stand 1 hr at 37°C at pH levels ranging from 5.2 to 9.8, and found greatest activity in the range pH 6.6 to pH 7.6 when the milk was subsequently incubated with the substrate at pH 8.9. Hence it appears that normal milk is in an optimum pH range for stability of the lipases. The same pH range of optimum stability was found in whole milk.

The stability of the lipase system in raw, lyophilized skimmilk has also been investigated.³⁰⁸ This powder stored at 4°C showed no loss in activity over a 7-month period when assayed at pH 6.6, 7.9, and 8.5.

SOME EFFECTS OF LIPOLYSIS

The most serious effect of lipolysis is the appearance of the so-called rancid flavor. The fatty acids and their soaps which are thought to be implicated in the flavor have been studied in an effort to assess the role of the individual acids in the overall rancid flavor picture.

Scanlan *et al.*³⁰⁵ reported that only the even-numbered fatty acids from C₄ to C₁₂ account for the contribution of fatty acids to the flavor, but that no single acid exerts a predominating influence. Another study has implicated the sodium and/or calcium salts of capric and lauric acids as major contributors to the rancid flavor.⁵ Butyric acid, assumed to be the compound most intimately associated with the flavor, was not singled out in either study as being especially involved.

Besides changing the natural flavor of milk, lipolysis may produce a variety of other effects. One of the most noticeable of these is the lowering of surface tension as lipolysis proceeds.^{39,72,81,204,353-356} Fatty acids, and more especially their salts, and mono- and diglycerides, being good surface-active agents, depress the surface tension of milk (see under Methods). Milkfat obtained from milk subject to lipase action also gives lower interfacial tensions with water than does milkfat obtained from nonlipolyzed milk.^{31,82}

Rancid milk decreases the quality of cream, butter, and buttermilk made from it, and a limit on the acid degree value of the fat of milk from which butter is eventually to be made has been proposed.¹⁶⁰

The higher saturated fatty acids have been noted to inhibit rennet action, whereas the lower fatty acids enhance it. The inhibitory effect of the higher acids could be nullified by CaCl₂.³⁵⁴

As little as 0.1% rancid milkfat proved to be a very effective foam depressant during the condensing of skimmilk and whey.³⁵ This effect was attributed to the mono- and diglycerides.

Lipolytic action has been observed to occur in composite samples preserved with mercuric chloride and decreased the reading of the Babcock test as much as 0.15%.²³⁵

An inhibitory effect of rancid milk on the growth of *S. lactis* has been reported. Koestler *et al.*¹⁹⁸ claim that rancid milk significantly inhibits the growth of bacteria in general and of *S. lactis* in particular. It has been stated that rancidity in milk may reach such a degree as to actually render the product sterile.⁹ Tarassuk and Smith³⁵⁶ attributed the inhibitory effect of rancid milk to changes in surface tension, but Costilow and Speck^{49,50} believe that the inhibition is due to the toxic effect of the individual fatty acids.

Although rancidity is a serious defect in market milk, it has also been utilized profitably. Whole milk powder made from lipase-modified milk has generally met acceptance among chocolate manufacturers. It is used as a partial replacement for whole milk because it imparts a rich, distinctive flavor to milk chocolate, other chocolate products like fudge and compound coatings, caramels, toffees, and butter creams.³⁸¹

METHODS FOR DETERMINING LIPASE ACTIVITY

A number of methods are available for following lipase activity. Although numerous modifications and variations have been introduced, the basic methods may be listed as: (1) titration of the liberated fatty acids, (2) changes in surface tension, (3) colorimetric determination of the fatty acids, (4) use of gas-liquid chromatography, and (5) use of radioactive substrates.

Titration

Titration of the fatty acids formed by the action of the milk lipase system has been the most widely used procedure. Titration has been conducted directly on the reaction medium either manually¹²⁰ or automatically,²⁶³ in the presence of added organic solvents,^{81,266,267} and after separation of the lipid phase by extraction,¹⁰⁶ distillation,^{101,289} churning,^{101,354} or adsorption of the medium followed by elution of the fatty acids.¹⁴⁰ All these techniques have their shortcomings. The most widely used laboratory methods appear to be the silica gel extraction¹⁴⁰ and the pH-stat methods,²⁶³ and in the field, the method of Thomas *et al.*^{359a}

Surface Tension

Efforts have been expended to apply surface-tension measurements for following lipolysis in milk.^{72,81,348,352,354-366} As mentioned earlier, the hydrolysis products resulting from lipase action are strongly surface-active. Tarassuk and Regan³⁵² have stated that the lowering of surface tension resulting from lipolysis is the most distinct change differentiating rancid from nonrancid milk. However, a great many variables influence the surface tension of milk, such as the elaboration of structurally different mono- and diglycerides and their concentration.

Colorimetry

Copper⁷¹ or cobalt²⁵⁵ soaps of long-chain fatty acids ($\geq C_{12}$) are soluble in chloroform and can be determined quantitatively by colorimetric determination of the extracted metal. This method has been used in some nondairy lipase investigations, but could conceivably be used as such or modified for use in milk lipase work. It is very sensitive.

Another sensitive colorimetric procedure is that of Mackenzie *et al.*,²³⁴ which utilizes the dye Rhodamine B to form benzene-soluble complexes with fatty acids. Nakai *et al.*²⁵⁰ developed a rapid, simple method for screening rancid milk based on the above procedure. The test is said

to detect rancid milk with an acid degree value above 1.2. Like the copper or cobalt soap method, the Rhodamine B reagent is also limited to the longer-chain fatty acids.

Gas-Liquid Chromatography

Gas-liquid chromatography (GLC) affords both a qualitative and, if adequate internal standards are used, a quantitative analysis of the products of lipolysis. It is necessary, however, first to isolate the acids by a suitable method and then inject them as free acids or as esters. The partial glycerides can be isolated by thin-layer chromatography and can also be determined by GLC of suitable derivatives. The acid(s) remaining in the partial glycerides can be identified readily by GLC following transesterification. Jensen and his co-workers have utilized these techniques in their studies on lipase specificity.^{164,166}

Radioactive Substrates

Koskinen *et al.*,²⁰⁵ Luhtala *et al.*,²³¹⁻²³² and Scott³¹¹ have used labeled triglycerides as substrates for milk lipases. This method, which is extremely sensitive, requires that the acids released by lipase action be isolated uncontaminated with any tagged glycerides. It also requires the preparation of labeled substrate, and, of course, counting equipment.

Miscellaneous

A manometric technique utilizing a Warburg apparatus has been used to follow esterase activity. The carbon dioxide liberated from sodium bicarbonate by the fatty acids is measured.³⁷³

An agar diffusion procedure has been utilized for screening microorganisms for lipolytic enzymes. The presence of lipase is indicated by clear zones in the turbid media.²²⁴

Lipid autoxidation in fluid milk and a number of its products has been a concern of the dairy industry for a number of years. The need for low-temperature refrigeration of butter and butter oil, and inert-gas or vacuum packing of dry whole milks to prevent or retard lipid deterioration, in addition to the loss of fluid and condensed milks as a result of oxidative deterioration have been major problems of the industry.

The autoxidation of milk lipids is not unlike that of lipids in other edible products. However, the complex composition of dairy products, physical state of the product (liquid, solid, emulsion, etc.) presence of natural anti- or pro-oxidants, as well as processing, manufacturing, and storage conditions tend to influence both the rate of autoxidation and the composition and percentage of autoxidation products formed.

The literature dealing with the autoxidative mechanism involved in lipid deterioration has been concerned with investigations on pure unsaturated fatty acids and their esters. The reactions involved, however, are representative of those occurring in lipids and lipid-containing food products.

AUTOXIDATION MECHANISM

The initial step in the autoxidation of unsaturated fatty acids and their esters is the formation of free radicals. Although the initiation of such radicals is not completely understood, the resulting free-radical chain reaction has been elucidated in the investigations of Farmer and Sutton,⁸⁹ and others.^{23,32} In the case of monounsaturated and non-conjugated polyene fatty acids—the acids of significance in milk-fat—the reaction is initiated by the removal of a hydrogen atom from the methylene (α -methylene) group adjacent to the double bond (I). The resulting free radical, stabilized by resonance, adds oxygen to form peroxide-containing free radicals (II); these in turn react with another mole of unsaturated compound to produce 2 isomeric hydroperoxides in addition to free radicals (III) capable of continuing the chain reaction.