

SOME FACTORS AFFECTING THE SOLAR-ACTIVATED FLAVOR
OF HOMOGENIZED MILK AND THE ISOLATION AND
CHARACTERIZATION OF A MINOR WHEY PROTEIN
FRACTION WHICH IS CAPABLE OF BEING
SOLAR-ACTIVATED

By

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INTRODUCTION

The solar-activated flavor is one of the major problems in the distribution of homogenized milk. The flavor defect occurs frequently, and gives rise to consumer complaints. At the present time no easy or practical means for preventing its development in commercially produced homogenized milk is available. The term solar-activated flavor as used throughout this study embraces a number of flavors such as "sunshine," "burnt," "burnt-feather," "burnt-protein," "cabbage," "mushroom," and/or even "medicinal."

Many investigators have shown that homogenized milk was far more susceptible to off-flavors as a result of exposure to daylight than the same milk not homogenized. The occurrence of the solar-activated flavor in homogenized milk exposed to daylight was rather disheartening in view of the gratifying discovery that homogenization retarded or inhibited the development of the copper-oxidized flavor. Thus, while homogenization retarded susceptibility of milk to one undesirable flavor, it enhanced the development of another off-flavor, if and when certain conditions prevail. Although the solar-activated flavor has caused much concern among the commercial dairy plant operators, the available data regarding the various factors involved in the development of the solar-activated flavor and an easy and practical method to prevent its inception are limited.

The purpose of the work reported herein were: (a) to study the various factors related to the off-flavor development; (b) to attempt to find a practical method to prevent the activated flavor development and

further; (c) to attempt to establish the identity of the constituent affected when a solar-activated flavor develops.

REVIEW OF LITERATURE

The deleterious effect of sunlight upon the flavor of milk has been recognized for many years. Browne (1899) early observed that oxidative rancidity was catalyzed by the exposure of the butterfat to the light. Since this early observation, various investigators, Hammer and Cordes (1920), Frazier (1928) and Henderson and Roadhouse (1934) have studied the effect of light upon the flavor of milk as being associated with a lipid oxidation. Hammer and Cordes (1920) reported that "off" flavors developed in certain milk samples after 10-minute exposure to light and further, that a definite tallowy flavor appeared after 45 minutes exposure to light. They noted also that an abnormal flavor developed rapidly in skimmilk upon exposure to sunlight. The flavor that developed in the exposed skimmilk was not the typical tallowy flavor. These workers discovered that the "off" flavor development could be retarded by the use of brown glass bottles. However, their use resulted in increased milk temperatures, thus favoring bacterial growth. In the light of our present knowledge it appears that the "off" flavor that Hammer and Cordes (1920) observed in exposed skimmilk was in reality the activated flavor so common today in commercially produced homogenized milk.

Frazier (1928) found that diffused daylight acted as a catalyst in the oxidation of the butterfat. He postulated that although the heavy glass of the milk bottle screened out the ultra-violet rays, it did, nevertheless, allow to pass through the longer rays which exerted the catalytic effect.

Tracy and Ruehe (1931) found from their studies of flavors in market milk that milk exposed to sunlight for short exposure periods in uncolored glass bottles developed the typical tallowy flavor. They observed that as the period of exposure of the milk to sunlight was increased, a point was eventually reached where the tallowy flavor was masked by the burnt flavor. They believed that the presence of metallic salts was not a factor in the development of the burnt flavor, and that skim milk and low-fat milks were more susceptible to the burnt flavor than were whole milk and cream. The burnt flavor described by Tracy and Ruehe (1931) was attributed to the action of sunlight upon the milk proteins.

Henderson and Roadhouse (1934) stated that cream exhibited a greater susceptibility to oxidation when exposed to diffuse or direct sunlight.

Doan and Myers (1936) observed that skim milk, whole milk and buttermilk could be protected from the catalytic action of sunlight by the use of paper milk containers. However, they found that paper milk bottles offered no protection against the tallowy flavors caused by sunlight. The authors postulated from the above findings that the photochemical reactions producing the tallowy flavor and the burnt flavor in milk were separate and distinct.

The study of the solar-activated flavor prior to the acceptance of homogenized milk as a marketable product received little attention. Since the solar-activated flavor was not a serious flavor defect in nonhomogenized milk, but rather more noticeable in skim milk, the urgency for a complete study of the activated flavor was not deemed necessary.

The phenomenal acceptance of homogenized milk in America during the past decade (to the extent that in some plants 100 percent is homogenized) has extended the possibilities of the occurrence of the defect. Hood and White (1934) early pointed out that homogenized milk was far more susceptible to off-flavors as a result of exposure to daylight than the same milk not homogenized. This observation has since been substantiated and/or reported by many workers. (Doan 1937a, 1937b, 1938, 1943; Tracy 1936, 1938, 1948; Corbett and Tracy 1937, 1939; Flake, Weckel and Jackson 1939; Dahle 1941; Babcock 1942; Henderson 1944; and Burke 1948.)

Tracy (1936) in a discussion of homogenized milk stated that homogenization was a factor in the case of milk exposed to sunlight since homogenized milk would acquire an off-flavor sooner than nonhomogenized milk. He found that when a bottle of each of the two milks was exposed to either direct or indirect sunlight for 10-15 minutes, the nonhomogenized milk developed a slight burnt flavor whereas the homogenized milk had developed a much more pronounced burnt flavor. Tracy (1936) theorized that while light rays would oxidized the butterfat, the effect of the sunlight on the milk proteins was responsible for the activated or burnt flavor.

Although the development of the solar-activated flavor gives rise to consumer complaints in the homogenized milk product, much of the work has been carried out using skim milk or whey.

Weckel, Jackson, Haman and Steenbock (1936), in an effort to determine the effect of irradiation on the flavors of milk, exposed milk to irradiated energy for various periods. The flavor that developed following irradiation in the incipient stage was described as "flat." Upon continued exposure, this flavor gradually changed into what the authors

described as "burnt," "burnt feather," "burnt protein" or "mushroom flavor." They concluded that the activated flavor must be distinguished from the papery, cardboardy, tallowy flavors which they believed resulted from the action of radiant energy or metals on the lipids in milk. Weckel, Jackson, Haman and Steenbock (1936) in reference to the division of the spectrum responsible for the off-flavor stated:

The effect is due to radiation from that part of the spectrum which is known to have an antirachitic effect as well as to parts of the spectrum devoid of such properties. An analysis of the emission of the various arcs permits the conclusion that energy ranging in wave length from 2,600-3,100 Angstrom units is less active in flavor production than energy of wave length less than 2,600 Angstrom units.

Josephson (1946) is in disagreement as to the wave length responsible for the off-flavor development. He stated that the sunlight flavor would develop on very cloudy days when little, if any, ultra-violet light penetrated the outer atmosphere. Josephson (1946) employed specially prepared exposure cells and light filters of known transmission and concluded that no protection against the off-flavor development was afforded even when all light below 5,900 Angstrom units was excluded. He reported that for complete protection against the off-flavor development all light below 6,200 Angstrom units must be excluded. He believed the active wave lengths of light were within 5,900 and 7,400 Angstrom units. Since the heavy glass of the milk bottle would tend to filter out the ultra-violet light rays, it seemed logical to assume that the range proposed by Josephson (1946) was the effective range.

Weckel and Jackson (1936); Flake, Weckel and Jackson (1939); and Flake, Jackson and Weckel (1940) are chiefly responsible for the few facts we know concerning the chemistry of the activated flavor compound.

The authors concluded that the constituent affected when a solar-activated flavor develops is protein in nature. Flake, Jackson and Weckel (1940), in an attempt to determine specifically the constituent affected, subjected casein, lactalbumin and various amino acids to ultra-violet radiation. They concluded that albumin, and to a lesser degree casein, acquired a flavor and odor typical of that which develops in milk similarly exposed to radiation. Of the amino acids studied, cystine, methionine, tryptophane and histidine were found to develop flavors suggestive of irradiated milk. The authors' attempts to measure the effect of ultra-violet light upon the protein structure by means of the Van Slyke amino nitrogen and nitrogen distribution determinations were not successful since the analytical procedures used were not sensitive enough to show a measurable structure change. Flake, Jackson and Weckel (1940) obtained concentrated solutions of dialyzable substances from milk and exposed them to irradiation. These dialyzable solutions upon irradiation gave rise to a disagreeable flavor and odor which were definitely not typical of the activated flavor of excessively irradiated milk. The authors also carried out dialytic experiments and concluded that the removal by dialysis of a large portion of the dialyzable substances of milk did not alter its susceptibility to the development of the activated flavor and odor.

Ansbacker, Flanigan and Supplee (1934) suggested the possibility that sulfur compounds might be involved in the activated flavor formation. Upon subjecting a foaming agent of milk to ultra violet light they noted an odor which was similar to that of over-irradiated milk. These workers theorized that the basic mechanism involved the mobilization of sulfhydryl groups. Flake, Jackson and Weckel (1940) further indicated the possible

role of sulfur in the development of the activated flavor when they observed that the intensity of the activated flavor was accentuated by heating milk to approximately 180°F. which is in the temperature range at which the cooked flavor becomes evident.

Doan and Myers (1936) believed that the burnt flavor due to solar radiation originated in the casein-and albumin-free serum of milk. Flake, Jackson and Weckel (1940) were able to concentrate a "fraction" that they believed responsible for the activated flavor development. They did not analyse the concentrated fraction. However, they did secure a positive nitroprusside test after reduction with potassium cyanide, which indicated the presence of disulfides. The authors reported that when a very small amount of the concentrated fraction was added to milk it imparted to the milk a flavor and odor very similar to that of milk exposed to radiation. Keeney (1947) was able to induce the activated flavor in a casein-, albumin-, and globulin-free milk serum. He succeeded also in concentrating a heat-flocculable, dialyzable substance which he believed to be one of the components responsible for the sunshine flavor common to homogenized milk.

PROCEDURE

The milk used in these studies was obtained from the Michigan State College dairy and experimental barns and the college creamery. All milk was holder pasteurized at 143°F. for 30 minutes, after which the milk was homogenized at 2,500 pounds pressure in a laboratory model, 25 gallon-per-hour homogenizer. The homogenizer was flush-washed with one-half gallon of 150° to 160°F. water after processing each sample to prevent any possible intermixing of samples. Following homogenization, the samples were ice colled at once to 50°F.

In an attempt to keep the source of radiant energy uniform all exposed samples were exposed to clear solar radiation between the hours of 10 a.m. and 2 p.m.

Isolation of Minor-Protein Fraction

The isolation procedure of Aschaffenburg (1946) was modified to meet the experimental problems encountered in this study. One gallon of freshly separated skim milk was rennet-coagulated at 26.7° C. (80° F.). The coagulum was cut into 0.5-inch cubes and heated to 50° C. (122° F.) to expel the whey. The whey was separated by vacuum filtration, transferred to a 4-liter beaker and heated to 95° C. (203° F.) for one hour to remove the heat-coagulable proteins. A mechanical stirrer was employed during the heating process to insure uniform heat distribution. Following the heat treatment, the mixture was filtered through a milk filter cloth and the resulting serum was clarified by centrifugation at 50,000 r.p.m. for 15 minutes in a Sharpless steam-turbine supercentrifuge. The centrifuged

serum was crystal-clear and was assumed to be free of the heat-coagulable proteins. The clarified serum was treated with 34.5 g. of C. P. ammonium sulfate per 100 ml. of serum. After standing for three hours, the precipitate was separated by centrifugation at 2,000 r.p.m. for 30 minutes. The resulting precipitate was washed in about one-fourth of its volume of distilled water and again re-separated by centrifugation. The washed precipitate was dispersed again in a small volume of distilled water, transferred to a cellophane dialyzing bag and dialyzed for 24 hours against running tap water and then for an additional 24 hours against slow-dripping distilled water. The non-dialyzable fraction was removed and dried from the frozen state under a high vacuum. The weight of the freeze-dried sample was 3.768 g. The yield was 0.10 percent.

Amino Acid Determinations

Amino acid determinations were carried out microbiologically by using Lactobacillus arabinosis, Streptococcus faecalis and Leuconostoc mesenteroides P-60. The media used in the various determinations were essentially the same as those described by Sauberlich and Baumann (1946) with the exception of those used for isoleucine and methionine, which were prepared according to the method of Kuiken et al. (1943) and Lyman et al. (1946). The hydrolyzates for the determination of arginine, cystine, glutamic acid, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine and valine were prepared according to the method of Stokes et al. (1945). One gram of the material was dispersed in 25 ml. of 6N HCl and autoclaved for eight hours at 15 pounds pressure. The hydrolyzates were cooled, neutralized to pH 6.6-6.8 with 18N NaOH, made up to 100 ml., filtered, covered with a few drops of toluene and stored in the refrigerator until analyzed.

The enzymatic digestion procedure of Wooley and Sebrell (1945) was used for the tryptophan assay. One gram of the material was weighed into a 100 ml. volumetric flask, 20 mg. of pepsin and 40 ml. of 0.1N H_2SO_4 were added and the flasks incubated at 37° C. for 24 hours with constant shaking. The contents of the flasks were then transferred to 100 ml. beakers and 3.0 g. of K_2HPO_4 were added to each beaker and the pH adjusted to 8.4 with 3N NaOH. The solutions were transferred to 100 ml. volumetric flasks and 20 mg. of trypsin were added and allowed to incubate at 40° C. for 24 hours with constant shaking. The contents of the flasks were cooled, adjusted to pH 7.0, diluted to 100 ml., centrifuged, filtered, preserved with toluene and stored in the refrigerator.

DL-Configurations of isoleucine, leucine, methionine, phenylalanine, threonine and valine were used in the preparation of standards for these amino acids, whereas, the L-configurations were used for the preparation of the standards for arginine, cystine, glutamic acid, histidine, lysine and tryptophan. In all cases the assays were run after the proper dilutions had been made.

RESULTS

I. The Susceptibility of Individual Cows' Milk to the Development of the Solar-activated Flavor

Quart samples of morning's milk collected from individual cows of the milking herds at the Michigan State College Dairy and Experimental Barns were pasteurized, homogenized and cooled as outlined under procedure. Each sample was then divided into three lots: Lot 1 served as a control; Lot 2 was exposed to clear solar radiation for 30 minutes between the hours of 10 a.m. and 2 p.m. during the first weeks of September; and Lot 3 was exposed under the same conditions, but for 60 minutes. Immediately after exposure the samples were dark stored at 40° F. for 48 hours. Flavor examinations were made at 24 and 48 hours by experienced judges who did not know the identity of the samples.

Susceptibility of individual cow and breed milk to solar activation.

The susceptibilities of milk from cows on pasture to the solar activated flavor are shown in table 1. The results comprise three trials on each cow at three-day intervals and include milk from nine Ayrshires, 12 Brown Swiss, 10 Guernseys, 11 Holsteins and four Jerseys. The data indicate that of the 46 cows whose milk was studied, 14 of them, or 30 percent, were non-susceptible to the solar-activated flavor in all trials after homogenization and exposure to solar radiation; while the milk from two Guernsey cows, Nos. 71 and 75, exhibited a non-susceptible tendency in two of the three trials. The correlation between breed and susceptibility of milk from individual cows in regard to the solar-activated flavor seems slight. Of the milk studied, 36, 33, 23 and 20 percent of that from the

Holstein, Ayrshire, Brown Swiss and Guernsey breeds respectively proved non-susceptible to solar activation. Studies on Jersey milk were limited since only four Jerseys were in lactation. However, of these four, two of them, or 50 percent, yielded milk susceptible to solar activation.

From the data obtained there appears to be no correlation between solar activation and stage of lactation. The results outlined in table 1 show that the cows producing non-susceptible milk represented, in general, all stages of lactation.

Fortunately, during the course of this study milk from the experimental herd of 20 Holsteins that were on dry feed for a period of time was available for investigation. Since approximately 30 percent of the cows on pasture yielded milk which upon pasteurization, homogenization, and sun exposure was stable to solar activation, this herd afforded the opportunity to study the relation of non-pasture or dry feed on the solar-activated flavor of the milk produced. The results of this phase of the study are given in table 2. The data indicate that milk from cows on dry feed is more susceptible to the activated flavor than from cows on pasture. Milk from the Holstein breed on pasture, reported above, exhibited a non-susceptible tendency to solar activation in 36 percent of the milks studied. However, all the milk studied from cows not on pasture feeding showed a susceptibility to solar activation.

During the course of this study an off-flavor developed in a few solar-exposed samples which was not typical of the true solar-activated flavor. It is noted by symbol Z in table 1. This atypical flavor is best described as "unclean," "nauseating," and as of a decomposed-protein nature suggesting that of milk sometimes noted from cows having a serious

physiological disturbance. This off-flavor has been noted previously, in samples brought to the laboratory for flavor examination, but heretofore was not associated with solar activation. The off-flavor is so distinct and different from the easily recognized true sunshine flavor that no suggestion is forthcoming that it is light induced. It is not improbable that some of these off-flavors in milk such as encountered in this study and which have heretofore given rise to consumer complaints are due to exposure to daylight.

Influence of time of exposure and period of storage on solar activation. The time of exposure and length of storage seem to have some effect on the intensity of the activated flavor. A 30-minute exposure to solar radiation in most cases resulted in a more intensified flavor development than did a 60-minute exposure. This may indicate a partial decomposition of the flavor component. From the data obtained and presented in tables 1 and 2 the intensity of flavor development after 24 hours appears to be very slight. However, some inconsistencies were noted. This would seem to indicate a maximum reaction rate is reached within the first 24 hours after exposure.

Relation of percentage fat. There seemed to be no correlation between the fat percentage and the susceptibility of the milk to the development of the activated flavor (tables 1 and 2). Activated flavors were noted both in low-and in high-fat milk.

TABLE 1.

Susceptibility Of Milk From Cows On Pasture To Solar
Activation When Homogenized, By Breeds

			Activated flavor* at								
		Stage of	24 hours when exposed for			48 hours when exposed for					
Cow	Trial	Fat				0 min.	30 min.	60 min.	0 min.	30 min.	60 min.
(no.)	(no.)	(%)	(mo.)	AYRSHIRE							
225	1	4.7	2	+++	+	-	+++	++	++	++	
	2			++	++	-	++	++	++	++	
	3			++	++	-	++	++	++	++	
228	1	2.9	2	+	+	-	++	++	++	++	
	2			++	+	-	++	++	++	++	
	3			+	+	-	++	++	++	++	
221	1	3.5	3	-	-	-	-	-	-	-	
	2			-	-	-	-	-	-	-	
	3			-	-	-	-	-	-	-	
222	1	3.9	6	-	-	-	-	-	-	-	
	2			-	-	-	-	-	-	-	
	3			-	-	-	-	-	-	-	
234	1	3.7	6	Z	Z	-	Z	Z	Z	Z	
	2			Z	Z	-	Z	Z	Z	Z	
	3			Z	Z	-	Z	Z	Z	Z	
232	1	4.0	7	Z	Z	-	Z	Z	Z	Z	
	2			Z	Z	-	Z	Z	Z	Z	
	3			Z	Z	-	Z	Z	Z	Z	

TABLE 1 (continued)

233	1	5.6	7	-	-	-	-	++	++	++
	2			-	+	-	-	++	++	++
	3			-	+	+	-	++	++	++
212	1	6.5	11	-	-	-	-	-	-	-
	2			-	-	-	-	-	-	-
	3			-	-	-	-	-	-	-
230	1	4.0	11	-	+++	+	-	+++	+++	+++
	2			-	+	+	-	++	++	++
	3			-	++	++	-	+++	+++	+++
BROWN SWISS										
327	1	4.2	2	-	+++	+	-	+++	+	+
	2			-	++	++	-	++	++	++
	3			-	++	++	-	++	++	++
353	1	4.7	2	-	++	+	-	++	++	++
	2			-	++	+++	-	+++	+++	+++
	3			-	++	+++	-	+++	+++	+++
359	1	3.8	2	-	+++	+++	-	+++	+++	+++
	2			-	+++	+++	-	+++	+++	+++
	3			-	+++	+++	-	+++	+++	+++
366	1	4.3	3	-	Z	Z	-	Z	Z	Z
	2			-	Z	Z	-	Z	Z	Z
	3			-	Z	Z	-	Z	Z	Z
331	1	3.8	4	-	++	+	-	++	+	+
	2			-	++	++	-	++	++	++
	3			-	++	++	-	++	++	++

TABLE 1 (continued)

459	1	3.4	3	-	+	+	-	+	+
	2			-	+++	+	-	++	+
	3			-	+	+	-	+	+
517	1	3.9	6	-	++	+	-	++	+
	2			-	+++	++	-	+++	++
	3			-	++	+	-	++	+
446	1	3.8	7	-	-	-	-	-	-
	2			-	-	-	-	-	-
	3			-	-	-	-	-	-
467	1	2.9	8	-	++	++	-	++	++
	2			-	++	+	-	+++	++
	3			-	++	+	-	++	++

JERSEY									
176	1	5.5	1	-	++	+	-	+++	++
	2			-	+	+	-	+	++
	3			-	+	+	-	++	++
192	1	5.1	2	-	-	-	-	-	-
	2			-	-	-	-	-	-
	3			-	-	-	-	-	-
184	1	4.6	3	-	+++	++	-	+++	++
	2			-	+	+	-	++	++
	3			-	++	++	-	++	++
172	1	5.8	6	-	-	-	-	-	-
	2			-	-	-	-	-	-
	3			-	-	-	-	-	-

*KEY: - No activated flavor +++ Pronounced activated flavor
 + Slight activated flavor Z Peculiar, unclear, nauseating flavor
 ++ Distinct activated flavor

TABLE 2

Cows Not On Pasture To Solar Activation
When Homogenized (Holstein Breed)

Cow	Trial	Fat (%)	Stage of lactation (mo.)	Activated flavor* at					
				0 min.	30 min.	60 min.	0 min.	30 min.	60 min.
(no.)	(no.)	(%)	(mo.)						
A55	1	3.0	21 days	-	+++	++	-	++	+++
	2			-	++	++	-	++	++
A73	1	3.2	1		+++	+++		+++	+++
	2				+++	+++		+++	+++
A80	1	3.4	1		+++	++		+++	+++
	2				+++	+++		+++	+++
A60	1	3.2	2		++	++		++	++
	2				+	++		+	++
A62	1	3.0	2		+	+		+++	+
	2				+	+		++	+
A66	1	3.4	2		+++	+		+++	++
	2				++	++		++	++
A72	1	3.2	2		++	++		++	++
	2				++	++		++	++
A76	1	3.4	2		++	++		+++	++
	2				++	+++		++	+++

TABLE 2 (continued)

A61	1 2	3.6	2	- -	++ +	++ +	++ +	- -	++ +	++ +	++ +
A26	1 2	3.4	3	- -	++ +	++ +	++ +	- -	++ +	++ +	++ +
A33	1 2	3.5	4	- -	++ +	++ +	++ +	- -	++ +	++ +	++ +
A53	1 2	3.0	4	- -	++ +	++ +	++ +	- -	++ +	++ +	++ +
A99	1 2	3.7	5	- -	++ +	++ +	++ +	- -	++ +	++ +	++ +
A27	1 2	3.3	6	- -	++ +	++ +	++ +	- -	++ +	++ +	++ +
A46	1 2	3.5	6	- -	++ +	++ +	++ +	- -	++ +	++ +	++ +
A61	1 2	3.5	7	- -	++ +	++ +	++ +	- -	++ +	++ +	++ +
A12	1 2	3.8	8	- -	++ +	++ +	++ +	- -	++ +	++ +	++ +
A42	1 2	3.7	9	- -	++ +	++ +	++ +	- -	++ +	++ +	++ +

TABLE 2 (continued)

A48	1	3.9	9	-	++	++	-	+++	+++
	2			-	+	++	-	+	++
A40	1	3.8	11	-	++	+	-	++	++
	2			-	++	+	-	++	+

*KEY: - No activated flavor
 + Slight Activated flavor
 ++ Distinct activated flavor
 +++ Pronounced activated flavor

II. The Role of Oxidation and the Effectiveness of Certain Treatments on the Solar-activated Flavor of Homogenized Milk

The mixed milk used in this study was pasteurized and homogenized as outlined under procedure. Antioxidants used and/or antioxidative treatments included: (a) Ascorbic acid, (b) nordihydroquaiaretic acid, (c) alpha tocopherol, (d) hydroquinone, (e) hydrogen peroxide and (f) high-temperature heat treatment. The antioxidants studied were added to the milk following homogenization. The samples were then exposed for 30-, 60-, and 120-minute intervals after which they were stored at 40° F. for 48 hours. Organoleptic determinations for flavor were made at 24- and at 48-hours storage. The nitroprusside test used in these studies was carried out in accordance with the method of Josephson and Doan (1939).

Ascorbic acid. The results obtained in 10 trials on the effect of various levels of ascorbic acid in preventing the solar-activated flavor in homogenized milk are shown in table 3. The organoleptic determinations for flavor made after 24 hours are not included in the table since the flavor determinations at 48-hours storage were representative of the results obtained at 24-hours storage.

In general, ascorbic acid at the concentrations used had no preventive effect on the solar-activated flavor. The mechanism for the oxidation of ascorbic acid by solar radiation appears to occur in such a manner as to accelerate the oxidation of the milk constituent responsible for the solar-activated flavor. This mode of action seems to be in direct contrast to the mechanism postulated for the copper-induced oxidized flavor, in which the ascorbic acid retards or prevents the oxidized flavor development, the ascorbic acid being oxidized preferentially by the copper present.

Nordihydroguaiaretic acid (N.D.G.A.). Since ascorbic acid exhibits a synergistic rather than a true antioxidative action, a series of trials were conducted to determine the action of nordihydroguaiaretic acid, a true antioxidant, both with and without added ascorbic acid, on solar radiation.

The results obtained in 10 trials on the protective action of nordihydroguaiaretic acid alone toward solar activation are summarized in table 4. The data show that the addition of 25 mg/l N.D.G.A. was sufficient to prevent the development of the solar-activated flavor after 30- and 60-minute exposures, and in eight of the 10 trials after 120-minute exposure. Addition of $12\frac{1}{2}$ mg/l of N.D.G.A. exhibited a protective action in seven of the 10 trials after a 30-minute exposure, but had no protective action after 60- and 120-minute exposures to solar radiation. The addition of 75 mg/l N.D.G.A. exhibited a bitter taste in the milk in all trials and exposures. These results indicate that (a) the solar-activated flavor can be retarded by the addition of 25 mg/l of N.D.G.A. and (b) that the mechanism for the development of the solar-activated flavor is oxidative in nature.

Nordihydroguaiaretic acid in combination with ascorbic acid. Since ascorbic acid is a synergist capable of donating its hydrogen ions to regenerate an antioxidant, a series of trials were conducted to study the effect of nordihydroguaiaretic acid in combination with ascorbic acid.

The results obtained in 10 trials on the inhibitory action of N.D.G.A. and ascorbic acid in combination are given in table 5. These results demonstrate that when N.D.G.A. is added in combination with ascorbic acid the threshold level of N.D.G.A. of 25 mg/l (table 4) must be increased to

secure protective action against the solar-activated flavor. In these trials, 75 mg/l of N.D.G.A. in the presence of 35 mg/l of ascorbic acid, protected the milk against solar activation. This quantity is three times the threshold value of N.D.G.A. when used alone. The ascorbic acid appears to reduce the efficiency of N.D.G.A. at all exposure times. The bitter flavor noted when 75 mg/l N.D.G.A. alone were added (table 4) did not appear when 35 mg/l ascorbic acid were used in combination.

Alpha tocopherol and hydroquinone. Alpha tocopherol and hydroquinone have long been accepted as antioxidants in the food industry. Since the solar-activated flavor of homogenized milk appears oxidative in nature, knowledge of the specific inhibitory effect of these antioxidants seemed desirable.

The results obtained in 10 trials on the protective action of alpha tocopherol and hydroquinone are given in table 6. The data show that, in general, hydroquinone in concentration of 0.0005 (five p.p.m.) percent was more effective in preventing the solar-activated flavor than was alpha tocopherol, at 50 mg/l. However, complete retardation of the solar-activated flavor was not exhibited when alpha tocopherol and hydroquinone were added in combination; also, no beneficial action not shown by hydroquinone itself, was exhibited.

Hydrogen peroxide. Krukovsky and Guthrie (1945, 1946) reported that ascorbic acid was a key factor in the development of the copper-induced oxidized flavor on non-homogenized milk and that complete, rapid destruction of ascorbic acid resulted in a retardation of the oxidized flavor. From our studies (tables 3 and 5) it was shown that added ascorbic acid, either alone or in combination with N.D.G.A., had no beneficial effect

in inhibiting the solar-activated flavor of homogenized milk. It was deemed advisable, therefore, to determine if complete, rapid destruction of ascorbic acid would prevent the solar-activated flavor in the homogenized product. Consequently, 0.028 ml. of 30 percent H_2O_2 was added to milk prior to pasteurization and homogenization to oxidize the naturally-occurring vitamin C. Ascorbic acid determinations were made 30 minutes after the addition of the H_2O_2 to check the completeness of this oxidation.

The results comprising seven trials on the effect of adding H_2O_2 before pasteurization and homogenization on the solar-activated flavor are presented in table 7. The data show that 0.028 ml. of 30 percent H_2O_2 added previous to pasteurization and homogenization prevented the solar-activated flavor in all trials at 30- and at 60-minute exposures. However, the milk thus treated and exposed developed an old milk-powder or rubber-like flavor. When 25 mg/l of ascorbic acid was added after the H_2O_2 treated milk was pasteurized and homogenized, the solar-activated flavor developed in five of the seven trials at 60-minutes exposure. By the addition of 2 mg/l of riboflavin along with 25 mg/l of ascorbic acid to the hydrogen peroxide-treated milk the solar-activated flavor could be further intensified. In this respect Reed (1949) reported as follows:

Riboflavin absorbs light of the visible portion of the spectrum, and this light promotes the oxidative breakdown of riboflavin and of other reducing substances, such as ascorbic acid. Light of 436 milli-microns wave-length accelerates this oxidation in the presence of riboflavin. The most rapid oxidation takes place at pH 6.5 which is approximately the pH of fresh milk.

High-temperature heat treatment. Gould and Sommer (1939) reported that the production of reducing substances in milk at high temperatures

is capable of preventing the copper-induced oxidized flavor. To acquire information on the effect of high-temperature heat treatment (176° F. for five minutes) in preventing the solar-activated flavor of homogenized milk, a series of trials were conducted.

The results, representing 10 trials, on the influence of high-temperature heat treatment on stabilizing the milk against solar activation are given in table 8. Strikingly, the pronounced typical cooked odor found in milk after heating to 176° F. for five minutes was noticeably lacking after 60-minutes exposure to solar radiation. After 24-hours storage the cooked flavor was replaced by the activated flavor, showing that the reducing compounds were not present, perhaps in sufficient quantities to inhibit the solar activation or else they were readily oxidized. Thus, the protective action of the high-temperature treatment against copper-induced oxidized flavor of non-homogenized milk was not exhibited when homogenized milk was similarly treated and exposed to solar radiation. The addition of 25 mg/l ascorbic acid did not alter these results materially. On the other hand, the addition both of ascorbic acid and of riboflavin increased the incidence of the solar-activated flavor. These results would seem to indicate that the reducing substances produced by high-temperature heat treatment resulting in the cooked flavor are oxidized quite rapidly by solar radiation.

Cysteine hydrochloride. Since the cooked flavor in high heat-treated homogenized milk was dissipated after exposure to solar radiation, a study was conducted to see if, in the absence of the cooked flavor dissipated by solar radiation, a nitroprusside test would yield more exacting

information. To this end the cooked flavor in milk was produced by the addition of from 20 to 40 mg/l of cysteine hydrochloride, a recognized sulphhydryl containing compound.

The results representing three trials are presented in table 9. Here it is clearly shown that all the samples before exposure gave a positive nitroprusside test while after 30-, 60-, and/or 120-minutes exposures a positive test could not be obtained. This would seem to substantiate the observations that the cooked flavor components are oxidized by solar radiation.

TABLE 3

Influence of Ascorbic Acid on the Development of the
Solar-activated Flavor in Homogenized Milk

Trial (no.)	Expo- sure (min.)	Activated flavor* in exposed homogenized milk when various amounts (mg. / l.) of ascorbic acid were added:						
		Control	0	35	50	75	100	200
1	0	-						
	30		+	-	+	+	+	+
	60		+	?	?	?	?	?
	120		+	+	+	+	+	+
2	0	-						
	30		+	+	+	+	+	+
	60		+	+	+	+	+	+
	120		+	+	+	+	+	+
3	0	-						
	30		+	-	+	+	+	+
	60		+	+	+	+	+	+
	120		+	+	+	+	+	+
4	0	-						
	30		+	+	+	+	-	+
	60		+	+	+	+	+	+
	120		+	+	+	+	+	+
5	0	-						
	30		+	?	?	+	+	+
	60		+	+	+	+	+	+
	120		+	+	+	+	+	+
6	0	-						
	30		+	?	+	+	+	+
	60		+	+	+	+	+	+
	120		+	+	+	+	+	+
7	0	-						
	30		+	+	+	+	+	+
	60		+	+	+	+	+	?
	120		+	+	+	+	+	+
8	0	-						
	30		+	+	-	?	?	+
	60		+	+	+	+	+	+
	120		+	+	+	+	+	+

TABLE 3 (continued)

9	0	-						
	30		+	+	?	+	+	+
	60		+	?	?	+	+	+
	120		+	+	+	+	+	+
10	0	-						
	30		+	+	+	+	+	+
	60		+	+	+	+	+	+
	120		+	+	+	+	+	+

+KEY: - No activated flavor
 ? Questionable activated flavor
 + Activated flavor

TABLE 4

Influence of Nordihydroguaiaretic Acid in the Absence
Of Added Ascorbic Acid on the Development of the
Solar-activated Flavor in Homogenized Milk

Trial (no.)	Expo- sure (min.)	Activated flavor* in exposed homogenized milk when various amounts (mg. / liter) of N.D.G.A. were added:				
		Control	0	12.5	25	75
1	0	-				
	30		+	-	-	Bitter
	60		+	+	-	Bitter
	120		+	+	-	Bitter
2	0	-				
	30		+	-	-	Bitter
	60		+	+	-	Bitter
	120		+	+	-	Bitter
3	0	-				
	30		+	+	-	Bitter
	60		+	+	-	Bitter
	120		+	+	-	Bitter
4	0	-				
	30		+	+	-	Bitter
	60		+	+	-	Bitter
	120		+	+	-	Bitter
5	0	-				
	30		+	-	-	Bitter
	60		+	?	-	Bitter
	120		+	+	?	Bitter
6	0	-				
	30		+	+	-	Bitter
	60		+	+	-	Bitter
	120		+	+	-	Bitter
7	0	-				
	30		+	-	-	Bitter
	60		+	+	-	Bitter
	120		+	+	-	Bitter
8	0	-				
	30		+	-	-	Bitter
	60		+	?	-	Bitter
	120		+	+	?	Bitter

TABLE 4 (continued)

9	0	-					
	30		+	-	-	Bitter	
	60		+	+	-	Bitter	
	120		+	+	-	Bitter	
10	0	-					
	30		+	-	-	Bitter	
	60		+	+	-	Bitter	
	120		+	+	-	Bitter	
<hr/>							
*KEY:	-	No activated flavor					
	?	Questionable activated flavor					
	+	Activated flavor					

TABLE 5

Influence of Nordihydroguaiaretic Acid in the Presence of
35 mg. / Liter of Added Ascorbic Acid on the Development
of the Solar-activated flavor in Homogenized Milk

Trial (no.)	Expo- sure (min.)	Activated flavor* in exposed homogenized milk when various amounts (mg. / liter) of N.D.G.A. were added:				
		Control	0	12.5	25	75
1	0	-				
	30		+	+	+	-
	60		+	+	+	-
	120		+	+	+	-
2	0	-				
	30		+	+	+	?
	60		+	?	+	-
	120		+	+	+	-
3	0	-				
	30		+	+	+	-
	60		+	+	-	-
	120		+	+	+	-
4	0	-				
	30		+	?	?	-
	60		+	+	+	-
	120		+	+	+	-
5	0	-				
	30		+	+	?	-
	60		+	+	+	-
	120		+	+	+	-
6	0	-				
	30		+	?	+	-
	60		+	+	+	-
	120		+	+	+	?
7	0	-				
	30		+	+	+	-
	60		+	+	+	-
	120		+	+	+	-
8	0	-				
	30		+	+	?	-
	60		+	+	?	-
	120		+	+	+	-

TABLE 5 (continued)

9	0	-				
	30		+	?	+	-
	60		+	+	?	?
	120		+	+	+	?
10	0	-				
	30		+	+	+	-
	60		+	+	+	-
	120		+	+	+	-

*KEY: - No activated flavor
 ? Questionable activated flavor
 + Activated flavor

TABLE 6

The Influence of Alpha Tocopherol and Hydroquinone on the Development of the Solar-activated Flavor in Homogenized Milk

Trial	Exposure	Activated flavor* in exposed homogenized milk when alpha tocopherol and hydroquinone were added to homogenized milk					
		Control (not exposed)	Alpha tocopherol		Hydroquinone		Alpha tocopherol plus hydroquinone
			0	50	0	0.0005	50 (mg. / l.) + 0.0005
(no.)	(min.)		(mg. / l.)	(%)	(mg./l.)		(%)
1	0	-					
	30		+	+	+	-	-
	60		+	+	+	-	-
2	0	-					
	30		+	+	+	?	-
	60		+	+	+	+	-
3	0	-					
	30		+	+	+	-	-
	60		+	+	+	-	-
4	0	-					
	30		+	?	+	-	-
	60		+	+	+	-	-
5	0	-					
	30		+	+	+	+	?
	60		+	+	+	+	+
6	0	-					
	30		+	?	+	-	-
	60		+	?	+	+	-
7	0	-					
	30		+	?	+	-	-
	60		+	+	+	?	-
8	0	-					
	30		+	+	+	-	?
	60		+	?	+	-	+

TABLE 6 (continued)

9	0	-					
	30		+	-	+	-	?
	60		+	?	+	-	+
10	0	-					
	30		+	?	+	-	-
	60		+	?	+	-	?

*KEY: - No activated flavor
 ? Questionable activated flavor
 + Activated flavor

TABLE 7

The Effect of Treating Homogenized Milk with Hydrogen Peroxide
and the Subsequent Addition of Ascorbic Acid and Riboflavin
on the Development of the Solar-activated Flavor

Series (no.)	Treatment	Exposure (min.)	Activated flavor* at 24 hours in trial						
			1	2	3	4	5	6	7
I	Addition of 0.028 ml. of 30% H ₂ O ₂ per liter to milk prior to pas- teurization and homo- genization	0 30 60	- * *	- * *	- * *	- * *	- * *	- * *	- * *
II	Addition of 25 mg./ l. ascorbic acid to perox- ide-treated milk.	0 30 60	- ? +	- + +	- + +	- ? +	- + +	- + +	- + +
III	Addition of 25 mg./ l. ascorbic acid and 2 mg./l. riboflavin to peroxide-treated milk.	0 30 60	- ++ +++	- ++ +++	- +++ ++	- ++ +++	- ++ ++	- ++ ++	- ++ ++

*KEY: - No activated flavor
 ? Questionable activated flavor
 * Powder milk-rubber like flavor
 + Slight activated flavor
 ++ Distinct activated flavor
 +++ Pronounced activated flavor

TABLE 8

The Effect of High Temperature Treatment (176° F.--5 min.) and the Subsequent Addition of Ascorbic Acid and Riboflavin on the Development of the Solar-activated Flavor in Homogenized Milk

Trial (no.)	Expo- sure (min.)	Activated flavor* when milk is pasteurized by high temperature treatment on the subsequent addition of ascorbic acid (25 mg./l) and riboflavin (2 mg./l)			
		Control	0	Ascorbic acid added	Ascorbic acid and riboflavin added
1	0	cooked			
	60		?	+	+
2	0	cooked			
	60		+	+	+
3	0	cooked			
	60		+	+	+
4	0	cooked			
	60		+	+	+
5	0	cooked			
	60		?	?	+
6	0	cooked			
	60		-	+	+
7	0	cooked			
	60		+	+	+
8	0	cooked			
	60		+	?	+
9	0	cooked			
	60		+	?	+
10	0	cooked			
	60		-	?	+
*KEY:		-	No activated flavor		
		?	Questionable activated flavor		
		+	Activated flavor		

TABLE 9

The Effect of Solar Radiation on the Sulfhydryl Group of Cysteine Hydrochloride as Measured by the Nitroprusside Test and Organoleptic Flavor Determinations

Trial (no.)	Expo- sure (min.)	Activated* flavor when cysteine HCl (mg./l.) is added to homogenized milk		Nitroprusside test**	
		20	40	before exposure	before exposure
1	0	cooked	cooked	+	
	30	+	-	+	-
	60	+	+	+	-
	120	+	+	+	-
2	0	cooked	cooked	+	
	30	+	?	+	-
	60	+	+	+	-
	120	+	+	+	-
3	0	cooked	cooked	+	
	30	?	?	+	-
	60	+	+	+	-
	120	+	+	+	-

*KEY: - No activated flavor
? Questionable activated flavor
+ Activated flavor

**KEY: - Negative nitroprusside test
+ Positive nitroprusside test

III. Effect of Deaeration, Surface Area of Fat Globules and Relation of the Kreis Test

The milk used in this study was pasteurized, homogenized and cooled as outlined under procedure. In the deaeration studies, the ice-cooled, 50° F. milk was divided into three lots: Lot I was used as a control; Lot II was not deaerated but was exposed to solar radiation for one hour, then stored at 40° F. for 48 hours; and Lot III was deaerated in a 500 ml. flask under partial vacuum, then similarly exposed and stored.

The milk used in the surface-area studies was mixed, pasteurized non-homogenized milk obtained from the College Creamery. The milk was divided into three lots: Lot I served as a control; Lot II was exposed to the sun for 60 minutes; while Lot III was similarly exposed then homogenized after exposure at 2,500 pounds pressure. All samples were stored at 40° F. for 24 hours and then examined for the activated flavor.

The homogenized milk used in the Kreis test was divided into five lots: Lot I served as a control; Lot II was exposed to solar radiation; Lots III and IV were treated with 35 and 50 mg./l of ascorbic acid respectively; while Lots V and VI were treated with 12.5 and 25 mg./l of nordihydroquaiarectic acid (N.D.G.A.) respectively. The milk to be exposed to solar radiation was further divided into three lots which were exposed for 30, 60 and 120 minutes respectively. After 24 hours storage at 40° F. the samples were examined organoleptically for the off-flavor and chemically, using the Kreis test, to note oxidation.

Deaeration. The results, representing five trials, obtained on the effect of deaeration on the solar-activated flavor in homogenized milk are shown in table 10. The data show that the solar-activated flavor did not develop when the milk was deaerated and then exposed to solar radiation.

However, when air was incorporated into the deaerated and exposed milk and then reexposed to solar radiation for 60 minutes, the milk developed the typical solar-activated flavor. The exposed milk not deaerated exhibited the typical solar-activated flavor, while that serving as a control was indistinguishable from that deaerated and exposed. These results would seem to substantiate the observations made under section two to the effect that the development of the solar-activated flavor in homogenized milk results from an oxidative process.

Increase in fat globule-surface area. Although many observations have been made showing a greater intensity of activated flavor development in homogenized than in nonhomogenized milk similarly exposed to daylight, no data were noted showing the potential activated flavor development of exposed milk when the surface area of the fat globules was increased by homogenization subsequent to exposure. Such a study would seem to show whether surface area of the fat globules was a factor in the development of the off-flavor.

The results, representing five trials, are presented in table 11. The data show that the nonhomogenized milk exposed to the sun (Lot two) did not develop an activated flavor in two of the five trials and only a slight off-flavor in the remaining three trials. On the other hand, portions of the same milk homogenized immediately following exposure (Lot three) and then stored developed a very strong activated flavor in all five trials. These results would seem to indicate that total surface area of the fat globules may be an important factor in the development of the solar-activated flavor.

Relation of the Kreis test. Since the solar-activated flavor has been shown to be oxidative in nature, a clue in regard to the constituent oxidized was sought by the use of the Kreis test. The chief phospholipid of milk, lecithin, is postulated to be the constituent oxidized when the copper-induced oxidized flavor develops, and further that the milk fat itself becomes oxidized during this process, following its induction period. The Kreis test denotes oxidative rancidity with the formation of color due to the presence of epihydrin aldehyde. Although the absence of color formation in itself is not definite proof of the absence of oxidative rancidity, the Kreis test remains a very good presumptive test. To this end studies were made.

The results on the relation of the Kreis test to the development of the solar-activated flavor in homogenized milk, representing 10 trials, are given in table 12. The data indicate that a positive Kreis test did not develop in any milk not exposed to solar radiation. A comparison of the flavor and the Kreis test examinations seems to indicate a trend between a positive organoleptic taste sensation and a positive Kreis test. The failure of a positive Kreis test to correspond with a positive solar-activated flavor in all cases does not, of course, preclude the possibility of a "lipid-fraction" oxidation, but it does, however, leave the way clear for further studies on the isolation and characterization of the constituent affected when a solar-activated flavor develops.

TABLE 10

The Effect of Deaeration on the Development of the Solar-activated Flavor in Homogenized Milk

Lot (no.)	Treatment of milk	Trial (no.)	Activated flavor* at	
			24 hours	48 hours
1	Control - not deaerated or exposed	1	-	-
		2	-	-
		3	-	-
		4	-	-
		5	-	-
2	Not deaerated - exposed to solar radiation for 60 min.	1	+++	+++
		2	+++	+++
		3	++	+++
		4	+++	+++
		5	+++	+++
3	Deaerated and exposed to solar radiation for 60 min.	1	-	-
		2	-	-
		3	-	-
		4	-	-
		5	-	-
4	Deaerated and exposed to solar radiation for 60 min. then treated so as to incorporate air and reexpose for 60 min.	1	+++	+++
		2	+++	+++
		3	+++	+++
		4	+++	+++
		5	+++	+++

*KEY: - No activated flavor
 ++ Distinct activated flavor
 +++ Pronounced activated flavor

TABLE 11

The Effect of Surface Area of the Fat Globule as Influenced by Homogenization, on the Development of the Solar-activated Flavor

Lot (no.)	Treatment of milk	Trial (no.)	Activated flavor* at 24 hours
1	Control - not exposed	1	-
		2	-
		3	-
		4	-
		5	-
2	Exposed to solar radiation for 60 min.- not homogenized (Normal surface area)	1	-
		2	+
		3	+
		4	-
		5	+
3	Exposed to solar radiation for 60 min. and then homogen- ized (Increased surface area after exposure)	1	+++
		2	+++
		3	++
		4	+++
		5	+++
*KEY: - No activated flavor			
+ Slight activated flavor			
++ Distinct activated flavor			
+++ Pronounced activated flavor			

TABLE 12
The Relation of the Kreis Test to the Development of the Solar-activated Flavor in Homogenized Milk

Trial	Ex- po- sure	Activated flavor* and reaction to the Kreis test** when various levels (mg./l.) of ascorbic acid and N.D.G.A. were added:											
		Ascorbic acid						N.D.G.A.					
		Control	0	35	50	0	12.5	25					
(no.)	(min.)	Acti- vated fla- vor	Kreis test	Acti- vated fla- vor	Kreis test	Acti- vated fla- vor	Kreis test	Acti- vated fla- vor	Kreis test	Acti- vated fla- vor	Kreis test	Acti- vated fla- vor	
1	0	-	-	-	-	-	-	-	-	-	-	-	
	30	+	+	-	+	+	+	+	+	-	-	-	
	60	+	?	?	?	+	?	+	+	-	-	-	
	120	+	+	+	+	+	+	+	+	+	-	-	
2	0	-	-	-	-	-	-	-	-	-	-	-	
	30	+	+	+	+	+	+	+	+	-	-	-	
	60	+	+	+	+	+	+	+	+	-	-	-	
	120	+	+	+	+	+	+	+	+	-	-	?	
3	0	-	-	-	-	-	-	-	-	-	-	-	
	30	+	+	-	+	+	?	+	+	+	+	-	
	60	+	+	+	+	+	+	+	+	-	-	?	
	120	+	+	+	+	+	+	+	+	+	-	-	
4	0	-	-	-	-	-	-	-	-	-	-	-	
	30	+	+	+	+	+	+	+	+	+	+	-	
	60	+	+	+	+	+	+	+	+	+	+	?	
	120	+	+	+	+	+	+	+	+	+	+	+	

IV. Isolation and Characterization of a Whey Constituent Capable of Producing the Solar-activated Flavor

Since previous studies using the Kreis test (section III) demonstrated that the activated constituent was probably not lipid in nature and further since the observations of many investigators have demonstrated that solar radiation does have destructive effects upon proteins and is capable of producing unpleasant odors and flavors, a whey constituent was isolated, as detailed under procedure, to ascertain the possibility of its susceptibility to off-flavors through solar radiation. Approximately 200 mg. of the freeze-dried material was dispersed in 400 ml. of distilled water and divided into two lots. Lot I was not exposed to solar radiation but Lot II was exposed for one hour. Both of the samples were stored for 24 hours at 40° F., after which time they were examined organoleptically for the activated flavor.

The data obtained from the solar radiation of aquasols prepared from five different isolations are given in table 13. The results show that aquasols prepared from the freeze-dried powder (hereafter referred to as a minor-protein fraction) were readily photosensitized to impart the typical solar-activated flavor which is characteristic of homogenized milk treated in the same manner.

Characterization of the compound. The lyophilized minor-protein fraction is a very light, fluffy, white crystalline powder having a distinct sheen. Its crystalline appearance may have resulted from the method of drying. It has a bland taste and is devoid of odor. The compound has surface-active properties, is water dispersible and, in this respect, is similar to Aschaffenburg's (1946) sigma-protease.

Aquasols made from the minor-protein fraction have the following qualitative characteristics: positive biuret, Millon, xanthroproteic and Molisch reactions. The aquasols have an isoelectric zone between pH 4.1-4.3.

Standard microchemical methods were used to determine carbon, hydrogen, nitrogen, phosphorus, sulfur and ash in the anhydrous sample. The factor of 6.38 was used to convert nitrogen to protein. The elementary composition of the anhydrous minor-protein fraction is presented in table 14. The carbon, hydrogen, nitrogen, phosphorus, sulfur and ash content of Aschaffenburg's (1946) sigma-protease, and casein (1918b), lactalbumin (1918a), beta-lactoglobulin (1945), pseudoglobulin and euglobulin (1946) are included for comparative purposes. An examination of these data show that the minor-protein fraction is not identical with sigma-protease, casein or any of the characterized whey proteins. The minor-protein fraction contains considerably less carbon and nitrogen and a higher ash content than the other milk proteins. The elementary analysis of the minor-protein fraction shows that the oxygen to nitrogen ratio is approximately 3:1, whereas, the ratio is less than 2:1 in all of the other milk proteins. The relatively high sulfur content of this fraction, plus the positive Molisch reaction, furnishes evidence to substantiate the postulation of Keeney (1947), namely, the substituents necessary to develop the activated-flavor embrace an aldehyde-sulfur reaction. The minor-protein fraction, however, is a different compound from that isolated by Aschaffenburg (1946) as is indicated by his elementary analysis, negative Molisch test and different isoelectric zone (pH 4.5).

Amino acid composition of the minor-protein fraction. The microbiological techniques employed are outlined under procedure. The analytical results obtained from the microbiological analysis of the essential amino acids and cystine and glutamic acid are given in table 15. The amino acid composition of casein, lactalbumin, beta-lactoglobulin, pseudoglobulin and euglobulin are also included for comparison. The data on the minor-protein fraction show the analytical agreement obtained from the analysis of three isolations from different mixed milks. All of the values show good agreement, indicating the reproducibility and homogeneity of separate isolations.

The minor-protein fraction differs markedly from the other milk protein fractions in its amino acid content when all of the values are expressed as grams per 100 g. of anhydrous, ash-free protein. The amount of arginine, glutamic acid, histidine, leucine, phenylalanine and tryptophan is markedly less than that reported for the other identified milk proteins, whereas the amount of threonine and valine is significantly higher than that reported for casein, lactalbumin and beta-lactoglobulin. The cystine content of the minor-protein fraction is more than twice as high as that found in casein but significantly less than that found in the other milk proteins. The isoleucine, lysine and methionine content of the minor-protein fraction is about one-half of that found in casein, lactalbumin and beta-lactoglobulin but nearly equivalent amounts are present in pseudo- and euglobulin. The amount of arginine, cystine, histidine, leucine, phenylalanine, threonine and tryptophan is markedly less than that reported for pseudo- and euglobulin whereas the valine content is approximately the same.

The amounts of cystine, histidine and tryptophan and leucine and lysine are present in about equal concentrations in the minor-protein fraction. The lack of similarity between the amino acid content of the minor-protein fraction and the other recognized milk proteins is evident. There is no information available on the amino acid composition of the other proteins shown to be present in whey by electrophoretic analysis (1946).

Minimum molecular weight of the minor-protein fraction. The minimum molecular weight (\underline{M} min.) can be calculated by the equation:

$$(\underline{M} \text{ min.}) = \frac{\underline{R}_a \times \underline{M}_a \times 100}{(\%) a} \quad (1)$$

\underline{R}_a is the integer representing the number of specific amino acid residues in the molecule, \underline{M}_a is the molecular weight of the amino acid, and $(\%) a$ is the percent of amino acid in 100 g. of the anhydrous, ash-free protein. In the determination of (\underline{M} min.) of the minor-protein fraction, the amino acid present in the smallest concentration (tryptophan) was used to make the adjustment to integers within three percent of the whole number. The figures used for the calculation of (\underline{M} min.) are presented in table 16. (\underline{M} min.) was calculated for each amino acid and for sulfur. The value 70,300 represents the average value obtained from the 12 amino acids and sulfur. The values reported in the literature for (\underline{M} min.) of casein and beta-lactoglobulin are included for comparative purposes. The yield in percent and moles per 10^5 g. of protein are given in columns two and three in table 16. In column four, the molar ratios calculated on the basis of one tryptophan molecule are obtained by dividing by the number of moles of tryptophan (4.26). In column five, these molar ratios are multiplied by three and all figures are rounded out to the nearest integer within

three percent. The only amino acid which deviated more than three percent was methionine (5%). Data are also included in table 16 for the comparison of the literature values of the number of amino acid residues in casein and beta-lactoglobulin.

TABLE 13

The Effect of Solar Radiation on the Development of the
Activated Flavor in Aquasols Prepared from the Minor-protein
Fraction in Whey (after 1-hr. exposure and 24-hr. storage at 40°F.)

Sample (no.)	Activated flavor	
	Lot I (unexposed)	Lot II (exposed)
1	-	+
2	-	+
3	-	+
4	-	+
5	-	+

TABLE 14

Comparison of Analytical Data of the Minor-protein Fraction with that of Sigma-protease, Casein, Lactalbumin, Beta-lactoglobulin, Pseudoglobulin and Euglobulin

Constituent	Minor-protein fraction (%)	Sigma- ¹ protease (%)	Casein ² (%)	Lact- ³ albumin (%)	Beta		Euglob- ⁵ ulin (%)
					Lacto ⁴ globulin (%)	Pseudo- ⁵ globulin (%)	
C	44.2	49.85	53.11	52.51	53.39	51.94	53.95
H	6.35	7.0	7.05	7.10	7.22	6.96	7.12
N	10.00	13.95	15.65	15.43	15.60	15.29	16.05
P	0.27	0.68	0.82	trace	-	-	-
S	1.30	0.72	0.82	1.92	1.60	1.00	1.01
Ash	6.9	3.2	-	-	-	0.45	0.05
O (diff.)	30.98	24.60	22.55	23.04	22.19	24.36	21.82
O:N, ratio	3:1	2:1	2:1	2:1	2:1	2:1	2:1

¹Aschaffenburg (1946)

²Osborne (1918b)

³Osborne (1918a)

⁴Brand (1945)

⁵Smith (1946)

TABLE 15

The Essential Amino Acids, Cystine and Glutamic Acid Content of the Minor-protein fraction, Casein, Lactalbumin, Lactoglobulin, Pseudoglobulin and Euglobulin
(All Amino Acid Values Expressed as Grams per 100 g. of Anhydrous, Ash-free Protein)

Constituent	Minor-protein fraction			Casein (6) ¹	Lactal- ² bumin	Beta- ³		Eu- ⁴
	Isol. 1	Isol. 2	Isol. 3			Lacto- ³ globulin	Pseudo- ⁴ globulin	
Arginine	1.69	1.71	1.71	4.10	3.50	2.88	3.50	4.90
Cystine	0.83	0.88	0.87	0.34	4.10	2.29	3.00	3.20
Glutamic acid	13.54	13.05	13.31	22.40	-	19.50	-	-
Histidine	0.90	0.88	0.89	3.10	2.00	1.58	2.14	1.89
Isoleucine	3.62	3.71	3.64	6.10	-	8.40	3.10	3.10
Leucine	5.53	5.79	5.82	9.20	15.00	15.60	9.10	10.40
Lysine	5.86	5.76	5.59	8.20	9.00	11.40	7.20	6.30
Methionine	1.34	1.34	1.35	2.80	2.80	3.22	1.08	0.98
Phenylalanine	2.30	2.40	2.34	5.00	5.60	3.50	3.80	3.60
Threonine	6.66	6.56	6.47	4.90	5.30	5.80	10.10	10.50
Tryptophan	0.88	0.85	0.87	1.20	2.30	1.94	2.70	2.40
Valine	9.96	9.74	9.52	7.20	4.00	5.80	9.40	10.40
Total nitrogen	15.67	15.67	15.67	15.63	-	15.60	-	-
Nitrogen % *	9.76	10.14	10.07	-	-	-	-	-

¹Gordon (1949)²Hlock and Bolling (1945)³Brand (1945)⁴Smith (1948)

* Percent nitrogen in anhydrous minor-protein fraction.

TABLE 16

The Number of Amino Acid Residues in the Minor-protein Fraction as Compared to Casein and Beta-lactoglobulin

Constituent	Yield %	Minor-protein fraction			Casein ¹	Beta- Lacto- globulin ²
		Moles per 10 ⁵ g. protein	Molar ratio when Tryptophan =			
1	2	3	4	5	6	7
Arginine	1.70	9.80	2.30	7	24	7
Cystine (1/2)	0.86	7.20	1.69	5	3	8
Glutamic acid	13.30	90.40	21.20	64	38	24
Histidine	0.89	5.70	1.34	4	20	4
Isoleucine	3.66	27.90	6.55	20	47	27
Leucine	5.71	43.60	10.20	31	70	50
Lysine	5.74	39.30	9.20	28	56	33
Methionine	1.34	9.00	2.10	6	19	9
Phenylalanine	2.35	14.20	3.30	10	30	9
Threonine	6.56	55.10	12.90	39	41	21
Tryptophan	0.87	4.26	1.00	3	6	4
Valine	9.74	83.30	19.60	59	61	21
Sulfur	1.40	43.80	10.30	31	-	21
(M min.)				70,300	12,800	42,020

¹Gordon (1949)

²Brand (1945)

V. The Electrophoretic Analysis of a Contributing Minor-protein Fraction

It has been reported previously (section IV) that a minor-protein fraction had been isolated from skimmilk, after the major proteins had been removed, which is capable of being photosensitized to produce the typical solar-activated flavor of homogenized milk. Furthermore, the elementary analysis and the percentage composition of the amino acids in the minor-protein fraction differed markedly from the other characterized whey proteins. Since the characterization and properties of this compound indicated that it was not a previously recognized whey protein, it was thought desirable to make an electrophoretic examination of the minor-protein fraction.

Various other investigators, Deutsch (1947), Smith (1946, 1948) and Stanley et al. (1950), have studied whey proteins electrophoretically, but their methods of preparing the whey proteins varied considerably and none was comparable to the method of preparation employed in this study.

Freshly separated skimmilk obtained from the Michigan State College creamery was rennet-coagulated according to a procedure outlined previously under procedure. Figure 1, however, shows graphically the fractionation procedure employed in the isolation of the various whey protein fractions which were examined electrophoretically.

Electrophoretic analysis of the minor-protein fraction. One percent solutions of the minor-protein fraction (fig. 1, fraction III) were prepared by dissolving a definite amount in four different buffer solutions and then dialyzing them to osmotic equilibrium at 5° C. for 24 hours, or until constant conductivity was reached on both sides of the membrane.

The electrophoretic resolution of the minor-protein fraction in the various buffers was then studied at pH ranges from 3.3 to 9.3 with a Perkins-Elmer Tiselius Electrophoresis apparatus at 1.2° C. The electrophoretic patterns of unit magnification of both the ascending and descending boundaries obtained from the electrophoretic examination of the minor-protein fraction are illustrated in figure 2. The horizontal arrows in figures 2 and 3 indicate the direction of migration. The diagrams on the left side of figures 2 and 3 represent ascending boundaries while those on the right represent descending boundaries. The tail of the arrow in both cases signifies the position of the starting boundary.

Electrophoretic analysis of whey proteins and heat-coagulated-whey serum proteins. In an effort to follow the various steps in the isolation of the minor-protein fraction electrophoretically, a series of patterns were obtained from the whey proteins and heat-coagulated-whey serum proteins. These two protein fractions were obtained in the course of the isolation of the minor-protein fraction and were removed and dried from the frozen state under high vacuum (fig. 1, fractions I and II). It should be mentioned that prior to lyophilization a precipitate was formed during the dialysis of the whey against water which was discarded.

Samples of the whey proteins (fig. 1, fraction I) were made up in concentrations of 0.22, 0.24 and 1.0 percent in three different buffer solutions and dialyzed to osmotic equilibrium at 5° C. for at least 24 hours.

The serum protein fraction (fig. 1, fraction II) was made up in one percent concentration in veronal-citrate buffer and dialyzed as indicated above. The veronal-citrate buffer suggested by the work of Stanley et al.

(1950) was used because poor electrophoretic resolution was evident from the use of acetate and phosphate buffers (fig. 3).

The resulting patterns at unit magnification of both the ascending and descending boundaries obtained from the electrophoretic analysis of the whey and heat-coagulated-whey serum proteins are shown in figure 3.

Any screening effect due to opalescence has been eliminated in the photographic reproductions of both figures.

Fig. I FLOW SHEET DIAGRAM FOR FRACTIONATION OF MILK PROTEINS

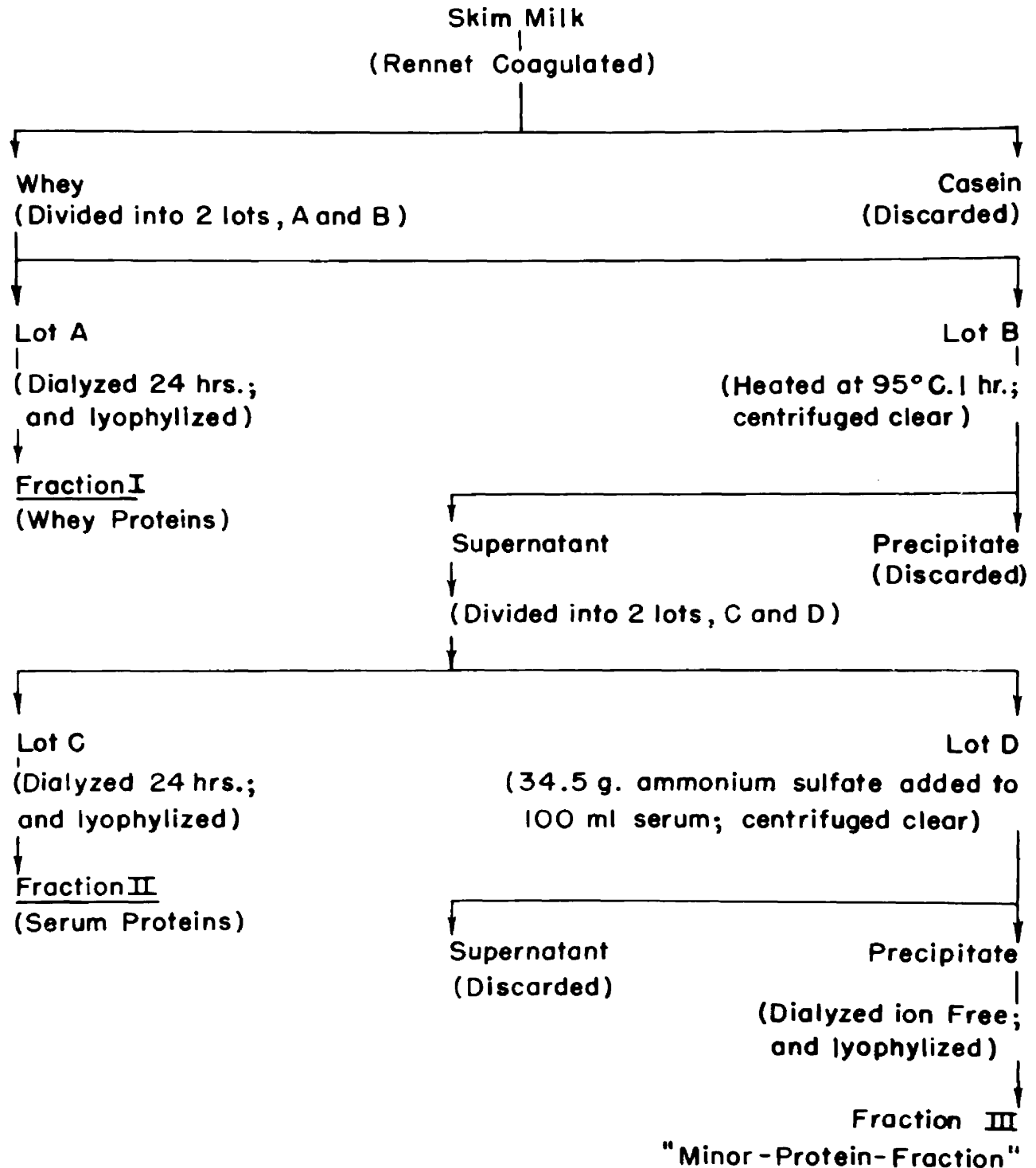


Fig.2 ELECTROPHORETIC PATTERNS FROM 1% SOLUTIONS OF MINOR -PROTEIN-FRACTION AT VARIOUS pH AND BUFFER MEDIA

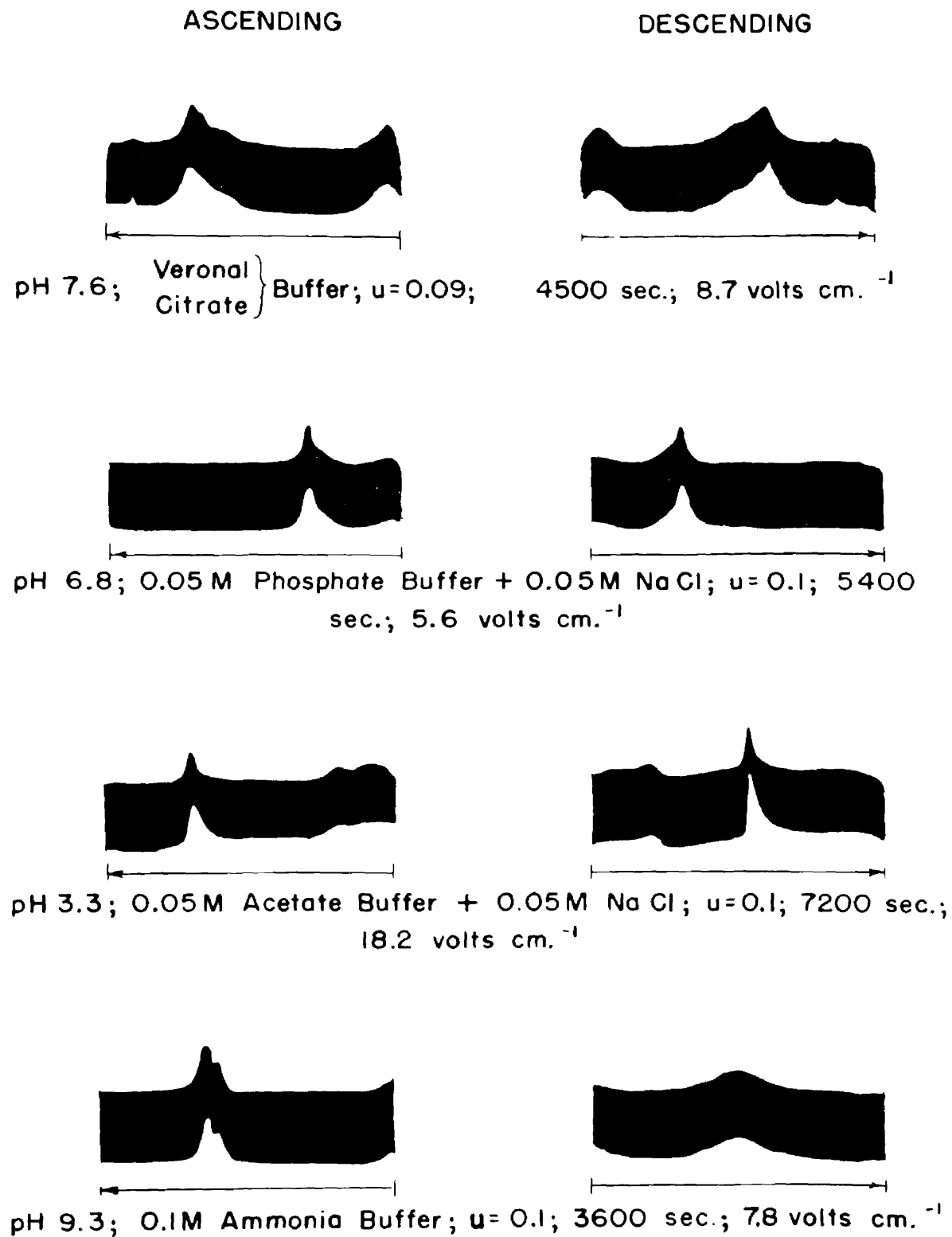
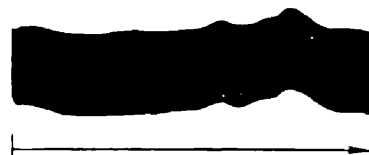
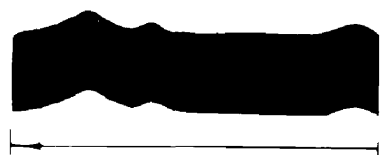


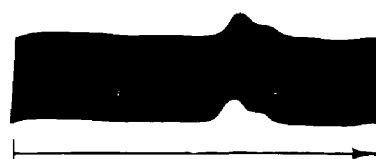
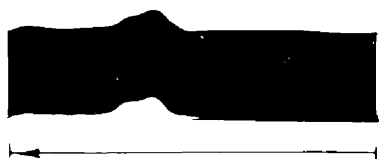
Fig.3 ELECTROPHORETIC PATTERNS OF WHEY AND HEAT COAGULATED WHEY SERUM PROTEINS.

ASCENDING

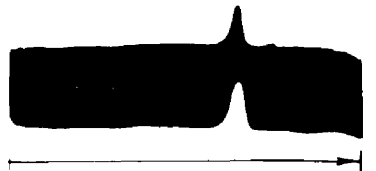
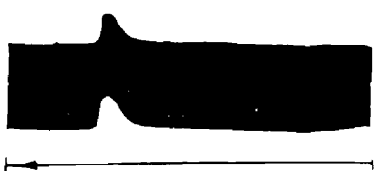
DESCENDING



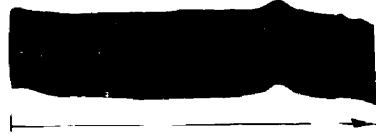
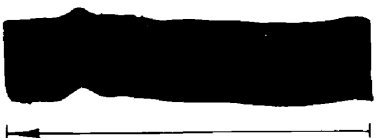
pH 7.6; Veronal - Citrate Buffer; $u=0.09$; 5500 sec.; 9.1 volts cm^{-1} ;
Whey Protein Concentration 1.0%.



pH 6.8; 0.05M Phosphate Buffer + 0.05M NaCl; $u=0.1$; 1800 sec.
8.1 volts cm^{-1} ; Whey Protein Concentration 0.24%.



pH 3.3; 0.05M Acetate Buffer + 0.05M NaCl; $u=0.1$; 5000 sec.;
5.8 volts cm^{-1} ; Whey Protein Concentration 0.22%.



pH 7.6 Veronal - Citrate Buffer; $u=0.09$; 4500 sec.; 9.1 volts cm^{-1} ;
Serum Protein Concentration 1%.

DISCUSSION

Many data are found in the literature relative to the factors responsible for and associated with the copper-induced oxidized flavor. Recorded data, however, relative to those factors associated with the solar-activated flavor such as susceptibility of individual cow's milk, antioxidant treatments, deaeration and surface area of the fat globules were not noted.

The data presented in section I showed that of the 46 cows on pasture feeding whose milk was studied 30 percent, were non-susceptible to the production of the solar-activated flavor after homogenization and exposure to solar radiation. However, all the milk from cows not on pasture feeding showed a susceptibility to solar activation. This observation is similar to that reported by many workers on the copper-induced oxidized flavor. The variations as regards the solar-activated flavor development within individual cows both of the same breed and of different breeds cannot be fully explained. However, it may indicate that under certain conditions some of the mammary secretory areas may not function properly. The higher incidence of off-flavor development found when cows are on dry feed can be attributed to the lack of green feed in the diet.

The use of antioxidants and other treatments (discussed in section II) seemed to indicate that the development of the solar-activated flavor was oxidative in nature. This fact was substantiated by the deaeration studies. The results presented in section III showed that the total surface area of the fat globules appears to be an important factor in

accentuating the intensity of the activated flavor development. The Kreis-test for oxidative rancidity did not confirm a lipid-fraction as the constituent oxidized by solar radiation, giving rise to the activated flavor development. This reaction, however, did not definitely preclude the possibility that a lipid-fraction was involved in the oxidation. Since non-homogenized milk does not develop the solar-activated flavor to the same intensity in a given time as does homogenized milk, and since the lipid-fraction has not been shown definitely to be the constituent affected, the key to the development of the activated flavor may be found in the selective rearrangement of the fat globule membrane following homogenization. The constituent affected may be a protein constituent adsorbed on the fat globule. Since homogenization gives rise to increased protein adsorption on the fat globule, this possible selective protein adsorption on the increased fat surface may explain the marked increase in the intensity of the solar-activated flavor in the milk that was homogenized following exposure.

Isolation and Characterization of a Minor-protein Fraction

A whey protein fraction has been isolated and characterized (section IV) which possesses the ability, after being photosensitized, of producing the characteristic solar-activated flavor commonly found in homogenized milk. These findings are in accord with the observations of Doan and Myers (1936) and Keeney (1947) that the solar-activated flavor originates in a serum component that remains after the removal of all of the major milk proteins. Keeney (1947) was able to produce the solar-activated flavor in a casein-, lactalbumin- and globulin-free serum and it may have been construed by some to mean that the flavor originated from

a protein-free serum. The isolation of a minor-protein fraction from the "protein-free" serum stresses the possible importance of milk proteins other than the major protein fractions. Since biological systems are dynamic rather than static, the possibilities and relationships of other minor proteins and/or protein transformation products in milk and whey are of fundamental interest. The minor-protein fraction obtained from the "protein-free" serum may be a normal constituent of milk, or it may have been due to an abnormal functioning of the mammary glands, or it may conceivably be accounted for by some degradation product of milk protein due to heat.

Electrophoretic Analysis of the Minor-protein Fraction

An inspection of the four patterns obtained for the minor-protein fraction shows that this fraction does not necessarily represent a homogeneous compound (fig. 2). The curves seem to indicate that at least two components or complexes are present in the so-called minor-protein fraction. There was no pH range or buffer system used in this investigation that appeared to suggest otherwise. The best resolution and enantiography for this fraction occurred when the veronal-citrate buffer system was used at pH 7.6 rather than at any other pH or buffer system. This conclusion is also evident from studies reported by Stanley et al. (1950) and Deutsch (1947).

Preliminary calculations showed that when mobility values of the major components were plotted as ordinates against the pH values of the buffer system as abscissas an isoelectric zone of the minor-protein fraction was evident at pH 3.7 to 4.4. This range is in good agreement with the

isoelectric range of 4.1 to 4.3 previously obtained by minimum solubility-pH relationships (section IV).

Inasmuch as this study was intended only to establish preliminary data on the electrophoretic character of the minor-protein fraction, it was not considered desirable to measure the individual mobilities and percentage composition for each component until further controlled work could be completed.

The patterns obtained on both the whey and heat-coagulated-whey serum proteins (fig. 3) in veronal-citrate buffer show conclusively that some of the components are lost after heat treatment of the whey. This would indicate that the heat labile components of whey are relatively well separated by this fractionation procedure. As was mentioned previously (section V), some precipitation occurred when the whey (fig. 1, fraction I) was dialyzed against water. The discarding of this precipitate may account for the small number of components in this whey fraction than would otherwise be anticipated.

The degree of resolution and enantiography was not affected noticeably by the concentration of the whey proteins when the veronal-citrate buffer was used. A preliminary pattern (not shown) of this fraction at a concentration of 0.24 percent showed essentially the same resolution as that obtained when the concentration was one percent.

A comparison of the electrophoretic pattern obtained at pH 7.6 in the veronal-citrate buffer of the serum protein (fraction II, fig. 3) with the pattern of the minor-protein fraction in the same buffer and at the same pH (fig. 2) shows that the two complexes are dissimilar.

SUMMARY AND CONCLUSIONS

Approximately 30 percent of the milk from individual cows on pasture failed to develop a solar-activated flavor following pasteurization, homogenization and 30- and 60-minutes sun exposures. Homogenized milk from all of the cows on dry feed was susceptible to solar activation. There appears to be no correlation between breed, stage of lactation, and fat percentage and the susceptibility of milk to develop this off-flavor. Milk from some cows on summer pasture, which upon pasteurization, homogenization and exposure to sun, yielded a nauseating flavor distinctly unlike the true activated flavor.

The addition of ascorbic acid had no preventive effect on the development of the solar-activated flavor of homogenized milk.

The addition of 25 mg./l of nordihydroguaiaretic acid alone, or 75 mg./l in combination with ascorbic acid, prevented the activated-flavor development in homogenized milk after 60-minutes exposure to solar radiation.

Alpha tocopherol and hydroquinone added separately or in combination did not offer complete protection to homogenized milk against the off-flavor development.

Homogenized milk treated with hydrogen peroxide to destroy the naturally occurring ascorbic acid rapidly, prior to pasteurization and homogenization, did not develop a typical solar-activated flavor when exposed to solar radiation.

The development of the solar-activated flavor was not retarded or prevented by high-temperature (176° F. for five minutes) heat treatment.

The typical cooked odor noted in adequately heat treated homogenized milk was dissipated after a 60-minutes exposure to solar radiation. When the cooked flavor was produced by the addition of cysteine hydrochloride and exposed to solar radiation for various periods of time, oxidation of the sulfhydryl group of the cysteine hydrochloride was indicated by a negative nitroprusside test.

Deaeration studies on homogenized milk indicate that the development of the solar-activated flavor results from an oxidative process.

Increasing the surface area of the fat globules of milk by homogenization subsequent to exposure accentuates the development of the solar-activated flavor and indicates that the surface area of the fat globules is a factor in the development of the off-flavor.

A definite correlation between a positive organoleptic taste sensation of the solar-activated flavor in homogenized milk and a positive Kreis reaction was not obtained in all cases. The identity of the constituent affected when the solar-activated flavor develops in homogenized milk was not established by the Kreis test.

A minor-protein fraction has been isolated from skimmilk after the major proteins had been removed, which is capable of being photosensitized to produce the typical solar-activated flavor of homogenized milk.

The elementary analysis, amino acid composition, isoelectric zone and the average minimum molecular weight ($\bar{M}_{min.}$) have been determined. From the data, a ($\bar{M}_{min.}$) of 70,300 was calculated along lines of orthodox organic chemistry. The determination of 12 amino acids shows that

the minor-protein fraction contains the following amino acid residues:

Arg₇, Cys₅, Glu₆₄, His₄, Ileu₂₀, Leu₃₁, Lys₂₈, Met₆, Phe₁₀, Thr₃₉, Try₃ and Val₅₉ (the first three letters of each amino acid is used as the symbol).

Electrophoretic analyses of the minor-protein fraction in various buffers and at various hydrogen-ion concentrations indicate that this fraction is composed of at least two components or complexes.

Best electrophoretic resolution and enantiographic patterns of all of the whey proteins were effected with veronal-citrate buffer at a pH of 7.6.

Electrophoretic patterns obtained with whey and heat-coagulated-whey serum proteins indicate that the heat labile whey components were effectively removed by the fractionation procedure employed in this work.

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