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ABSTRACTS:

IN THIS ISSUE

GRAM NEGATIVE BACTERIA ASSOCIATED WITH SLOUGHING, A SOFTENING OF CALIFORNIA RIPE OLIVES. R. H. VAUGHN, A. D. KING JR., C. W. NAGEL, H. NG, R. E. LEVIN, J. D. MCMILLAN & G. K. YORK II. *J. Food Sci.* **34,** 224–227 (1969) —A softening of olives, called "sloughing," has occurred during the processing of California "ripe" olives for many years. Control of the spoilage was accomplished largely by reducing the washing period for the olives to a maximum of three days. The gram negative bacteria associated with the spoilage include species allocated to five different genera: *Aerobacter, Escherichia, Paracolobactrum, Aeromonas,* and *Achromobacter*. The characteristics of these bacteria, their ability to cause softening of olives, and the kinds of pectolytic enzymes they produce are described.

RAPID DETERMINATION OF PROLINE IN GRAPES AND WINES. C. S. OUGH. *J. Food Sci* **34,** 228–230 (1969)—A direct dilution technique instead of the usual benzene extraction increases the speed and accuracy of the proline determination. No significant interference of other amino acids in amounts found in grapes and wine would normally be expected. The method is applicable to most biological material as long as lysine, hydroxyproline, tryptophan and glutamine are not present in amounts greatly exceeding that of proline. The relative standard deviation between pairs of samples of grape juice averaged about 3% and for wine about 4%.

OCCURRENCE AND PROPERTIES OF ENZYMES ASSOCIATED WITH MEVALONIC ACID SYNTHESIS IN THE ORANGE. V. H. POTTY. *J. Food Sci.* **34,** 231–234 (1969)—Techniques were developed to obtain active enzyme preparations from orange fruit. Acetyl-CoA synthetase and aceto-acetyl-CoA thiolase, two enzymes in mevalonic acid pathway, were detected in juice vesicles, albedo and peel preparations. Hydroxymethylglutaryl-CoA synthase and corresponding reductase were not found. In absence of hydroxymethylglutaryl-CoA synthase, acetoacetyl-CoA was found to be metabolized via 3-hydroxybutyryl-CoA dehydrogenase by orange fruit.

ENZYMIC ACTIVITY DURING GROWTH OF VANILLA FRUIT. 1. Proteinase, Glucosidaseβ, Peroxidase and Polyphenoloxidase. CARLOS WILD-ALTAMIRANO. *J. Food Sci.* **34,** 235–238 (1969)—Proteinase activity decreases with pod age. The activities of glucosidase, peroxidase and polyphenoloxidase increase with stage of growth reaching a maximum near or at ripening. Possibly these enzymes play some role in reactions taking place in curing vanilla, either directly or indirectly supplying the initial products to be transformed in those responsible for color and odor. Partially purified polyphenoloxidase shows a ratio catecholase: cresolase activity of 8.9.

EFFECT OF PROCESSING ON RECOVERY OF POLIO VIRUS FROM INOCULATED FOODS. N. D. HEIDELBAUGH & D. J. GIRON. *J. Food Sci.* **34,** 239–241 (1969)—Foods were inoculated with polio virus and then subjected to either freeze dehydration, exposure to gamma irradiation, storage at 4°C, or storage at 20°C. Recoverability of the virus was determined following the processing. In general, virus recoverability was reduced by each treatment, but complete loss occurred only in highly acid foods held at 20°C for 96 or 168 hr.

HEAT GELLING PROPERTIES OF MYOSIN, ACTOMYOSIN AND MYOSIN-SUBUNITS IN A SALINE MODEL SYSTEM. K. SAMEJIMA, Y. HASHIMOTO, T. YASUI & T. FUKAZAWA. *J. Food Sci.* **34,** 242–245 (1969)—The heat gelling properties of these protein solutions do not run parallel with those of saline model systems composed of these proteins and stroma. Actin does not exert any influence on the binding properties of the system, but when F-actin and myosin A were both present, the resulting binding properties were considerably improved. Since heavy and light meromyosins have little influence on the binding properties, it may be concluded that an intact molecule of myosin is required for development of binding properties upon heating.

COMPOSITION OF MONTMORENCY CHERRY ESSENCE. I. Low-Boiling Components. E. E. STINSON, C. J. DOOLEY, V. J. FILIPIC & C. H. HILLS. *J. Food Sci.* **34,** 246–248 (1969)—The low-boiling neutral components of a commercial Montmorency cherry essence were isolated and identified by gas chromatography, mass spectrometry and chemical methods. Ethanol and methanol comprised 9 and 0.5% of the essence, respectively. Other low-boiling components included acetaldehyde, diethyl ether, propionaldehyde, acetone, isobutyraldehyde, methyl acetate and ethyl acetate. The concentration of each component in the essence was determined.

ASCOSPORE PRODUCTION BY Byssochlamys fulva. D. F. SPLITTSTOESSER, M. C. CALDWELL & M. MARTIN. *J. Food Sci.* **34,** 248–250 (1969)—The effect of different variables on ascospore production was studied quantitatively. Maximal populations were obtained in 5% malt extract broth, pH 2–3, after an incubation of 7–14 days at 30°C. Tests on nine different fruit and vegetable juices showed that eight afforded good sporulation. Prune, grape and pineapple yielded the greatest numbers. Plating methods were found to underestimate the true ascospore populations because of spore dormancy and because a majority of the asci each containing eight ascospores, remained intact.

INFLUENCE OF FREE FATTY ACIDS ON SWEET CREAM BUTTER FLAVOR. M. R. MCDANIEL, L. A. SATHER & R. C. LINDSAY. *J. Food Sci.* **34,** 251–254 (1969)—A threshold pattern for the even-numbered free fatty acids (FFA) in butter depended on chain-length. Butyric acid had the lowest total average flavor threshold (AFT) of the more volatile FFA, and the total AFT values increased as chain-length increased through hexanoic and octanoic acids. A soapy aftertaste predominated at and above the AFT for decanoic and dodecanoic acids. Threshold values decreased from octanoic acid through dodecanoic acid as the chain-length increased. The determination of AFT values for FFA in butter allowed an estimation of the importance of fatty acids in butter flavor. Mixture threshold results support the concept that flavor components interact at subthreshold concentrations. Decreased preference was shown for butter containing suprathreshold levels of total FFA.

CHARACTERIZATION OF ISRAEL LEMON OIL AND DETECTION OF ITS ADULTERATION. ABRAHAM LIFSHITZ, Y. STEPAK & H. B. BASKER. *J. Food Sci.* **34,** 254–257 (1969)—Samples of genuine Israel lemon oil were examined by the usual standard methods and by gas-liquid chromatography. Variations are discussed and evaluated statistically. Chi-square equations were computed for the evaluation of samples of doubtful origin. Sophisticated adulterations of the order of 10% were clearly detectable.

MEAT PIGMENT CHANGES IN INTACT BEEF SAMPLES. G. L. ZIMMERMAN & H. E. SNYDER. *J. Food Sci.* **34,** 258–261 (1969)—Reflectance spectrophotometry was used to study pigment changes in beef samples that (1) had been oxygenated and wrapped with an oxygen-impermeable film, (2) had been treated with ferricyanide and (3) had either treatment (1) or (2) plus malonic acid. Treatment (1) showed a metmyoglobin (MetMb) accumulation of 30% in 30 min, followed by reduction of MetMb. Malonic acid inhibited oxygen utilization and MetMb-reducing activity (MRA). Treatment (2) showed MRA that was inhibited by the presence of oxygen or malonic acid. Malonic acid inhibited oxygen utilization rather than directly inhibiting MRA.

HEAT OF RESPIRATION OF FRESH PRODUCE AS AFFECTED BY CONTROLLED ATMOSPHERE. R. TOLEDO, M. P. STEINBERG & A. I. NELSON. *J. Food Sci.* **34,** 261–264 (1969)—A calorimeter was devised to measure the amount of heat evolved by respiring products during continuous flushing in air and controlled atmosphere. The heats of respiration in air determined with this calorimeter were in good agreement with values reported in the literature for these products. Values obtained for the heat of respiration of peas, lima beans, cut sweet corn, and apples in controlled atmosphere were from 28 to 32% of their heat of respiration in air.

TASTE THRESHOLDS OF BUTTER VOLATILES IN DEODORIZED BUTTEROIL MEDIUM. T. J. SIEK, I. A. ALBIN, L. A. SATHER & R. C. LINDSAY. *J. Food Sci.* **34,** 265–267 (1969)—Thresholds of mixtures of each of the five classes of saturated aliphatic volatiles of butter (free fatty acids, normal aldehydes, methyl ketones, gamma-lactones, and delta-lactones) were studied. A homologous series mixture of free fatty acids (C_2 to C_{12}) showed a high degree of synergism with respect to taste thresholds. Synergism was also exhibited by a methyl ketone mixture (C_3 through C_{15}). Of the compounds studied, diacetyl, methyl sulfide, *n*-hexanal, butyric and caproic acids and gamma-undecalactone had relatively low thresholds.

INCIDENCE AND GROWTH OF SOME HEALTH-RELATED BACTERIA IN COMMERCIAL FRESHWATER CRAYFISH (GENUS PROCAMBARUS). R. T. LOVELL & J. A. BARKATE, *J. Food Sci.* **34,** 268–271 (1969)—Coliforms, *E. coli,* fecal streptococci, coagulase-positive staphylococci, *Salmonella,* and *C. botulinum* type E were found in 100, 92.6, 94.1, 3.0, 3.0, and 0%, respectively, of replicate samples of freshwater crayfish collected from 22 commercial crayfishing areas in Louisiana. All of these organisms except *C. botulinum* grew in raw and cooked crayfish flesh and in a commercial-type crayfish product at 25 but not at 5°C. *C. butulinum* type E produced toxin in all three substrates at 30 and 5°C. However, after 72 hr in the raw flesh and 96 hr in the cooked flesh the pH increased to 8.0 and the toxin was inactivated; in the commercial product the pH decreased to 5.7 and the toxin remained active.

SENSORY EVALUATION OF LAMB AND YEARLING MUTTON FLAVORS. O. M. BATCHER, A. W. BRANT, & M. S. KUNZE. *J. Food Sci.* **34,** 272–274 (1969)—Differences in flavor attributable to age of animal and to sex were detected when broth from samples of lamb and yearling mutton meat were served to panel members in triangle tests. No differences were detected in slices of roasted, broiled, or braised meat scored by the panel. Significant differences in flavor intensity of wether and ram meat served as patties in triangle tests were present only in the patties containing 20% added fat. Full natural flavor of cooked meat slices was not associated with either cover fat thickness of the cut or with fat content of the muscle.

FREE AMINO ACIDS AND OTHER NITROGENOUS SUBSTANCES OF TABLE GRAPE VARIETIES. W. M. KLIEWER. *J. Food Sci.* **34,** 274–278 (1969)—Arginine, proline, glutamic acid and alanine were the most prominent amino acids in the juices of 28 table varieties of grape. Eight amino acids accounted for 74 to 96% of the total free amino acids. Total free amino acids at early and late fruit maturity ranged from 1.04 to 5.53 and 1.24 to 6.45 m *M*/100 ml juice respectively. Amino acid fraction N ranged from 60 to 90% of total N. The eight amino acids accounted for 60 to 96% of the amino acid fraction Kjeldahl N and 37 to 85% of total Kjeldahl N, and arginine accounted for 15 to 50% of total N.

INFLUENCE OF TEMPERATURE ON SOME BIOCHEMICAL CHARACTERISTICS OF Pseudomonas ASSOCIATED WITH SPOILAGE OF CHICKEN. C. R. REY, A. A. KRAFT, R. G. SEALS & E. W. BIRD. *J. Food Sci.* **34,** 279–283 (1969)—Growth, survival and production of the green fluorescent pigment, pyoverdine, and extracellular proteinase and lipase activities were used as indices of the ability of pseudomonads to produce spoilage. The four isolates differed in their ability to perform the metabolic functions mentioned. Growth and enzyme activity were more extensive at 5° than at 15°C. Survival of the cultures was better at −18° than at −29°C. Impairment of pyoverdine secretion was observed after exposure of the organisms to freezing temperatures, but the activity of the extracellular enzymes was not affected at temperatures below 0°C.

CLASSIFICATION OF SOME ESTERASES OF THE GREEN BEAN (PHASEOLUS VULGARIS L.). TERYL B. PUTNAM & M. W. MONTGOMERY. *J. Food Sci.* **34,** 283–286 (1969)—Aqueous extracts of green beans hydrolyzed the acetyl, propionyl and *n*-butyryl esters of glycerol, phenol, sodium 2-naphthol-6-sulfonate and choline, but not triolein or soluble long-chain esters of sodium 2-naphthol-6-sulfonate. Optimum esterase activity occurred at pH 7.2. By means of substrate and inhibitor specificities, three esterases were identified; one was classified as an arylesterase (EC 3.1.1.2) and two as carboxylesterases (EC 3.1.1.1).

CAROTENOID DEGRADATION IN BLEACHED PAPRIKA. ROSALITA R. DE LA MAR & F. J. FRANCIS. *J. Food Sci.* **34,** 287–290 (1969)—The sunlight bleaching of paprika samples resulted in a loss of nearly 96% in total extractable pigment expressed as beta-carotene. The qualitative change afforded by bleaching involved the presence of 17 more pigments than that observed in unbleached Domestic paprika. There were 54 and 37 pigments isolated from bleached and unbleached paprika, respectively. Of these, only 33 and 21, respectively, were completely or tentatively identified. Sixteen known pigments were found in both samples. The fractionation of diffraction grades of paprika showed a great variation in pigment distribution.

SULFHYDRYL CONTENT OF EXCISED CHICKEN BREAST MUSCLE DURING POSTMORTEM AGING. K. A. CALDWELL & HANS LINEWEAVER. *J. Food Sci.* **34,** 290–291 (1969)—Concentrations of thiol compounds in excised chicken *pectoralis superficialis* were measured over 6 hrs postmortem by use of the Ellman reagent. Data reveal no significant change in sulfhydryl concentration during this time. Results do not support the view of a correlation between sulfhydryl content and the reactions associated with rigor or with tenderization.

NICOTINAMIDE AND NICOTINIC ACID IN COLOR PRESERVATION OF FRESH MEAT. J. L. KENDRICK & B. M. WATTS. *J. Food Sci.* **34,** 292–294 (1969)—Nicotinamide (NAm) protected the metmyoglobin (MetMb) reducing activity and oxygen consumption of ground beef or pork on refrigeration or freezer storage. Nicotinic acid (NA) increased MetMb in aerobically refrigerated ground meats, while NAm, particularly in combination with ascorbic acid, decreased it. In model systems and meats, hemochrome formation occurred with reduced myoglobin and either NA or NAm, but more readily with NA and at lower pH values. No hemochrome was formed in aerobically stored meats with NA or NAm even with ascorbic acid present.

DISTRIBUTION OF ARSENIC RESIDUES BY ACTIVATION ANALYSIS. J. R. GEISMAN, W. E. CAREY, W. A. GOULD & E. K. ALBAN. *J. Food Sci.* **34,** 295–298 (1969)—The development of mechanized harvesting of vegetables has been accompanied by the use of defoliants for chemical mowing. One of the most common defoliant materials is sodium arsenite. Chemical methods for detecting residues of this compound are sophisticated and involve elaborate techniques. A method to detect arsenic by activation analysis has been developed. No prior chemical separations or treatments are necessary except to remove moisture. The method is reproducible and has been utilized for assay of samples of soil, plant material, raw and processed products.

REESE H. VAUGHN, A. DOUGLAS KING,[a] CHARLES W. NAGEL,[b]
HENRY NG,[a] ROBERT E. LEVIN,[c] JAMES D. MACMILLAN[d] and GEORGE K. YORK II
Department of Food Science and Technology, University of California, Davis, California 95616

Gram Negative Bacteria Associated with Sloughing, a Softening of California Ripe Olives

SUMMARY——*A softening of olives, called "sloughing," has occurred during the processing of California "ripe" olives for many years. The spoilage is characterized by the softening and ultimate sloughing of at least part of the skin and tissue from the pit of the olive. Control of the spoilage was accomplished largely by reducing the washing period from the customary four to a maximum of three days. The gram-negative bacteria associated with the spoilage include species allocated to five different genera:* Aerobacter, Escherichia, Paracolobactrum, Aeromonas, *and* Achromobacter. *The characteristics of these bacteria, their ability to cause softening of olives, and the kinds of pectolytic enzymes they produce are described.*

INTRODUCTION

A SOFTENING of olives, commonly called "sloughing" in the industry, was first called to the attention of the senior author in the fall of 1937. Because of the sporadic nature of the outbreaks, however, it was not possible, until comparatively recently, to observe under commercial conditions, outbreaks of "sloughing" from inception until the ultimate spoilage of all or nearly all of the olives under process. Severe outbreaks occurred during the fall of 1958 and the spring of 1959 in several different plants. These outbreaks occurred in sufficient number to determine, without doubt, that the spoilage started during the final stages of the washing process used to remove the sodium hydroxide remaining after the alternate lye-treatments and oxidation used to produce the dark color of the ripe olives. This defect most frequently occurs during the warmer fall and spring months.

The spoilage involves the softening and ultimate sloughing of at least a part of the skin and tissue from the pits of the olives (Fig. 1). Under commercial conditions [see Vaughn (1946) and Cruess (1958) for detailed discussions of the processing of California ripe olives] the olives do not start to soften appreciably until the third day of the washing period, but the texture has been very severely degraded by the end of the usual four or more days of washing. This latter observation suggested that the spoilage might be reduced or eliminated by reducing the length of the washing period. The sloughing was promptly controlled when the leaching cycle was reduced to a maximum of three days. However, there was still occasional evidence of incipient softening so a study was initiated to determine the microbiology of the spoilage. This report describes the microbiology of the sloughing and characterizes the gram-negative bacteria known to be associated with the softening.

EXPERIMENTAL

Source of olives

The sloughed olives investigated were of commercial origin and represented the Ascolano, Obliza, Manzanilla, Mission and Sevillano varieties from the major production areas of the San Joaquin and Sacramento Valleys. The spoilage outbreaks detected were studied in the field. Each time aliquot samples of the spoiled olives and wash waters were collected aseptically and taken to Davis for additional investigations.

When in vitro reproduction of the spoilage was attempted, "standard" size commercial

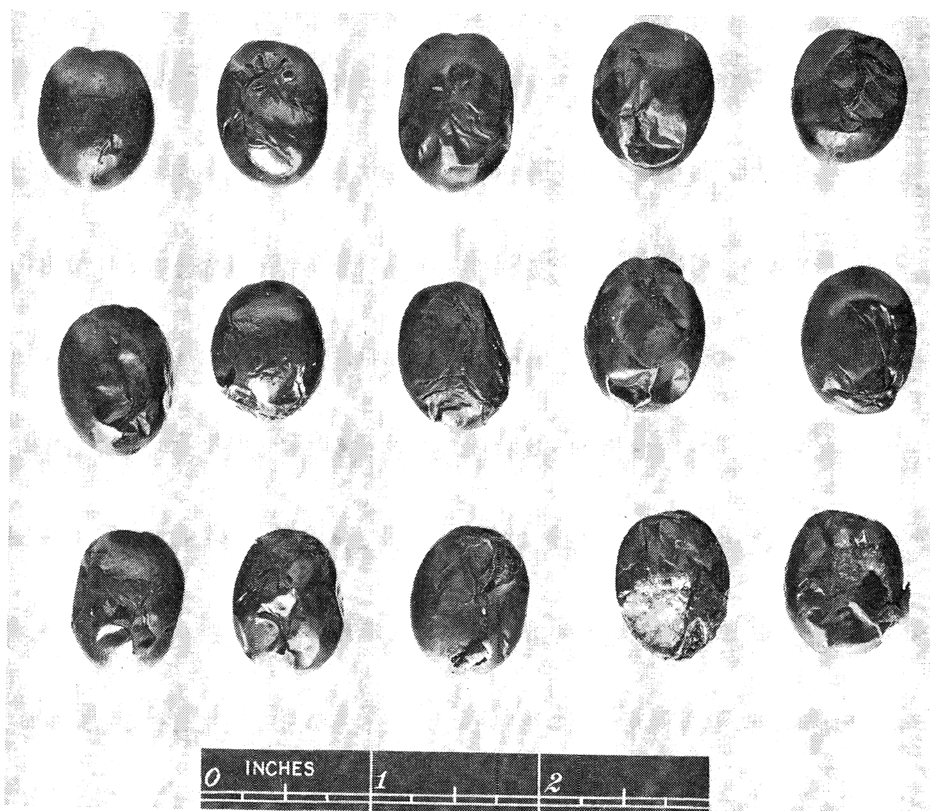

Fig. 1—Various stages in the sloughing spoilage.

Present addresses:
[a] USDA Western Utilization R&D Div., 800 Buchanan Street, Albany, California 94710
[b] Department of Horticulture, Washington State University, Pullman, Washington 99164
[c] Department of Food Science and Technology, University of Massachusetts, Amherst, Massachusetts 01003
[d] Department of Biochemistry and Microbiology, Rutgers, The State University, New Brunswick, New Jersey 08903

Manzanilla canned ripe olives were used.

Detection of pectinolysis

Demonstration in vitro of pectolytic enzymes that might be present in the wash waters first involved dialysis of 100 ml of sample with tap water overnight. Then, after concentration to 10 ml by evaporation, the sample was tested for activity on pectin N.F. and polygalacturonic acid No. 3491 (both products from Sunkist Growers). Pectin methyl esterase (PME) was detected by the qualitative method described by McComb et al. (1958) except that the "cup-plate" technique was substituted for the filter paper disks they had used. The presence of polygalacturonase (PG) was demonstrated by the "cup-plate" quantitative assay method described by Nagel et al. (1961a). Later, after the discovery of bacterial polygalacturonic *trans* eliminase (PATE) by Nagel et al. (1961b, 1962), it became clear that this method was not a specific assay for either *trans*-eliminases or hydrolytic polygalacturonases because both kinds could be present in the same sample. Therefore, analysis of crude wash water dialysates subsequently involved detection of *trans*-eliminase activity as described by Nagel et al. (1962).

At the same time aliquots of the wash waters were streaked onto nutrient and eosine methylene blue polypectate gels (Vaughn et al., 1957) to detect the presence of pectolytic bacteria.

After it was found that the presence of large populations of pectolytic organisms always were present in the wash waters showing pectolytic activity, enzyme assay was discontinued to concentrate on the bacteria presumed to be responsible for the sloughing spoilage. It was soon found that the eosine methylene blue gel was selective only when the gram negative bacteria predominated in a sample. To correct this problem the crystal violet gel (King et al., 1961) was developed and used to isolate gram negative pectolytic bacteria from the spoiled samples, processing vat walls, floors, etc., in the commercial plants. This new gel used together with the older nutrient gel permitted an approximation of "total" organisms, "total" gram negative bacteria and, by difference, gram positive and negative pectolytic and nonpectolytic bacteria by counting the plates and noting whether depressions were formed under and around the colonies.

Texture determinations

The Christel texture meter (Hartmann et al., 1959) equipped with a 0 to 100 psi gauge was used to determine the texture of the processed olives involved in this study.

Isolation and purification of the bacteria

All but two of the 19 cultures to be described here were isolated from typical samples of sloughing olives. One of the two was obtained from dehydrated, powdered garlic and the other was recovered from rotted lettuce leaves. Most of the cultures had originally been isolated on the eosine methylene blue gel. The cultures all were purified by restreaking on the crystal violet gel two or more times and then a final time on Levine's eosine methylene blue agar.

Identification of the bacteria

Conventional methods were used for generic and specific identification of the bacteria. General references used included Levine (1954), Breed et al. (1957), Society of American Bacteriologists (1957), and Skerman (1959). When necessary the original literature was consulted. These latter references will be cited in connection with specific differential tests.

Reproduction of the spoilage

Since the commercial spoilage occurred during the washing period before salt was added, salt-free "standard" size (also known as "small" and "select") Manzanilla olives were used. These were commercially processed and canned in No. 10 (603 × 700) lacquered cans, retorted at 116–117°C for 1 hr, cooled, and returned to the laboratory for use.

To reproduce the softening in the laboratory the salt free olives first were repacked in plain tin baby food (202 × 214) or 8-oz juice cans (211 × 304) and covered with hot (90°C) tap water leaving the appropriate headspace. After seaming, one end of each can was covered with a hood made by pressing a 6-in. square of heavy aluminum foil tightly over the end and down the sides of the can. The hood functions like the lid of a petri dish.

After retorting at 116–117°C for 1 hr and cooling, the "hooded" cans of olives were inoculated under aseptic conditions in an inoculation room. First, the foil hood was raised from the lid of the can. Then the lid was flamed and punctured with a flamed beverage can opener. The opened can then was seeded with the desired inoculum, the hood replaced, and the inoculated olives were incubated at 20°C. After incubation the cans were opened, the olives were pitted, sliced in half, and 40 g of each lot were tested for loss in firmness with the Christel texture meter. At the same time a portion of the liquid was checked to determine that the bacteria comprising the original inoculum were present without contamination.

The use of cans as containers is imperative if texture of the olives is being stressed. The use of conventional plugged tubes or flasks and sterilization in the usual manner causes a marked change in texture of the olives resulting from tissue softening and damage caused by "steam pocket" formation in the flesh of the fruits.

RESULTS

DURING the course of accumulation of the descriptive information required to identify the gram negative, pectinolytic bacteria it was possible to eliminate some genera from consideration either by morphological or physiological differences. Solely on the basis of oxygen requirements, glucose and lactose fermentation, and type of flagellation there appeared to be three different groups of bacteria. Their descriptions follow.

The coliform bacteria

Eleven of the cultures had characteristics commonly associated with the coliform group of bacteria. All cultures were facultatively anaerobic, lactose fermenting, nonsporulating, short, gram negative, rod-shaped bacteria. The generic allocations were made on the basis of the Voges-Proskauer and Koser's citrate tests. Species separation followed the classification recommended by Levine et al. (1934), in which primary emphasis was placed on the production of hydrogen sulfide and indole in suitable differential media, and the fermentation of esculin, glycerol, salicin, and starch in a standard basal medium. The differentiation of the pectinolytic coliform bacteria is shown in Table 1.

Eight of the isolates were considered to be *Aerobacter aerogenes* types although two cultures fermented starch with acid production but no visible gas after 2 weeks of incubation at 30°C and two of the cultures did not grow in Koser's citrate medium even when incubated for 6 days. Although the ability to utilize citrate as a sole source of carbon in an otherwise inorganic medium is considered to be a primary characteristic of the genus, strains of *Aerobacter* are found occasionally that do not possess this ability (Ruchhoft et al., 1931). Despite this discrepancy, the two cultures were typical of *A. aerogenes* in other respects and thus were

Table 1—Differentiation of the coliform bacteria.

Character	Number of positive reactions			
	Aerobacter aerogenes 8 strains	*Aerobacter cloacae* 1 strain	*Escherichia intermedia* 1 strain	*Paracolobactrum aerogenoides* 1 strain
Motility	0	1[1]	1[1]	1[1]
Voges-Proskauer	8	1	0	1
Citrate[2]	6	1	1	1
H_2S	0	0	0	0
Indole	8	0	1	0
Glycerol	8	0	0	0
Esculin	8	0	1	1
Salicin	8	1	1	1
Starch	8[3]	0	0	1

[1] Peritrichous flagella.
[2] Citrate incubated 6 days at 30°C.
[3] Acid only produced by 2 strains.

Table 2—Some characteristics of the gram negative bacteria allocated to the genera Aeromonas and Achromobacter.

Taxonomic Allocation:→	*Aeromonas liquefaciens*					*Achromobacter*		
Culture No.:→ Morphological and physiological tests	46	156	160	163	175	34	44	178
Flagella	polar	polar	polar	polar	polar	none	Peritrichous	none
Avg. cell size (μ)	1.6 × 0.8	2.4 × 0.8	1.6 × 0.8	2.4 × 0.8	1.6 × 0.8	1.6 × 0.8	2.4 × 0.8	1.6 × 0.8
Gelatin liquefaction	+	+	+	+	+	+	+	−
Colony pigment[1]	white	yellow	cream	cream	cream	white	white	white
Nutrient broth[2]	PST	RST	PST	PST	RST	ST	ST	PST
NO_3 reduction	+	+	+	+	+	−	−	+
Urea decomposition	−	−	−	−	−	−	−	−
Starch hydrolysis	+	+	+	+	+	+	−	+
Litmus milk[3]	AD	RC	AD	AD	AD	N	RN	RCD
Indol production	+	+	+	+	+	−	−	−
Methyl red reaction	+	+	+	+	+	−	+	+
V.-P reaction	+	−	+	+	+	−	−	+
Citrate utilization	−	+	+	+	+	−	−	+
Glucose[4]	AG	AG	AG	AG	AG	−	−	A
Lactose	AG	AG	AG	AG	AG	A	A	A
Maltose	AG	AG	AG	AG	AG	A	A	−
Sucrose	AG	AG	AG	AG	AG	−	A	A
Glycerol	AG	−	AG	AG	AG	−	−	−
Mannitol	AG	AG	AG	AG	AG	AG	AG	A
Salicin	AG	AG	AG	AG	AG	AG	AG	A
Starch	AG	−	AG	AG	AG	−	−	−

[1] Pigment of cultures grown on nutrient agar.
[2] P, pellicle; R, ring; S, sediment; and T, turbidity.
[3] A, acid; C, curd; D, digested; N, neutral; and R, reduced.
[4] A, acid and G, gas.

allocated to that species. One culture had all of the characteristics considered to be typical of *Aerobacter cloacae.* Another coliform isolate was identified as *Escherichia intermedia* on the basis of the characteristics shown in Table 1. The one remaining culture was classified as *Paracolobactrum aerogenoides* solely on the basis that it fermented lactose slowly and visible gas production was not evident until 6 days of incubation after inoculation.

The group of pseudomonads

It was evident that five of the pectinolytic cultures were pseudomonads because all were motile and the cells had one polar flagellum. All of the cultures were allocated to the genus *Aeromonas* because they fermented both glucose and lactose with visible gas production. They also differed from species of *Zymomonas* because they grew well under aerobic conditions and four of the five cultures also produced acetyl methyl carbinol (V.-P. positive). In respects other than flagellation the five isolates closely resembled the coliform species allocated to the genus *Aerobacter* although the fermentation of carbohydrates was less active.

Four of the five cultures grew well at 0°C, producing visible colonies on nutrient agar plates within two weeks, thus indicating their psychroduric nature, although the optimum temperature for growth of all five was 30°C.

As seen by the information contained in Table 2, three of the cultures (160, 163, and 175) were almost identical in characteristics, varying only in average cell size and type of growth in nutrient broth. The other two cultures (46 and 156) varied in culture pigmentation, growth in nutrient broth, action in litmus milk, the Voges-Proskauer reaction citrate utilization and glycerol fermentation. It is interesting that strain 156 was able to cause hydrolysis of starch agar when grown in a petri dish but showed no evidence of fermentation of that substrate as indicated by acid or gas formation in a liquid medium.

On the basis of polar flagellation and the fermentation of glucose and lactose with acid and gas production we have considered allocation to the species *Aeromonas liquefaciens* the most appropriate for these five cultures, especially since their pectolytic activity would suggest that they probably would attack plant rather than animal tissues. We have no evidence that these cultures are pathogenic to plants or to mice, frogs, carp, eels and other fishes. It is to be remembered that this species is reported as generally found in certain marshes and swamps (Breed et al., 1957). This knowledge lends some credence to the recognition of *Aeromonas liquefaciens* and *Aeromonas punctata* as separate species but also indicates that more work is needed to clarify the extent of pectolytic activity in the genus.

The genus Achromobacter

As is to be seen from Table 2, the three remaining cultures (34, 44 and 178) are quite variable in their characteristics and might well represent three different species. The cultures did not produce gluconic acid from glucose and did not oxidize ethanol to acetic acid so the two nonmotile cultures did not belong to any of the genera of the order *Pseudomonadales.* They did not attack agar, alginates, or chitin. They did not produce pigment on nutrient agar and did not actively ferment carbohydrates with acid and gas production. Therefore, the isolates were tentatively allocated to the genus *Achromobacter.*

Clear cut, specific allocation of these cultures is impossible at the present time. The pectinolytic activity of these three free-living forms is the problem. Certainly, a monographic study of free-living gram-negative pectolytic bacteria is indicated.

Pectolytic enzymes produced by the bacteria

It was demonstrated that all of the cultures produced at least two extracellular pectolytic enzymes. The one, a pectin methyl esterase (PME), caused the deesterification of pectin. The other, a pectic acid *trans* eliminase (PATE), caused the further degradation of the pectic acid formed when pectin was demethylated by the PME and resulted in the formation and accumulation of unsaturated digalacturonic acid as the major endproduct.

No attempt was made to determine whether the PATE was an endo-polygalacturonic acid *trans* eliminase (Nagel et al., 1961b) or an exo-polygalacturonic acid *trans*-eliminase (Macmillan et al., 1964) and no separation techniques were used to see if the hydrolytic bacterial polygalacturonase might be present in the

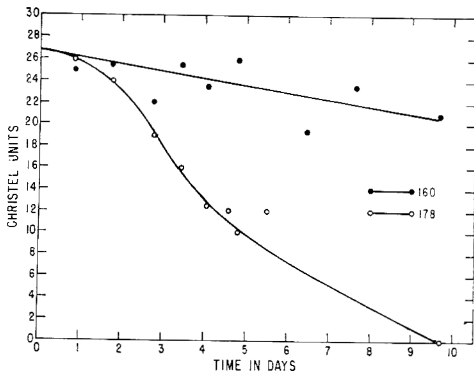

Fig. 2—The rate of softening of olives caused by slow and rapid action. #160- Aeromonas liquefaciens *#178* - Achromobacter sp.

crude cell-free preparations. These investigations were thought to be more pertinent to other studies related to these bacteria (see discussion). In any event, the observations on the occurrence of the two pectolytic enzymes strongly support the association of the bacteria in question with the sloughing spoilage of olives described above.

Softening of olives and other plant tissues

All of the cultures were observed to cause softening of olives to a greater or lesser degree. Some caused such rapid loss of firmness in the olives that it had been reduced by about half in four days and by ten days was no longer measurable by the Christel texture meter. Others caused only slight softening even after ten days incubation. Even so, the damage caused by these slow cultures occurred at the stem end of the olives and caused a softening of the tissue and puckering of the skin around the area of the former stem site so that these olives also would be classified as cull fruit. These differences in rate of softening are shown in Figure 2.

Five of the different bacteria representing one strain of *Aerobacter aerogenes*, four isolates of *Aeromonas liquefaciens* and one culture of *Achromobacter* sp. caused the rapid type of softening of olive tissue. The remainder of the cultures produced the slow type of softening.

Further proof of the ability of all of the cultures to soften plant tissues was obtained by growing the cultures on previously sterilized 10 mm diameter plugs of carrots and potatoes cut so they provided a slanted surface for inoculation. Controls included sterile wedges plus wedges inoculated with known nonpectolytic bacteria. The bacteria under question in this study all softened the two kinds of vegetable wedges, but at a considerably slower rate than the olives. In this case even the five extremely active cultures mentioned above caused only slight softening in six days of incubation and it was not until 18 days that 11 of the inoculated wedges were soft as compared to the controls when probed with a straight inoculating needle.

DISCUSSION

THERE IS NO question that the various bacteria isolated in this study cause an undesirable softening of olives. However, there is one question still unanswered. The rupture of the skin of the olives did not occur to any extent under the conditions of the experiments just described. Why not? There are two obvious explanations. The conditions of the experiments did not provide for the build-up of turgidity that might, under commercial conditions, cause enough physical pressure to result in a bursting of the skin of the individual fruits. On the other hand, it might be that the weakening and ultimate rupture of the skin of the olives is caused by the development of cellulase by microorganisms capable of living in the storage brines used for olives. It is known (unpublished results of Vaughn et al., 1965) that free-living cellulase-producing microorganisms occur in the brines covering olives in storage.

The explosive softening of olives undergoing spoilage as described earlier possibly is easier to explain. The reason for the extremely rapid softening of olives is that the pectolytic bacteria produce more degrading enzymes under poor conditions of nutrition (Hsu et al.). These authors found that very slow feeding of the substrates glucose glycerol or pectic acid to a culture of *Aeromonas liquefaciens* (strain number 160 of this study), greatly increased the amount of PATE formed.

Certainly by the time the olives have reached the washing period they should have little readily utilizable substrates left with crude fiber, olive oil, pectic materials and polyphenolic compounds being the most abundant and also more resistant to attack. The more utilizable substrates glucose, a product of partial alkaline hydrolysis of the bitter glucoside oleouropein (Simpson et al., 1961), and traces of the sodium salts of organic acids produced during fermentation in brine storage and, possibly, some of the polyphenolic acid components resulting from glucoside breakdown must provide the initial energy for the bacteria at a rather slow rate during leaching of the olives to remove the sodium hydroxide residue.

It is thought, on the basis of the in vitro studies already cited, that the set of conditions just described stimulates the production of the enzymes that start degradation of the pectic substances. Once these are sufficiently degraded they then leach into the wash water to provide more food for the bacteria. The normal and unsaturated di-galacturonic acids as well as the monomer compounds are utilized readily by all of the bacteria included in this study.

This explanation becomes all the more plausible when one considers that the nutritionally richer carrot and potato plugs were softened at a much slower rate than the olives.

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C. S. OUGH
Department of Viticulture and Enology, University of California
Davis, California 95616

Rapid Determination of Proline in Grapes and Wines

SUMMARY—An improved method for the rapid determination of free proline in grapes and wine is given. No significant interference from other amino acids in amounts found in grapes and wine would normally be expected. The method is applicable to most biological material as long as lysine, hydroxyproline, tryptophan and glutamine are not present in total amounts greatly exceeding that of proline. Lysine caused greatest interference and this was only 6% error on equal mole amounts. A direct dilution technique instead of the usual extraction of reaction product by benzene increased accuracy and speed of this method. The relative standard deviation between pairs of samples of grape juice averaged about 3% and for wine about 4%.

INTRODUCTION

CHINARD (1952) reported on the acidic ninhydrin reaction as a photometric method for proline determination. Troll et al. (1955) improved upon the original method by the use of benzene to extract the color reaction product. The use of excess glycine was suggested by Wren et al. (1965) to stabilize enhancing effects of this compound. Kimura (1967) investigated the effects of glutamine and developed a method of correcting proline determination for errors caused by high amounts of glutamine.

Other interfering substances were studied by the above authors but perhaps the most extensive work was that of Messer (1961). He checked the effects of most of the natural occurring amino acids and also some analogs which might be present and interfere. A large group of the amino acids cause some interference and others enhanced the effect of proline. In his tests, all of the test amino acids were present in 10- to 20-fold greater concentrations than the proline. He also studied a large group of peptides and concluded that they would have little real effect on the measurements.

The presence of rather large amounts of proline in grapes and wine has been reported on by Castor et al. (1956) and by Lafon-Lafourcade et al. (1959) using microbiological assay methods. Nassar et al. (1966) using paper chromatography techniques found high amounts of proline and higher amounts of arginine in Thompson Seedless grapes. Other amino acids were present in much smaller amounts.

It was pointed out by Van Slyke et al. (1941) that the time of heating, necessary to develop complete reaction between amino acid and ninhydrin, was related to the ninhydrin concentration and the pH. The higher the ninhydrin concentration the less time and the lower the pH the longer heating period needed. Troll et al. (1955) used equal volumes of glacial acetic acid, test solution and ninhydrin mixture in aqueous acid solution. They heated for 30 min at about 95°C. Kimura (1967) also used these solutions and reaction times. The color reactions were extracted with benzene and measured. Lashkhi et al. (1967) used about the same reagents except a few drops of formic acid in place of glacial acetic and a few drops of 2 *N* hydrochloric acid. They centrifuged their color mixture prior to benzene extraction, indicating some precipitation and hence loss of the reaction color product.

Chinard (1952) noted that ornithine gave a yellow color with acid ninhydrin. Troll et al. (1955) found by using Permutit the interfering substances of lysine and ornithine could be quantitatively removed. They also noted cysteine could cause interference. Kimura (1967) studied the interference of glutamine and found that the color developed from 70 μg of glutamine was equivalent to that from 1 μg of proline at 517 mμ. He further noted that glycine intensification effect, reported on by Wren et al. (1965), can be compensated for by the addition of known amounts of glycine and rerunning the determination. Kruze et al. (1967) report that hydroxyproline and tyrosine yield interfering color products with acidic ninhydrin reaction. Lashkhi et al. (1967) indicate that only hydroxyproline and tryptophan of the naturally occurring amino acids will give color reaction products which will interfere with the proline determination.

MATERIALS & METHODS

Grape juice and wines

The grapes, both red and white wine varieties of the species *Vitis vinifera*, used in these studies were grown in the University vineyards at Davis and Oakville. Standard procedures were used to make the wines. All juices were stored at 32°F under toluene prior to analysis. No special filtration or other treatment was given other than normal settling and decanting.

Reagents

The amino acids were obtained from Nutritional Biochemical Corp. or H. & M. Chemical Co. Ltd. and were C.P. grade. No repurifications were made. Deionized water was used for dilutions. Methyl cellosolve was purchased from J. T. Baker Co. Ninhydrin (1,2,3,triketohydrindene) was that of Eastman Organic Chemicals and was made up as a 3% solution (wt/vol) in methyl cellosolve. Formic acid was Baker and Adamson Reagent grade. Benzene (thiophene free) was obtained from Mallinckrodt Chemical Works. Isopropanol was reagent grade from Eastman Organic Chemicals and was diluted 1:1 by volume with water. Dowex 50 × 8 [H^+] resin was used for routine ion exchanging to separate amino acids.

Procedure

Samples of amino acids, grape juices or wines were diluted to contain between 0.05 and 0.50 μ moles/ml of proline. One-half ml of these diluted samples plus 0.25 ml of formic acid and 1 ml of 3% ninhydrin solution were added to screw-cap test tubes (15 × 150 mm). The tubes were stoppered tightly and put into boiling water bath for 14 to 15 min. The tubes were cooled in a 70°F water bath for 5 to 10 min and while cooling, 5 ml of 1:1 isopropanol-water solution added. Absorbance readings at 517 mμ were made after diluted samples had cooled at least 5 min and before 30 min from time of removal from the heated baths. If Klett-Summerson colorimeter was used, the readings were taken using either a #50 or #54 filter. Loss of developed color with time is at the rate of about 2%/hr.

Benzene extractions were made on the samples after heating and cooling. The extractions were made in the same reaction tubes and the benzene decanted off through #2 Whatman filter paper to clear the extracts.

A blank was made and carried through all the steps for either method by the use of ½ ml of water instead of the test solution.

Instruments used

A Klett-Summerson colorimeter was used with standard Klett tubes and filters. A Bausch and Lomb. Spectronic 505, recording

spectrophotometer was used to determine the continuous spectra from 440 to 590 mμ.

Statistical tests

The calculations of standard deviations, relative standard deviations, etc. were made as outlined by Snedecor (1956).

RESULTS & DISCUSSION

Conditions

Tests in this laboratory showed that in aqueous solutions in the presence of acid an orange precipitate readily forms which is difficult to dissolve during the benzene extraction. However, a far more stable color product results if methyl cellosolve is used for the ninhydrin solvent. One ml of this ninhydrin solution will keep 2 to 4 times the suggested maximum amount of reaction product in solution. Also, it was noted that the reaction could be done in a boiling water bath, leading to a shorter heating time (15 min) and a more uniform result.

Uniform and maximum color values were found between 12 and 15 min heating at 100°C. Color losses begin after 15 min heating with a loss of about 1% of the color per minute over 15 min and to 30 min. The addition of larger amounts of formic acid ($^1/_2$ the volume of the test mixture) give more uniform color product; and if the benzene extraction was used, it greatly increased the solubility of the color product in the benzene. Only a few quick mixings are needed to completely extract the color compound from the reaction mixture.

Further, it was noted that under the above conditions it was now possible to dilute the reaction mixture directly with isopropanol-water (1:1) and not be troubled with the benzene extraction.

Trials showed that with the suggested conditions, maximum color products resulted and the color reaction product remained dissolved.

Although the physical methodology is similar to that proposed by Moore et al. (1954) the chemical reactions are not. Under their conditions all amino acids present react with the ninhydrin, while in this reaction the proline is the major reactant to give a colored product.

Interference

In grapes, and especially in wine, the amount of proline is generally the greatest or nearly so of all the amino acids. The possibility of interference from hydroxyproline was investigated. The results are shown in Fig. 1. The hydroxyproline yields a color reaction product roughly 100 times less intense per mole than proline. Contrary to the results reported by Lashkhi et al. (1967) the color product is extracted by the benzene. Since it is present in grape juice and wine in very small amounts compared to proline, it would not be a detectible interference.

Table 1—Interference effects of various amino acids on proline measurement

Known amino acids,[1] μ moles/ml		Calculated as proline: moles/ml	Calculated as proline: % Response[2]
Proline	0.42, 0.84	0.42, 0.84	100, 100
Lysine	0.80, 10.0	0.05, 0.44	6.2, 4.4
Tryptophan	0.80, 10.0	0.02, 0.14	2.5, 1.4
Histidine	0.80, 10.0	0.00, 0.024	0.00, 0.24
Hydroxyproline	0.4, 5.0	0.00, 0.045	0.00, 0.9
Glutamic acid	0.4, 5.0	0.00, 0.00	0.00, 0.00
Glycine	0.4, 5.0	0.00, 0.00	0.00, 0.00
Proline 0.42 + Lysine 0.40		0.445	105.9
Proline 0.42 + Tryptophan 0.40		0.415	98.9
Proline 0.42 + Histidine 0.40		0.415	98.9
Proline 0.217 + Hydroxyproline 5.0		0.220	101.3
Proline 0.217 + Glutamic acid 5.0		0.223	102.7
Proline 0.217 + Glycine 5.0		0.198	91.2

[1] All solutions in water.
[2] Calculated as percent of proline present; or as moles of calculated proline/mole of amino acid tested × 100 if no proline present.

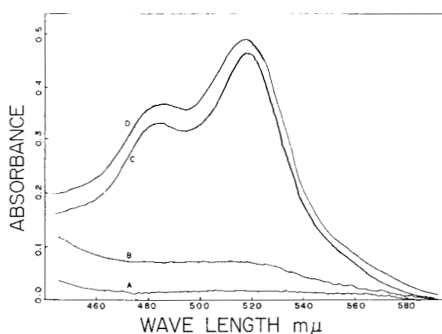

Fig. 1—Plots of benzene-extracted color products of A, blank; B, 5 μ moles/ml of hydroxyproline; C, 0.2 μ moles/ml proline; and D, equal volume mixture of B and C. A "Spectronic 505" was used.

Several other amino acids in water solutions were investigated for interference effects both independently and with proline. Table 1 gives a summary of the results. The only amino acid which gave appreciable color was lysine. On equal-mole basis, this gave about 6% as much color as the proline. Tryptophan gave detectible interference but less than lysine. The glycine caused less color and this is explainable. Wren et al. (1965) showed that the rate of color development was speeded by the addition of glycine and that the color loss after maximum color formation was also more rapid. Since maximum color formation is reached in 15 min, the addition of large amounts of glycine would be expected to lower the color under these working conditions. Neither hydroxyproline nor glutamic acid at over 20-fold the concentration of proline caused significant interference.

Another set of tests adding glutamic acid, glycine and hydroxyproline at 400 μ moles/100-ml concentration to three samples of grape juice containing low, medium and high amounts of proline caused no significant differences in proline values measured. This is a far greater concentration of glycine and hydroxyproline than would normally be found in grape juice or wine. Glutamic acid has been reported (Castor, 1953) at this level or slightly higher in grape juice.

Ion-exchange and dilution

Evaluating the inherent error and time losses involved in ion-exchanging, the possibility that this step could be omitted was considered. Five juices and 59 wines were ion-exchanged using the usual practice based on the work of Moore et al. (1951). Samples of the juice and wines were diluted directly with water to the same volume. The juice solutions were tested for proline, using the benzene extraction procedure. No significant variation was found between measurements of samples ion exchanged and nonion exchanged (Table 2).

Table 2—A comparison of ion-exchange vs. direct measurements of grape juice and wine

Substance tested	Number of samples	Mean proline values, μ moles/ml: Ion exchange	Mean proline values, μ moles/ml: Direct	Standard error, population differences
Juice[1]	5	0.190	0.208	0.009
Wine[2]	59	0.273	0.270	0.005

[1] Using benzene extraction procedure.
[2] Using direct dilution procedure.

A second comparison, using the direct dilution of the color reaction product by isopropanol-water diluent, was made on 59 wine samples. The results indicate that

no advantage is gained by ion exchange and no significant differences in the measurements result. These results indicate that for this type of measurement, ion-exchange is neither necessary nor desirable.

Measurements

A comparison of the error involved using the benzene extraction vs the direct dilution technique with standard solutions is made in Table 3. As expected with the

Table 3—Estimation of the error of proline measurement using benzene extraction or direct dilution of the developed color

	Benzene extraction			Isopropanol-water diluent		
μ moles proline/ml	Number of tests	Standard deviation Klett units	Relative standard deviation, %	Number of tests	Standard deviation Klett units	Relative standard deviation, %
0.1	6	2.7	3.5	4	0.5	0.9
0.2	6	2.6	1.6	4	1.0	0.9
0.3	4	4.1	1.8	4	1.2	0.7
0.4	6	5.2	1.9	4	2.5	1.1
0.5	4	2.6	0.8			
1.0	4	5.0	0.9			

more direct method, lower variations result. The coefficients of variation are about one half as large for the direct method.

The standard error of estimate for the calibration curve (0.00–0.40 μ moles/ml-range) is ±0.04 μ moles proline/ml. The standard error of estimate for two sets of calibration data (0.00–0.40 μ moles/ml range) done independently and with different reagent was ±0.009 μ moles proline/ml.

Table 4 gives recovery data for known amounts of proline added to several samples of grape juices and wines using the direct measurement and then dilution with isopropanol-water technique. The recoveries are within acceptable limits.

A number of test samples of grape juice and of wine were analyzed for proline using both the benzene extraction and direct methods. The results are shown in Table 5. The standard deviations and relative standard deviations are for the differences between pairs of measurements. Each pair was for an individual sample. The calculated amounts of proline in the samples ranged from 0.0 μ moles per 100 ml to 2500 μ moles/100 ml. The relative deviations may seem unreasonably larger than those for the standard solutions but these values are based on differences between pairs and not differences from a mean and are roughly twice as large than if calculated from a mean. Comparing the benzene extraction and the direct dilution techniques, the relative size of the coefficients of variations remain about the same as the previous comparison made using standard solutions.

Table 5—Error between paired samples of grape juice and of wines using benzene extraction and direct dilution of developed color

		Benzene extract			Isopropanol-water diluent		
Range measured, μ moles/ml	Substance tested	No. of Pairs	Standard deviation, μ moles	Relative standard deviation, %	No. of Pairs	Standard deviation, μ moles	Relative standard deviation, %
0.000–0.199	Juice	7	0.016	10.7	21	0.004	4.0
	Wine				18	0.005	6.7
0.200–0.450	Juice	9	0.010	2.8	5	0.004	1.6
	Wine				10	0.008	2.3

Table 4—Recovery of added proline from grape juices and wines using the direct measurement on the non ion-exchanged samples

Material	Proline, μ moles/100 ml	Proline added, μ moles/100 ml	Proline found, μ moles/100 ml	Proline recovered μ moles/100 ml	%
Grape juices					
1	280	420	704	424	101
2	570	420	992	422	100
3	2840	420	3276	436	104
Wines					
1	205	2100	2220	2015	96
2	820	1050	2000	1180	112
3	325	3150	3385	3150	100
4	570	1050	1695	1125	107
5	1290	2100	3340	2050	98

An extensive report on the proline composition of grapes and wines has been published by Ough (1968).

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V. H. POTTY[a]
USDA Southern Utilization R&D Div., ARS, Fruit and Vegetable Products Laboratory, Winter Haven, Florida 33880

Occurrence and Properties of Enzymes Associated with Mevalonic Acid Synthesis in the Orange

SUMMARY—Active enzyme preparations were obtained from mature orange fruit in a quest for the various enzymes associated with terpenoid synthesis. These preparations were examined systematically for the presence of enzymes involved in the biosynthetic pathway to mevalonic acid. Orange fruit was shown to contain acetyl-CoA synthetase (EC.6.2.1.1) forming acetyl coenzyme A from acetate. It also contained the iodoacetamide-sensitive acetoacetyl-CoA thiolase (EC.2.3.1.9) the enzyme responsible for the synthesis of acetoacetyl coenzyme A. Hydroxymethylglutaryl-CoA synthase (EC.4.1.3.5) and the corresponding reductase (EC.1.1.1.34), however, were not found in preparations from orange fruit. Nonetheless, acetoacetyl coenzyme A, formed by the thiolase, was further metabolized in the mature fruit via 3-hydroxybutyryl-CoA dehydrogenase (EC.1.1.1.35), a constituent enzyme in lipid biosynthesis. These results suggest that mature orange fruit derives mevalonic acid or other necessary basic intermediates for terpenoid biosynthesis from other sources.

INTRODUCTION

THE ROLE of enzymes in the natural ripening of citrus fruit has never been adequately explored, particularly in relation to biogenesis of flavor. The lack of knowledge of the enzymic make-up in citrus fruit is exemplified by the review of Braverman (1949) who discussed enzymes in general as minor constituents of citrus juice. So far, very few enzymes have been reported to be present in citrus fruit and these are amply reviewed in Agricultural Handbook No. 98 (Agricultural Research Service, 1956) and more recently by Joslyn et al. (1961).

The recent attempts to use active mitochondrial preparations, isolated from juice sacs of mature oranges, for flavor improvement in orange juice concentrates (Attaway et al., 1966) is a progressive step. The characteristic flavor of citrus fruit is generally attributed to terpenoid compounds which are predominantly present in the volatile citrus essence (Stanley, 1958). A proper understanding of the sequential reactions leading to the formation of terpenoids would contribute to the ultimate goal of flavor improvement of processed citrus products with enzymes. The present study is an outcome of such a supposition.

Ever since the discovery by Tavormina et al. (1956) that mevalonic acid is an intermediate in cholesterol biosynthesis, there has developed an increasing interest in the role of this compound both in sterol and terpenoid syntheses (Popjak, 1959; Purcell et al., 1959; Ritting et al., 1959).

A number of laboratories have investigated the biogenesis of mevalonic acid from acetate or acetyl coenzyme A by yeast and animal systems (Brodie et al., 1960; Lynen et al., 1958; Rudney et al., 1959). These investigations indicated that acetoacetyl coenzyme A and β-hydroxy-β-methylglutaryl coenzyme A are intermediates in the formation of mevalonic acid.

Millerd et al. (1953) as early as 1953 reported that plants contain enzymes, not only for the formation of acetyl coenzyme A, but also for the conduct of the subsequent condensation reaction to form acetoacetyl coenzyme A. Seifter (1954) reported occurrence of coenzyme A in plants, including orange. The biosynthesis of mevalonic acid usually proceeds by the condensation of one mole of acetoacetyl coenzyme A with one mole of acetyl coenzyme A to form hydroxymethylglutaryl coenzyme A, followed by reduction, by a pyridine nucleotide linked reductase, to mevalonic acid. The mechanism of acetate activation and the subsequent related enzymatic transformations remain to be elucidated in fruit tissues.

As a first step in the investigation of enzymology of isoprenoid formation from acetate, this study includes data pertaining to acetate activation and acetoacetyl coenzyme A formation in addition to hydroxymethylglutaryl coenzyme A synthesis and mevalonic acid formation by mature orange fruit preparations.

EXPERIMENTAL

Preparation of enzyme extracts

Valencia oranges having Brix/acid ratio of more than 8 were selected for the present study. Cleaned fruit, carefully peeled and freed of adhering albedo, were fast-frozen by dropping into liquid nitrogen. Using a hand-press and applying light pressure, the brittle chunks were broken down to individual juice sacs which were carefully separated from segment membranes and seeds, and stored at —50°C. Peel and albedo tissues were separately frozen and stored at —50°C. Particular care was taken to scrape off the adhering albedo from the peel before being frozen. These fast-frozen materials were used for enzyme preparations as needed. Weighed amounts of frozen vesicles were thoroughly mixed in liquid nitrogen with requisite amounts of 1 *M* tris buffer, pH 10 (as predetermined to give a final pH of 8) and were comminuted in a stainless steel Waring blendor for a few seconds.

All operations were conducted at the temperature of liquid nitrogen (—196°C). The frozen powder was thawed to about 4°C, homogenized in a Potter-Elvehjem glass homogenizer and strained through a double-layered cheese cloth. The strained juice was then spun in a Lourdes Beta-fuge with 9RA Rotor at 5,000 rpm for 30 min. The supernatant was either used as such or purified further for enzymatic studies.

Enzyme assays

The activities of the following enzymes under study were determined on duplicate samples by the methods described below. Absorptivity was measured on Beckman DB recording spectrophotometer or Beckman DU spectrophotometer with Gilford modifications. Three-ml silica cuvettes with 1-cm light path were used.

Acetate kinase activity (EC.2.7.2.1) was measured by the rate of formation of acetyl phosphate (Rose et al., 1955); phosphate acetyl transferase (EC.2.3.1.8) was assayed by the rate of disappearance of acetyl phosphate (Stadtmann, 1955); acetyl-CoA synthetase (EC.6.2.1.1) was determined by both hydroxylamine trapping method (Jones et al., 1955) as well as oxalacetate trapping system (Beinert et al., 1953).

Orange fruit preparations were also examined for the presence of acetyl-CoA hydrolase (EC.3.1.2.1) by the method of Gergely (1955). The rate of disappearance of acetoacetyl coenzyme A in presence of coenzyme A was taken as a criterion for the assay of acetoacetyl-CoA thiolase (EC.-2.3.1.9) (Stern, 1955).

[a]NRC-Postdoctoral Research Associate.

Hydroxymethylglutaryl-CoA synthase (EC.4.1.3.5) was assayed by recording the decrease in absorption at 310 mμ on the addition of acetyl coenzyme A to acetoacetyl coenzyme A (Carr, 1962). The reductase, transforming hydroxymethylglutaryl coenzyme A to mevalonic acid (EC.1.1.1.34) was assayed with reduced nicotinamide adenine dinucleotide phosphate ($NADPH_2$), according to Knappe et al. (1959).

The alternate metabolism of acetoacetyl coenzyme A via 3-hydroxybutyryl-CoA dehydrogenase using $NADH_2$ (EC.1.1.1.35) was evaluated by measuring the loss of absorption at 340 mμ on acetoacetyl coenzyme A addition to $NADH_2$, the system being controlled by blanking against a cuvette measuring $NADH_2$ oxidase activity (Lynen et al., 1955). The presence of mevaldate reductase (NADP) (EC.1.1.1.33) in the preparation was tested by quantitating the formation of $NADPH_2$ from NADP in presence of added mevalonic acid.

A protein fraction, precipitated by 90% saturation with ammonium sulfate was examined for the presence of enzymes capable of incorporating acetate ions into mevalonic acid. This enzyme fraction (about 10 mg protein) was incubated with 10 μmoles $NADPH_2$, 5 μmoles coenzyme A, 10 μmoles $MgCl_2$, 50 μmoles potassium acetate, 20 μmoles reduced glutathione and 200 μmoles tris buffer (pH 8) in a final volume of 5 ml. After 2–3 hr incubation at 30°C, the pH of the reaction system was raised to more than 12. After 15 min, concentrated HCl was added to lower the pH to less than 2. At the end of 15 min, mevalonic acid, in its lactone form, was extracted into diethyl ether in a two-step extraction procedure as described by Linn (1967). The residue, after evaporation of ether, was extracted with small amounts of acetone and the mevalonic lactone in the extract was estimated by either gas liquid chromatography (GLC) on diethyl glycol succinate column (Hinse et al., 1967) or thin layer chromatography (TLC) on $CaCO_3$-Supercel with benzene as developing solvent (Linn, 1967). In some experiments acetate-2-^{14}C (20 μc) was included in the incubation system and 50 μmoles of DL-mevalonic lactone were added at the end of the incubation period. The mevalonic acid fraction, separated on TLC was counted in a Packard Tri-Carb Scintillation spectrometer (Model 4322).

Protein estimations

Proteins were estimated in samples by measuring turbidity, produced by 2.5% sulfosalicylic acid, at 600 mμ wave length and referring to a standard curve obtained with crystalline egg albumin (Layne, 1957).

Chemicals

Acetic-2-^{14}C acid was purchased from New England Nuclear Corporation, Boston, Massachusetts. Hydroxymethylglutaryl coenzyme A was prepared using a yeast fraction as outlined by Ferguson et al. (1959). All other chemicals were obtained from commercial sources and were of highest purity available.

Table 1—Acetyl-CoA synthetase of orange fruit.

Enzyme preparation	μMoles of acetyl coenzyme A formed by 1 mg protein in 20 min. Hydroxylamine method[1]	Oxalacetate trapping system[2]
Vesicular extract	0.12	—
Amm. sulfate fraction I 0–0.3 saturation	<0.10	<0.010
Amm. sulfate fraction II 0.3–0.6 saturation	0.24	0.179
Amm. sulfate fraction III 0.6–0.9 saturation	<0.10	0.013

[1] The reaction system contained in a total volume of 3 ml, 10 μmoles ATP, 20 μmoles pot. acetate, 100 μmoles tris buffer (pH 7.4), 200 μmoles neutral hydroxylamine, 10 μmoles $MgCl_2$, 1 μmole coenzyme A and 0.2 to 1 mg enzyme protein. After 20 min reaction was stopped with 1 ml of 10% trichloracetic acid and acethydroxamate was estimated in the supernatant as the ferric complex.

[2] The reaction system consisted of 100 μmoles tris buffer (pH 7.4), 20 μmoles pot. acetate, 10 μmoles $MgCl_2$, 10 μmoles reduced glutathione, 10 μmoles ATP, 1 μmole coenzyme A, 0.3 μmole NAD, 50 μg each of malic dehydrogenase and citrate synthetase, 10 μmole L-malic acid and 0.1 to 1 mg enzyme protein in a final volume of 3 ml. Acetyl CoA was calculated from the increase in absorption at 340 mμ.

RESULTS & DISCUSSION

Acetate activation

Two possible mechanisms of acetate activation were examined, i.e., acetyl-CoA synthetase and acetate kinase. As the results in Table 1 show orange fruit is able to synthesize acetyl coenzyme A from acetate via acetyl-CoA synthetase. The hydroxylamine method, although commonly used for the detection of this enzyme, was found not to be very sensitive. But the oxalacetate-trapping system provided an excellent method for assaying acetyl-CoA synthetase activity even in low concentrations. The only disadvantage was that it could not be employed for crude preparations. The second mechanism of acetate activation via acetate kinase and phosphate acetyl transferase was absent in these preparations. It has been generally found that acetyl-CoA synthetase is distributed over a broad spectrum of living species, while acetate kinase system has been observed so far only in the microbial kingdom (Rose et al., 1955).

Table 2 illustrates the relative distribution of acetyl-CoA synthetase activity in the whole fruit. The 0.6–0.9 saturated ammonium sulfate precipitates of extracts from juice sacs, albedo and peel were used since the enzyme activity was largely concentrated in this fraction. The fraction isolated from juice vesicles is considerably more active in forming acetyl coenzyme A from acetate, than those from albedo and peel. Seifter (1954) reported that coenzyme A content in orange pulp is considerably higher than that in other portions of the fruit. Thus, the ready availability of coenzyme A might be responsible for the presence of more coenzyme A-utilizing enzymes such as acetyl-CoA synthetase in the juice vesicles.

Although the enzyme activity observed in the present study is somewhat lower than that obtained with enzymes isolated from other sources, nevertheless, it does represent a clear demonstration of the presence of this enzyme in orange fruit. No attempts were made to purify the enzyme beyond ammonium sulfate fractionation. Low acetyl-CoA synthetase activity was observed also by Millerd et al. (1953) in spinach leaf and a variety of other plant tissues. In a recent report Garth-Everson et al. (1962) noted with isolated chloroplast preparations that acetyl-CoA synthetase activity contribute relatively little to the formation of lipids compared to incorporation of CO_2 by photosynthetic fixation. This could be a reason why plant tissues possess very low acetyl-CoA synthetase activity since bulk of their energy requirements are derived via CO_2 assimilation by photofixation.

The possibility that an acetyl-CoA hydrolase system depressed the detection of acetyl coenzyme A was examined. But none of the preparations used for the present study showed hydrolase activity.

Acetoacetyl coenzyme-A formation

A centrally important reaction of acetyl coenzyme-A is that involving the formation of acetoacetyl coenzyme A. The equilibrium of the reaction, 2 acetyl coenzyme A $\rightleftharpoons$ acetoacetyl coenzyme A + coenzyme A, mediated by acetoacetyl-CoA thiolase lies far to the left, providing a convenient assay of the enzyme by monitoring the rate of backward reaction. Table 3 presents evidence for the existence of acetoacetyl-CoA thiolase in orange juice vesicles. The enzyme activity was found to be concentrated in a fraction precipitating between 0.3 and 0.6 am-

Table 2—Distribution of acetyl-CoA synthetase in whole orange fruit.

Enzyme source	μMoles of acetyl CoA formed in 20 min by 1 mg enzyme protein: Hydroxylamine method	Oxalacetate trapping system
Juice vesicles	0.22	0.17
Albedo	<0.10	0.02
Peel	<0.10	0.03

Amm. sulfate fractions obtained between 0.3 and 0.6 saturation were used. Other details as in Table 1.

Fig. 1—Effect of iodoacetamide on acetoacetyl-CoA thiolase activity. Details are as in Table 3. 32 μg of enzyme protein was used.

Fig. 2—The metabolic fate of acetoacetyl coenzyme A in orange fruit. The reaction system contained 200 μmoles tris buffer (pH 8), 0.2 μmole acetoacetyl coenzyme A, 10 μmole reduced glutathione, 10 μmole $MgCl_2$ and 200 μg of enzyme protein (0.3 to 0.6 amm. sulfate saturated ppt) in a final volume of 3 ml. Optical density was recorded for 2 min before making the indicated additions.

Table 3—Acetoacetyl-CoA thiolase activity of orange fruit.

Enzyme preparation	μMoles of acetoacetyl coenzyme A consumed in 1 min by 1 mg protein
Vesicular extract	64×10^{-3}
Amm. sulfate fraction I 0–0.3 saturation	35×10^{-3}
Amm. sulfate fraction II 0.3–0.6 saturation	126×10^{-3}
Amm. sulfate fraction III 0.6–0.9 saturation	0
Alcohol fraction 67% alcohol ppt.	281×10^{-3}

Each cuvette contained, in a total volume of 3 ml, 200 μmoles tris buffer (pH 8), 8 μmoles $MgCl_2$, 1 μmole coenzyme A, 10 μmoles reduced glutathione and 0.2 μmole acetoacetyl coenzyme A. The reaction was started by introducing enzyme (0.05 to 0.2 mg protein) and the rate was measured by recording absorption at 310 mμ.

Table 4—Distribution of acetoacetyl CoA thiolase activity in whole orange fruit.

Enzyme source	μMoles of acetoacetyl CoA consumed in 1 min by 1 mg protein
Juice vesicles	282×10^{-3}
Albedo	46×10^{-3}
Peel	125×10^{-3}

The proteins, precipitated with ethyl alcohol at a final concentration of 67% were used as enzyme source. Other details were same as in Table 3.

monium sulfate saturation. About fourfold increase in specific activity was obtained by precipitation with ethyl alcohol. The enzyme activity was also observed in albedo and peel tissues, though to a lesser extent (Table 4).

Acetoacetyl-CoA thiolase of orange was found to be inhibited by iodoacetamide which at a concentration $15 \times 10^{-4}M$ inactivates the enzyme almost completely under the conditions of the experiment (Fig. 1). Lynen (1953) also observed similar inhibition of thiolase activity by iodoacetamide and implicated the involvement of sulfhydryl groups in activity of the enzyme.

Formation of mevalonic acid

The next step in the biosynthesis of mevalonic acid is the formation of hydroxymethylglutaryl coenzyme A by the corresponding synthase. An exhaustive search of various enzyme preparations from orange fruit failed to show up any hydroxymethylglutaryl-CoA synthase activity. This could mean a possible block in the sequential reaction leading to mevalonic acid.

The final step in this sequence is the $NADPH_2$-dependent reductive transformation of hydroxymethylglutaryl coenzyme A to mevalonic acid by the corresponding reductase. The orange fruit preparations which were tested for the reductase activity were found to lack this

Table 5—3-Hydroxybutyryl-CoA dehydrogenase activity of orange fruit.

Enzyme preparation	μMoles of $NADH_2$ used in 1 min by 1 mg enzyme protein[1]
1. Vesicular extract	12.3×10^{-3}
2. Dowex treated extract[2]	27.3×10^{-3}
3. 0.3–0.6 Saturated amm. sulfate fraction	83.3×10^{-3}
4. Same as 3 but dialysed[3]	98.1×10^{-3}

[1] Each cuvette contained in a total volume of 3 ml, 200 μmoles tris buffer (pH 8), 0.25 μmoles $NADH_2$, 0.2 μmoles acetoacetyl coenzyme A and 0.05 to 0.2 mg enzyme protein. Rate of decrease in optical density recorded at 340 mμ.

[2] The vesicular extract was treated with Dowex 1 (Cl^-) for 30 min.

[3] The fraction precipitated between 0.3 and 0.6 amm. sulfate saturation, was dialysed against 0.001 tris buffer (pH 7.4) for 3 hr at 4°C.

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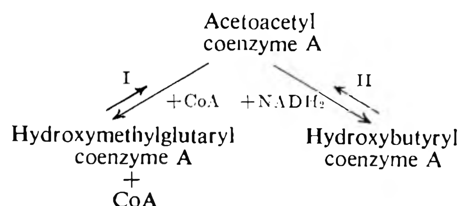
enzyme. The absence of the two above enzymes is confirmed by the inability of orange vesicular preparations, when incubated with acetate and other necessary cofactors, to form mevalonic acid as tested by GLC, TLC and radioisotopic techniques. Moreover, these preparations do not show mevaldic acid reductase activity which precludes the possibility that mevalonic acid was lost from the system as mevaldic acid.

Alternate metabolism of acetoacetyl coenzyme A

Acetoacetyl coenzyme A, once formed, could go either into isoprenoid route or lipid pathway depending on the nature of cofactors available.



The absence of reaction I in orange makes reaction II the probable route for the metabolism of acetoacetyl coenzyme A. A protein fraction precipitating between 0.3 and 0.6 ammonium sulfate saturation showed sizeable 3-hydroxybutyryl-CoA dehydrogenase activity. Thus, the acetoacetyl coenzyme A formed by thiolase would participate in this reaction (Fig. 2). While addition of acetyl coenzyme A to the system had no effect on the reaction, coenzyme A and $NADH_2$ increased the rate of disappearance of acetoacetyl coenzyme A.

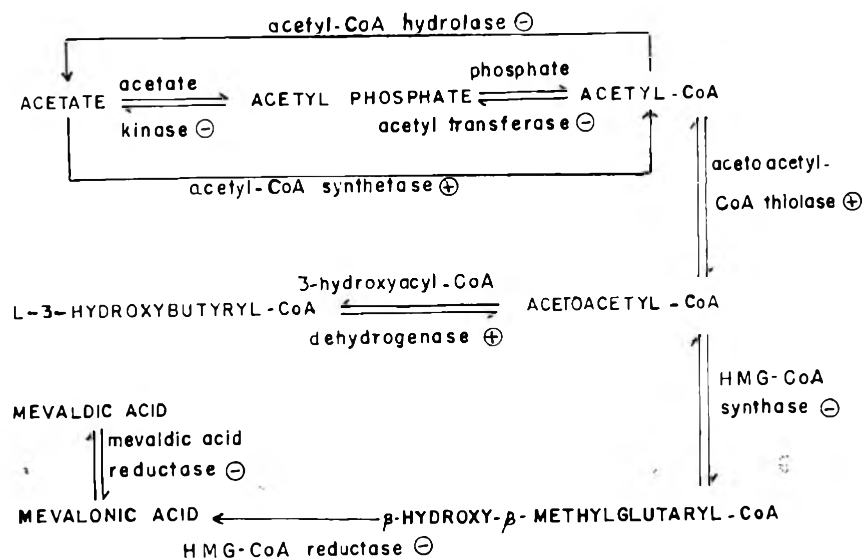


Fig. 3—Enzymes involved in mevalonic biosynthesis in orange fruit +, present; —, absent.

CONCLUSION

THE FINDINGS, summarized in Figure 3, point out that orange fruit is defective in the enzymic make-up from the standpoint of mevalonic acid synthesis. But it definitely possesses a mechanism to synthesize higher fatty acids from acetate.

Future efforts will be directed towards identifying the basic intermediate from which the fruit builds up the more complicated terpenoid molecules and the nature of enzymes involved in such transformations.

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Mention of brand names does not imply endorsement.

CARLOS WILD-ALTAMIRANO
Departamento de Ingeniería Bioquímica, Escuela Nacional de Ciencias Biológicas, Instituto Politécnico Nacional
México, D. F., México

Enzymic Activity during Growth of Vanilla Fruit 1. Proteinase, Glucosidase , Peroxidase and Polyphenoloxidase

SUMMARY—A preliminary study has been made of these enzymic activities along the growth of vanilla pods looking for information about biochemical reactions which lead to the production of the aroma. Proteinase activity decreases with pod age. The activities of glucosidase, peroxidase and polyphenoloxidase increase with stage of growth reaching a maximum near or at ripening. Possibly these enzymes play some role in reactions taking place in curing vanilla, either directly or indirectly supplying the initial products to be transformed into those responsible for color and odor. Partially purified polyphenoloxidase shows a ration catecholase-cresolase activity of 8.9.

INTRODUCTION

VERY LITTLE is known about the biochemical reactions taking place during ripening and curing of the fruit of vanilla (*Vanilla planifolia*), and which lead to the conditions required for obtaining aromatic extracts. Balls, et al. (1941), observed that the traditional methods of processing vanilla beans to produce the aroma caused a marked increase in the rate of evolution of carbon dioxide from the tissues. They suggested that the basis of the curing might be an accelerated rate of overall oxidation. They also found that vanilla beans contain a large amount of peroxidase, enzyme which alongside of polyphenoloxidase and phenols remains after the curing process is finished.

Arana (1944) undertook a study involving a comparative evaluation of the various commercial methods of curing vanilla beans in which an attempt was made to correlate the quality of the product with the chemistry of the curing. She reports data of moisture, glucovanillin and vanillin contents, phenol value, as well as the activities of the enzymes glucosidase and peroxidase.

Curing of other plant products like tea, cacao and tobacco presents similar problems, to those encountered in vanilla (Forsyth, 1964). Forsyth, et al. (1963), reviewed the existent data on the biochemistry of cacao and the mechanism of curing and pointed out the importance of phenolic constituents of the seeds with regard to color and flavor. Concerning enzyme contents a host of catalyzers, e.g., invertase, amylase, glucosidase, various oxidases, catalase, peroxidase and a proteinase have been reported. However, Ciferri (1931) failed to detect proteinase activity in cacao.

Forsyth, et al. (1963), comment that since protein hydrolysis is known to occur during fermentation (De Witt, 1961), the presence of a proteinase in cacao beans may be accepted, in accordance with an earlier finding of Brill (1915). These observations led us to presume the existence of a proteinase in vanilla, which we did confirm. In addition to the activity of this enzyme, we present the activities of glucosidase, peroxidase and polyphenoloxidase. These activities were determined at various stages of growth of vanilla pods. At the same time, an attempt was made to purify proteinase and polyphenoloxidase to investigate some of their properties.

EXPERIMENTAL

Material

Vanilla pods were obtained from a plantation located at Gutierrez Zamora in Veracruz State, México. They were collected monthly from June through October, from early stages of development until full ripening of the pods.

Reagents

All chemicals utilized in the preparation of buffers, etc. were of analytic reagent grade.

Enzymic substrates

Casein and hemoglobin were used as substrates for determining proteolytic activity. Casein, prepared according to Hammarsten was obtained from Merck, Darmstadt. Bovine hemoglobin was prepared from beef blood by the method of Anson (1939). Guaiacol and hydrogen peroxidase from Mallinckrodt were employed in the assay of peroxidase. The substrate for glucosidase was *p*-nitrophenyl D-glucopyranoside (PNPG) grade A from Calbiochem. In determining polyphenoloxidase activity the following chemicals were utilized: catechol, resorcinol, hydroquinone, pyrogallol, phloroglucinol and tyrosine from Eastman Organic Chemicals; 3,4 dihydroxphenylalanine, chlorogenic acid from Sigma Chemical Co.; phenol from Baker, and *o*- and *p*-cresol from Coleman and Bell Co. All of these reagents were of the best grade available.

Detergent

Polyoxyethylene lauryl ether (Brij 35) utilized for solubilizing vanilla proteinase, was purchased from Atlas Powder Co.

Gel filtration and ion exchanger materials

Sephadex G-50, G-75 and DEAE Sephadex A 50 were obtained from Pharmacia Fine Chemicals, Inc., Uppsala, Sweden.

PROCEDURES

Preparation of acetone powder

One hundred g of vanilla pods were macerated with 100 ml of 50% aqueous acetone at $-5°C$ in a blendor and then filtered through cheesecloth. The residue was washed twice with 100 ml of 80% acetone and once more with pure acetone filtering through porous glass funnel (coarse). The final residue was spread over filter paper and let dry in the cold room. The dry powder was kept in a vacuum desiccator at 5°C.

Determination of proteolytic activity

The action of proteinase on casein was measured by the procedure of Kunitz (1947) as further modified by Castañeda Agulló (1956) to make it suitable to small amounts of enzyme. The time of incubation was 18 hr at 37°C unless otherwise indicated. In controls trichloroacetic acid solution was added prior to the addition of the enzyme. The increase in absorbancy at 280 mμ was read in a Zeiss Spectrophotometer Model PMQ II. The activity unit utilized throughout this work is the activity of a preparation for which $\Delta A_{280} = 1$ under the stated conditions. For the digestion of hemoglobin at various pH values, the Anson (1938) method was followed.

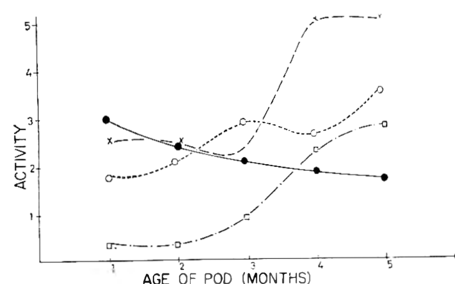

Fig. 1—Enzymatic activity as a function of vanilla pod age. Crosses: polyphenoloxidase units × 10^{-1}. Filled circles proteinase units. Open circles: glucosidase, concentration of substrate hydrolyzed M × 10^{-8}. Squares: peroxidase equivalent to HRP mg/ml × 10^{-3}.

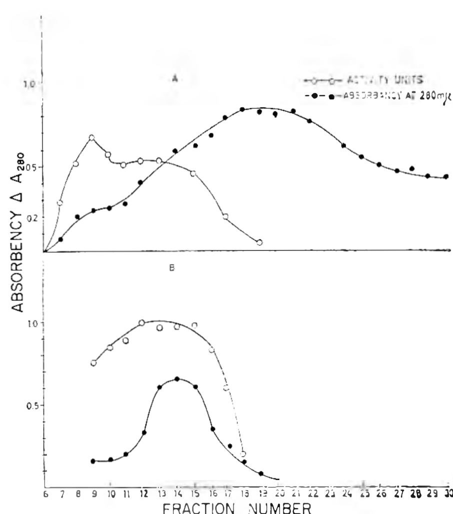

Fig. 2—Purification of vanilla proteinase by columns of DEAE-Sephadex A —50. (A) first elution, (B) second elution of (A) fractions 7 to 17. Filled circles: absorbancy at 280 mμ. Open circles: activity units.

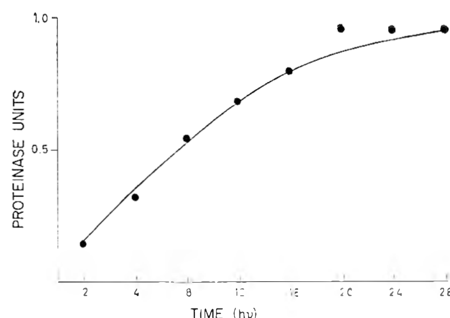

Fig. 3—Time of casein hydrolysis by purified vanilla proteinase. The reaction mixture (2 ml) contained 1% casein and 80 μg/ml enzyme. Temp. 37° C, pH 8.

The proteolytic activity was determined in extracts of acetone powder prepared this way: to 50 mg powder 5 ml of 0.25*M* phosphate buffer pH 6.5 was added, the suspension stirred 5 min and then filtered.

Assay of peroxidase

This enzyme was assayed in extracts prepared by grinding 25 g fresh pods with 25 ml 0.05*M* acetate-acetic acid buffer pH 5 containing 0.05*M* EDTA, solution which was used by Dávila, et al. (1966) to extract proteinase peroxidase activities from the latex of *Pileus mexicanus*. The activity was measured by the procedure of Maehly, et al. (1954). The following formula of these authors was employed to calculate the concentration of peroxidase in mg per ml assuming the same molecular weight and specific activity for horseradish and vanilla peroxidase (provisionally and for the sake of convenience).

$$\frac{\text{mg Perox}}{\text{ml}} = \frac{1}{t} \times 0.98 \times 10^{-8} \times 44{,}000 \times \text{dilution}$$

In the above equation t stands for the time required for the enzyme to change the absorbancy at 470 mμ of a guaiacol solution by 0.05 units.

Polyphenoloxidase activity

This was measured according to the procedure of Patil, et al. (1965), in extracts obtained from acetone powder treated with 0.1 phosphate buffer pH 5.7 at the proportion of 10 ml to 1 g. They define one activity unit as that which makes the absorbancy at 265 mμ of catechol or chlorogenic acid decrease at the rate of 0.01 per min starting 15 sec after enzyme addition. The conditions maintained in the present investigation were the same stated by Patil, et al. (1965), excepting the concentration of ascorbic acid which was 5.6 × $10^{-5}M$, that is, twice as much as that used by Patil, et al.

Activity of glucosidase

This was extracted from acetone powder with 0.2*M* phosphate buffer pH 7 (10 ml to 1 g). The assay was conducted by a procedure similar to that of Lederberg (1950) for galactosidase but using PNPG as substrate instead of ONPG (*o*-nitrophenyl D-galactoside). The time of incubation of the enzyme-substrate mixture was 30 min but the activity is expressed in moles of *p*-nitrophenol liberated in 10 min.

Determination of protein content in the extracts

This was accomplished by the method of Lowry, et al. (1951), and by analysis of protein nitrogen by micro Kjeldahl (Legget, 1962).

RESULTS

Enzymic activity during pod growth

The activity of proteinase, peroxidase, glucosidase and polyphenoloxidase was followed through the age of 5 months, from June to October. Figure 1 shows the change in activity of the four enzymes as a function of growth. While proteolytic activity decreases with pod age, that of peroxidase is elevated markedly from the third month onward. The activity of both polyphenoloxidase and glucosidase also increases with age, but less notably.

Purification of proteinase

Various extracting solutions were tested to extract the maximum activity of acetone powder and the one which gave best results was 0.25*M* phosphate buffer pH 6.5 containing 0.25% polyoxyethylenelauryl ether (Brij 35).

In a first attempt to purify vanilla proteinase, we utilized extracts of acetone powder obtained from three month old pods. Under these conditions, peroxidase activity always accompanied that of proteinase. We failed to separate both activities by fractionating the extracts through columns of either Sephadex G-50, G-75 or carboxymethycellulose. However, it was possible to obtain a proteinase preparation free from peroxidase activity taking advantage of the previous observation that at 1 month of age vanilla pods showed slight activity of peroxidase whereas proteinase activity was maximum. Consequently, pods of less than a month were used as raw material for the preparation of acetone powder and extraction of proteinase.

A description of the technique utilized 4 g acetone powder prepared as described under procedures were extracted with 90 ml 0.25*M* phosphate buffer p 6.5 with 0.25*M* Brij. Out of this extract, 80 ml were reduced to about 20 ml with the aid of a Büchi Rotavapor and applied to column of DEAE-Sephadex A-50 (140-400 mesh) previously stabilized in 0.02*M* phosphate buffer pH 6.6. No difference was observed in the elution pattern and activity of the final preparation, when the extract was dialyzed prior to chromatographic separation. The dimensions of the column were 3.4 cm. × 20 cm. The elution was carried out with 0.01*M* phosphate buffer pH 6.6 at a rate of 0.25 ml/min, collecting 5 ml fractions.

The elution diagram is presented in Fig. 2a. The proteolytic activity was found in fractions 7 to 17. These were mixed together recovering 110 ml solution which was then reduced to 39 ml and fractionated again through DEAE-Sephadex with the same eluant used before. The elution is shown in Fig. 2b. The fractions with proteolytic activity, i.e., from 10 to 17 were gathered and the volume reduced to 25 ml. This stock solution which contained 0.96 mg/ml protein and 6.4 activity units/ml was used.

The degree of purification of proteinase at each stage is shown in Table 1.

Table 1—Calculations of the yield in the extraction of the proteinase

	Volume of buffer, ml	Units/ml	Units/mg	Total Units	Yield, %	Purification factor
Extraction from acetonic powder. Original volume	80	2.24	1.06	170		
First elution step from DEAE Sephadex. Volume reduced at vacuum	39	4.48	2.58	174	97	2.4
Second elution step from DEAE Sephadex. Volume reduced at vacuum	25	6.40	6.60	160	89	6.2

The yield was 28 mg protein with proteolytic activity per 100 g fresh pods.

Electrophoresis in acrylamine

The procedure of Ornstein, et al. (1962), was followed. The power supply was adjusted to 5 MA and 200 V. For a run 1 ml stock solution was freeze-dried and dissolved in 0.5 ml 5% sucrose solution. In each of three columns 0.03, 0.05 and 0.1 ml was placed. In Tris buffer pH 8 the samples migrated to the anode and gave rise to a single band, suggesting the possibility that the enzyme is homogeneous.

Effect of pH and time course of casein hydrolysis catalyzed by vanilla proteinase

Fig. 3 shows the course of casein hydrolysis at pH 8, up to 28 hr. The liberation of soluble products absorbing light at 280 mμ reaches a maximum at about 20 hr and then remains constant. No significant difference was observed when the casein pH was varied in the range of 7 to 10.

Effect of temperature on casein hydrolysis

The reaction rate increases when temperature is elevated up to 37°C and then the curve levels off within the range studied (Fig. 4).

Michaelis Menten constant

Considering a molecular weight of 33,600 for casein (Burk, et al., 1930) a value, $1.72 \times 10^{-4} M$ was obtained for K_m at pH 8 and 37°C.

Figure 5 shows the double reciprocal plot from which the above datum was calculated.

Digestion of hemoglobin

Hemoglobin is hydrolyzed by vanilla proteinase at an increasing rate as pH varied from 6 to 9 (Fig. 6).

Effect of heavy-metal ions

EDTA (ethylenediamino tetracetate) and cyanide on the proteolytic activity. Since many plant proteinases contain thiol groups essential for their activity, we investigated, whether the activity of vanilla proteinase depends upon the presence of such groups. The results showed (Table 2) that there is no significant effect of any of the reagents tested, suggesting that in the present case thiol groups apparently do not play a catalytic function.

Table 2—Effect of thiol reagent, EDTA and Cyanide ion on proteolytic activity

Reagent	Concentration	A_{280}
	Control	0.90
$HgCl_2$	$10^{-3}M$	0.80
	$10^{-4}M$	0.86
	$10^{-5}M$	0.92
$PbCl_2$	$10^{-3}M$	0.92
	$10^{-4}M$	0.92
	$10^{-5}M$	0.93
p-Chloro Hg benzoate	$10^{-3}M$	0.87
	$10^{-4}M$	0.89
	$10^{-5}M$	0.93
EDTA	$0.05M$	0.71
CN^-	$0.05M$	0.96

Extraction and purification of vanilla polyphenoloxidase

As judged from the color of the final product, it may be inferred that an intense oxidative activity acting on phenolic compounds operates along the process of curing of ripe pods of vanilla. This led us to select polyphenoloxidase as one of the most promising enzymes to investigate. We followed, with some modifications, the technique of Patil, et al. (1965), for purifying polyphenoloxidase of potato peeling. Cold acetone (3 L) was added to 1 kg mature pods (4–5 months of age) cut in pieces of about 1 cm and the mixture let stand overnight at −15°C. After decanting the supernatant liquid the frozen tissue was ground in a blendor with two volumes of fresh acetone at −15°C. The residue was filtered through cheesecloth, washed with cold acetone and then extended over filter paper until dry. The resultant acetone powder was submitted to a series of treatments as shown in Fig. 7.

Table 3 presents the specific activities, yield and purification degree of the various fractions obtained. The most active fraction, namely 2 C which is 16.6 times as active as the original extract was utilized in the assay of cresolase and cate-

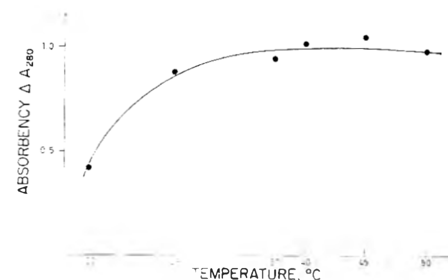

Fig. 4—Effect of temperature on casein hydrolysis catalyzed by vanilla proteinase.

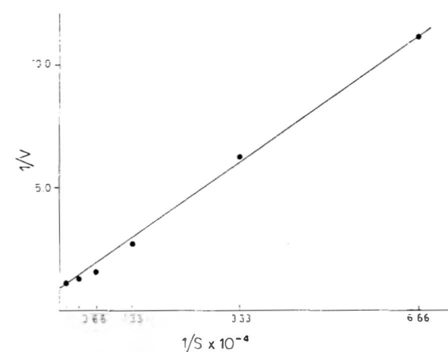

Fig. 5—Double reciprocal plot for the hydrolysis of casein catalyzed by purified vanilla proteinase. Temp. 37°C, pH 8. Calculated K_M/1.72 × 10^{-4} mol/L.

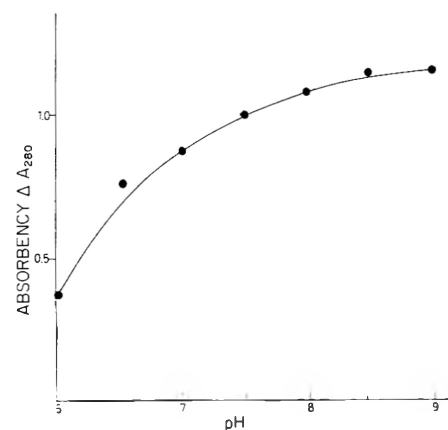

Fig. 6—Digestion of hemoglobin by vanilla proteinase at different pH.

Table 3—Calculations of the yield in the extraction of polyphenoloxidase

	Volume, buffer/ ml	Units[1]/ ml	Units/mg	Total	Yield, %	Purification factor
First extract	570	120	60	68 400		
First precipitate solution						
1A	220	200	—	44 000	64	
1D	160	120	—	19 200		
1E	6	900	523	5 400		8.6
Second precipitate solution						
2A	110	130	—	14 300	48 1D + 2A	
2C	5	1250	1000	6 250	17 1E + 2C	16.6

[1] Measured as catecholoxidase activity.

cholase activity on a series of substrates. Results are given in Table 4.

The ratio of catecholase to cresolase activity for vanilla polyphenoloxidase is 8.9 whereas those obtained for its counterparts in cacao and *Polyporus* are 2.2 and 3.4, respectively (Moore, et al., 1952). The best three substrates for vanilla peroxidase are catechol, pyrogallol and caffeic acid.

DISCUSSION

THE TREND of variation of activity with pod growth of the various enzymes studied might be considered as an index of the relative importance of the metabolic processes taking place at a given stage. For instance, proteolysis apparently becomes less important as ripening approaches. The increase of the other activities assayed suggests that at the time maturity is reached carbohydrate degradative reactions and oxidative activities acquire more preponderance. The absence of peroxidase activity in pods of less than a month of age raises the question as to whether this enzyme (1) is not synthesized at early stages of growth or (2) is present but inhibited. This is a problem to be elucidated by further work.

Even though the polyphenoloxidase obtained from vanilla shows a ratio catecholase: cresolase of 8.9, this enzyme still can be considered as an hydroxylant one as compared to other phenolases with greater values for this ratio. It is possible that glucosidase, peroxidase and especially polyphenoloxidase contribute in some way or another to the formation of the various products which bestow on vanilla its characteristic aroma and color.

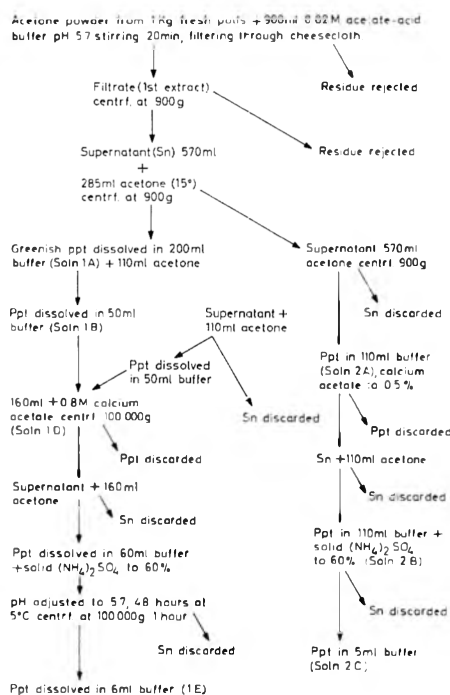

Fig. 7—Extraction and purification of polyphenoloxidase from the fresh pods of Vanilla.

Table 4—Activity of polyphenoloxidase on different substrates

Substrates, 2.8 × 10^{-4}*M*	Units/mg
o-Diphenols	
Catechol	1000
Resorcinol	48
Hydroquinone	80
Pyrogallol	960
Phloroglucinol	56
3,4-Dihydroxyphenylalanine	360
Caffeic acid	320
Chlorogenic acid	440
Quercetin	120
Monophenols	
Phenol	48
Tyrosine	64
p-cresol	112
o-cresol	56
Vainillin	80

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NORMAN D. HEIDELBAUGH[a] and DAVID J. GIRON
USAF School of Aerospace Medicine, Aerospace Medical Division (AFSC)
Brooks Air Force Base, Texas

Effect of Processing on Recovery of Polio Virus From Inoculated Foods

SUMMARY—The recovery of polio virus inoculated into a variety of foods was studied as a function of four food processing operations: freeze dehydration, "pasteurization" by exposure to Cobalt-60 gamma irradiation, refrigerated storage at 4°C, and storage at 20°C. In general, a decrease in virus recovery was noted following each of the operations. Complete loss in recoverability was observed only in highly acid foods (below pH 2.9) stored at 20°C for 96 or 168 hr. Use of a DEAE Sephadex chromatographic column was found to have potential application for separation of virus foods.

INTRODUCTION

INGESTION OF certain foods has been epidemiologically associated with outbreaks of several acute and chronic viral diseases (Becker, 1966; Berg, 1964; Cliver, 1966; Dack, 1964; Lemon, 1964). These findings suggest that low concentrations of viruses infective to man can gain entrance to foods during their production, processing, or preparation. While it is known that viruses do not multiply outside the living cell, little is known concerning the survival of virus in processed foods.

Information on the survival of virus in processed foods is necessary to an understanding of the epidemiology of food-borne virus diseases. Food processess designed for the destruction or control of bacteria, fungi, or molds cannot be assumed to remove or destroy food-borne viruses (Heidelbaugh et al., 1968a).

In the studies reported here, polio virus was inoculated into foods, and the recoverability of the virus was determined after the foods were subjected to a food processing operation. The processing operation employed was either: freeze dehydration, "pasteurization" by exposure to Cobalt-60 gamma irradiation, refrigerated storage at 4°C, or storage at 20°C. The foods were selected as representing types that would normally be considered for processing by the respective operation employed.

MATERIALS & METHODS

Viruses

Polio virus Type I strains, Mahoney (PIM), and LS_c ^{2}AB Sabin (PIS), were used in this study. The PIM stock suspension contained 1×10^8 plaque-forming units (PFU)/ml. The PIS stock contained 5×10^7 PFU/ml. The stock viruses were suspended in Eagle's basal medium (Eagle, 1955) with Hanks' balanced salt base (Hanks et al. 1949). Both stock suspensions were stored at −70°C until used. Virus dilutions were made in Hanks' balanced salt solutions (BSS).

Virus titration

The S_3 line of HeLa cells was used to propagate and titrate the virus by plaque count technique using a nutrient agar overlay. The cells were grown in suspension in Eagle's basal medium with Hanks' balanced salt base supplemented with 10% calf serum. The monolayers were prepared 24 hr prior to use by seeding plaque bottles with $4\text{–}6 \times 10^6$ cells in 10 ml of medium. The plaque bottles were tightly closed glass bottles presenting approximately 75×12 mm of flat surface area. Appropriate tenfold serial dilutions of the samples to be titrated were prepared using Hanks' BSS, and 0.2 ml of each dilution was added to each of 4 bottles. The monolayers were then incubated for 30 min at 37°C after which they were overlayed with Eagle's medium containing 1% agar. After 48 hr of incubation, the cultures were stained by adding 5 ml of 0.01% neutral red in Hanks' BSS to each bottle for 30 min. Plaques were counted 4–12 hr following staining.

Inoculation of food and preparation for recovery of virus. Each test sample of food consisted of 9 g of food inoculated with 1 ml of the virus stock being studied. Following inoculation and processing, each food sample was prepared for recovery of virus by taking the entire sample and blending in a sterile mortar and pestle while diluting with 90 ml of Hanks' BSS. This preparation was poured through sterile gauze following which the filtrates were centrifuged for 10 min at $2{,}700 \times G$ in 100 ml centrifuge bottles. The supernatant fluid was decanted and filtered through a Millipore filter (HA 47 mm 0.45μ) using a stainless steel wire support. All filtrates were titrated by determining the PFU/ml in triplicate or quadruplicate for each of 4 tenfold serial dilutions in Hanks' BSS. The resulting titers were reported as titer of food filtrate.

Preparation of Sephadex column eluate for virus titration. To study the usefulness of a Sephadex column for separation of virus from foods, some of the triturated and filtered inoculated food samples were titrated directly, and also after elution from a Sephadex column. The column was prepared using DEAE Sephadex A-25, medium grade, which was allowed to swell in water. Following swelling, the fines were removed and the gel was washed in sequence by 0.5*N* hydrochloric acid, water, 0.5*N* sodium hydroxide, water, and neutralized with 0.5*N* hydrochloric acid. The gel was then suspended in M/15 phosphate buffer (pH 7.5) and packed by pouring the suspension into 14 cm $\times$ 1.2 cm glass columns. No attempt was made to sterilize either the column or the gel.

Two ml of the food filtrate were entered into the top of the column and allowed to flow through the column by gravity. The elution of all columns was carried out at room temperature with M/15 phosphate buffer at pH 7.5. The first 3 ml eluate was discarded and the next 5 ml were collected (Giron, et al., 1964). Virus content of this eluate was determined and the resulting titer reported as titer of DEAE Sephadex eluate of food filtrate.

Recovery of virus from foods after freeze dehydration. Five foods were prepared according to guides for the production of foods intended for space flight feeding systems (Hollender et al., 1965). These foods were German potato salad (pH 4.9), beef and vegetables (pH 5.7), beef pot roast (pH 5.8), chicken with gravy (pH 6.1), and salmon salad (pH 6.1). Two 9 g samples of each of the five foods were inoculated with 1 ml of the stock suspension of PIM.

Following inoculation, each food was split into two samples, both of which were frozen by holding at −20°C for 24 hr. One sample of each frozen food was selected at random and freeze dehydrated for 24 hr at a pressure between 50 and 150 μ of Hg with a platen temperature of 21.8°C and a condenser temperature of −51°C. In this manner, all dehydration was performed on the samples simultaneously. Following dehydration the foods were immediately placed in individual jars with tightly fitting screw caps and held at −20°C until it was convenient for virus titration. It was assumed that no change in virus recoverability occurred during the −20°C storage. Randomly selected specimens of the dehydrated and nondehy-

[a] Current address: Department of Nutrition and Food Science, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139.

drated foods were titrated within a 4-week period following dehydration. The dehydrated samples were rehydrated immediately prior to titration by adding the quantity of water specified in the production guides. This entire sequence of procedures was performed three times and virus titers reported as arithmetic mean values.

Recovery of virus from fish fillets after exposure to Cobalt-60 gamma irradiation. Fourteen 9 g samples of frozen dressed Whiting fish were inoculated with 1 ml of the PIS stock suspension. Following inoculation the food samples were irradiated using a 6-kilocurie Cobalt-60 source (Hardy et al., 1965) of gamma irradiation. The samples were removed for virus recovery after 0, 150, 300, 375, 450, 525 and 600 kilorad total dose was obtained. Dose rate was 1 kilorad/min. Both filtrates and DEAE Sephadex column eluates of the filtrates of the irradiated fish were titered to determine virus recoverability.

Suspensions of the virus were prepared by dilution in Hanks' BSS to contain about the same titer as that recovered from the non-irradiated fish (4.2×10^6 PFU/ml). These suspensions were irradiated at the same time as the fish fillets with the following total dosages: 0, 75, 150, 225, 300, 375, 450, 525 and 600 kilorads. Following irradiation the suspensions were titrated both directly, and after passage through a Sephadex column.

Each fish fillet specimen and virus suspension was contained in an individual glass bottle packed in melting ice (0°C) at all times during the irradiation. This entire sequence of procedures was performed three times and virus titers reported as arithmetic means.

Recovery of virus from foods following storage at 4 and 20°C. Eight 9 g samples of each of five different foods were inoculated with 1 ml of stock suspension of PIS. The five foods consisted of four commercially prepared items and one experimental food. The commercial foods were canned jellied cranberry sauce (pH 2.7), reconstituted frozen concentrated orange juice (pH 2.9), mayonnaise (pH 4.2) and pork "junior food" (pH 5.6). The experimental food (pH 7.1) was a nutrient defined formula, which had been previously developed for physiological studies (Heidelbaugh et al., 1968b). The nutrient defined formula diet is formulated as follows: dry powdered skim milk, 40 g; sucrose, 20 g; lactose, 20 g; dextrose and maltose, 100 g; coffee whitener, 100 g; sodium caseinate, 17.5 g; sodium chloride, 1.5 g; and magnesium oxide, 0.1 g. This food was reconstituted before addition of virus. The pH of all foods was determined using a Beckman Zeromatic pH meter.

Following virus inoculation, half of the food samples were stored at 4°C and the other half at 20°C. Individual samples were stored for 48, 96, and 168 hr. Following storage the recoverability of virus was determined by titration of DEAE Sephadex column eluates of filtrates of the foods. This entire sequence of procedures was repeated once with virus titers reported as arithmetic means.

RESULTS

Polio virus survived the freeze dehydration process in all the foods tested. The results of these studies are presented in Table 1. The average titer of recoverable virus was between 3 to 4 logs less in the freeze dehydrated food compared to the food that was not dehydrated. The loss in titer of the stock suspension diluted in Hanks' BSS was equivalent to the loss in the foods.

Table 1—Recovery of polio virus[1] from foods after freeze dehydration[2]

Food	Titer without dehydration	Titer after dehydration
German potato salad	3.0×10^{6}[3]	1.5×10^3
Beef and vegetables	3.0×10^6	6.0×10^2
Beef pot roast	3.0×10^6	5.9×10^2
Chicken with gravy	2.6×10^6	2.9×10^2
Salmon salad	2.3×10^6	6.9×10^2
1 ml virus stock in 9 ml Hanks' balanced salt solution	4.0×10^6	2.0×10^3
1 ml virus stock in 9 ml water	1.5×10^5	4.0×10^1

[1] Nine g samples of food inoculated with 1 ml of virus stock suspension containing 1×10^8 plaque forming units (PFU)/ml polio-virus type I Mahoney strain in Eagle's basal medium with Hanks' balanced salt base.

[2] Freeze dried at 50 to 150 μ of Hg pressure, condenser temperature −51°C, platen temperature 21.8°C.

[3] Mean PFU/ml of quadruplicate titrations of each sample from three studies.

In the studies of recovery of polio virus from irradiated fish fillets, it was observed that a total dosage of 600,000 rads was sufficient to produce approximately 99% reduction in recoverability of the virus. These results and the results of the studies of virus recovery from the suspension are shown in Table 2. Within the fish fillet samples the average percentage recovery of virus from the eluates of the DEAE Sephadex was high compared to recovery directly from the food filtrates. A correlation coefficient (Bancroft, 1957) of ±0.14 was calculated for the relationship between the change in titer in the irradiated fish filtrates and that of the irradiated suspensions of virus.

Table 3 shows the results of the recovery of polio virus from the DEAE Sephadex column eluates of filtrates of foods stored at 4 and 20°C for 0, 48, 96, 168 hr. Compared to controls virus recovery was generally high from all foods at 4°C with exception of the highly acid foods, cranberry sauce and orange juice. A comparable pattern of recovery was observed in the group of foods held at 20°C except the pork product, which exhibited a higher retention of recoverable virus than the controls. After 48 hr all the foods stored at both temperatures studied exhibited an increasing degree of spoilage.

DISCUSSION

The results of virus recovery from the foods following freeze dehydration suggest that for the foods studied, this processing operation may be expected to reduce polio virus recoverability by about 99.9%. The average percentage of recovery of virus from the food was not appreciably different from the recovery observed in the Hanks' BSS.

The recovery of virus from the freeze dehydrated stock suspension diluted with water was unexpected. Polio virus has been considered as relatively labile during laboratory lyophilization. This suggests a possible difference in the effects on virus survival from the freeze dehydration conditions studied compared to usual procedures employed for virus lyophilization.

The total dosage of Cobalt-60 gamma irradiation given in this study was in the range of those usually considered effective for "pasteurization" of foods (i.e. 300,000 to 600,000 rads). The poor correlation between rate of loss of virus in the fillet samples compared to the virus in the suspensions suggest a significantly greater loss in recoverability of virus from the food. The data show an approximately tenfold greater drop in virus titer in the fish fillet compared to that of the suspen-

Table 2—Recovery of polio virus from suspensions and fish fillets exposed to Cobalt 60 gamma irradiation

Total irradiation in kilorads	Suspension of Virus[1]: Titer of suspension	Suspension of Virus[1]: Titer of DEAE sephadex eluate of suspension	Inoculated Fish Fillets[2]: Titer of fish filtrate	Inoculated Fish Fillets[2]: Titer of DEAE Sephadex eluate of fish filtrate
0	4.2×10^{6}[3]	4.2×10^6	4×10^6	5.3×10^6
75	3.2×10^6	2.3×10^6		
150	1.6×10^6	1.6×10^6	6×10^5	1.6×10^5
225	1.4×10^6	9.2×10^5		
300	7.5×10^6	4.2×10^5	2.2×10^5	9.3×10^4
375	3.8×10^5	2.3×10^5	1.1×10^5	3.2×10^4
450	2.2×10^5	2.0×10^5	4.7×10^4	2.0×10^4
525	1.5×10^6	1.7×10^5	3.6×10^4	6.3×10^3
600	1.1×10^6	1.0×10^5	2.3×10^4	4.0×10^3

[1] Polio virus type 1 strain LS_c 2AB in Eagle's basal medium with Hanks' balanced salt base.

[2] Nine-g samples of Whiting fish fillets inoculated with 1 ml of virus stock suspension containing 5×10^7 plaque forming units (PFU)/ml polio virus type 1 strain LS_c 2AB.

[3] Mean PFU/ml of triplicate titration of each sample from three studies.

Table 3.—Recovery of polio virus[1] from DEAE Sephadex column eluates from foods stored at 4 and 20°C

Food	0 hr		48 hr		96 hr		168 hr	
	4°C	20°C	4°C	20°C	4°C	20°C	4°C	20°C
Pork	$4.5 + 10^{6}$[2]	5.1×10^6	2.3×10^6	1.4×10^6	2.1×10^6	1.5×10^6	1.4×10^6	2.4×10^6
Reconstituted diet Formula	4.8×10^6	4.1×10^6	1.4×10^6	8.0×10^5	2.2×10^6	4.5×10^6	1.3×10^6	5.0×10^4
Cranberry sauce	3.5×10^6	4.0×10^6	2.0×10^4	3.6×10^4	2.7×10^4	2.3×10^3	2.0×10^4	$<1 \times 10^1$
Mayonnaise	4.3×10^6	2.8×10^6	2.1×10^6	3.4×10^5	2.4×10^6	2.1×10^5	1.6×10^6	1.6×10^4
Orange juice	3.0×10^6	4.2×10^6	6.2×10^4	1.6×10^2	5.0×10^4	$<1 \times 10^1$	5.7×10^4	$<1 \times 10^1$
Virus control[3]	5.0×10^6	4.5×10^6	3.5×10^6	2.1×10^5	5.0×10^6	2.4×10^5	1.0×10^6	2.1×10^5

[1] Nine g samples of food inoculated with 1 ml of virus stock suspension containing 5×10^7 plaque forming units (PFU)/ml polio virus type 1 strain LS_c
[2]AB in Eagle's basal medium with Hank's salt base.

[2] Mean PFU/ml of triplicate titrations of each sample from two studies.

[3] Stock virus diluted 1:10 in Hanks' balanced salt solution.

sion following 600 kilorad dosage of irradiation. The food environment during irradiation accelerated virus loss compared to loss in the Hanks' BSS suspension. Since all food samples and suspensions were held at approximately 0°C during irradiation, and since recovery of virus from the nonirradiation fish filtrate and suspensions were equivalent, it might be suggested that reactions secondary to irradiation occurring in the fish were detrimental to virus recovery.

The results of the recovery of polio virus from the refrigerated foods suggested that acidity and/or protein content of the food can affect the recoverability of this virus compared to recoverability from suspensions in Hanks' BSS. Otherwise the recovery of virus from refrigerated foods was similar to that reported by Lynt (1966). Similar results were obtained (Heidelbaugh et al., 1967) using Mengovirus in a group of four foods (ranging in pH from 2.4 to 7.1) stored for 14 days at 4°C. In those studies no virus could be detected in the food having pH 2.4 (commercial cola beverage) while the other foods retained in excess of 90% of their original 4.2×10^8 PFU/ml titer after the storage period. In all of these storage studies there was no indication that spoilage of the foods affected virus recovery.

Titers of public health significant virus as high as those studied in this report probably never occur in actual foods. Reductions in virus populations of the order of magnitude reported here may well provide adequate safety margins. Accurate assessment of the impact of food processing operations on the public health aspects of viruses in foods awaits qualitative and quantitative epidemiological data in public health food virology.

It should be emphasized that some of the apparent changes in virus titers following processing operations could result from change in the adherence of virus particles to the food and/or change in the ability of the virus to form plaques under the conditions studied. Measure of virus recovery by methods like those reported here is not necessarily a function of virus infectivity. This suggests the need for a better method for separation of virus particles from foods and the correlation of the results of that method with results of detection of virus infectivity by feeding trials.

In the study of the pork and orange juice, the use of the DEAE Sephadex column was essential for the titration of virus since monolayers of tissue culture cells were frequently destroyed if the food filtrate was not purified by passage through the column. This study also confirms the relatively high percentage of recovery of virus from filtrates passed through a DEAE Sephadex column. This method of separation of infective virus from foods would be useful when highly purified eluates are desired.

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K. SAMEJIMA & Y. HASHIMOTO
The College of Dairy Agriculture, Nishinipporo, Ebetsu
Hokkaido, Japan
T. YASUI & T. FUKAZAWA
Department of Animal Science, Faculty of Agriculture, Hokkaido University, Sapporo
Hokkaido, Japan

Heat Gelling Properties of Myosin, Actin, Actomyosin and Myosin-subunits in a Saline Model System

SUMMARY—Myosin and actin were isolated from rabbit skeletal muscle. The gelation of myosin, actin and actomyosin, as well as of heavy and light meromyosins derived from myosin by trypsin treatment, by heat was studied in various systems. The data indicate that the heat gelling properties of these protein solutions do not run parallel with those of saline model systems composed of these proteins and stroma.

Actin does not exert any influence on the binding properties of the system, but when F-actin and myosin A were both present the resulting binding properties were considerably improved. Since heavy and light meromyosins have little influence on binding properties, it may be concluded that an intact molecule of myosin is required for development of binding properties upon heating.

INTRODUCTION

IN MANUFACTURING cured meat products such as sausages, water-holding and binding properties of meat when cooked have been thought to be important factors influencing product quality. On the basis of the manufacturing process of sausages, it may be supposed that a portion of the myofibrillar proteins is dissolved with sodium chloride before heat processing, suggesting a close relationship between solubility and extractability of salt-soluble proteins in muscle and product quality. Although changes in the water-holding properties of muscle under various conditions have recently received a great deal of attention (Wierbicki et al., 1957; Hamm, 1957, 1959, 1960), these studies have not included salt (e.g. potassium or sodium chloride) solubility studies at salt levels similar to those obtained in sausage manufacture. Nevertheless, the relationship of the myofibrillar proteins to quality has been suggested by these studies.

Denaturation studies (Yasui et al., 1958, 1960, 1964a, 1964b) on isolated myosin and actomyosin from muscle, and studies (Hashimoto et al., 1959; Fukazawa et al., 1961a, 1961b) on experimental sausages prepared from myofibrils from which certain myofibrillar proteins such as myosin, actin and tropomyosin were removed with specific solvents for each, respectively, clearly showed that myosin and actomyosin, actin-myosin complex, were essential among the myofibrillar proteins in developing binding properties of the product.

These results indicate that physicochemical changes of myosin and actomyosin upon heating may play a role in determining the binding properties of sausages. In fact, a heat gelling test for salt-soluble proteins from muscle was devised by Trautman (1966) to study subtle protein changes occurring in muscle, though the result indicated no relationship between variation and product quality.

The aim of this report is to study the cause and diversity of heat-gelling properties of isolated myosin, actin and actomyosin in saline-model systems.

The results obtained have shown that the heat gelling of the proteins in a simple model system does not run parallel with a gel-forming ability in a more complex model system and that only whole molecules of myosin can give an invariable influence on heat-induced gelation of the systems.

Actin alone has been found to have no effect, but exerts a significant effect on heat gelation when combined with myosin.

MATERIALS & METHODS

Preparation of myosin A

Myosin A was prepared by extracting fresh rabbit skeletal muscle according to the method described by Perry (1955).

Preparation of myosin B

Myosin B was prepared by treating the rabbit skeletal muscle with the Weber-Edsall solution for 24 hr and purifying it by repeating the usual "dilution precipitation method" two or three times.

Preparation of actin

G-actin was prepared from acetone-treated rabbit muscle after applying the method of Mommaerts (1952), and transformed to F-actin in 0.1*M* KCl solution.

Preparation of heavy meromyosin (HMM) and light meromyosin (LMM)

Meromyosins were prepared by treatment of 10 mg/ml solution of myosin A in 0.5*M* KCl at pH 7.0 with trypsin (1:200 weight ratio) freshly dissolved in cold water. Meromyosins were isolated from the resulting solution by the method of Szent-Györgyi et al. (1960), after 10 min of digestion at 25°C. The reaction was stopped by the addition of a 1.3-fold weight excess of soybean trypsin inhibitor freshly dissolved in cold water.

Crude LMM (ATPase activity showed about a half value of myosin A) was precipitated by dialyzing trypsin-treated myosin A against 10 ~ 15 vol 10 m*M* phosphate buffer for 12 hr. The precipitate was gathered by centrifuging for 15 min at 12,000 rpm and dissolved in 0.02*M* borate buffer (pH 8.3) in 0.15 *M* KCl solution.

HMM was precipitated by holding the supernatant after dialyzing against 57% ammonium sulfate solution. The above-mentioned crude LMM might be contaminated with myosin and HMM, because it showed 0.5 times as high ATPase activity as mother myosin. Then LMM was further purified by adding F-actin to the crude LMM preparation and centrifuging out the resulting acto-HMM and -myosin (Lowey et al. 1962).

Preparation of LMM fraction 1 (LMM Fr. 1)

Light meromyosin fraction 1 was prepared from LMM according to the method described by Szent-Györgyi (1960). Three vol ethanol (95% v/v) were added to LMM in 0.6*M* KCl solution at room temperature and the mixture was allowed to stand for 2–3 hr. The protein was quantitatively precipitated and collected by centrifuging for 20 min at 4,000 rpm. The precipitate was resuspended in the initial volume of 0.6*M* KCl and dialyzed against 0.6*M* KCl for 24 hr. The protein was precipitated at low ionic strength ($\mu = 0.1$). Light meromyosin fraction 1 was obtained by dissolving the precipitate in the initial volume of 0.6*M* KCl.

Preparation of stroma

Myosin free stroma. Myosin-free stroma was prepared by treating minced matured hen skeletal muscle with the Hasselbach-Schneider solution (1951) for 20 ~ 30 min

and squeezing with gauze. This extraction procedure was repeated three times. The resulting Gohst fibrils were washed with water, treated with acetone and dried in the air at room temperature.

Myosin and actin free stroma. The fresh myosin-free stroma was treated twice with 0.6*M* KI solution for 1–2 hr (Huxley et al., 1960) and squeezed with gauze. The residue was washed with water and then with acetone and dried as before.

Determination of ATPase activity

ATPase activity was measured in 0.5*M* KCl at 25°C and 20 m*M* tris-maleate (pH 7.0) in the presence of 5 m*M* $CaCl_2$, 1.0 m*M* ATP and 0.1 mg protein/ml. Aliquots of the reaction mixture were removed at measured intervals of time and the reaction stopped by the addition of 10% TCA. The amount of liberated inorganic phosphate was measured by the method of Martin et al. (1949).

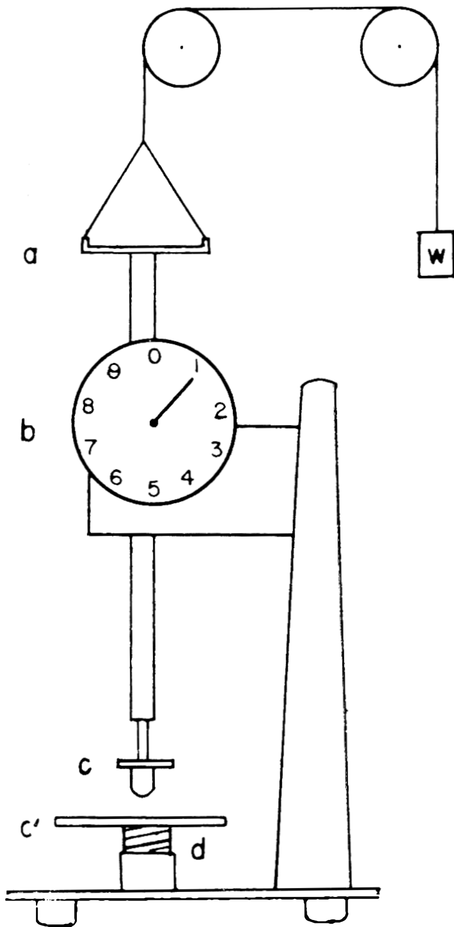

Fig. 1—The gel-strength tester. A sample in a glass container was placed on the plate (c), and the end of the sliding bar (c) was lowered until it touched the surface of the sample. Then the pointer of the circular scale (b) was adjusted to 0 by turning the plate (c). Next, increasing weights were added at (a), and the readings of the pointer were recorded 1 min after the addition of each weight (reading A). Then, the reading of the pointer when the end of the rod (c) reached to the bottom of the glass container was recorded (reading B). Then tensile strength values were calculated from these data as follow: Tensile strength = reading A/reading B × 100.

Electron microscopic observation

The particles of the gel were fixed in 2.5% glutaraldehyde in phosphate buffer (pH 7.0), and then postfixed in 1% OsO_4 in Veronal buffer (pH 7.2). After dehydration in graded series of alcohol solution followed by propylene oxide, the materials were embedded in Epon 812 (Luft, 1961). The sections were stained with 1% uranyl acetate followed by lead citrate and then examined in a Hitachi HU-11 B electron microscope operated at 75 Kv.

Heat-gelling properties

The qualitative heat-gelling test was carried out according to Trautman (1966). Calculated concentrations (~20 mg/ml) of myosin A and B, F-actin, HMM, LMM and LMM Fr. 1 in 0.6*M* KCl and 10 m*M* buffer (pH 7.0) were pipetted into 5-mm diameter glass test tubes. The tubes were transferred to a 60°C water bath, held for 30 min and finally transferred to a 11°C water bath. After 15 min, each tube was then inverted. The tubes in which all the content of the protein heated remained when inverted were expressed as + and negative ones as —.

Evaluation of gel-strength in the systems containing stroma

Two milliliters of the protein preparations in 0.6*M* KCl buffered at pH 7.0 or 6.0 were mixed with 1 g of stroma described above in a glass container whose inside diameter and depth were 2.0 and 1.5 cm, respectively, and equilibrated for 30 min. The slurry was heated at 60°C for 30 min and then quickly cooled in ice. In accord with the heat-gelling property of the protein, the inside materials showed various conditions after heating; e.g., control (stroma plus 0.6*M* KCl and buffer) remained as it was and myosin A or myosin B formed a plug holding stroma together in the center of the container. The gel-strength of the systems was evaluated with a device reported by Fukazawa et al. (1961a). The sensitivity of the tester has been improved ten times as high as previously reported by cancelling out the weight of the sliding bar (Fig. 1). Relative values of tensile strength were plotted against weight used, and grams of weight added to reach 50% tensile strength [W50(G)] were taken to compare gel-strength of the cooked systems.

In the process of this work, it became essential to establish standard conditions for protein concentration vs gel-strength. Figure 2 shows the effect of protein concentration on degree of gel-strength of the systems in which hide powder (Merck Co., Germany) was used in place of stroma. Differences in gel-strength due to protein species become apparent at comparatively low concentration of proteins used. To standardize conditions for this experiment and to save protein samples, protein concentration of 4 mg/ml were used for the remainder of this study. Although the conditions of the experiments were standardized as closely as possible, the grams of weight at 50% tensile strength differ from one preparation of stroma to another, so that the curves from different stroma preparations may be compared only in general shape and not in absolute values. However, when stroma from the same source was used, the data obtained were reproducible. Averages of three experiments on stroma from the same source were used throughout this study.

Turbidity measurement

Myosin A solutions containing 0.6 mg/ml protein, 0.5*M* KCl and 20 m*M* tris-maleate buffer at pH 7.0 with or without 5 m*M* thioglycol, 1 m*M* Na_2SO_3 or 0.05 m*M* *p*-mercuribenzoate were incubated at temperatures from 20–70°C for 15 min. The coagulation of myosin A due to heating was followed by the increase in turbidity of the solution under various conditions. Turbidity was determined by measuring the optical density of the protein solution in a 1-cm cell at 370 mμ using a Hitachi photoelectric spectrophotometer (EPU-2A).

Water-holding capacity

The samples used for the measurement of tensile strength were centrifuged at 10,000 rpm for 1 hr and the volume of the separated water was measured in a graduated cylinder. Then the water-holding capacity was given by [1-(volume of water separated/volume of water initially added)] ×100.

Determination of protein concentration

Protein concentrations were determined by a micro-Kjeldahl or a biuret procedure.

ATP was purchased from Sigma Chemical Co. Other reagents used were of the best reagent grade available.

RESULTS

Heat-gelling properties in simple model systems

Figure 3 illustrates specific ATPase activities of various myosins used in this

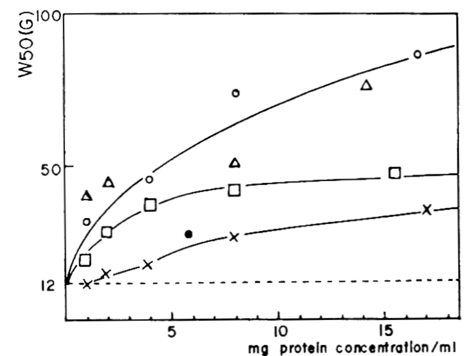

Fig. 2—The effect of protein concentrations on gel-strength of the systems composed of hide powder and myosin A, its derivatives and F-actin after heat treatment at 60°C and pH 7.0.

- - - - Hide powder alone (control: 1 g of hide powder plas 2 ml of 0.6 M KCl with 10 mM phosphate buffer.)

- △ *Myosin A*
- □ *LMM (crude)*
- ● *F-actin*
- ○ *Myosin B*
- × *HMM*

study. When complexed with F-actin, the specific activity decreased, since F-actin does not have any ATPase activity under this condition. Conversely, if myosin A molecule is split by trypsin treatment and divided into its globular head (HMM) and helical rod (LMM), specific ATPase activity of the former remarkably increased. Light meromyosin is believed to possess no ATPase activity. The purified LMM obtained almost no activity. Further purification by ethanol fractionation of LMM completely eliminates the activity. LMM and LMM Fr. 1, however, possess the solubility characteristics of mother myosin A and are soluble only at ionic strengths greater than $\mu = 0.25$, while HMM is water-soluble (Szent-Györgyi, 1953). Using high enough concentrations (20 mg/ml) to cause heat gelation, if any (Trautman, 1966), heat-gelling properties of the proteins above described and of F-actin were tested. After heat treatment at 60°C and pH 7.0 for 15 min, myosin A and B and HMM solutions formed gels which remained in the test tube when inverted. Other proteins formed a flocculated coagulum or became turbid, but showed no sign of gelation. It should be emphasized that, despite the fact that LMM and LMM Fr. 1 have the solubility characteristics of myosin A, they exert no effect on heat gelation.

Examination of the gels by electron microscopy indicates that, in all cases, islands of protein aggregates were scattered evenly and held water inside (Fig. 4a,b,c), suggesting the formation of a network system upon heating. These results are summarized in Table 1, along with sulfhydril content in each protein. The results give an impression that the ATPase active site or the region nearby in myosin A molecules might play a role in the binding properties of cured comminuted meat products upon heating.

Heat-gelation in complexed model system

The apparent relation of ATPase activity to heat-gelation seen in the sample model systems does not accurately depict what happens in sausage emulsions upon heating, since the binding properties of sausage develop in the presence of two other protein groups, sarcoplasmic and stroma proteins. Since there is little possibility of participation of the binding or heat gelling property of the former on the basis of accumulated evidence (Fukazawa et al., 1961 and Trautman, 1966), the effect of stroma proteins on the heat-gelation of aforementioned proteins has been studied in this article. The gel-strength of heated model systems in which 1 g of stroma and the proteins (4 mg/ml) in 6 ml of 0.6*M* KCl and 10 mM buffer at pH 6.0 are presented and compared in Fig. 5.

When myosin A-free stroma was used, the order of resulting gel-strength is myosin A > myosin B > F-actin > HMM > LMM Fr. 1 (Fig. 5a). In the case of myosin A and actin-free stroma, however, the decreasing order of gel-strength is myosin B, myosin A, HMM, LMM, F-actin and LMM Fr. 1 (Fig. 5b).

Two questionable aspects may arise from the results shown in Fig. 5. One is that the weight necessary to reach the half value of relative tensile strength was much higher in Fig. 5b than in Fig. 5a, especially when the effective proteins are present. The other is that the order of myosin A and B with regard to gel-strength is reversed depending on the use of two different stroma preparations from the same muscle source.

The first point may be explained by the results listed in Table 2, showing the difference in the water-holding capacity

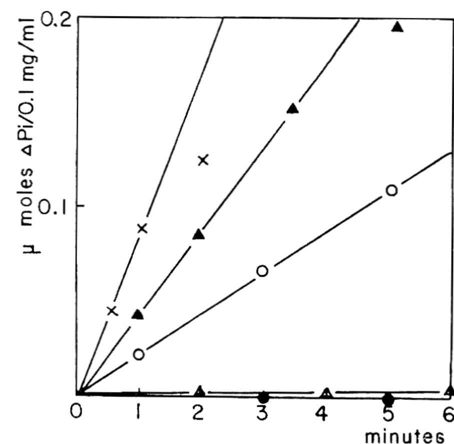

Fig. 3—ATPase activities of myosin A and its derivatives.
× HMM
○ Myosin B
● LMM Fr. 1
▲ Myosin A
△ LMM

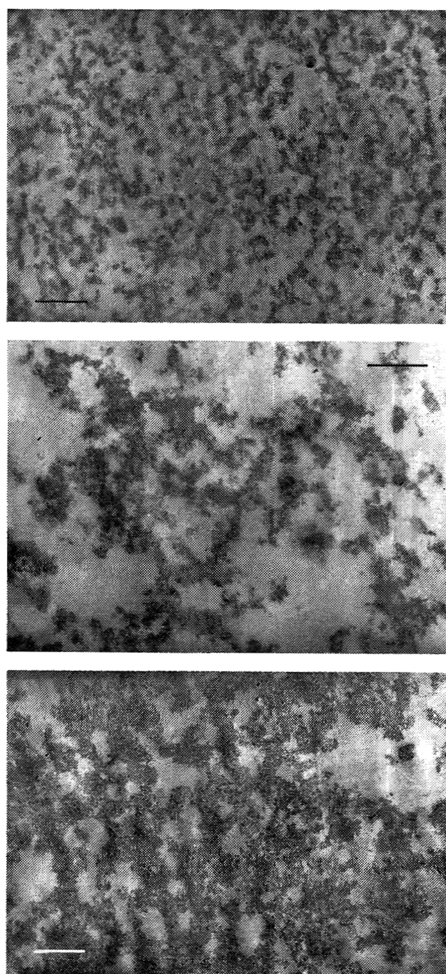

Fig. 4—Electron micrographs of heat-induced gels. Top, Myosin A; center, Myosin B; bottom, HMM. Bar-length in each photograph is 0.5 μ.

Table 1—ATPase activity, heat gelling property and sulfhydryl contents. +, Positive; −, Negative; c, Coagulation; quoted from 1,3,5 (Szent-Gyorgyi, 1959); 2,6 (Barany, 1959), and 4 (Lowey, 1962).

	ATPase activity	Heat Gelling test	$SH/10^5g$
Myosin A	+	+	7.4[1]
Myosin B	+	+	6.3[2]
LMM	−	−	4.3[3]
LMM Fr. 1	−	−	4.0[4]
HMM	+	+	8.5[5]
F-actin	−	C	3.0[6]

Table 2—Water-holding capacity of the systems composed of stroma and myosin A, myosin B and LMM after heat-treatment at 60°C and pH 6.0.

	Myosin-free stroma	Myosin, actin-free stroma
Myosin A	48.3, %	41.2
Myosin B	47.0	43.6
LMM	40.8	37.5

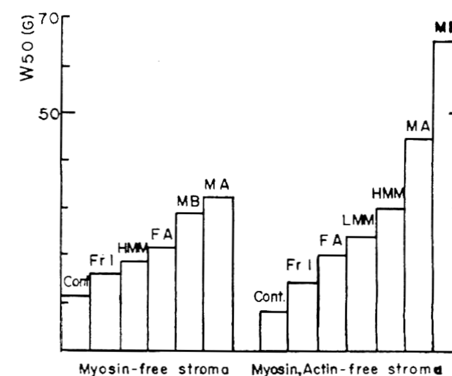

Fig. 5—Gel-strength of the systems composed of stroma and myosin A, its derivatives and F-actin after heat-treatment at pH 6.0 and 60°C.
a, Myosin-free stroma;
b, Myosin-and actin-free stroma.

of the gel formed by heat treatment. Water concentrations of heat-induced gels containing myosin-free stroma are higher than those containing of myosin- and actin-free stroma, thus indicating the decrease in hardness of the gel in the former. As for the second, it may be said that complex formation of actin and myosin might play a role in this phenomenon, since the main component of myosin B is the actomyosin complex and the effect of myosin A and B on the gel-strength depends on the presence or absence of actin in the stroma used.

DISCUSSION

The results obtained from experiments on heat gelation of isolated protein solutions appear to implicate the gelation with ATPase activities as well as sulfhydril content of those proteins. If SH-groups are involved in gelation, formation of a network system (Fig. 4) might result from formation of intermolecular disulfide bridges due to oxidation of sulfhydril groups on native protein molecules.

Using an amperometric titration method with $AgNO_3$, Hamm et al. (1965) investigated the changes in sulfhydril and disulfide groups of myofibrils and actomyosin during the course of heat coagulation and reported that oxidation of SH to S-S was not observable up to 70°C, though the coagulation had already occurred from 45°C. In fact, the conventional turbidity measurements on myosin solution in 0.5*M* KCl during the course of heat coagulation (Fig. 6) clearly indicate that neither a reducing reagent such as thioglycol or Na_2So_3 nor a SH-blocking reagent such as *p*-chloromercuribenzoate prevent the solution from coagulating, but rather accelerates the reaction up to 60°C.

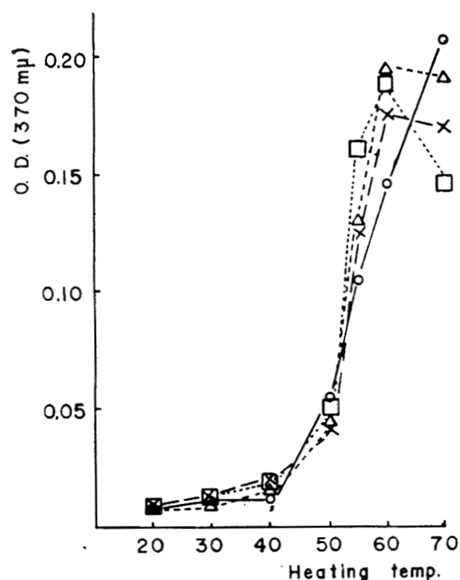

Fig. 6—Changes in turbidity of myosin A solution upon heating at pH 7.0 (20 mM tris).

○ *Control*
Δ *Thioglycol 5mM*
× *Na_2SO_3 1 mM*
□ *p-chloromercuribenzoate 0.05 mM*

This supports the conclusion by Hamm et al. (1965) that the heat coagulation of myofibrillar proteins is not due to the oxidation of SH-groups but to the intermolecular association of other side groups on the molecules. Therefore, in spite of its apparent coincidence with enzymic and chemical characteristics of myofibrillar proteins, gelation induced by heat would be achieved through other ways than is expected from the results in Table 1.

Experiments on the physical properties of the gel formed in more complex model systems composed of stroma and myofibrillar proteins have shown contradictory results to those from simple model systems. Contrary to the results in Table 1, HMM demonstrates little influence on heat-gelling properties of the complex model systems.

On the other hand, LMM which is believed to govern the solubility of myosin A (Szent-Györgyi, 1953) has been shown to have no effect on the heat-gelling properties of any system tested (Table 1 and Fig. 5). It is of interest that the subunits of myosin A have little to do with the heat gelling-properties, whereas the converse is true for parent myosin A. This may suggest that a whole molecule of myosin A is needed for the development of this property and that care should be taken in the use of meat tenderized by proteolytic enzymes in sausage manufacture.

F-actin itself exerts little influence on heat-gelling properties (Table 1 and Fig. 5). The result is consistent with the conclusion stated by Fukazawa et al. (1961). However, it was found in this study that F-actin improves the gel-strength when complexed with myosin A. In the system of myosin A- and actin-free stroma plus proteins, myosin B (natural actomyosin) forms a harder gel with more water content than myosin A (Fig. 5b) and *vice versa* in the system of myosin A-free stroma plus proteins (Fig. 5a). This suggests the complementary effect of F-actin on improvement of gel property which is commonly given by myosin A in most systems.

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E. E. STINSON, C. J. DOOLEY, V. J. FILIPIC[a] and C. H. HILLS
USDA Eastern Utilization R&D Div., ARS, Philadelphia, Pa. 19118

Composition of Montmorency Cherry Essence. 1. Low-boiling Components

SUMMARY—*The low-boiling neutral components of a commercial Montmorency cherry essence were concentrated by distillation. Individual components were separated and identified by combined gas chromatography and mass spectrometry. The identifications were confirmed by gas co-chromatography with known compounds. Ethanol and methanol were the most abundant low-boiling substances. These compounds were estimated to comprise 9 and 0.5% of the essence, respectively. The next most abundant compound was acetaldehyde. Other compounds present included diethyl ether, propionaldehyde, acetone, isobutyraldehyde, methyl acetate and ethyl acetate. The estimated concentrations of these compounds in the original essence are given in each instance.*

INTRODUCTION

A NUMBER of investigations have been made on the volatile constituents of cherries and related products. Nelson et al. (1939) identified benzaldehyde in Montmorency cherry juice and isolated a small amount of a yellow oil with an odor they considered suggestive of geraniol. Serini (1957) identified 2,3-butylene glycol and acetylmethyl carbinol from sweet cherries. Waser et al. (1937) found terpineol in cherry wine. Mohler (1934) found that the flavor components of cherry brandy included a low-boiling fraction containing aldehydes and esters, an intermediate boiling fraction of unidentified substances that produced the characteristic cherry brandy aroma and a high-boiling fraction that contained high-boiling alcohols, benzaldehyde, coumarin and vanillin. More recently, Lovric (1962) identified hydroxymethylfurfural in heat-processed red tart cherries.

The development of gas chromatography and its combined use with mass spectrometry has led to increased knowledge of many flavor compositions. Mehlitz et al. (1962), Kovacs et al. (1964) and Spanyar et al. (1964) made gas chromatographic studies of the volatile materials of cherries but the compounds were not identified.

This paper is the first of a series on the composition of Montmorency cherry aroma. The objective is to improve the flavor of the processed fruit, as most production of this variety is used for baking or other heat treatment processes. The present paper is concerned with the identification of the low-boiling constituents of Montmorency cherry essence, a commercial flavor concentrate produced by stripping and rectification of Montmorency cherry juice.

[a] Deceased, May 19, 1968.

MATERIALS & METHODS

Cherry essence

Commercial grade 150-fold Montmorency cherry essence was obtained from the A. F. Murch Company, Paw Paw, Mich., and was prepared by the procedure developed by Claffey et al. (1958). The degree of vaporization of the juice was 25%. The essence had a strong, pleasant "cooked cherry" aroma typical of pasteurized cherry juice. It was shipped in 1 gal polyethylene bottles and stored in these containers at 34°F until used.

Gas-liquid chromatography

Two gas-liquid chromatographic units were used in this investigation. The first assembly, used principally for analytical determinations, consisted of an F&M 720 gas-liquid chromatograph equipped with the F&M 700 Module (F&M Corporation, Avondale, Pa.). This combination permitted dual column operation with flame ionization detectors. Stainless steel columns containing either Carbowax 20-M or "Tris" (1,2,3-tris-2-cyanoethoxypropane) liquid phases were used for the gas chromatography.

The main column for the analytical determinations was 50′ × 1/8″ O.D. containing 10% Carbowax 20-M (the standard "Hi-Pak" column produced by the F & M Corp.). A second pair of columns was used, consisting of stainless steel, 6′ × 1/8″ O.D., and containing 20% "Tris" on 80–100 mesh Chromosorb Z support. These were also purchased from the F & M Corporation.

The second gas chromatograph was an F & M 810 unit used in conjunction with the mass spectrometer Model 21-103C Consolidated Electrodynamics, Inc. Columns containing the same liquid phases (Carbowax 20-M and "Tris") were used for preparative scale separations with this instrument. Both the thermal conductivity and flame ionization detectors were used, the latter with a 1:100 stream splitter. A portion of the gas emerging from the exit port of the gas chromatograph was conducted to the mass spectrometer through heated conduit tubes.

Infra-red analysis

Materials were collected for analysis in a Perkin-Elmer 237B by passing the effluent gas from the exit port of the gas chromatograph through a "U"-shaped length of capillary tubing with the loop immersed in a dry ice-acetone bath.

Classification tests

Qualitative organic classification tests were used together with gas chromatography to indicate the presence of various functional groups. Decrease in the gas chromatographic peak height after reaction indicated the presence of that particular functional group in the compound responsible for that peak. The following classification tests were used (Howard et al., 1967): carbonyl compounds, hydroxyammonium chloride; olefins and aldehydes, potassium permanganate. No satisfactory test for esters has been found. Alcohols were identified by their 3,5-dinitrobenzoate derivatives (Shriner et al., 1956).

Preparation of concentrate

The low-boiling volatile materials were isolated and concentrated by three successive distillations. A 36″ glass-spiral Widmer column (Labglass, Inc., Vineland, N.J.) was used for the first two distillations. The third distillation utilized an 18″ spinning band column (Nestor Faust Manufacturing Corporation, Newark, Del.). The outlets in all three distillations were protected by a trap immersed in a dry ice-acetone bath. Mechanical stirring was used in all instances to improve efficiency of distillation. Representative results from the distillation of 3370 ml of essence were as follows:

Distillate No. 1—distillation of original essence, collecting all material distilling up to 77°C (i.e., including ethanol). Vol. = 325 ml.
Distillate No. 2—distillation of above distillate, collecting all material distilling up to 70°C (i.e., eliminating most ethanol, but not methanol). Vol. = 18.8 ml.
Distillate No. 3—distillation of distillate No. 2, collecting all material distilling up to 60°C (eliminating most of methanol). Vol. = 1.1 ml.

Table 1—Low-boiling components present in Montmorency cherry essence.

Peak no.	R acetaldehyde	Identity	Estimated concentration in original essence	Method of identification[1]
1	0.73			
2	0.87	Diethyl ether	140 ppm	MS, GC
3	...	Acetaldehyde	485 ppm	MS, GC
4	1.22	Propionaldehyde	less than 5 ppm	MS, GC
5	1.31	Acetone and isobutyraldehyde	16 ppm each (estimated on this column)	MS, GC MS, GC
6	1.37	Methyl acetate	32 ppm	MS, GC
7	1.46	...	...	
8	1.69	Ethyl acetate	295 ppm	MS, GC
9	1.83	Methanol	5000 ppm (0.5%)	MS, GC, IR, CHEM
10	2.02	Ethanol	90,000 ppm (9.0%)	MS, GC, IR, CHEM

[1] MS = mass spectrometry; GC = gas chromatography; IR = infrared; CHEM = chemical derivatives.

RESULTS & DISCUSSION

Quantitative analysis

The concentrations of methanol and ethanol present in the original 150-fold essence were determined by a series of chromatographic runs using propanol-1 as an internal standard according to the method of Dal Nogare et al. (1962). Known quantities of propanol-1 were added to the original essence and also to known mixtures of methanol and ethanol. The concentrations of the alcohols were estimated by comparing the heights of their respective peaks with the height of the propanol-1 peak.

Figure 1 shows the chart obtained by injection of a sample of 150-fold essence directly onto the 50′ × 1/8″ O.D. Hi-Pak column containing 10% Carbowax 20M. The numbers assigned to the peaks correspond to the numbers in Table 1.

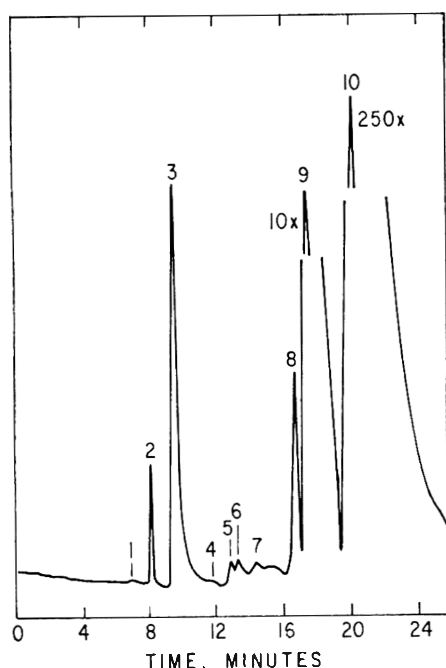

Fig. 1—Chromatogram of low-boiling components in original Montmorency cherry essence using 50′ × 1/8″ Carbowax 20M column at 65°C.

The two major peaks were identified as methanol and ethanol using fractions isolated by gas chromatography. Their identifications as methanol and ethanol by mass spectra were confirmed by infrared analysis and the melting points of their 3,5-dinitrobenzoate derivatives (melting points of 104–105°C and 90–91°C, respectively; unaltered by admixture of the corresponding derivatives of the known compounds). It was estimated that the original essence contained approximately 9.0% ethanol and 0.5% methanol.

The high concentrations of methanol and ethanol that were found in 150-fold cherry essence may not be representative of the composition of the juice of fresh, sound fruit. Ethanol, for example, may be formed by reactions occurring after the fruit is crushed, either by fermentation of sugars or by hydrolysis of esters. Thus a slight build-up to 0.06% ethanol by incidental fermentation would result in 9.0% ethanol in the 150-fold essence after concentration. Traces of methanol could arise from the enzymic cleavage of the methoxy groups present in cherry pectins.

The low concentrations of the minor components in the original essence made it necessary to concentrate them by the series of distillations described in Materials and Methods. This also reduced the preponderance of ethanol and methanol in the concentrates. The final product, Distillate No. 3, was chromatographed and the results are shown in Figure 2. The components were identified by mass spectral analysis of the gas emerging from the Carbowax 20M and the "Tris" columns. The concentration of each component was estimated by comparing the peak height with that produced by a standard solution containing 100 ppm. of that component. A 50′ × 1/8″ Carbowax 20M column was used because of the excellent separation and the sharpness of the peaks obtained. The compounds identified in the distillate are indicated in Table 1, together with the estimated concentrations in the original essence and the retention distances relative to acetaldehyde on a Carbowax 20M column. The identity of Peak 1 has not been established.

The presence of diethyl ether (Peak 2) was unexpected as this compound is not a product of normal plant metabolism. The possibility that it represents a breakdown product of ethanol arising during the analysis from degradation of ethanol in the injection port was eliminated by a duplicate determination injecting purified ethanol into the volume. It is possible that diethyl ether is formed by degradation of ethanol during distillation.

Acetaldehyde (Peak 3) was the main component emerging before methanol. The odor of acetaldehyde was easily detected in control solutions that contained the same concentration as found in the original essence. Peak 4 was identified as propionaldehyde.

Acetone and isobutyraldehyde (2-methylpropanal) formed a single peak (Peak 5) on the column containing Carbowax 20M (Fig. 2) but formed well separated peaks on the "Tris" column. The peak heights on the latter column indicated that acetone and isobutyraldehyde are present in approximately equal amounts in cherry essence. Methyl acetate, the component of the next peak, was somewhat more abundant. The identity of Peak 7 has not been established.

Ethyl acetate, next to acetaldehyde, is the most abundant of the minor components of cherry essence boiling below methanol. It occurred on the Carbowax 20M column adjacent to the methanol peak, which tended to obscure it. However, on the "Tris" column it occurred as a distinct, separate peak.

Several of these components have definitely perceptible odors in control solutions at the levels at which they occur in cherry

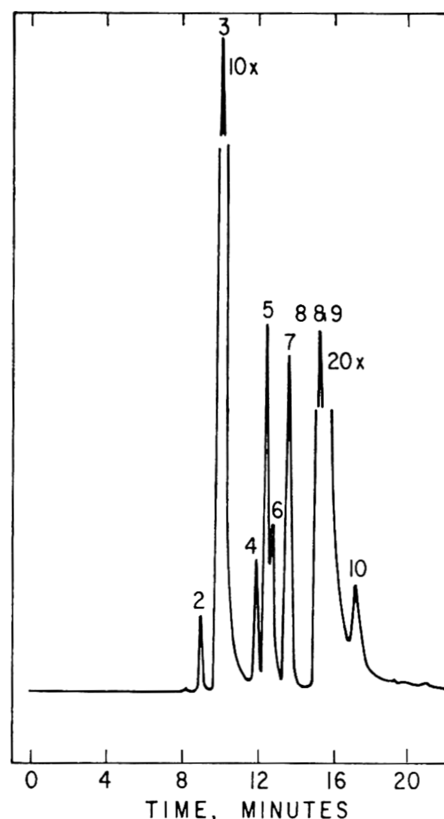

Fig. 2—Chromatogram of concentrated distillate containing low-boiling components in Montmorency cherry essence, using 50′ × 1/8″ Carbowax 20M column at 75°C.

essence. Their relative contribution to the total "cherry" aroma in the presence of the other components has not yet been evaluated.

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D. F. SPLITTSTOESSER, MARK C. CADWELL and MARSHA MARTIN
New York State Agricultural Experiment Station, Department of Food Science and Technology, Cornell University, Geneva, New York 14456

Ascospore Production by Byssochlamys fulva

SUMMARY—The effect of different variables on ascospore production was studied quantitatively. Maximal populations were obtained in 5% malt extract broth, pH 2-3, after an incubation of 7-14 days at 30°C. Tests on nine different fruit and vegetable juices showed that eight afforded good sporulation. Prune, grape and pineapple yielded the greatest numbers. Plating methods were found to underestimate the true ascospore populations because of spore dormancy and because a majority of the asci, each containing eight ascospores, remained intact.

INTRODUCTION

THE GENUS *Byssochlamys*, which encompasses two species *B. fulva* and *B. nivea*, is unique in that it produces ascospores sufficiently heat-resistant to survive the thermal processes commonly given highly acid foods. The mold was first recognized as a cause of canned fruit spoilage in Great Britain (Olliver et al., 1933), and for about 20 years the problem appeared to be restricted to that country. In recent years, however, *Byssochlamys* has been found in Switzerland (Lüthi et al., 1952), Canada (Yates et al., 1963), Australia (Spurgin, 1964), Holland (Put, 1964), and the United States (Michener et al., 1966). In this country the mold apparently has not been a serious cause of food spoilage even though it may be a common contaminant of grapes (Michener et al., 1966) and perhaps other fruits.

A number of studies have been concerned with sources of contamination (Olliver et al., 1934; Hull, 1939; Put, 1964) and heat resistance (Hirst et al., 1933; Gillespy, 1938; Hull, 1939). Little information is available, however, regarding the effect of cultural conditions on ascospore generation. Such data are important because they may point to methods for controlling contamination of foods and also are a prerequisite for the production of the large crops of ascospores needed for heat resistance determinations and for studies on spore physiology. The objective of this research, therefore, was to evaluate procedures for enumerating ascospores and to study the effect of different variables on the numbers produced.

EXPERIMENTAL

THE CULTURE was isolated from a commercial, thermally processed grape drink. The isolate matched the description of *Byssochlamys* with respect to appearance of colonies, conidia and asci, and in the heat resistance of its ascospores. Its tawny pigmented colonies indicated it to be *B. fulva* rather than *B. nivea* (Brown et al., 1957).

A relatively simple method was developed for studying the effect of different variables on ascospore yields. The inoculum, usually a suspension of ascospores, was added by pipette to 5 ml of broth contained in 150 × 16 mm culture tubes. The tubes were not agitated during incubation, and thus the fungal mat developed on the surface of the broth, which had an area of approximately 1.5 cm². Ascospores were enumerated by blending the broth and mat in a micro-homogenizer (Ivan Sorvall, Inc.) until the material appeared homogeneous, usually 2–3 min at 50,000 rpm. The homogenate then was heated for 1 hr at 70°C to destroy conidia and hyphal fragments and to activate dormant ascospores. Appropriate decimal dilutions were

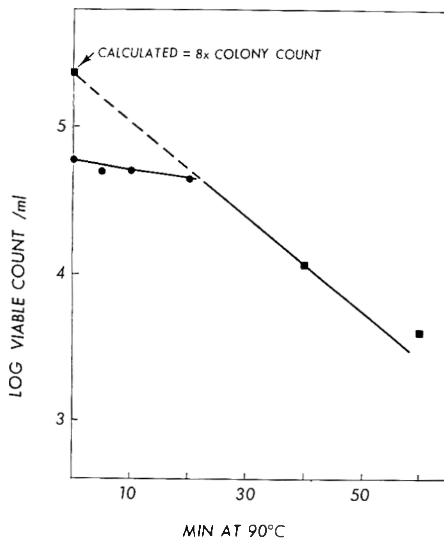

Fig. 1—Thermal death of asci heated in distilled water.

plated on Difco potato dextrose agar of pH 5.6. Plates having 30–80 colonies were counted after an incubation of 2 days at 30° C.

Total growth was determined by measuring the turbidity of homogenates that were resuspended in distilled water following centrifugation for 10 min at 5,000 rpm. Optical densities were read in Spectronic-20 colorimeter at 540 mμ.

RESULTS & DISCUSSION

Ascospore enumeration

As later data will illustrate, the plating of individual tube cultures afforded relatively reproducible ascospore counts, and thus the effect of different variables could be readily measured. The colony counts, however, underestimated the actual number of ascospores present in the homogenate by a rather large factor. The reasons for the low figures were that many of the asci remained intact, and, secondly, a significant percentage of the ascospores appeared to possess a dormancy that prevented their germination when they were introduced into a nutrient medium.

Microscopic counts obtained with a haemocytometer revealed that 90% of the asci remained intact after 2 min of blending in the micro-homogenizer. Asci rather than ascospores, therefore, were being enumerated, and, since each ascus contains eight spores, the number of active ascospores in the heated blendate was approximately 8-fold higher than the colony count. This difficulty in rupturing asci has been observed with other cultures of *Byssochlamys* (Gillespy, 1946; Michener et al., 1966), and, therefore, our isolate was not unique in this respect.

The fact that the asci also remained intact when suspensions were heated at a lethal temperature was reflected when survival data were plotted. It can be seen (Fig. 1) that only a slight reduction in the viable count occurred during the first 20 min at 90°C, but that following this period a more rapid death rate was obtained. However, when the curve was extrapolated to an 8-fold greater initial count, which represented the true active ascospore population, the data suggest a single destruction rate throughout the 60 min heating period. The explanation for these results, of course, is that all eight spores within an ascus must be destroyed before the colony count is affected.

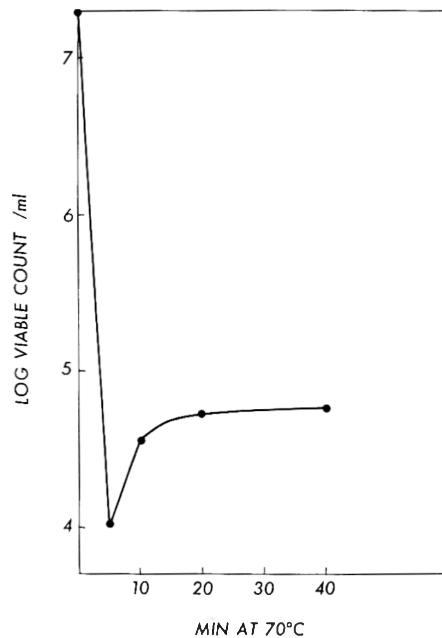

Fig. 2—Changes in the viable count of a blendate during heating at 70°C.

Heating homogenates at 70°C resulted in an initial rapid decrease in the viable count followed by an increase (Fig. 2). The data show that most of the heat-labile material were destroyed during the first 5 min of heating and that spore activation was about complete after 20 min. The heat activation data differ from those of Hull (1939) who found that 120 min at 70°C and 10 min at 75°C were required for maximal germination. At present, it is not known whether the optimal heat treatment is a strain characteristic or whether it is a factor influenced by nutritional or other cultural conditions.

It appears that even under the best conditions only a fraction of the asci formed visible colonies. Thus, when direct microscopic counts were compared with plate counts, only 6.6% of the unheated asci produced colonies, while a recovery of 18% was obtained when some of the same suspension was heated at 70°C prior to plating. Extending the incubation of the plates beyond 2 days did not result in a significant increase in recovery. It is not known whether the 18% figure was typical for most trials because usually the low level of asci plus the high concentration of conidia and hyphae in the homogenate prevented microscopic counts. Dormancy may be a common property of *Byssochlamys* since a low percent germination also has been observed with *B. nivea* ascospores when heat activated in a variety of menstrua (Yates et al., 1968).

Ascospore generation

A comparison of tryptone-glucose-yeast extract, potato dextrose and malt extract broths showed that the latter afforded the higher ascospore populations. The relative counts in 10, 5 and 1% malt extract (Difco) solutions were 100, 78 and 2.2, respectively. The 5% solution was adopted as the standard medium since it yielded almost as many ascospores as the higher level.

Maximal spore populations were obtained in malt extract broth after an incubation of 7–14 days at 30°C. Longer incubations often resulted in a decrease in counts, probably because some of the ascospores had germinated.

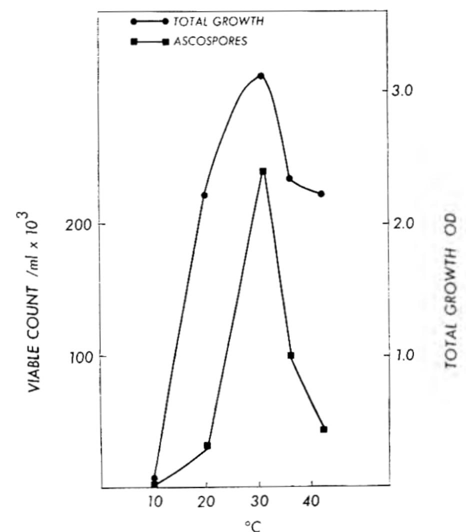

Fig. 3—Effect of temperature on total growth and ascospore production. Cultures incubated 15 days in 5% malt extract broth.

The optimal temperature for growth and ascospore production was about 31°C (Fig. 3). At this temperature, but not at the others, the peak ascospore population was already reached at 7 days' incubation. Thus the rate of spore generation, as well as total numbers, was favored by this temperature. The data also show that temperature was more critical for sporulation than for total growth. While small devia-

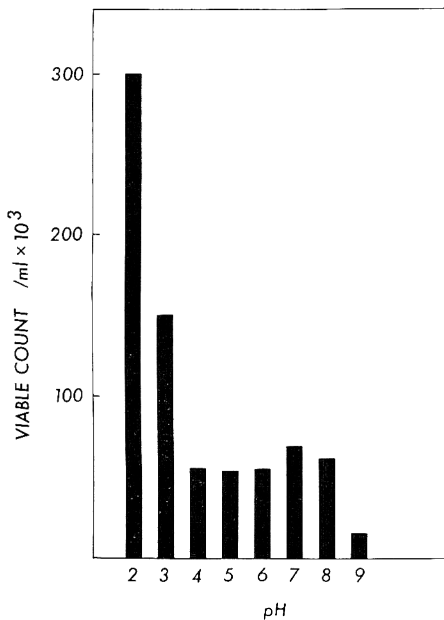

Fig. 4—Effect of pH of the culture medium on ascospore production. Cultures incubated 15 days at 30°C.

tions from the optimal temperature caused a marked reduction in the number of ascospores, relatively good vegetative growth was obtained over the range of 20-40°C. Total growth doubled between 7 and 15 days' incubation at 31°C, suggesting that vegetative growth proceeded without additional sporulation. It is possible, of course, that during this period spore formation and germination occurred at similar rates with the overall effect that the spore population remained relatively constant.

Another factor affecting ascospore production was the pH of the medium. When the reaction of malt extract broth was adjusted with sodium hydroxide or an acid, the optimal value was found to be below pH 3, and similar spore yields were obtained over the wide pH range of 4-8 (Fig. 4). The hydrogen ion concentration rather than the type of acid appeared to be the important factor since comparable results were obtained with hydrochloric and tartaric acids.

The suitability of different acid foods as a medium for spore formation was evaluated. The objective was to learn if lines processing certain foods might be more susceptible to a buildup of ascospores. Commercial juices, devoid of added preservatives, were selected for these trials. Samples were cultured throughout an incubation of 32 days at 30°C. The results (Table 1) showed that most of the juices permitted good sporulation and that the prune, grape and pineapple juices afforded the highest counts.

Table 1—Ascospore production in different commercial juices.

Juice	pH	Viable count	Relative count
Cranberry	2.7	56×10^2	0.13
Concord grape	3.3	18×10^6	43
Apple	3.6	70×10^4	17
Prune	3.9	42×10^6	100
Orange	3.9	78×10^4	19
Pineapple	3.4	20×10^5	48
Vegetable cocktail	4.4	46×10^4	11
Tomato	4.4	84×10^4	20
Sauerkraut	3.5	56×10^4	13

Cranberry juice, which contained relatively few ascospores, also failed to support good vegetative growth. The presence of benzoic acid, a natural constituent of cranberries, may have been responsible.

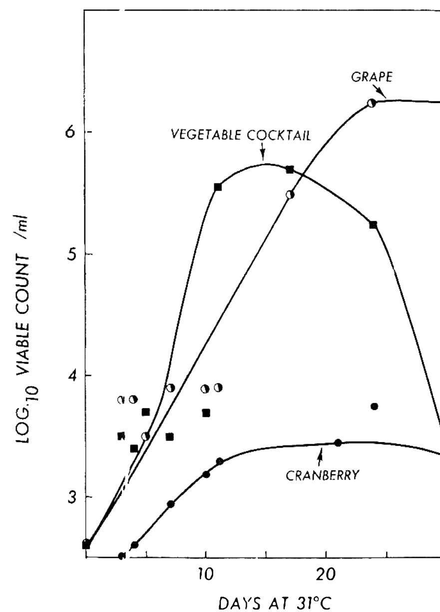

Fig. 5—The rate of ascospore production in different juices.

The data in Table 1 represent maximal counts obtained during the 32-day period. As illustrated in Figure 5, differences in rates of ascospore formation between juices were noted. The fastest sporulation occurred in tomato juice where the count increased from an initial 400 per ml to 51,000 in only 3 days. Prune juice, which ultimately yielded the most spores, did not initiate rapid sporulation until sometime after the 11th day of incubation. The highest counts in the apple, orange and sauerkraut juices were found on the last day of sampling. Extending the incubation of these juices beyond 32 days, therefore, might have resulted in still higher numbers.

The reason juices were selected for growth studies was they stimulated the solubles that accumulate on the surfaces of processing equipment. The fact that most juices supported good sporulation and that *B. fulva's* optimal temperature is that of many processing plants in summer makes one wonder why the organism does not present more of a problem. Three explanations come to mind: (1) The mold may not be widely distributed in this country and, therefore, only by chance finds its way into processing lines; (2) Because 7 days or longer usually are required for significant ascospore formation, the mold is removed by cleanups before this occurs; and (3) The mold may be inhibited by aciduric organisms. Some of the normal contaminants of processing lines may prevent sporulation or growth by competing for nutrients or producing metabolic products toxic to the mold. These are areas that will be studied in future work.

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M. R. MCDANIEL,[a] L. A. SATHER and R. C. LINDSAY
Department of Food Science and Technology, Oregon State University, Corvallis, Oregon 97331

Influence of Free Fatty Acids on Sweet Cream Butter Flavor

SUMMARY—A threshold pattern for the even-numbered free fatty acids (FFA) in butter depended on chain-length. Butyric acid had the lowest total average flavor threshold (AFT) of the more volatile FFA, and the total AFT values increased as chain-length increased through hexanoic and octanoic acids. A soapy after-taste predominated at and above the AFT for decanoic and dodecanoic acids. Threshold values decreased from octanoic acid through dodecanoic acid as the chain-length increased. The determination of AFT values for FFA in butter allowed an estimation of the importance of fatty acids in butter flavor. Mixture threshold results obtained support the concept that flavor components interact at subthreshold concentrations. Decrease in preference was shown for butter containing suprathreshold levels of total FFA.

INTRODUCTION

ENDOGENOUS levels of free fatty acids (FFA) are believed to contribute to the flavor of sweet cream butter. Excessive concentrations of volatile short-chain FFA are recognized as the cause of hydrolytic rancidity flavor in dairy products. The FFA content of several sweet cream butteroils has been reported by Bills et al. (1963) and Iyer et al. (1967). Their results showed considerable variations between samples. Scanlan et al. (1965) found that even-numbered fatty acids from butyric to dodecanoic accounted somewhat equally for the fatty acid contribution to rancid milk flavor.

FFA thresholds for butyric through dodecanoic have been determined in water (Patton et al., 1964; Siek et al., 1968) and for butyric through lauric in edible oil (Feron et al., 1961). The threshold for added butyric acid has been determined in deodorized butteroil (Siek et al., 1968) and added butyric and hexanoic acid thresholds have been determined in milk (Patton, 1964).

The purpose of this investigation was to find the thresholds for the C_4-C_{12} FFA in fresh sweet cream butter. This was accomplished by determining the amount of each individual FFA to add to butter in order to reach its added threshold value. This added value was combined with the endogenous level of each acid in the butter to give a total threshold value.

A second objective was to demonstrate the synergistic flavor interaction for mixtures of FFA in butter. Day et al. (1963) previously have discussed additive interactions of other flavor compounds at subthreshold concentrations.

Finally, the flavor preference for butters containing added FFA was determined.

[a]Present Address: International Flavor and Fragrances, Union Beach, N. J.

EXPERIMENTAL

Fatty acids

Reagent grade (Eastman Distillation Products) butyric, hexanoic, octanoic, decanoic and dodecanoic acids were used. Butyric, hexanoic and octanoic acids were vacuum distilled in a glass distillation apparatus (Siek et al., 1968). Decanoic and dodecanoic acids were purified by three consecutive ethanol-water recrystallizations. All acids were examined by thin-layer and/or gas chromatography, and were found to be at least 99.5% pure.

Sweet cream butter

High quality fresh sweet cream secured from commercial sources was pasteurized and churned in the University Dairy Products Laboratory, and was immediately frozen and held at −23°C until used. The storage time interval never exceeded 28 days.

Three lots of butter were used throughout the flavor panel testing: Lot I for individual FFA thresholds, Lot II for interaction thresholds and Lot III for preference evaluations.

FFA analysis

The procedure followed for FFA analysis was that of Bills et al. (1963), an adaptation of the original method reported by Hornstein et al. (1960), except that FFA were isolated from intact butter instead of isolated milk fat.

Preparation of butter test samples

Sweet cream butter was allowed to warm at ambient temperature to a workable state (ca. 16°C). Measured amounts of FFA were added at levels to include concentrations below and above the anticipated flavor threshold. After a thorough mixing by kneading in a plastic bag, the butter was formed into long-pound units and stored at 10°C for 24 hr prior to testing. Prior to serving, the butter was cut into patties and placed on coded paper butter chip plates. The serving temperature of the butter was approximately 16°C.

Threshold panel selection

A series of 21 triangle tests, using varying levels of added butyric acid in sweet cream butter, was presented to 25 people. From these tests 14 judges were chosen for their ability to detect butyric acid in butter. Judges selected for the panel scored correct responses on 50% or more of the triangle tests. The judges were experienced panel members but were not trained butter judges. Each judge was trained to detect individual fatty acid flavors when each acid was added at a high level to sweet cream butter.

Conflicts prevented three judges from participating throughout the entire testing period. New judges were evaluated by triangle tests as mentioned above, and were added to the panel as needed.

Individual compound threshold panel tests

Tests were conducted at 10:00 am and 3:00 pm. Samples were served on a tray to the judges seated in light controlled testing booths. Each tray contained two reference samples, marked "0" and "Ref," and six test samples coded with three-digit randomly selected numbers. The "0" reference contained no added fatty acid and the "Ref" references contained a high level of the fatty acid being tested. The yes-no type ballot was similar to that described by Patton et al. (1957) and Wyatt et al. (1965).

The fatty acid tested on a given day was identified on the ballot.

For butyric, hexanoic and octanoic acids the judges' task was to indicate whether or not they could detect a difference between the "0" reference and the coded butter samples. Due to the persistent soapy after-taste associated with decanoic and dodecanoic acids, duo-trio tests (Amerine et al. 1965) were used for determining the thresholds of these fatty acids. All threshold tests were replicated three times.

Mixture threshold panel tests

Interaction thresholds were determined by the same procedure as for the individual thresholds of butyric, hexanoic and octanoic acids. Mixtures of decanoic and dodecanoic acids were tested by triangle tests (Amerine et al. 1965). Instead of adding equal subthreshold amounts of each fatty acid in the mixtures as reported by Day et al. (1963), mixtures were prepared according to ratios based on individual thresholds. For example, the ratio of the individual thresholds of butyric and hexanoic acids was approximately 1:5, and butter samples were prepared with dilutions of fatty acids in this ratio. Tests were replicated at least twice.

Table 1—FFA analyses of butter and milk fat samples (average of duplicate trials).

	Concentration in butter			Concentration in milk fat				
	mg/kg			mg/kg[1]			mg/kg[2]	
Acid	I	II	III	IV	V	VI	VII	VIII
4:0	39.1	46.3	66.4	9.3	17.1	24.5	36.0	38.4
6:0	42.2	47.8	26.4	5.8	10.7	13.5	17.8	17.0
8:0	0.0	26.8	7.4	20.3	17.8	14.8	22.8	17.8
10:0	201.4	87.1	167.1	54.7	41.5	38.0	38.8	59.0
12:0	175.5	60.7	95.1	121.6	89.2	80.6	115.4	10.4
14:0	216.9	124.4	147.5	346.6	278.5	263.5	301.2	219.2
16:0	512.1	354.6	429.3	1084.0	710.2	641.1	620.2	650.8
18:0	197.9	78.5	182.3	320.1	197.2	202.9	329.2	282.8
18:1	659.5	316.0	568.4	1078.0	725.3	616.1	1050.8	1019.2
18:2	146.2	37.8	90.4	107.3	102.8	77.9	127.4	118.8
18:3	49.4	18.0	87.1	38.3	40.2	20.9	73.6	76.8

[1] Iyer et al. (1967).
[2] Bills et al. (1963).

Table 2—Average flavor thresholds of individual free fatty acids C_4-C_{12} in sweet cream butter.

FFA	Added AFT in mg/kg[1]	Total AFT in mg/kg[1,2]	Total AFT in moles (× 10^{-4}) FFA / kg
C_4	11.4 ± 2.1	50.5 ± 15.8	5.7
C_6	51.5 ± 9.8	93.7 ± 29.7	8.1
C_8	454.6 ± 87.1	454.6 ± 144.2	31.5
C_{10}	161.6 ± 37.9	363.0 ± 140.9	21.1
C_{12}	127.9 ± 30.0	303.4 ± 117.8	15.1

[1] 95% confidence limit on AFT.
[2] Endogenous FFA level (Table I, Butter Sample I) plus added FFA.

Table 3—Flavor thresholds of C_4-C_{12} free fatty acids in various media.

	Threshold concentration in ppm				
FFA	Added sweet cream butter	Total sweet cream butter	Water[1]	Neutral Edible oil[2]	Milk[1]
4:0	11.4	50.5	6.8	0.6	25
6:0	51.6	93.8	5.4	2.5	14
8:0	454.7	454.7	5.8	350.0	—
10:0	161 [illegible]	[illegible]3.0	3.5	200.0	—
12:0	12[illegible]	[illegible]4	...	700.0	—

[1] Patt[illegible]
[2] Fer[illegible]

Preference panels

Student preference panels of 150 members were conducted for each individual FFA in butter. Students serving on the panel were not selected for taste acuity. A hedonic scale from like extremely (nine) to dislike extremely (one) was used. Each student panel member was given four coded butter samples and a hot roll. One sample was sweet cream butter and the remaining three butter samples contained varying amounts of added fatty acids.

Statistical analysis

A linear regression of correct positive responses on FFA concentration was run on each replication of the individual and mixture threshold panel data, and on the duo-trio panel data. The FFA concentration at the 50% level of positive responses was reported as the average flavor threshold (AFT). In mixture threshold interactions determinations, each component was tested separately for significance because the mixture ratios were approximations of individual thresholds.

For statistical analysis of the mixture threshold data, a transformation was made to approximate common variances for all compounds by dividing the AFT of each compound in a mixture by the AFT obtained when the compound was evaluated by itself. A pooled variance term from the individual and mixture AFT was used in the *t* test for interaction significance. Confidence limits of 95% were calculated for the 50% positive response threshold.

The triangle tests were evaluated by using the 5 and 1% levels from the "Significance in Triangular Test Tables" in Amerine et al. (1965). The preference test data were analyzed by analysis of variance and the least significant difference (LSD) determined at both the 5 and 1% levels.

RESULTS & DISCUSSION

RESULTS of FFA analyses of the three sweet cream butter samples used for the panel testing are presented in Table 1. Values for butteroil samples analyzed by Iyer et al. (1967), and Bills et al. (1963) are given also for comparison.

The levels of short-chain fatty acids found in butter were considerably higher than those reported for butteroil. This is probably due to the fact that intact butter samples were used rather than isolated milk fat. The short-chain fatty acids would partition more favorably into the aqueous phase, and thus would be measured more completely when intact butter is analyzed instead of isolated milk fat.

Results of the individual fatty acid threshold panels are presented in Table 2. Values are presented for both added and total FFA levels at the AFT in the butter. Butyric and hexanoic acids were generally detected by both odor and taste when added to butter samples. The results show that as chain length increased the AFT increased to a maximum for octanoic acid. Decanoic and dodecanoic acids had lower thresholds than octanoic acid, and were detected primarily by their characteristic soapy after-taste. Interpretation of the AFT values on a molar basis rather than a weight basis gave the same general threshold pattern (Table 2).

The interpretation of threshold data are difficult when the product already contains an endogenous level of the class of compounds tested. The amount of FFA present in salt or acid form in butter depends on the pH of the medium and the pKa of the acids involved. Although the Henderson-Hasselbach equation is most applicable for ideal aqueous systems, it can be used to predict approximate salt-acid ratios in complex biological systems. Therefore, butter with a pH of 6.5 would yield a 45:1 salt to acid ratio. This is probably one reason for butyric and hexanoic acids having lower thresholds in water than in milk even though milk contains endogenous FFA. Table 3 summarizes threshold data for fatty acids in various media.

The threshold values for both the added AFT and the total AFT of mixtures of FFA in butter are presented in Table 4. The *t*-test for interaction significance compares the AFT of each individual FFA alone with the AFT of each FFA in the mixture.

In this type of statistical evaluation, it is possible for zero one or more of the components in a mixture to show a significant flavor interaction. The calculated *t*-values for the mixture of butyric and octanoic acids illustrate this point. The *t*-values for butyric and octanoic acids at their added AFT levels were −4.481 and −4.684, respectively. These relatively large negative *t*-values reveal that the concentrations of both acids at their AFT in the mixture were significantly lower (at 0.01% level) than the concentration of each at its AFT alone. When total AFT's were evaluated for the same mixture, a *t*-value of −2.371 was obtained for octanoic acid. This showed that there was a significant flavor interaction at the 0.05% level, but not at the 0.01% level. On the other hand, the *t*-value for butyric acid in the same mixture was +0.035. This positive *t*-value indicates that the concentration of butyric acid in the mixture at the total AFT was higher than its concentration at its AFT alone, i.e., no flavor interaction effects were noted.

Many negative *t*-values were found for components of the mixtures which were not statistically significant. These *t*-values, although not statistically significant at either the 0.05 or the 0.01% level, indi-

Table 4—Average flavor thresholds of mixtures of free fatty acids in sweet cream butter.

FFA mixture	Added AFT[1] mg/kg	Calculated t-value	Total AFT[2] mg/kg	Calculated t-value
Butyric, Hexanoic				
Butyric	4.5 ± 2.6	−4.077[3]	50.7 ± 19.6	+0.020
Hexanoic	16.7 ± 12.1	−4.566[3]	64.5 ± 36.4	−1.271
Butyric, Octanoic				
Butyric	4.6 ± 2.1	−4.481[3]	50.9 ± 16.0	+0.035
Octanoic	171.4 ± 87.0	−4.684[3]	217.9 ± 144.0	−2.371[4]
Butyric, Decanoic				
Butyric	7.2 ± 2.1	−2.711[3]	53.5 ± 16.0	+0.027
Decanoic	140.9 ± 30.9	−0.862	228.2 ± 114.9	−1.512
Butyric, Dodecanoic				
Butyric	6.8 ± 2.6	−2.695[3]	52.6 ± 19.6	+0.168
Dodecanoic	[illegible].0 ± 30.0	−2.781[3]	130.8 ± 117.8	−2.114[4]
Hexanoic, Octanoic				
Hexanoic	[illegible]3.2 ± 12.1	−5.007[3]	61.0 ± 36.4	−1.421
Octanoic	148.2 ± 106.6	−4.542[3]	174.9 ± 176.5	−2.505[3]
Hexanoic, Decanoic				
Hexanoic	25.2 ± 12.1	−3.444[3]	73.0 ± 36.4	−0.898
Decanoic	132.8 ± 37.9	−1.095	201.8 ± 140.9	−1.650
Hexanoic, Dodecanoic				
Hexanoic	26.0 ± 12.1	−3.349[3]	73.7 ± 36.4	−0.870
Dodecanoic	71.5 ± 30.0	−2.714[3]	132.7 ± 117.8	−2.091[4]
Octanoic, Decanoic				
Octanoic	367.8 ± 106.6	−1.287	394.9 ± 176.5	−0.534
Decanoic	173.2 ± 37.9	+0.443	260.3 ± 140.9	−1.051
Octanoic, Dodecanoic				
Octanoic	335.6 ± 106.6	−1.760[4]	362.4 ± 176.5	−0.825
Dodecanoic	84.0 ± 30.0	−2.110[4]	143.4 ± 117.8	−1.959[4]

[1] AFT with 95% confidence limits.
[2] Endogenous free fatty acid level (Table 1, Butter Sample II) plus added FFA.
[3] Significant at 0.01% level ($t = -2.457$ for 30 degrees of freedom).
[4] Significant at 0.05% level ($t = -1.697$ for 30 degrees of freedom).

Table 5—Preference panels mean flavor scores of FFA concentrations in sweet cream butter.

Added mg/kg	Total[1] mg/kg	Mean score	LSD
	Butyric		
0.0	66.4	6.45	
8.2	74.6	6.44	0.01 = 0.3276
16.5	82.9	6.39	0.05 = 0.2492
33.0	99.4	5.93	
	Hexanoic		
0.0	26.4	6.24	
40.0	66.4	6.38	0.01 = 0.3368
80.0	106.4	6.06	0.05 = 0.2562
160.0	186.4	5.96	
	Octanoic		
0.0	7.4	6.44	
576.4	583.8	5.96	0.01 = 0.3721
929.5	936.9	5.19	0.05 = 0.2831
1639.0	1646.4	5.15	
	Decanoic		
0.0	167.1	6.56	
112.0	279.1	6.55	0.01 = 0.3575
225.0	392.1	6.16	0.05 = 0.2720
450.0	617.1	5.56	
	Dodecanoic		
0.0	95.1	6.62	
82.5	177.6	6.67	0.01 = 0.3239
165.0	270.1	6.53	0.05 = 0.2465
330.0	425.1	6.08	

[1] Endogenous FFA level from Table 1, Butter Sample III plus added FFA.

cate a general tendency for the fa ty acids to participate in flavor interactions. Considering only added AFT data, butyric and hexanoic acids always exhibited significant flavor interactions. The same was true for the soapy-tasting dodecanoic acid containing mixtures. Octanoic acid showed significant flavor interactions for all added AFT mixtures except that of octanoic and decanoic acids. Decanoic acid did not exhibit any significant interaction in any of the mixtures.

When total AFT's were evaluated (Table 4), only octanoic acid in mixtures with butyric and hexanoic acids and decanoic acid in mixtures with butyric, hexanoic and octanoic acids showed significant flavor interactions. While butyric, hexanoic and dodecanoic acids appeared to be highly significant when considering added AFT values, only octanoic and dodecanoic acids were found to be significant in flavor interactions at the total AFT. These data emphasize the importance of consideration of endogenous levels of [illegible] (and other flavor compounds) present in the testing medium.

The added AFT values for the mixture of the soapy-tasting decanoic and dodecanoic acids were determined by triangular tests to be 96.9 and 76.7 ppm, respectively, and the total AFT values were 184.0 and 137.4 ppm, respectively (at the 95% confidence limit).

Caution should be used in comparing these data directly with the values given in Tables 2 and 4. Triangular test thresholds are determined by significance tables where $p = 1/3$, and thus are sample number dependent. If the 50% positive response level were used instead of the 95% significance level, AFT levels probably would be lower than those observed using triangular tests.

The results of the preference panel tests on butter containing the respective FFA added at approximate threshold and higher levels are given in Table 5. For butyric acid, the panel did not show a significant decrease in preference until a total level of 99.4 ppm was attained. This level was about twice the total AFT obtained for butyric acid by the difference panel (Table 2). However, for the remaining fatty acids studied, the total concentrations at which significant preference flavor score decreases occurred were within the confidence limits established by the individual FFA threshold determinations (Table 2).

The determination of AFT values for FFA in butter allows an estimation of the importance of each acid in butter flavor. For example, the total AFT of octanoic acid was much higher than that usually found in fresh sweet cream butter. Even though high levels of octanoic acid showed significant flavor interactions, the levels found in fresh butter would indicate that it is of only minor importance in fresh butter flavor. However, octanoic acid may be highly important in hydrolytic rancidity flavors in dairy products. This study has demonstrated interactions between pairs of compounds in the presence of endogenous levels of the same class of compounds as well as other non-acid flavor components in sweet cream butter. It is probable that all contributing flavor components interact to yield complete sweet cream butter flavor.

In summary, FFA thresholds in butter show a threshold pattern dependent on chain-length. Butyric acid exhibited the lowest total AFT for the more volatile FFA, and the AFT values increased as chain-length increased for hexanoic and octanoic acids. A soapy after-taste predominated at and above the AFT for decanoic and dodecanoic acids. Threshold values decreased from octanoic acid through dodecanoic acid as the chain-length increased. Mixture threshold results support the concept that flavor components interact at subthreshold concentrations. Endogenous FFA levels must be considered when testing products already containing FFA as part of their natural flavor. A decrease in preference was found for butters containing suprathreshold levels of added FFA.

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ABRAHAM LIFSHITZ and Y. STEPAK
Department of Biochemistry, Tel-Aviv University, Tel-Aviv, Israel
H. B. BASKER
Braverman Citrus Products Research Laboratory, Rehovot, Israel

Characterization of Israel Lemon Oil and Detection of its Adulteration

SUMMARY—Samples of genuine Israel lemon oil were examined by the usual standard methods and by gas-liquid chromatography. The variations obtained are discussed, and were evaluated statistically. Chi-square equations were computed for the evaluation of samples of doubtful origin. Sophisticated adulterations of the other of 10% were clearly detectable.

INTRODUCTION

OF THE three principal citrus essential oils traded internationally, the highest prices are paid for lemon oil, and therefore some risk of adulteration is always present. The characterization of genuine Israel lemon oil by means of a statistical appraisal of the relationship between some of the physical and chemical parameters routinely tested and by G.L.C. examination studied on samples from three successive seasons are here reported. It is hoped that similar investigations will be undertaken by institutions in the other main growing areas.

In a review of some of the "classical" methods of analysis of citrus oils, Safina (1962) noted that these provided a framework only, within which wide variations were commonly found. Gas-liquid chromatography has been used for the analysis of lemon oils (Bernhard, 1960; Montes, 1962; MacLeod et al., 1964) but has not been systematically used to accumulate data on their intraseasonal variation, nor as a systematic tool for the detection of adulteration.

EXPERIMENTAL

Materials

For the statistical appraisal of the relationships between the physical and chemical parameters routinely tested, samples of genuine Israel lemon oil were drawn from seven citrus-processing factories operating under the general supervision of one of us. The samples are considered representative of the entire growing area in Israel. They were examined after settling of any insoluble suspended material had been completed, and before having been packed for storage, *i.e.*, between one and two months after production. All the oils were produced by the cold-press method (Braverman, 1963).

During the 1964–65 processing season, 15 such samples were examined; during the 1965–66 season, 9 samples and during the 1966–67 season, 15 samples were examined.

For gas-liquid chromatography, 30 samples of cold-pressed Israel lemon oil were drawn during the course of the 1967–68 processing season, directly at the clarifying centrifuges at four factories located in the central (main) growing region, and were examined after settling had been completed, as above.

Methods of examination

Details of the "classical" methods of examination performed are given in the Israel Standard (S.I. 180, 1959). The nonvolatile residue was obtained after heating on a boiling water bath at atmospheric pressure and is expressed as a percentage by weight. The specific gravity, refractive index and optical rotation were determined at ambient temperature and were corrected to 20°C. The acid value was determined by titration. The total aldehyde content was determined by the hydroxylamine hydrochloride method, and is expressed as the percentage by weight of citral.

Gas-chromatographic examination was performed by injection of 1 μl aliquots in a Packard Model 7621 gas chromatograph equipped with a flame ionization detector. A 6-ft column by $^1/_4$ in. o.d. packed with 20% Carbowax 20 M on 60- to 80-mesh Chromosorb P was used. Gas flows were: nitrogen, 14 ml/min; hydrogen 40 ml/min; and air 500 ml/min. Injection-port temperature was 200°C; oven temperature was 171°C; and detector temperature was 210°C. The detector potential was 250 volts, and chart speed was set at 30 in./hr.

RESULTS AND DISCUSSION

Standard specifications

The limiting ranges of the various parameters specified by the Israel Standard (S.I. 180, 1959) are shown in Table 1.

Table 1—Standard specifications for Israel lemon oil

Nonvolatile matter (% w/w)	Maximum 4
Specific gravity (20°C)	0.850 to 0.864
Refractive index (20°C)	1.473 to 1.476
Optical rotation $[\alpha]_D^{20}$	+63° to +71°
Acid value (mg KOH per g)	Maximum 0.6
Citral content (% w/w)	2.0 to 3.0

The values obtained on examination of the samples are shown in Table 2. It will be seen that one of the 39 samples lies outside the limits for specific gravity and that all 39 comply with the limits for nonvolatile matter, refractive index, optical rotation, acid value and citral content. The mean values obtained are shown in Table 3 and the standard deviations calculated are shown in Table 4.

The results were treated by analysis of variance. The differences between the seasonal means of optical rotation were found to be very significant ($P < 0.0005$). The differences between the seasonal means of acid value may be significant ($p < 0.025$).

Correlations were sought between the values obtained on analysis of the samples, and those of statistical significance are shown in Table 5, together with some others of interest. It is seen that none of the three pairs of parameters where significant correlations were found (optical rotation and acid value, optical rotation and citral content, nonvolatile matter and acid value), were equally significant during all the seasons examined. The negative relationship found between optical rotation and acid value is of particular interest.

Gas chromatography

Two of us have identified some of the constituents of Israel lemon oil by gas-chromatographic techniques (Lifshitz and Stepak unpublished data). A typical trace is shown in Fig. 1 in which seven characteristic peaks have been numbered serially. The heights of these peaks were recorded on all the samples examined and are shown in Table 6; the means, standard deviations and coefficients of variation of the heights are shown in Table 7. Correlations were sought between the heights of the peaks listed, and those of statistical significance are shown in Table 8.

Chi-square test

The χ^2 test was performed by constructing an equation as follows:

(a) The mean and the standard deviation of the values of each relevant parameter ($\bar{x}_i$, σ_{xi}) were calculated.

(b) The covariance of each pair of relevant parameters was calculated.

Table 2—Detailed results of standard examination of samples of Israel lemon oil

Season	Non-volatile matter, % w/w	Specific gravity, 20°/20°C	Refractive index, n_D^{20}	Optical rotation, $[\alpha]_D^{20}$	Acid value, mg KOH / 1g oil	Citral content, % w/w
1964–65	2.7	0.8528	1.4747	65.3	0.40	2.16
	2.6	0.8516	1.4746	70.0	0.30	2.38
	3.1	0.8500	1.4751	69.6	0.35	2.47
	4.0	0.8508	1.4753	67.7	0.40	2.54
	3.1	0.8509	1.4747	68.3	0.30	2.43
	2.7	0.8512	1.4741	67.0	0.30	2.60
	2.7	0.8506	1.4754	66.8	0.40	2.59
	3.0	0.8516	1.4750	69.0	0.40	2.86
	3.8	0.8502	1.4744	67.2	0.40	2.74
	2.7	0.8504	1.4749	68.3	0.40	2.61
	3.0	0.8512	1.4744	69.3	0.30	2.49
	2.2	0.8508	1.4747	69.6	0.24	2.77
	2.3	0.8503	1.4753	67.9	0.20	2.68
	2.3	0.8509	1.4737	66.5	0.40	2.52
	2.3	0.8541	1.4743	66.1	0.45	2.66
1965–66	2.5	0.8506	1.4758	67.0	0.30	2.87
	2.4	0.8506	1.4746	67.5	0.30	2.73
	2.6	0.8535	1.4756	63.1	0.47	2.17
	3.3	0.8511	1.4741	65.4	0.47	2.76
	3.2	0.8533	1.4747	67.4	0.50	2.76
	2.1	0.8549	1.4751	66.3	0.39	2.84
	2.3	0.8499	1.4751	67.9	0.29	2.77
	2.2	0.8511	1.4754	66.3	0.36	2.47
	3.2	0.8520	1.4752	68.7	0.47	2.85
1966–67	3.3	0.8512	1.4738	69.1	0.31	2.66
	2.5	0.8528	1.4746	69.0	0.26	2.37
	2.8	0.8513	1.4751	69.2	0.26	2.37
	3.2	0.8566	1.4737	68.3	0.29	2.40
	3.1	0.8531	1.4750	66.7	0.43	2.37
	2.7	0.8517	1.4744	69.8	0.28	2.58
	2.9	0.8506	1.4743	69.8	0.31	2.76
	3.1	0.8514	1.4741	69.7	0.25	2.72
	3.1	0.8517	1.4744	70.0	0.32	2.62
	2.3	0.8540	1.4731	70.3	0.25	2.76
	1.9	0.8508	1.4749	70.0	0.23	2.80
	2.4	0.8520	1.4753	69.4	0.20	2.69
	2.9	0.8519	1.4749	69.2	0.40	2.65
	2.7	0.8525	1.4758	67.6	0.32	2.38
	3.4	0.8526	1.4757	69.9	0.40	2.40

Table 3—Mean values of various parameters obtained during different growing seasons

	Season			
	1964–65	1965–66	1966–67	3 Seasons
Nonvolatile matter	2.83	2.64	2.82	2.79
Specific gravity	0.851	0.852	0.852	0.852
Refractive index	1.475	1.475	1.475	1.475
Optical rotation	67.91	66.62	69.20	68.11
Acid value	0.35	0.39	0.30	0.34
Citral content	2.57	2.69	2.57	2.60

Table 4—Standard deviations of various parameters obtained during different growing seasons

	Season			
	1964–65	1965–66	1966–67	3 Seasons
Nonvolatile matter	0.526	0.467	0.413	0.465
Specific gravity	0.001	0.002	0.002	0.001
Refractive index	<0.0005	0.001	0.001	0.001
Optical rotation	1.419	1.642	0.988	1.637
Acid value	0.072	0.085	0.066	0.080
Citral content	0.173	0.228	0.168	0.187

(c) The matrix

$$A = \begin{pmatrix} \sigma_{X_1}{}^2 & \text{cov}(X_1,X_2) & \text{cov}(X_1,X_3) & \dots \\ \text{cov}(X_2,X_1) & \sigma_{X_2}{}^2 & \text{cov}(X_2,X_3) & \dots \\ \text{cov}(X_3,X_1) & \text{cov}(X_3,X_2) & \sigma_{X_3}{}^2 & \dots \\ \cdot & \cdot & \cdot & \\ \cdot & \cdot & \cdot & \\ \cdot & \cdot & \cdot & \end{pmatrix}$$

was formed.

Table 5—Some correlations obtained between the results of standard examination of samples of Israel lemon oil

Parameters	Season 1964–65	1965–66	1966–67	3 Seasons
Optical rotation and acid value	−0.52[1]	−0.32	−0.46	−0.59[2]
Optical rotation and citral content	+0.22	+0.79[1]	+0.66[3]	+0.26
Nonvolatile matter and acid value	+0.31	+0.75[1]	+0.63[1]	+0.39[1]
Number of samples	15	9	15	39

[1] Significant at 5% level.
[2] Significant at 0.1% level.
[3] Significant at 1% level.
Unsigned: not statistically significant.

Table 6—Detailed results of G.L.C. peak heights (mm) of samples of Israel lemon oil

Sample No.	Fraction No.[1] 1	2	3	4	5	6	7
1	84	29 1/2	44 1/2	159	81	71 1/2	111
2	80	20	38	149 1/2	75	64 1/2	105
3	77	27	40	162 1/2	90	76	112 1/2
4	78	30	42 1/2	173	90	76	118
5	78	26	32 1/2	177	78 1/2	73 1/2	117
6	82	24	42	154	79	72	108
7	81	23	42	152	77	71	107
8	79	26	38	167	78 1/2	71	111
9	76	27 1/2	38 1/2	160	75	67	106 1/2
10	81	20	30	149	81	68	100
11	79 1/2	27	39	157	88 1/2	66 1/2	112
12	82	25	32 1/2	169	81	67	102
13	86	28 1/2	41	159	91	75 1/2	111
14	78	27	36	158	82 1/2	83 1/2	118
15	80	16 1/2	32 1/2	155	88	89	115
16	79 1/2	18 1/2	32	150	93 1/2	86	114 1/2
17	76	14	31	144	82	81	110
18	81	21 1/2	24	156	81	75	109 1/2
19	90	28 1/2	41	166	80	73	107
20	90	24	40	177	84	87	101
21	75	18 1/2	37	164	66	60	100
22	75	15	34	163	70	65	98
23	75	12	38 1/2	165	73	69	99
24	77	25	34	172	76	72	100
25	81	32	40	160	82	73	105
26	80	22	31	150	78	69	102
27	78	29	36	160	80	81	109
28	80	21	35	155	80	82	109
29	79	24	32	165	75	70	107
30	76	28	39	160	75	67	106

[1] Fraction numbers correspond to peak numbers of Fig. 1 and Tables 7 and 8.

Table 7—Fractions identified in Israel lemon oil and employed here for statistical analysis

Fraction[1]	Compound	Peak characteristics: Mean height, mm	Standard deviation, mm	Coefficient of variation, %
1	*d*-Limonene	79.8	3.82	4.80
2	Decanal and/or Linalool	23.7	5.09	21.5
3	Linalyl acetate	36.5	4.65	12.7
4	Citronellol	160.3	8.33	5.20
5	Geranyl acetate	80.4	6.29	7.83
6	Neral	73.7	7.09	9.64
7	Geranial	107.7	5.76	5.34

[1] Fraction numbers correspond to peak numbers of Fig. 1 and Tables 6 and 8.

(d) The values of the inverse matrix were computed.

$$A^{-1} = \begin{pmatrix} b_{11} & b_{12} & b_{13} & \dots \\ b_{21} & b_{22} & b_{23} & \dots \\ b_{31} & b_{32} & b_{33} & \dots \\ \cdot & \cdot & \cdot & \\ \cdot & \cdot & \cdot & \\ \cdot & \cdot & \cdot & \end{pmatrix}$$

(e) Then, putting $\chi_i = X_i - \bar{x}_i$ and assuming that the parameters are normally distributed about their means

$$\chi^2 = b_{11}x_1{}^2 + b_{12}x_1x_2 + b_{13}x_1x_3 + \dots + b_{21}x_2x_1 + b_{22}x_2{}^2 + b_{23}x_2x_3 + \dots + b_{31}x_3x_1 + b_{32}x_3x_2 + b_{33}x_3{}^2 + \dots$$

with number of degrees of freedom equal to the number of parameters.

Comparing Tables 3 and 4, it will be seen that the coefficients of variation of the optical rotation and the citral content were 2.4 and 7.2% respectively. These parameters are not considered to be liable to change during industrial handling. Any adulterations of the type described below would not be expected to have any appreciable effect on either the specific gravity or the refractive index, and they are therefore not considered further even though their coefficients of variation were low. From Table 5 it will be seen too that some significance may be attached to the relationship between the optical rotation and the citral content. These considerations suggested that a useful χ^2 equation might be constructed on the basis of the values of these two parameters.

Thus

$$\chi_2{}^2 = 0.400a^2 + 30.5c^2 - 1.80ac \quad (1)$$

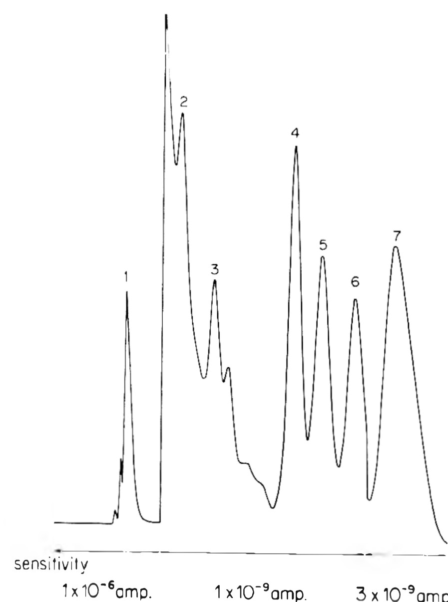

Fig. 1—Typical G.L.C. chromatogram of Israel lemon oil. Column: 20% Carbowax 20 M on 60- to 80-mesh Cromosorb P.

where a = optical rotation—68.11 degrees and c = citral content—2.60%.

A sample of Israel lemon oil can then be said to be from a different population from those examined,

(a) at the 95% confidence level, if, according to equation (1)

$$\chi_2^2 \geq 5.99$$

(b) at the 99% confidence level, if, according to equation (1)

$$\chi_2^2 \geq 9.21$$

This relationship is shown graphically in Fig. 2.

Half of all samples would be expected to fall within the limits of the ellipse equivalent to the $p = 0.50$ value of χ^2, 95% within the limits of the ellipse equivalent to the $p = 0.95$ value of χ^2 and 99% within the limits of the ellipse equivalent to the $p = 0.99$ value of χ^2.

From Table 7, it will be seen that fairly low coefficients of variation of the peak heights were obtained for fractions 1, 4, 7 and 5 (in order of increasing magnitude). Considerations similar to those above suggested that useful χ^2 equations might be constructed on the basis of the values of either three or four of these parameters. Thus

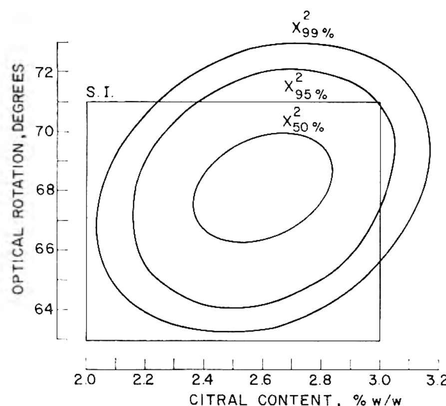

Fig. 2—Relationship between optical rotation and citral content of Israel lemon oil. Distribution of χ^2 according to equation (1).

$$\chi_3^2 = 0.070a^2 + 0.015d^2 + 0.030g^2 - 0.008ad - 0.002ag + 0.002dg \quad (2)$$

and

$$\chi_4^2 = 0.091a^2 + 0.015d^2 + 0.058e^2 + 0.059g^2 - 0.014ad - 0.072ae + 0.048ag + 0.010de - 0.006dg - 0.082eg \quad (3)$$

where

a = height of peak no. 1—79.8 mm
d = height of peak no. 4—160.3 mm
e = height of peak no. 5—80.4 mm
g = height of peak no. 7—107.7 mm

Table 8—Statistically significant correlations obtained between the peak heights of the fractions listed in Table 6

Fractions[1]	Correlation coefficient
1, 5	+0.367[2]
2, 3	+0.487[3]
5, 6	+0.718[4]
5, 7	+0.653[4]
6, 7	+0.578[4]

[1] Fraction numbers correspond to peak numbers in Fig. 1 and fraction numbers in Tables 6 and 8.
[2] Significant at 5% level.
[3] Significant at 1% level.
[4] Significant at 0.1% level.

A sample of Israel lemon oil can then be said to be from a different population from those examined,

(c) At the 95% confidence level, if, according to equation (2), $\chi_3^2 \geq 7.81$

(*d*) At the 99% confidence level, if, according to equation (2), $\chi_3^2 \geq 11.34$

(e) At the 95% confidence level, if, according to equation (3), $\chi_4^2 \geq 9.48$

(f) At the 99% confidence level, if, according to equation (3), $\chi_4^2 \geq 13.27$

A three-dimensional model would be required to illustrate equation (2). No real model can be drawn to equation (3).

Detection of adulteration

Samples of Israel lemon oil were deliberately mixed with varying proportions (in steps of 5 or 10%) of *d*-limonene, Israel orange oil, commercial lemon terpenes and commercial orange terpenes, with and without further additions of citral. Mixtures with distilled lemon oil and synthesized lemon oil (No. 320, Glidden Co., Jacksonville, Fla.) were also prepared. The limiting proportions of the admixed substances, which altered the results of the classical parameters sufficiently to cause them to lie outside the limits of the Israel Standard (S.I. 180, 1959), are shown in Table 9. Also in Table 9 are shown the limiting proportions of admixed substances which, on the basis of the χ^2 tests, according to equation (1) (Classical tests), (2) and (3) (GLC tests) do not form part of the same population, at the 99% confidence level; some such proportions at the 95% confidence level are shown in brackets. Sophisticated adulterations of the order of 20% were detected by equation (1), and of the order of 10% by equations (2) and (3).

Table 9—Percentages of some possible adulterants detectable by various methods[1]

	"Classical" tests			Gas chromatography	
	Optical rotation	Citral content	Equation (1)	Equation (2)	Equation (3)
d-limonene	25	25	10	10	10
+0.1% citral	25	25	10	10	10
+0.2% citral	25	30	10	10	10
+0.3% citral	25	30	10	10	10
Orange oil	15	n.d.[2]	15	15	15
+0.1% citral	15	n.d.	15	15	15
+0.2% citral	15	n.d.	15	15	15
+0.3% citral	15	n.d.	15	15	15
Commercial lemon terpenes	n.d.	30	20	(30) 40	20
+0.1% citral	n.d.	30	20	(20) 30	20
+0.2% citral	n.d.	35	20	(10) 30	20
+0.3% citral	n.d.	40	20	(20) 30	(20) 30
Commercial orange terpenes	n.d.	30	20	10	10
+0.1% citral	n.d.	30	20	10	10
+0.2% citral	n.d.	35	20	10	10
+0.3% citral	n.d.	40	20	10	10
Distilled lemon oil	n.d.	30	20	10	10
Synthesized lemon oil	n.d.	n.d.	(30) 40	10	10

[1] For the χ^2 test, the percentages detected at the 95% confidence level are shown in brackets; the remainder refer to the 99% confidence level.
[2] Not detectable even when 50% present.

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G. L. ZIMMERMAN and H. E. SNYDER
Department of Food Technology, Iowa State University, Ames, Iowa 50010

Meat Pigment Changes in Intact Beef Samples

SUMMARY—Reflectance spectrophotometry was used to study pigment changes in beef samples that (1) had been oxygenated and wrapped with an oxygen-impermeable film, (2) had been treated with ferricyanide and (3) had either treatment (1) or (2) plus malonic acid. Treatment (1) showed a metmyoglobin (MetMb) accumulation of 30% in 30 min, followed by reduction of MetMb. Malonic acid inhibited oxygen utilization and MetMb-reducing activity (MRA). Treatment (2) showed MRA that was inhibited by the presence of oxygen or malonic acid. Malonic acid inhibited oxygen utilization rather than directly inhibiting MRA.

INTRODUCTION

THE PRIMARY motivation for studying MetMb accumulation in fresh beef samples is the discoloration that develops in meat during storage. This discoloration restricts attempts to market fresh beef by a centralized prepackaging system. Recent work has shown the feasibility of using reflectance spectrophotometry as a nondestructive analysis technique for following changes in the myoglobin derivatives in beef samples (Stewart et al., 1965b; Snyder et al., 1967).

Two ideas have been used to explain the accumulation of MetMb in meat samples. One is the autoxidation of MbO_2 and of Mb, which results in MetMb. This reaction has a peculiar response to the partial pressure of oxygen in that decreased partial pressures enhance the oxidation. Extensive studies of autoxidation of myoglobin (George et al., 1952a; 1952b, and 1954) and autoxidation of hemoglobin (Brooks, 1935) in vitro have established the relationship between autoxidation rate and partial pressure of oxygen. These results do have relevance to meat discoloration because the initial observations of Brooks, which suggest an enhanced rate of autoxidation due to decreased oxygen pressure, were made on meat samples (Brooks, 1929). Robach et al. (1961) presented evidence that the effect of bacteria on MetMb accumulation is probably due to a decrease of partial pressure of O_2, which enhances the autoxidation.

A second idea pertinent to MetMb accumulation in meat samples is the ability of meat to reduce MetMb to Mb, which can oxygenate to form MbO_2. The MetMb-reducing activity (MRA) that exists in muscle has been studied in ground meat systems by Cutaia et al. (1964) and by Stewart et al. (1965b), Watts et al. (1966) and Hutchins et al. (1967).

We initiated this study of intact beef samples to examine some particular situations of MetMb accumulation and MetMb reduction. We were particularly interested in data that would help distinguish between autoxidation and loss of MRA as causes for MetMb accumulation. The particular situations were: MetMb accumulation following the wrapping of an oxygenated piece of meat with an oxygen-impermeable film, effects of respiratory inhibitors on MetMb accumulation and MRA, and MRA at the surface of meat samples treated with ferricyanide and made anaerobic.

There is considerable evidence that the respiratory activity of meat (and the effect of this activity on the partial pressure of oxygen) influences meat discoloration. For example, high partial pressures of oxygen tend to retard autoxidation but also tend to retard MRA. Since there are reports in the literature that high oxygen pressures inhibit or destroy the activity of sulfhydryl enzymes (Dickens, 1946; Rosenbaum et al., 1966), we attempted to inhibit enzymatic respiratory activity by using high oxygen pressures. The rationale was that inhibition of respiration would lead to a high partial pressure of oxygen throughout the meat sample. This should be conducive to maximum color stability although flavor stability through lipid oxidation might suffer.

EXPERIMENTAL

Apparatus

The instrument used for all reflectance measurements was a Beckman Spectrophotometer, DK-2A, with Model 24500 reflectance attachment. The integration sphere and reference plates were coated with MgO, and sample holders and reference plates were in the diffuse reflectance position.

A Bethlehem No. 614 table-top hyperbaric chamber was used for treating samples with high oxygen pressure. Oxygen was obtained from a commercial cylinder, and the chamber was flushed with oxygen before increasing the pressure. Temperature was controlled by placing the hyperbaric chamber and oxygen cylinder in a cold room at 2°C.

Reflectance techniques.

The relative amounts of myoglobin derivatives at the surface of meat samples can be measured by the reflectance techniques described by Stewart et al. (1965a) and by Snyder et al. (1967). In brief, this technique consists of treating meat samples to contain predominantly Mb, MbO_2, or MetMb at the surface (Snyder, 1965) and measuring the percentage reflectance at 474, 525 and 571 nm. The percentage reflectance measurements are converted to K/S values by using Table D of Judd et al. (1963). By forming ratios of the K/S values at the appropriate wave lengths and by plotting the ratios vs. percentage myoglobin derivative, it is possible to measure the percentage Mb, MbO_2 and MetMb at the surface of meat samples.

This measurement involves the assumption that a linear relationship exists between the K/S ratios and percentage myoglobin derivative. It has been shown that this assumption is valid for a model system containing myoglobin (Snyder et al., 1967).

The reflectance measurements and K/S ratios from this study agree well with ratios reported earlier (Stewart et al., 1965a; and Snyder et al., 1967). We found the K/S ratio for MetMb to be 0.59 at 571 nm/525 nm; for MbO2, K/S equaled 1.36 at 571 nm/525 nm and 0.88 at 474 nm/525 nm; and for Mb, K/S equaled 0.53 at 474 nm/525 nm.

Treatment of beef samples.

Slices of freshly cut beef round purchased from a local market were cut further in the laboratory to fit plastic sample boxes 2 × 1 1/2 × 1/2 in. The samples were 1/2 in. thick. The samples were over wrapped with 195-MSAD-80 (DuPont) or CryoVac L-200 films for oxygenation of the surface or with Saran (Dow) to exclude oxygen.

Inhibitors were added by pipetting the correct volume onto the surface and spreading the solution uniformly. For malonic acid additions, we found no difference between adding the free acid or the sodium salt of the acid. All inhibitors were used as either 1 ml of an 0.1 *M* solution or 0.5 ml of an 0.2 *M* solution. Potassium fericyanide was added as either 1 ml of a 1% solution or 0.5 ml of a 2% solution.

The actual concentration of the inhibitors throughout the meat is not known nor is the degree of diffusion of the inhibitors known.

RESULTS & DISCUSSION

Hyperbaric oxygen treatment.

We exposed fresh beef samples to 80 psig of oxygen for short time periods (ap-

proximately 12 hr) and for 12 days. The high oxygen pressure was sufficient to completely oxygenate samples so that no Mb could be observed beneath the surface. During the time that the samples were exposed to high oxygen pressure, there was no gross evidence of MetMb accumulation. The samples were observed for the presence of Mb and MetMb but no reflectance studies were made. The excessive oxygenation under hyperbaric oxygen led to different K/S ratios for MbO_2 than we observed in air.

The purpose for treating meat samples with hyperbaric oxygen was to test the possibility of inhibiting respiratory enzymes by oxidizing sulfhydryl groups. There was no indication of any permanent inhibition. After removing the 12-hr samples from the hyperbaric chamber, the meat continued to respire and MetMb accumulated at approximately the same rate as in control samples not exposed to high oxygen pressures. The samples treated for 12 days did not appear to have appreciable MetMb accumulated, but did have off-odors that may be attributed to lipid oxidation or bacterial spoilage, or both. Evidently, MetMb accumulation is retarded under these extremely high oxygen pressures, but no new practical or theoretical information was obtained.

MetMb accumulation by excluding oxygen.

Cutaia et al. (1964) presented data for accumulation of MetMb in ground beef samples that were allowed to oxygenate and then wrapped with an oxygen-impermeable film. Maximum MetMb accumulation amounted to 40 to 60%, occurred approximately 10 to 15 hr after excluding oxygen, and was followed by reduction to Mb.

We have noticed a similar, but much more rapid sequence of changes, when intact beef samples are allowed to oxygenate and then wrapped with Saran. Typical results are shown in Figure 1. MetMb accumulates to approximately 30% in 30 min followed by reduction to Mb. The temperature of the sample was 2°C at the start of the experiment, but it was held in the spectrophotometer at about 23°C during the experiment. The main effect of the sample gradually warming to 23°C is an increase in the rate of reduction to Mb. If samples are maintained at 2°C, the accumulation of MetMb is just as rapid but subsequent reduction to Mb is slower than at 23°C.

The rapid accumulation of MetMb, when oxygenated samples are deprived of oxygen, has been noted previously. The most common cause for excluding oxygen from previously oxygenated samples is putting the wrong side of the 195-MSAD-80 film next to the meat. When the film is not wetted, it is impermeable to oxygen and MetMb rapidly accumulates. Most investigators have attributed the rapid MetMb accumulation to the observation with purified MbO_2 solutions that autoxidation rates are about 3 times faster at 2 mm of oxygen than at 150 mm of oxygen (George et al., 1952b). In meat samples, however, MetMb accumulates about 100 times faster under reduced oxygen pressures when compared with air (30 min for 30% MetMb accumulations with oxygen excluded vs. about 2 days for 30% MetMb accumulation in air). Perhaps there is some factor other than autoxidation of myoglobin active in MetMb production in meat samples.

There is a gradient of oxygen pressure from approximately 150 mm Hg in air at the upper surface of a meat sample to zero mm Hg at some point beneath the surface of the meat sample. Consequently, there must be in every meat sample a pressure of oxygen similar to that existing for the samples shown in Figure 1. One would expect to see MetMb rapidly accumulating (30 to 60 min) in meat samples at some point beneath the surface; yet to our knowledge, such an accumulation is not observed. The reason MetMb does not accumulate rapidly beneath the surface of meat samples may be the presence of MetMb-reducing activity (MRA).

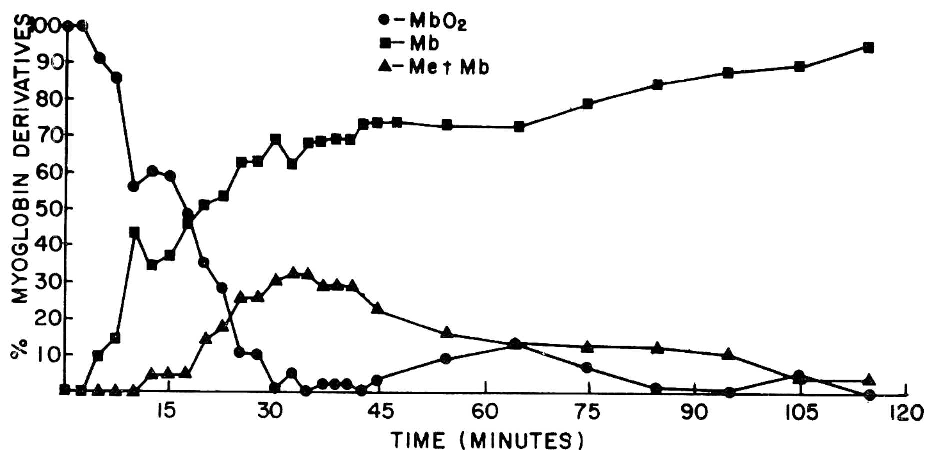

Fig. 1—Changes in myoglobin derivatives when beef samples predominant in MbO_2 are wrapped with Saran. Sample temperature was 2° at the start but samples were kept at 23°C. Results are averages of 2 samples.

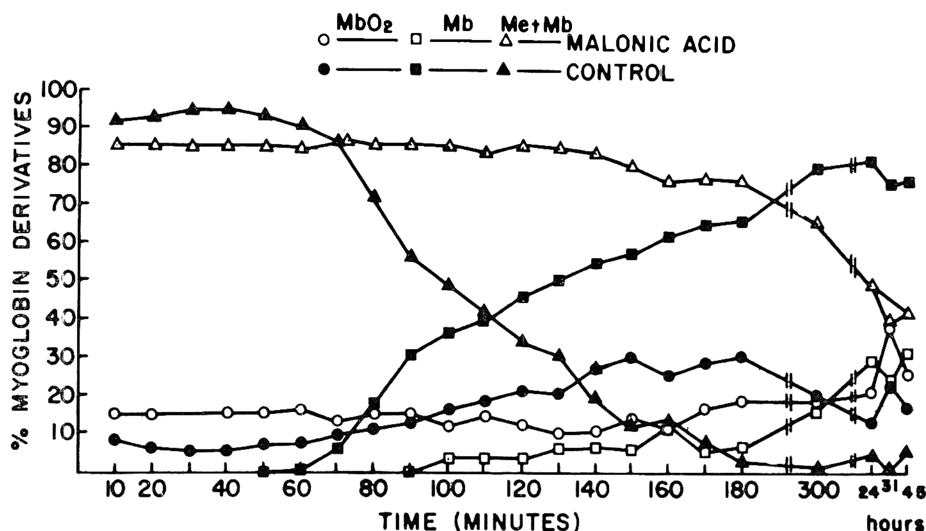

Fig. 2—Changes in myoglobin derivatives when beef samples predominant in MetMb are incubated anaerobically with and without 0.1 m M of malonic acid. MetMb was produced by ferricyanide. Sample temperature was 2°C at the start but samples were kept at 23°C. Results are averages for 14 samples.

Metmyoglobin reduction and malonate inhibition.

A study of the MRA in intact beef samples is shown in Figure 2. When beef samples are oxidized by addition of ferri-

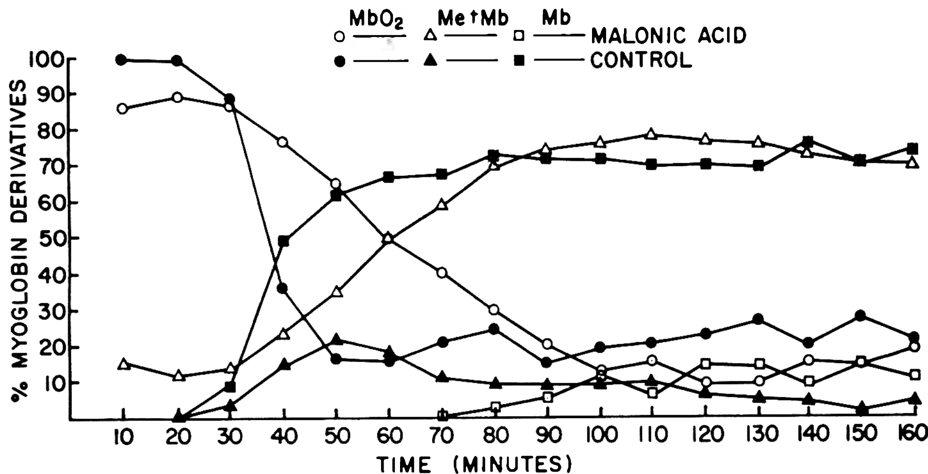

Fig. 3—Changes in myoglobin derivatives when beef samples predominant in MbO_2 are wrapped with Saran. Malonic acid added as 0.1 m M per sample. Sample temperature was 2°C at the start but samples were kept at 23°C. Results are averages of 4 samples.

cyanide and wrapped with Saran to exclude oxygen, a definite and rapid reduction of MetMb to Mb takes place. The rate of MRA is increased by increasing the temperature, and the rate is decreased by the presence of oxygen. The lag time of approximately one hr before MetMb reduction starts is thought to be due to residual oxygen in the packages.

The rate of utilization of oxygen by a piece of meat is closely related to meat color. In Figure 1, the appearance of MetMb is accelerated greatly by excluding oxygen. In Figure 2, the reduction of MetMb is delayed by the presence of oxygen. We were interested in the relationship between oxygen utilization, respiration by the meat samples and the oxidation or reduction of myoglobin derivatives.

Malonic acid is a known inhibitor of succinic dehydrogenase and, consequently, is useful as an inhibitor of oxygen utilization. Figure 2 shows that adding malonic acid to meat samples treated with ferricyanide greatly retards the MRA. Similarly, if malonic acid is added to meat samples wrapped in Saran to exclude oxygen (but not treated with ferricyanide), the main effect is delay of MRA. Figure 3 shows meat samples in the presence and absence of malonic acid, and the appearance of MetMb is roughly at the same rate for both sets of samples. The big difference between the samples is in the rate of MetMb reduction. For samples not treated with malonic acid, the reduction is essentially complete after 70 min (as is true also for the samples shown in Fig. 1) but for samples treated with malonic acid the reduction of MetMb has not even started at 70 min. The net result is the accumulation of much larger amounts of MetMb in the presence of malonic acid.

There are two likely and possible explanations for the results obtained in the presence of malonic acid. One possibility is that malonic acid somehow directly interferes with the reduction of MetMb, for example, by interfering with the production of NADH. The second possibility is that the action of malonic acid is more indirect and is linked to the presence of oxygen which would not be used up in those packages with malonic acid present.

An experiment was done to test whether the presence of oxygen was the main variable affected by malonic acid, or if some other variable was involved. Packages of meat were prepared with one-half of the sample treated with ferricyanide and malonic acid and the other half left untreated. The untreated part of the meat sample acted as an oxygen scavenger and depleted the package of oxygen. No quantitative data were obtained for these experiments but the results showed that meat samples treated with ferricyanide and malonic acid behaved the same as samples without malonic acid when oxygen was depleted by the untreated meat sample. A similar experiment was done with one-half of the meat sample treated with malonic acid and the other half receiving no treatment. Upon wrapping these samples with Saran, there was no obvious difference in the concentration of myoglobin derivatives between the two halves.

Thus, the malonic acid effect is nullified when oxygen is used up by an auxiliary system, and our conclusion is that malonic acid changes the concentration of myoglobin derivatives by its effect on oxygen concentration. Watts et al. (1966) tried malonic acid as an inhibitor of MRA in a ground meat system and found only "slight, variable inhibition." This result agrees with our results since Watts et al. (1966) also used an auxiliary system for decreasing the oxygen concentration in their packages (flushing with nitrogen). However, their conclusion that the meat had very little succinate present because malonic acid had little effect is not valid. In the presence of oxygen, malonic acid does inhibit further oxygen uptake, which means the meat must be utilizing succinic dehydrogenase for respiration under normal meat storage conditions.

We tried other respiratory inhibitors with the intact meat samples, but water suspensions of amytal, rotenone and thenoyltrifuoroacetone showed no activity; and the use of ethanol or propylene glycol as solvents for the inhibitors did not increase their activity.

Our results have not provided, as yet, data that would help to distinguish between autoxidation and MRA as causes for MetMb accumulation in meat samples. Nor is it possible to say unequivocally that both processes are involved. Certainly, the facts that MetMb normally accumulates in an air atmosphere and that MRA is inhibited by oxygen indicate that autoxidation is important. However, MRA may be active beneath the surface in the anaerobic layer of meat just adjacent to the oxygenated layer, and MetMb may accumulate only after MRA is lost.

The only valid conclusion at this stage of our knowledge is that more experimental data are needed to decide about the relative importance of MRA and autoxidation as causes for MetMb accumulation in fresh meat samples.

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R. TOLEDO, M. P. STEINBERG, & A. I. NELSON
Department of Food Science, University of Illinois
Urbana, Illinois 61801

Heat of Respiration of Fresh Produce as Affected by Controlled Atmosphere

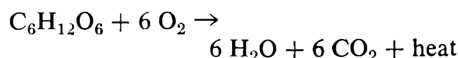
SUMMARY—The heat of respiration of fresh produce in controlled atmosphere (CA) storage was determined with a calorimeter to provide information on the refrigeration load imposed. The heat of respiration in air at the same temperature was also measured to enable direct comparison with the heat of respiration in CA. The calorimeter was a heavily insulated Dewar flask and the product was continuously flushed with either air or CA.

The values obtained for the heat of respiration in air were in good agreement with those reported in the literature for these products. The heat of respiration (Btu/Ton-24 hr) in optimum CA with the ratio in CA to that in air given as a percent was as follows: Wando peas at 48°F—6,690, 31%; Thorogreen lima beans—2,290, 30%; cut Victory Golden sweet corn—3,810, 32%; Red Delicious apples—525, 28%. It was concluded that the heat of respiration of fresh produce in CA can be estimated by assuming that it is about 30% that in air.

INTRODUCTION

IN RECENT YEARS, controlled atmosphere (CA) storage has been successfully used as an adjunct to refrigeration in extending the storage life of apples, pears and citrus fruits. Work on vegetables reveals its great potential in retarding postharvest changes and thus retain produce quality for a longer period. This may result from the fact that respiration in CA is much less than that in air (Lebermann et al., 1968).

In air, heat of respiration of a plant material may be measured in two ways: directly by calorimetric determination and indirectly by measuring CO_2 evolution and O_2 uptake. According to Langworthy et al. (1912), the heat evolved by a respiring product in producing a certain amount of CO_2 is equivalent to that produced by combustion of a hexose sugar in a calorimeter. The equation is:



$$C_6H_{12}O_6 + 6\ O_2 \rightarrow 6\ H_2O + 6\ CO_2 + \text{heat}$$

so that the respiratory quotient (vol. of CO_2/vol. of O_2) is 1.0 and the heat liberated is 2.58 cal/g CO_2.

These conclusions are not valid in case of CA storage; the equation is much more complicated with the formation of unknown intermediate oxidation products such as organic acids (Allendorf et al., 1954) and the constant of 2.58 cal/g CO_2 cannot be applied. Therefore, heat generation calculated from CO_2 production may be inaccurate and the direct calorimetric method should be used whenever the constant relating heat of respiration to CO_2 production has not been experimentally ascertained.

Not much work has been done on direct calorimetric determination of heat of respiration (Haller et al., 1932; Curtis et al., 1950). The very small amounts of heat generated and the environmental conditions that must be maintained for the live product require special considerations in the design of the calorimeter used. Early workers used insulated Dewar flasks to store the product and measured the temperature rise. The method was highly inaccurate and results were only of a qualitative nature (Pierce, 1912). Langworthy et al. (1911, 1912) described a large calorimeter for use in the study of heat of respiration of fruits; they obtained 2.63 cal/g CO_2 with a respiratory quotient of 1.04 for bananas. Green et al. (1941) devised a calorimeter for use on smaller samples. Their results verified those of Langworthy et al. (1912).

In this work, the first objective was to make a direct calorimetric determination of the heat of respiration of some products in CA. Second, based on these data, a common factor was sought which could be used to estimate the heat of respiration in CA from data on heat of respiration in air.

EXPERIMENTAL

Source and preparation of products

All products were grown on the University of Illinois farm in Urbana, Ill.

Wando variety peas and Thorogreen thin-seeded variety lima beans were harvested,

mechanically vined and shelled. Further cleaning was done by passing through a "Clipper" air cleaner and by washing twice in riffle and rotary reel washers operated in tandem. Victory Golden variety sweet corn was hand harvested, mechanically husked and cut. The cut corn was then washed as described for peas and lima beans. After washing, the vegetables were pre-cooled in ice water to about 40°F and excess water was drained. To eliminate the effect of product variability, samples for both air and CA were taken from the same lot.

The air sample, about 20 lb, was weighed to 0.1 lb and placed inside the calorimeter. The remainder of the pre-cooled product was continuously flushed with CA in stainless steel bins held at 34°F. Just before the calorimeter became available after about 48 hr, the bins were moved to room temperature and flushing with CA continued until the product warmed to 40°F. The CA sample, about 20 lb, was weighed to 0.1 lb and placed inside the calorimeter.

Red delicious apples were hand harvested and divided into two lots. Both lots were stored at 34°F, one in air and the other in CA for air and CA determinations, respectively. Determinations were made on air samples 3 and 26 weeks after harvest and on CA samples 4 and 18 weeks after harvest.

Source of controlled atmosphere

CA was obtained from a generator (Tectrol Division, Whirlpool Corporation, Benton Harbor, Mich.) which burned natural gas with the proper proportion of excess air in a catalytic combustion chamber. The CA considered optimum and used for the vegetables contained about 10% CO_2 and 2% O_2 (Lebermann et al., 1968). That taken as optimum for the apples was about 5% CO_2 and 2% O_2 (Smock, 1942; Allen et al., 1948), obtained by intermittently admitting air into the generator effluent.

The calorimeter

The calorimeter (Fig. 1) consisted of a 24 liter wide neck Dewar flask insulated with 4 in. of ground cork (18) and fitted with a paraffin impregnated, air-tight cork cover. The bottom of the flask held a wire screen which formed a plenum chamber to insure a uniform distribution of gas through the product. The calorimeter was installed inside a constant temperature box at 35° ± 2°F.

Air or CA was continuously flushed through the flask to cool the product and prevent the build-up of expired gases. A vane pump (3) sucked the purged gas into the system through a trap (2) and delivered it to a receiver (4) and a pressure regulator (8). This insured a constant rate of gas feed and smoothed out fluctuations in gas pressure due to pump action. Excess gas was bled off to the atmosphere through a valve (5) and a spring loaded blow-off valve (6) insured against excess pressure in the receiver. Flow rate was controlled by a needle valve (10) and a rotameter (11) indicated the rate at which purge gas was fed to the calorimeter. Pressure gages (7) and (9) indicated pressure in the system.

The purge gas then entered the constant temperature box and was measured by a wet-test meter (12). A pressure gage (13) indicated the pressure in the meter. The pressure regulator (8) maintained the meter at atmospheric pressure. Water condensed from gas leaving the meter was collected in a tube (14). The purge gas passed through a heat exchanger (15) cooled by ice water. The base of the heat exchanger was insulated with fiberglass insulation (16) to minimize temperature changes in the purge as it entered the flask. The purge gas was led to the plenum chamber, percolated through the product and left through an exit tube (21), where a thermoelectric dew point hygrometer (Cambridge Systems, Cambridge, Massachusetts) measured the dew point.

Inlet and exit temperatures of purge gas were measured by thermocouples (17 and 23). A reference junction was placed in ice water and EMF was measured by a potentiometer (Rubicon Instruments, Minneapolis Honeywell, Philadelphia, Pa.).

Product temperature was measured by means of five thermocouples mounted at fixed distances (not exactly uniform) on a rod (19). The rod was placed at the axis of the flask, and the thermocouple ends were distributed radially in different directions but at the same distance from the axis. A weighted average temperature of the product was calculated taking into account the amount of product represented by each thermocouple.

Calorimeter calibration

The flask was calibrated for heat losses. An Aminco mercury thermoregulator and a 7-watt light bulb were placed inside the flask. The system regulated power fed to the light bulb so the temperature inside the flask was maintained at ±0.15°F from the set point. The rate of power consumption was measured by a standard watt-hour meter. Periodic readings were taken of the meter until the power consumption per unit time leveled off. This quantity, converted to Btu per unit time, was the heat loss at the set point temperature.

A calibration curve for heat losses versus set point temperature was obtained. The equation of the linear portion of the curve was calculated to be: $Q_t = 0.37T_t - 13.101$; where Q_t is the heat loss in Btu/hr, and T_t is the average temperature inside the flask. The linearity held within the temperature range used here, 35 to 52.5°F, so the equation was used to calculate the heat loss at any flask temperature during the determination. In calculating the loss over a certain time period, T_t was taken as the average product temperature during this period.

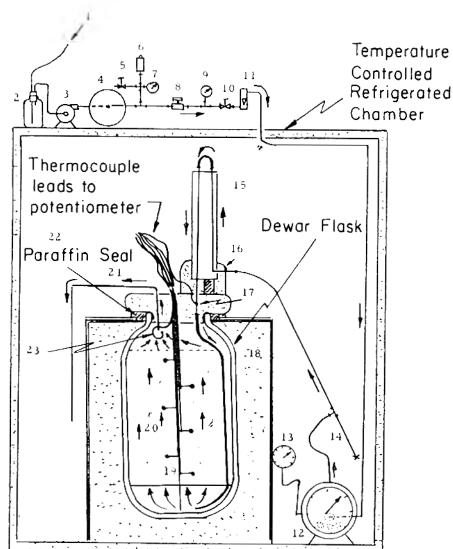


Fig. 1—The Calorimeter Assembly

Procedure

Prior to the start of a determination, the light bulb and the thermoregulator set at the determination temperature were inserted into the flask. When the watt-hour meter showed constant heat input by the light bulb, indicating steady state heat loss by the calorimeter to the surroundings, the bulb and regulator were removed and immediately replaced by product. The calorimeter was sealed and purge gas fed into the system. Flow rate was regulated so that the change in temperature of the product was kept to a minimum.

Periodic temperature readings were taken of the inlet gas, exit gas and product. Gas meter readings were periodically recorded. Exit gas was occasionally checked for saturation using the thermoelectric dew point hygrometer. Ice was periodically added to the heat exchanger. Inlet gas temperatures measured just after addition of ice and just before renewal of ice were averaged.

A slightly different procedure was followed in the determination of heat of respiration of apples. To insure that the purge gas left the flask saturated with water vapor, a small amount of water was placed inside the plenum chamber. The purge gas passed through this water and was therefore saturated as it passed through the product. Saturation was confirmed by periodic measurements of the dew point of the exit gas.

The moisture content of the products at the start and termination of each run was determined by the vacuum oven method (AOAC, 1965). The average value was then used in calculating the specific heat (Siebel, 1918).

Calculations

The heat of respiration was calculated by making a balance of the heat quantities entering and leaving the calorimeter flask. Thus:

$$\text{Heat of Respiration} = \text{Heat out} - \text{Heat in} + \text{Heat accumulation}$$

Heat leaving the calorimeter consisted of the sensible heat content of exit gas, of the latent heat of water evaporated from the product, and the heat loss from the flask to the surroundings. Heat entering the calorimeter consisted of the sensible heat content of inlet gas. Heat accumulation consisted of the gain or loss in the sensible heat of the product during the determination.

The sensible heat content of the inlet and exit gases was calculated by considering the dry gas and the water vapor portions separately. The datum temperature was chosen as 0°F for all calculations. Although the dry

gas passed through the calorimeter unchanged in weight, the water vapor content increased due to evaporation. Thus, for the inlet dry gas, $Q_s = WC_pT_i$, where Q_s is sensible heat content in Btu, W is the weight of dry gas in lb, T_i is inlet gas temperature in °F, and C_p is the specific heat of the dry gas, 0.24 Btu/lb °F. For the inlet water vapor, $Q_s = H_1C_wT_i$ where H_1 is the weight of vapor in lb and C_w is its specific heat, 0.444 Btu/lb °F. Calculations for the exit gas were similar except T_e, exit gas temperature in °F, was substituted for T_i, and H_2, weight of exit water vapor in lb, for H_1.

The latent heat content of the exit gas was calculated as the product of $(H_2—H_1)$, the difference in lb of water vapor entering and leaving the flask, and lambda, the latent heat of evaporation of water at the temperature of the exit gas, in Btu per lb.

Heat accumulation can either be positive or negative depending on whether the product temperature increased or decreased. This was calculated by: $Q_a = W_pC_{pr}\ (T_2—T_1)$; where W_p is the weight of product in the flask and T_2 and T_1 are the average product temperatures at the end and at the beginning of a period, respectively. C_{pr} is the specific heat of the product calculated by $C_{pr} = 0.008M + 0.2$ (Siebel, 1918), where M is the moisture content of the product in percent.

Typical experimental data collected from one replicate and the calculated values of the heat quantities are shown in Tables 1 and 2, respectively. The desired heat of respiration was finally calculated in terms of Btu/ton-24 hr.

Table 1—Data from one calorimetric determination of heat of respiration of peas in air

Weight 25.0 lb
Moisture content 81.4%
Specific heat of product (calculated) 0.852 Btu/lb °F

Reading no.	Time between readings, (hr), t	Purge gas (12.578 cf per lb dry gas), cu ft	Purge gas (dry), lb, W	Water in incoming gas, lb × 10^{-3}, H_1	Water in leaving gas, lb × 10^{-3}, H_2	Inlet gas temp., °F, T	Exit gas temp., °F, T_e	Average Product Temp., °F	
								Initial T_1	Final T_2
	7.1	35.425	2.816	11.04	18.73	32.85	46.30	49.654	51.000
2	4.0	60.015	4.771	19.71	27.58	33.17	46.78	51.000	51.705
3	2.9	46.925	3.731	14.81	24.51	33.17	47.09	51.705	51.647
4	7.5	138.475	11.009	44.37	77.61	33.55	47.82	51.647	50.568
5	4.5	81.460	6.476	26.16	46.56	33.60	48.32	50.568	50.033
6	5.5	99.150	7.883	31.50	56.10	33.36	48.07	50.033	49.435
7	4.5	84.940	6.754	27.29	46.87	33.62	47.40	49.435	48.976
8	3.0	53.015	4.215	16.65	29.04	33.03	47.24	48.976	48.714

RESULTS & DISCUSSION

The heat of respiration of shelled peas, shelled lima beans, cut sweet corn, and apples in air and in controlled atmosphere at each product temperature are shown in Tables 3 through 6.

Since heat of respiration is highly dependent upon the product temperature, comparisons between values of heat of respiration in air and CA must be made at the same temperature. The change in product temperature during the determination was only about 2°F. Within this short temperature range, a straight line approximation to the heat of respiration versus temperature curve can be assumed. The equation of a regression line was statistically determined from the data on heat of respiration and temperature (Steel et al., 1960). This equation enabled calculation of heat of respiration at a common temperature for both air and CA. These values are shown in the last line of Tables 3 through 6.

Table 7 shows the experimental results compared to literature values for heat of respiration in air. Values obtained for apples and peas in air storage agreed with the literature values. In the case of lima beans, the value was higher than that reported; this discrepancy could be due to differences in maturity, variety, climatic conditions during growth and microbiological contamination. Reported data for cut sweet corn could not be found.

The heat of respiration (Btu/ton-24 hr) in CA (Table 7) was 6,690 for shelled peas at 48°F, 31% that in air; for cut sweet corn at 40°F, it was 3,810, 32% that in air; for lima beans at 40°F it was 2,920, 30% that in air; and for Red Delicious apples at 35°F it was 525, 28% that in air.

These results show that the relationship of heat of respiration in CA to that in air was quite similar for the different products, varying only between 28 and 32%. Thus, the results indicate that if CA composition is optimum, the proportional reduction of the heat of respiration of a product in CA from that in air should be the same as for other products.

The similarity of this relationship is also indicated by CO_2 values found in the literature. Smock (1942) showed that at 32°F the CO_2 production of McIntosh apples in CA containing 5% CO_2 and 2% O_2 was equal to 42% of the CO_2 production in air. Allen et al. (1948), using the optimum CA composition for Bartlett pears at 32°F, obtained 46% of the CO_2 production in air. Groeschel et al. (1966), using CA containing 5% CO_2 and 2% O_2, obtained 47% the CO_2 in air for green beans at 45°F. Lebermann et al. (1968), using 2% O_2 and 10% CO_2 for broccoli at 45°F, obtained 40% the CO_2 production in air.

On the basis of these results, and from information available in the literature on the extent of reduction of CO_2 production by some fruits and vegetables due to storage in CA, the heat of respiration in CA of the optimum composition can be estimated from the heat of respiration in air by assuming a value 28 to 32% that in air.

This range of values for comparison of heat of respiration in CA with that in air is lower than the range of values in the literature obtained from CO_2 production as quoted just above. This may be explained on the basis that the respiratory quotient in CA may be expected to be dif-

Table 2—Calculations of heat of respiration for peas stored in air from data in Table 1

Reading no.	Heat in, dry gas (WC_pT_i), Btu	Heat in, water ($H_1C_wT_i$), Btu	Heat out, dry gas (WC_pT_e), Btu	Heat out, water ($H_2C_wT_e$) Btu	Water evaporated ($H_2 - H_1$) × 10^{-3}, lb	Heat of evaporation at exit temp. (λ), Btu/lb	Heat out, evaporated water ($H_2 - H_1$)λ, Btu	Heat out through flask $Q_f t$, Btu	Heat accumulated in product [W_pC_{pr} ($T_2 - T_1$)], Btu	Heat of respiration,[1] Btu/ton-24 hr
1	22.201	0.161	31.291	0.385	7.69	1067.05	8.206	40.264	28.653	23,349
2	37.984	0.290	53.569	0.573	7.87	1066.81	8.396	14.212	15.008	30,472
3	29.702	0.218	42.166	0.512	9.70	1066.65	10.347	17.779	−1.235	26,250
4	88.644	0.661	126.348	1.648	33.24	1066.21	35.441	44.695	−22.969	24,595
5	52.222	0.390	75.101	0.999	20.40	1065.94	21.745	25.465	−11.389	25,303
6	63.113	0.446	90.942	1.197	24.60	1066.06	26.225	29.969	−12.730	25,150
7	54.498	0.407	76.835	0.986	19.58	1066.46	20.881	23.634	−9.771	24,600
8	33.412	0.244	47.790	0.609	12.39	1066.56	13.215	15.354	−5.578	24,149

[1] Heat of respiration in Btu/ton-24 hr $= [WC_pT_e + H_2C_wT_e + Q_f t - WC_pT_i - H_1C_wT_i + W_pC_{pr}(T_2 - T_1) + (H_2 - H_1)\lambda] \times \frac{2000\ \text{lb/ton}}{25.0\ \text{lb}} \times \frac{24}{t\ \text{hr}}$.

Table 3—The heat of respiration at each of several temperatures of shelled green peas stored in air and in controlled atmosphere

Respiration in air		Respiration in controlled atmosphere (11% CO_2-1.8% O_2)	
Temperature, °F	Heat of respiration, Btu/ton-24 hr	Temperature, °F	Heat of respiration, Btu/ton-24 hr
51.67	26,200	48.74	7,490
51.35	30,500	48.03	6,740
51.10	24,600	47.46	6,470
50.33	23,300	47.04	6,680
50.29	25,300	46.69	5,990
49.73	25,100	46.30	6,140
49.20	24,600	45.95	6,590
48.84	24,100		
Heat of respiration calculated from the regression line equation			
48.00	22,600	48.00	6,690

Table 4—The heat of respiration at each of several temperatures of shelled lima beans stored in air and in controlled atmosphere

Respiration in air		Respiration in controlled atmosphere (11% CO_2-1.8% O_2)	
Temperature, °F	Heat of respiration, Btu/Ton-24 hr	Temperature, °F	Heat of respiration, Btu/ton-24 hr
41.51	10,890	40.76	3,560
40.81	10,070	40.60	3,880
40.43	9,160	40.39	2,750
40.07	11,180	40.08	2,810
39.48	9,750	39.78	3,360
38.99	9,200	39.45	1,490
38.75	9,230	39.11	2,200
38.48	8,160	38.87	1,800
—	—	38.74	3,090
Heat of respiration calculated from the regression line equation			
40.00	9,820	40.00	2,920

Table 5—The heat of respiration at each of several temperatures of cut victory golden sweet corn stored in air and in controlled atmosphere

Respiration in air		Respiration in controlled atmosphere (11% CO_2-1.8% O_2)	
Temperature, °F	Heat of respiration, Btu/ton-24 hr	Temperature, °F	Heat of respiration, Btu/ton-24 hr
40.86	12,830	40.23	3,990
40.56	13,410	40.11	3,588
40.28	11,660	41.51	4,050
42.26	11,320	41.27	3,550
41.08	12,470	41.10	4,200
40.27	13,760	41.01	3,990
39.87	12,170		
40.24	11,460		
39.93	10,370		
39.66	10,690		
Heat of respiration calculated from the regression line equation			
40.00	11,900	40.00	3,810

ferent from that in air and that a given CO_2 production does not result in the same heat evolution under both conditions as explained in the introduction. The present work is based on calorimetric measurements and should therefore be more authoritative than the literature values calculated from CO_2 evolution.

Table 6—The heat of respiration at each of several temperatures of apples stored in air and in controlled atmosphere

Respiration in air		Respiration in controlled atmosphere (3.8% CO_2-2.2% O_2)	
Temperature, °F	Heat of respiration, Btu/ton-24 hr	Temperature, °F	Heat of respiration, Btu/ton-24 hr
34.09	2,120	35.06	360
33.95	2,100	35.04	410
33.80	1,170	34.99	510
35.88	2,020	35.71	850
35.87	1,990	35.41	710
35.83	2,070	35.29	340
35.74	2,320	35.21	710
35.73	1,760	35.14	770
35.63	1,790	34.94	710
35.58	1,980	34.85	690
35.52	1,790	34.81	400
—	—	34.81	430
—	—	34.79	760
—	—	34.66	210
Heat of respiration calculated from the regression line equation			
35.00	1,900	35.00	525

LIST OF SYMBOLS

C_p = the specific heat of dry gas, 0.24 Btu/lb °F

C_w = the specific heat of water vapor, 0.444 Btu/lb °F

C_{pr} = the calculated specific heat of the product, Btu/lb °F

H_1, H_2 = weight of inlet and exit water vapor, respectively, lb

λ = the latent heat of evaporation of water, Btu/lb

M = the moisture content of the product, %

Q_a = the heat accumulation by the product, Btu

Q_f = heat loss from calorimeter, Btu/hr

Q_s = sensible heat content, Btu

T_f = average temperature inside the calorimeter flask as well as average product temperature, °F

T_i, T_e = inlet and exit gas temperatures, respectively, °F

T_2, T_1 = average product temperatures at the end and at the beginning of a period, respectively, °F

W = the weight of dry gas, lb

W_p = weight of product in the flask, lb

Table 7—Summary of heats of respiration of three vegetables and apples stored at refrigerated temperatures in air and in controlled atmosphere of optimum composition for the product

Product	Variety	CA Composition % CO_2	CA Composition % O_2	Temp., °F	Heat of respiration, Btu/ton-24 hr: Air, experimental values	Air, literature values	Controlled atmosphere	Comparison: CA/air, %
Peas	Wando	11	1.8	48	22,600	21,000 to 27,000	6,690	31
Sweet Corn, cut	Victory Golden	11	1.8	40	11,200	—	3,810	32
Lima Beans	Thorogreen	11	1.8	40	9,820	4,300 to 6,100	2,920	30
Apples	Red Delicious	3.8	2.2	35	1,900	450 to 1,850	525	28
							Avg.	30.3

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T. J. SIEK, INGA A. ALBIN, LOIS A. SATHER & R. C. LINDSAY
Department of Food Science and Technology
Oregon State University, Corvallis 97331

Taste Thresholds of Butter Volatiles in Deodorized Butteroil Medium

SUMMARY—Taste thresholds of 31 volatile compounds found in butter were measured in deodorized butteroil and thresholds of seven volatiles were measured in fresh butter. Thresholds of mixtures of each of the major classes of volatile compounds (free fatty acids from C_2 through C_{12}, gamma-lactones from C_7 through C_{11}, even-numbered delta-lactones from C_8 through C_{14} and methyl ketones from C_3 through C_{13} except C_{12} were determined as well as thresholds of single comoounds of these classes. Butyric acid, diacetyl, delta-decalactone, 2-nonanone, gamma-undecalactone and n-hexanal, oft-reported constituents of milk fat, had thresholds in butteroil of 0.66, 0.055, 1.4, 7.7, 0.95 and 0.19 ppm, respectively. The threshold of a mixture of free fatty acids from C_2 through C_{12} was 0.55 ppm. Synergistic interactions among methyl ketones and free fatty acids were pronounced and interactions among aldehydes were weak, while interactions among lactones were not apparent.

INTRODUCTION

MORE THAN 100 volatile compounds have been identified as natural constituents of butter or milk fat (Day et al., 1960; Forss et al., 1967; Jurriens et al., 1965; Langler et al., 1964, and Wong, 1963). A small number of these compounds are generally recognized as principal components of butter flavor. Different types of butter flavor concentrates are available from at least 41 commercial suppliers. None of these concentrates duplicates the complete natural flavor of butter, nor are all the compounds in these concentrates necessarily natural butter aroma constituents.

Human taste and odor thresholds of individual compounds are indexes of flavor (Patton et al. 1957). Thresholds of mixtures of compounds have been shown to complicate interpretations of flavor chemistry due to additive, synergistic and antagonistic interactions of mixtures (Day et al. 1963; Langler et al. 1964; Meijboom, 1964). Water thresholds of many butter volatiles are known. To complement the water threshold data, taste thresholds of key butter volatiles in butteroil medium and in butter itself were needed.

This study was undertaken to determine butteroil thresholds of individual compounds as well as mixtures of the prominent classes of volatile compounds found in butter. Compounds included were free fatty acids (even-numbered C_2 through $C_{18:1}$), delta-lactones (C_8, C_{10}, C_{12}, and C_{14}), gamma-lactones (C_7 through C_{11}), methyl ketones (C_3 through C_{11}, C_{13}, and C_{15}) and selected miscellaneous compounds which are known to be present in butter.

EXPERIMENTAL METHODS

Preparation of odor-free butteroil

Butteroil separated from fresh, melted sweet cream butter was filtered through Eaton & Dikeman No. 17 filter paper to remove the remaining free butter serum. The filtered oil was then vacuum steam-distilled in an all-glass apparatus designed to handle 10 to 12 L. of oil (Fig. 1). Earlier studies revealed that relatively high temperatures were required to remove butter flavor from the oil. In a typical vacuum steam distillation, melted oil was heated to 210° ± 10°C, held at this temperature for about 2 hr, then allowed to cool to 140°C. Antioxidant was added according to the method of Wyatt et al. (1965). The pressure above the oil, which increased with temperature, ranged from 9 to 23 mm (Hg), and was 1 mm or less at the pump end of the system. The amount of water distilled was 1/4 to 1/2 the oil volume.

Volatile compounds, as well as cholesterol and carotenoids, were removed by the distillation. The deodorized oil had a faint odor and a slightly sweet or nutty taste. A recognizable butter flavor, however, was not detectable.

Purification of compounds tested

For flavor studies, reagent grade chemicals whose normal boiling points were below 230°C were vacuum-distilled in an all-glass 10 to 50-ml capacity distillation apparatus. Lauric, myristic, palmitic and oleic acids were purified by several recrystallizations from ethanol-water. Other high boiling compounds were purified by preparative GLC, using a 1/4 inch × 4.5 ft aluminum column containing 80/120 mesh glass beads coated with 0.1% Apiezon H. Purity of the liquids, as determined by gas chromatography (150 feet × 0.01 in. I.D. stainless steel capillary coated with Ucon), was better than 99.8% except for 2-pentanone (99.6%).

Testing procedures

Glassware used in all tests was thoroughly cleaned and carefully inspected to exclude containers which had residual stains or odors. Stock solutions were prepared in 100-ml volumetric flasks by dissolving calculated amounts of purified compounds in deodorized oil at 38° ± 4°C and bringing to volume; four or five dilutions to lower concentrations were made from each stock solution. Compounds whose thresholds were measured in water were brought into solution by first dissolving the compound in ethanol and then dispersing the ethanol solution into water (subthreshold ethanol concentration was maintained).

The flavor tests were conducted by the procedure described by Wyatt et al. (1965).

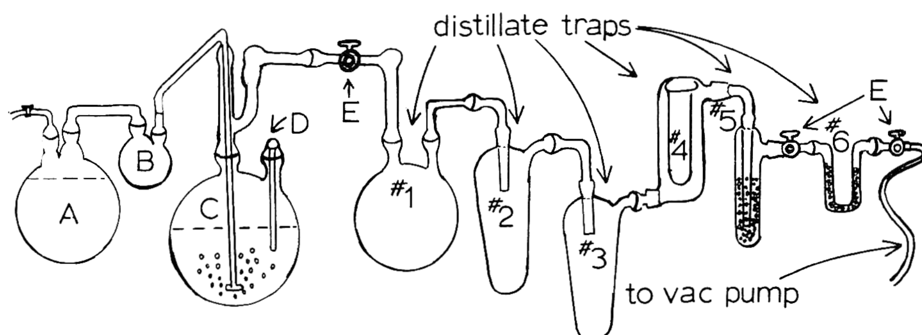

Fig. 1—Vacuum steam distillation apparatus used. A—steam reservoir, B—safety trap, C—substrate receptacle, D—thermometer well, E—stockcocks; traps #1 and #2—dry ice-acetone cooled; traps #3 through #6—liquid nitrogen cooled traps (traps #5 and #6 contain glass beads).

Table 1—Taste thresholds of selected butter volatiles measured in deodorized butteroil

Compound	Concentration in ppm	
	Threshold	Approximate level in fresh butter[1]
Butyric acid	0.66	9 to 38
Caproic acid	2.5[2]	6 to 18
Diacetyl	0.055	0.02 to 0.1
Dimethyl sulfide	0.009	0.02
Delta-decalactone	1.4	0.01 to 9
Ethyl acetate	22	4
Ethyl butyrate	0.60	<0.2
Ethyl hexanoate	0.85	<0.1
Acetaldehyde	0.11	0.7
n-Hexanal	0.19	0.14
n-Heptanal	0.75	0.16
n-Nonanal	1.0[3]	0.07

[1] Concentration values were from the following sources: fatty acids, Iyer et al. (1967); aldehydes, Day et al. (1960); and delta-decalactone by Forss et al. (1967) (0.01) and Jurriens et al. (1965). Other concentrations were estimated from GLC analysis of butter volatiles (unpublished data, T. J. Siek).
[2] From Patton (1964).
[3] From Hvolby (1962).

Table 2—Taste thresholds of free fatty acids in deodorized butteroil—individual compounds and mixtures

Fatty acid	Concentrations in ppm		
	Individual threshold[1]	Fatty acid in mixture at the mixture threshold[2] I	II
C_2	7.0	0.002	0.003
C_4	0.66	0.07	0.10
C_6	2.5	0.03	0.06
C_8	350	0.05	0.08
C_{10}	200	0.11	0.17
C_{12}	700	0.29	0.45
C_2–C_{12}	1260	0.55 = mixture threshold	
C_{14}	5000		814
C_{16}	10000		2203
C_{18}	15000		617
$C_{18:1}$	8000		1315
C_2–$C_{18:1}$	39260		5000 = mixture threshold

[1] Individual thresholds were taken from Feron et al. (1961) for C_6 through $C_{18:1}$; their value for C_4 was 0.60.
[2] Column I was a mixture through C_{12}; column II contained all fatty acids listed.

Four or five dilutions of the compound being tested were served to tasters along with a labeled blank (zero concentration) and a coded blank. The panel of judges consisted of 28 members of the Food Science and Technology staff with 20 members used per test; oil samples were served at 42° ± 3°C; and judges were not pre-selected for taste acuity, but were experienced in serving on flavor panels.

In the flavor booths, judges were asked to taste the two reference samples (zero concentration and the highest concentration) and then to taste at random the coded samples containing the flavor compound. The judges marked their ballot "plus" when the compound in question was detected in a sample. No time limit was imposed during tasting. If panel members misjudged the coded blank and/or coded maximum concentration sample, their ballot was excluded from the results. Otherwise, each "plus" answer was recorded. Taste tests were conducted within a week after the sample purification. Solutions of compounds tested were made on the day of the test. The 50% positive response level used by Patton et al. (1957) was calculated, so that direct comparisons might be made with reported 50% thresholds. Threshold concentrations are defined as concentrations of compounds in a given medium in parts per million.

For comparison, several thresholds were measured in fresh sweet cream butter. The butter samples were served from the refrigerator (4°C) as butter patties. Mixture solutions of several classes of butter volatiles were also prepared for testing. Mixture thresholds were established in the same manner as single-compound thresholds, the mixture threshold being the total volatile concentration which could be detected. Ratios of individual compounds were constant in each dilution of the mixture.

Threshold determinations usually required one or more preliminary tests to find the appropriate concentration range. In practice, panelists often sniffed all samples and tasted only those they could not categorize by sniffing. Panelists could usually detect one or two concentrations lower by tasting than they could by sniffing only. Meijboom (1964) found that for 31 aldehydes, threshold values for taste (measured in paraffin oil) were in all cases lower than those for odor.

RESULTS & DISCUSSION

Individual thresholds

Taste thresholds of several butter compounds are presented in Table 1 along with their reported or estimated concentrations in butter. Other individual thresholds are shown in Tables 2, 3, 4, and 5.

The threshold of delta-decalactone was measured five times (on five days) with some variation of panel members in each test. The threshold obtained was 1.4 ± 1.1 (σ). Greater precision was noted in repeated tests with other compounds: for 2-pentanone the threshold was 61 ± 3; gamma-undecalactone, 0.95 ± 0.22, and *n*-hexanal, 0.016 ± 0.003 (in water). Thresholds depend greatly on expertise and size of the panel used (Amerine et al. 1965, p. 182); thus differences in taste thresholds from different laboratories are probably due to differences in make-up of the panel. By observing general trends of a homologous series (Table 3) more validity can be ascribed to individual threshold values, and from these values a measure of relative flavor potential can be ascertained.

Table 3—Taste thresholds of methyl ketones in deodorized butteroil—individual compounds and mixtures

Carbon number	Concentration in ppm	
	Individual threshold[1]	Ketone in mixture at mixture threshold
C_3	125	0.09
C_4	30	0.09
C_5	61	2.6
C_6	—	0.09
C_7	15	3.5
C_8	2.5	0.09
C_9	7.7	2.6
C_{10}	11	0.09
C_{11}	100	5.3
C_{13}	182	3.5
C_{15}	—	5.3
C_3 to C_{15}		23 = mixture threshold

[1] Values obtained by Hvolby (1962) for C_4, C_8, C_{10}, C_{11}, and C_{13} were 30, 5, 10, 10, and 500 respectively.

Table 4—Taste thresholds of normal aldehydes in deodorized butteroil—individual compounds and mixture

Carbon number	Concentration in ppm	
	Individual threshold	Aldehyde in mixture at mixture threshold
C_5	0.30	0.16
C_6	0.19	0.16
C_7	0.75	0.16
C_8	0.9[1]	0.16
		0.64 = mixture threshold

[1] From Lea and Swoboda (1958); their value for *n*-hexanal was 0.3.

Table 5—Taste thresholds of lactones in deodorized butteroil—individual compounds and mixtures

Lactone	Concentration in ppm	
	Individual threshold	Lactone in mixture at mixture threshold[1]
γ-C_6	8.0	—
γ-C_7	3.4	0.47
γ-C_8	3.5	0.94
γ-C_9	2.4	0.94
γ-C_{10}	1.0	0.94
γ-C_{11}	0.95	0.94
		4.3 = mixture threshold
δ-C_8	3.0	0.54
δ-C_{10}	1.4	2.7
δ-C_{12}	95	5.4
δ-C_{14}	500	5.4
		14 = mixture threshold

[1] Gamma- and delta-lactone mixtures were measured separately.

Thresholds of mixtures

Thresholds of individual compounds and thresholds of mixtures of several classes of butter volatiles are given in Tables 2, 3, 4 and 5 (note that in Tables 2 to 5, individual compound concentrations are given above the mixture threshold concentration and individual thresholds are in a separate column). Methyl ketone, free fatty acid and aldehyde homologous series mixtures exhibit results similar to those reported by Langler et al. (1964). Interaction in the mixtures is apparent especially with fatty acid and methyl ketone mixtures (Tables 2 and 3), as at the mixture threshold, concentrations of individual compounds are subthreshold. Langler et al. (1964) refer to such effects as "synergistic." The synergistic effect was not pronounced with aldehydes and was not evident among lactones.

Table 6 gives the water, oil and butter taste thresholds of several selected compounds obtained in this study and by other investigators. The data show that oil thresholds are generally higher than water thresholds. Thresholds in butter are closer to oil thresholds than water thresholds. Factors influencing water and oil threshold differences have been discussed previously (Lea et al. 1958; Patton 1964).

CONCLUSIONS

Among the thresholds measured, those of several butter volatiles are lower (or nearly so) than their reported concentration in butter, and thus would be expected to contribute to sweet cream butter flavor Compounds that fall into this group are diacetyl, butyric and caproic acids, hexanal, acetaldehyde, dimethyl sulfide and possibly delta-decalactone. Levels of 2-hepatanone and 2-nonanone (Langler et al. 1964) could be high enough to influence flavor due to synergistic interactions. The aroma and flavor of fresh cream butter depend on a concentration balance of low threshold compounds reported herein (plus possibly some volatiles not as yet identified in butter), with little contribution to flavor by high threshold compounds. Synergistic interactions exhibited by mixtures probably play an important role in giving butter its unique flavor and aroma.

Table 6—Taste thresholds of representative volatile compounds in different media

Compound	Threshold concentration in ppm			
	Water	Oil	Milk	Butter
Ethyl acetate	6.6	22	4.7	—
Ethyl butyrate	0.015	0.60	0.016	—
Dimethyl sulfide	—	0.009	0.019[1]	0.17
Diacetyl	0.0054	0.055	0.014	0.032
2-Octanone	0.15	2.5	—	3.4
2-Decanone	0.19	11	—	9.3
Delta-decalactone	0.14	1.4		
Acetic acid	22	7.0		
Butyric acid	6.2	0.66	25[2]	
Caproic acid	15	2.5[2]	14	
Caprylic acid	5.8[2]	350[2]	23[3]	
Capric acid	3.5[2]	200[2]	28[2]	
n-Heptanol[4]	2.4	20	—	10
n-Dodecanal[4]	0.011	0.75[5]		
Acetaldehyde	1.3[6]	0.11		
n-Pentanal	0.07	0.30	0.13[7]	
n-Hexanal	0.016	0.19	0.05[7]	0.80
n-Heptanal	0.031	0.75	0.12[7]	0.90

[1] From Reddy et al. (1967).
[2] From Patton (1964); his values for acetic, butyric and caproic acids were 54, 6.8 and 5.4 respectively.
[3] Concentration that gives a rancid flavor (Scanlan et al. 1965); butyric and caproic acid values (rancid flavor) were 46 and 30 respectively.
[4] Not indigenous to butter.
[5] Lea et al. (1958) reported 0.9.
[6] From Berg et al. (1955).
[7] From Day et al. (1963).

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RICHARD T. LOVELL[a] **and JOHN A. BARKATE**[b]
Department of Food Science and Technology, Louisiana State University
Baton Rouge, Louisiana 70803

Incidence and Growth of Some Health-Related Bacteria in Commercial Freshwater Crayfish (Genus Procambarus)

SUMMARY—Collections of freshwater crayfish were obtained from 22 sources representing the major commercial crayfishing areas in Louisiana, and analyzed for coliforms, Escherichia coli, *fecal streptococci, coagulase-positive staphylococci,* Salmonella *and* Clostridium botulinum *type E. Growth patterns for* E. coli, Staphylococcus aureus, Streptococcus faecalis *and* Salmonella typhimurium *in raw and cooked crayfish flesh, and in a crayfish-containing commercial-type substrate were determined at 5, 25, and 37°C.* C. botulinum *type E toxin production was determined at intervals of storage in ice pack and at 5 and 30°C.*

Coliforms, E. coli, *fecal streptococci, coagulase-positive staphylococci,* Salmonella, *and* C. botulinum *type E were found in 100, 92.6, 94.1, 3.0, 3.0 and 0%, respectively, of the samples analyzed.* Staphylococcus aureus *and* Salmonella typhimurium *grew well in all three of the substrates at 25 and 37°C.* Streptococcus faecalis *and possibly* E. coli *showed suppressed growth in the raw flesh, but both grew well in cooked flesh and the commercial-type substrate. None of these four organisms grew at 5°C.* C. botulinum *type E produced toxin in all three of the substrates within 48 to 72 hours at 30°C and after 33 days at 5°C.*

Toxin was not produced at 56 days in ice-pack. The pH increased in the raw and cooked flesh, with extended storage time, to above 8.0 and the toxin became inactive. In the commercial-type substrate, the pH decreased to 5.7 and the toxin remained active. When the pH of the commercial-type substrate was adjusted to 8.5 with NaOH, the toxin was inactivated.

INTRODUCTION

FRESHWATER CRAYFISH of the genus *Procambarus* have been consumed for many years in southern Louisiana and other areas along the Gulf Coast. The size of the freshwater crayfish industry is shown by the facts that in 1965 the commercial catch in Louisiana, as reported by the Bureau of Commercial Fisheries, was 8.6 million lb and in 1966 the Louisiana State Board of Health licensed 34 crayfish processing plants.

For many years these freshwater crustaceans were marketed alive, and the consumer boiled them himself before he ate them. In recent years fresh, hand-peeled tail meat and precooked frozen crayfish products have become important retail products. Because a review of the scientific literature revealed no reports on the microbiology of freshwater crayfish (as a food), an investigation of the incidence and growth of some health-related microorganisms in crayfish and crayfish products appeared to be a logical first effort in studies relating to the microbiology of a food rapidly gaining commercial prominence.

Due to the great amount of hand labor involved in the peeling and preparation of crayfish products, there is ample opportunity to introduce into the product various microorganisms of public health significance—providing: (1) the organism is in the environment; and (2) proper sanitation practices are not followed scrupulously. Growth characteristics of these organisms, if or when introduced into crayfish meat and crayfish products, are of concern from the standpoint of establishing bacteriological standards and, more importantly, as potential public health hazards.

The following study was planned with two objectives in perspective: (1) to determine the extent to which several health-related bacteria are inhabitants of freshwater crayfish from commercial sources; and, (2) to ascertain the patterns of growth or toxin production of these fecal indicator or pathogenic organisms in crayfish flesh and formula foods containing crayfish. The organisms were *Escherichia coli*, fecal streptococci, coagulase-positive staphylococci, *Salmonella*, and *Clostridium botulinum* type E.

The commercial crayfish sources were natural (swamps, lakes, marshes and a flooded river basin) and man-made (rice fields and ponds constructed specifically for crayfish production) areas located in southern Louisiana. Bacteria growth studies were made with both raw and cooked crayfish tail meat (crayfish tail meat is usually marketed as a partially cooked product because the crayfish have been scalded prior to peeling) and a commercial-type formulation containing approximately 25% crayfish meat.

MATERIALS & METHODS

Survey

During the crayfish harvest season (February through June) of 1966 and 1967, a total of 66 collections of crayfish and water were obtained from 22 sites representing essentially all the major commercial crayfishing areas in Louisiana. A minimum of two collections was made from each site during the study. The crayfish were taken directly from traps, or from the fisherman's boat within a few hours following removal from the traps. They were transported alive in sterile glass jars to the laboratory for analysis. With each collection, five crayfish weighing 20 to 40 g each were placed into separate blender jars and comminuted with Butterfield's phosphate buffer solution in a 1:5 suspension for 2 min.

Serial dilutions or transfers to enrichment media were made from each blender jar with the following procedures being used for the various analyses: (1) *MPN of coliforms:* The procedure recommended by American Public Health Association (1966) was used; (2) *MPN of E. coli:* The American Public Health Association (1966) method was followed; (3) *MPN of fecal streptococci:* The American Public Health Association (1966) procedure was applied with microscopic examination of Gram-stained slides for conformation of identity as Gram-positive streptococci; (4) *Coagulase-positive staphylococci:* The AFDOUS method used by Surkiewicz (1966) for frozen food analysis was followed with modification. Decimal dilutions were placed in single tubes of 10% NaCl trypticase soy broth (TSB) and incubated for 48 hr at 37°C. The TSB was loop-streaked on mannitol egg agar (MEA) plates which were incubated for 24 hr at 37°C, and colonies showing a zone of precipitate were considered presumptively positive. Confirmation was made by the coagulase test using Bacto-Coagulase Plasma (Difco). (5) *Salmonella:* The procedure for detection of organisms of the genus Salmonella was the pre-enrichment method recommended by American Public Health Association (1966). Suspect colonies were tested with Bacto-Salmonella O antisera (Difco). (6) *C. botulinum type E:* The procedure involved the isolation of typical type E colonies with subsequent toxicity tests using anti-toxin- protected mice, similar to that described by Slocum (1964).

Fifty ml of an enrichment medium of trypticase glucose peptone (TGP) broth in a

[a] Present address: Auburn University, Auburn, Alabama.
[b] Present address: Ralston Purina Company, St. Louis, Missouri.

25 × 150 mm screw-cap tube was inoculated with 10 ml of the blended crayfish-phosphate buffer suspension and incubated at 30°C for 72 hr in anaerobic jars (Case, No. 2) under an atmosphere of 90% N and 10% CO_2. After incubation, the tube was shaken and a 1-ml sample was removed and placed into a sterile tube with an equal volume of absolute ethanol.

After standing for 1 hr at room temperature with occasional agitation, a loopful of the mixture was streaked onto a plate of liver-veal agar with 4% egg yoke (LVEY). In addition, approximately 0.2 ml of the mixture was transferred to a tube of TGP broth. The plates and the tubes were placed into separate anaerobic jars and incubated at 30°C. The plates were removed after 48 hr and examined for the presence of characteristic type E colonies. A control with *C. botulinum* type E organisms from a stock culture was run with each replication (usually one per week) of analyses.

Where colonies on the plates showed slight to marked resemblance to the control colonies, i.e., a precipitate in the medium and a "pearly" layer adjacent to and covering the colonies, the corresponding TGP tubes were allowed to incubate for 72 hr, and supernatant extract from the tubes was administered to mice for toxin determination.

The toxin assay procedure was as follows: 2 ml of the supernatant, adjusted to a pH of 6.2 by the addition of a gel-phosphate buffer (Duff et al., 1965), was added to each of two 16 × 125 mm screw-cap tubes along with 0.2 ml of a 10% solution of trypsin (Difco, 1:250 activity). The tubes were incubated for 45 min at 37°. One tube was heated for 11 min at 100°C, and the other was not heated. A total of 0.5 ml of the nonheated extract was injected into each of two 20-g mice (Carworth, Inc.) protected with 5 IU of type E antitoxin (Communicable Disease Center, Atlanta, Ga.), and an equal dose was injected into two unprotected mice. Then 0.5 ml of the heated extract was injected into each of two protected mice and two unprotected mice. Type E toxin was assumed to be present only when: (1) the unprotected mice receiving the nonheated extract died within 48 hr; (2) all protected mice survived; and (3) all mice receiving the heated extract survived.

For each analysis procedure, controls were run using organisms from pure culture collections. Pure cultures were obtained from the following sources: *Salmonella typhimurium* (Loeffler), American Type Culture Collection (ATCC), Number 13311; *Staphylococcus aureus,* ATCC, Number 9664; *Escherichia coli* (0127 B:8), Walter Reed Army Medical Laboratory. A. Abrams: *Streptococcus faecalis,* ATCC, Number 7070; *Clostridium botulinum* type E, strain 8E, Continental Can Company, C. F. Schmidt. The media used in this study were from Difco or Baltimore Biological Laboratories.

Growth and toxin production in crayfish foods

Growth patterns of *E. coli, Staphylococcus aureus, Streptococcus faecalis* and *Salmonella typhimurium* in raw and cooked tail flesh, and in a crayfish-containing commercial-type food product were determined at 5, 25 and 37°C incubation temperatures. *C. botulinum* type E toxin production in the three crayfish substrates was determined in ice-pack and at 5 and 30°C at various storage intervals.

The raw flesh substrate was prepared by immersing the live crayfish in boiling water for 10 sec (to facilitate removal of the shell from the tail muscle), washing the blanched crayfish vigorously with a detergent-sanitizing solution and rinsing with sterile water, and removing the shell and tail-vein with sterile forceps and scissors. Although this was done as aseptically as possible, the excised tissue was not expected to be sterile, so samples of the raw peeled tails were randomly selected and aerobic total plate counts were made on BHI agar to estimate the size of the residual microflora population in the raw flesh substrate.

The cooked flesh substrate consisted of conventionally peeled tails autoclaved for 15 min at 121°C after peeling. The commercial-type formulation was of the following composition: crayfish tail meat (25%); crayfish "fat" (1%); NaCl (1%); wheat flour (5%); onion, pepper, and celery (4%); cooking oil (3%); cayenne pepper and other spices (0.5%); and added water (60.5%). It was autoclaved for 15 min at 121°C prior to inoculation.

Inocula were prepared by growing *E. coli, Staphylococcus aureus, Streptococcus faecalis* and *Salmonella typhimurium* from the pure cultures in BHI broth. Cell concentrations in the inocula after 24 hr incubation at 35°C were measured turbidometrically, and cell suspensions in phosphate buffer solution containing approximately 10^5 organisms per ml were prepared. Three replicates of 20 g of each of the three substrates were placed into separate 6-oz Nalgene screw-cap jars and inoculated with 5 ml of a cell suspension of one of the test organisms. One of each replicate was incubated at 3, 25 and 37°C. Enumeration of the test organisms was made at 0, 6, 12, 18, 24, 36, 60 and 84 hr following inoculation.

The MPN methods reported above for the survey analyses were used for *E. coli, Streptococcus faecalis* (fecal streptococci), and *Salmonella typhimurium.* For *Staphylococcus aureus,* the plate count method described by Zebovith et al. (1955), using freshly prepared tellurite-glycine (TG) agar, was employed. To determine *C. botulinum* type E toxin production, 5 ml of a toxin-free washed-spore suspension (10^6 spores per ml in 0.85% NaCl solution) prepared as described by Schmidt et al. (1962) was added to 50 g of each of the substrates in blender jars and mixed for 1 min.

The mixture was transferred to Nalgene jars and placed into anaerobic jars and stored in ice-pack and at 5 and 30°C. Toxin assays and pH measurements were made according to the following schedule: 30°C—0, 24, 48, 72, 96 and 120 hr; 5°C—0, 7, 14, 21, 27, 30 and 33 days; ice-pack—0, 7, 14, 21, 35, 42, 49 and 56 days. The toxin assays were made by homogenizing 5 g of substrate from the inoculated pack in 5 g of sterile 0.85% NaCl solution, centrifuging, removing the supernatant and proceeding with mouse protection tests as described above.

Table 1—Summary of quantitative and qualitative analyses for health-related bacteria in freshwater crayfish from commercial sources

Organism	No. of samples	No. of positive samples	Per cent of samples having >3 MPN/g	>100 MPN/g	>1100 MPN/g	Average, MPN/g
Coliforms	66	—	100.0	95.0	91.0	60,000
E. coli	66	—	92.6	66.0	30.0	1,200
Fecal streptococci	66	—	94.1	46.0	0	120
Coagulase-positive staphylococci	66	2				
Salmonella	66	2				
C. botulinum	66	0				

RESULTS & DISCUSSION

Health-related bacteria in crayfish from commercial sources

Table 1 summarizes the bacterial analyses of the live crayfish from the 22 collection sites. Because the crayfish is a bottom feeder and often burrows into the mud, a very large coliform count was not unexpected. But the high incidence and high counts of *E. coli* and fecal streptococci in the crayfish were not anticipated, as most of the commercial sources did not appear to be connected to watersheds from populous areas. No published reports were found on the occurrence of these fecal indicator organisms in freshwater fish or shellfish from the Gulf Coast area.

It is well substantiated that *E. coli* is not a normal inhabitant of the intestinal tract of marine fish and shellfish, but is often found along with other coliforms in catches from polluted waters. In addition, the organisms of the fecal streptococci group, with a few exceptions (Mundt, 1963), have been associated with the intestinal tract of homeothermic animal species. Although most of these sources are located remote from populous areas, the high incidence of *E. coli* and fecal streptococci indicate extensive pollution of the commercial crayfishing areas. Improvements are essential in the sanitation of the production and harvest aspects of the crayfish industry. Fortunately, a growing percentage of the commercial crayfish catch appears to be coming from man-made sources where pollution can be controlled.

One collection site received effluent

containing raw sewage from several rural dwellings. This area, on two collection dates, provided the two *Salmonella*-positive (O antisera) samples indicated in Table 1. *Salmonella* species are widely distributed in nature, and their incidence may be relatively high in some populations of domestic animals such as in poultry (Sadler et al., 1965) and in swine (Williams, 1967).

The Salmonella Surveillance Report of the U.S. Public Health Service indicated that *Salmonella* have been found in several aquatic species from sources in the southern United States; however, it was pointed out by Martin (1966; private communication) of the Salmonella Surveillance Unit, Communicable Disease Center, Public Health Service, that marine and aquatic species living away from sources of human contamination are not likely to be infected with *Salmonella* organisms. *Salmonella* do not appear to be common contaminants of live freshwater crayfish from commercial sources in Louisiana. Apparently coagulase-positive staphylococci are not commonly found in commercial crayfish, either. The two samples showing positive tests for coagulase positive staphylococci (Table 1) were obtained from fishermen coming from remote areas in the Atchafalaya River basin and could possibly have been contaminated by the fishermen removing them from the traps.

The data indicate that live crayfish coming from commercial sources in the Gulf Coast area may be sources of *E. coli* or fecal streptococci in processed crayfish products if correct sanitation procedures are not followed rigidly in the processing plants. On the other hand, coagulase-positive staphylococci or *Salmonella* organisms found in the processed products will likely have been introduced in the processing plant

Prior to peeling, crayfish are scalded to facilitate removal of the shell from the tail muscle. Prolonged heating will cause the tail muscle to tear apart; consequently, in many cases processors may reduce the heating time enough that it is insufficient to destroy all the vegetative microorganisms. But, with adequate scalding and proper handling procedures in the plant (separation of the area where the live crayfish are received from the peeling and processing areas), *E. coli* and fecal streptococci from the live crayfish should not be carried through to the final consumer product.

C. botulinum type E was not found in crayfish samples collected from any of the 22 sites selected for this study. Foster (1966) reported that this organism is found in most parts of the world, and that it has been found in inland waters in this country in New York and Tennessee and in the Great Lakes. The incidence of *C.*

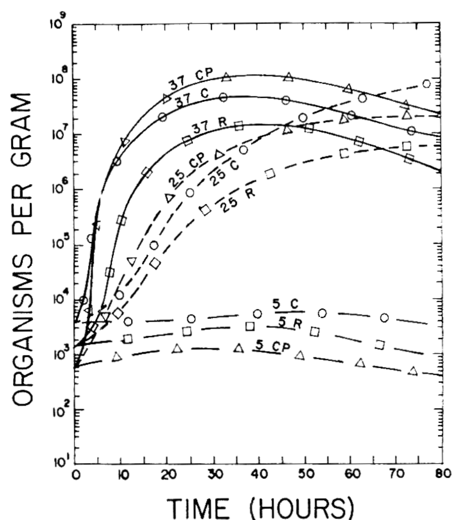

Fig. 1—Growth curves for *E. coli* in raw flesh (R), cooked flesh (C) and a commercial-type crayfish product (CP) at 5, 25 and 37°C.

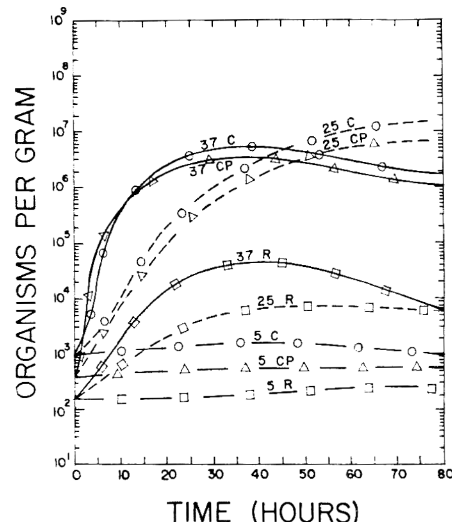

Fig. 2—Growth curves for *Streptococcus faecalis* in raw flesh (R), cooked flesh (C) and a commercial-type crayfish product (CP) at 5, 25 and 37°C.

botulinum type E in fish and shellfish from freshwater sources in the southern United States has not been reported, but a survey of the literature indicated that intensive studies have not been conducted in this respect. Ward et al. (1965) found *C. botulinum* type E in the Gulf of Mexico off the Texas coast.

Growth patterns

Figs. 1 through 4 show the growth curves, respectively, for *E. coli, Streptococcus faecalis, Staphylococcus aureus* and *Salmonella typhimurium* in raw flesh, cooked flesh and a commercial-type formulation, at incubation temperatures of 5, 25 and 37°C. The incubation period for samples stored at 5°C was extended to 21 days. There was no significant growth by any of the organisms in any of the substrates at 5°C. *Staphylococcus aureus* (Fig. 3) and *Salmonella typhimurium* (Fig. 4) grew well at 25 and 37°C in all three substrates. *E. coli* (Fig. 1) and *Streptococcus faecalis* (Fig. 2) grew well at these temperatures in the cooked flesh and commercial product, but growth in the raw flesh was suppressed slightly for *E. coli* and decidedly for *Streptococcus faecalis*.

It is unlikely that competitive inhibition by the residual microflora in the nonsterile raw flesh caused the suppression of growth, since the total aerobic plate count (BHI agar) of the raw flesh did not exceed 10^4 col/g after 72 hr incubation at

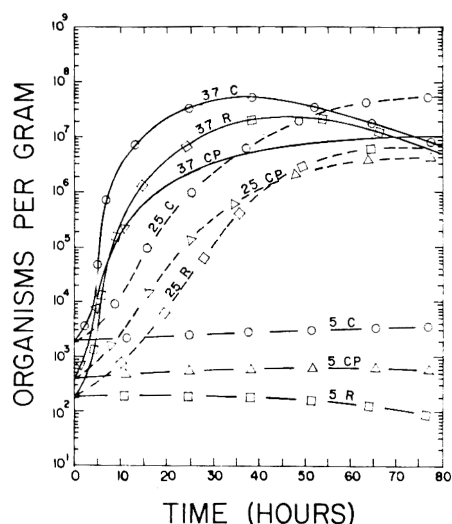

Fig. 3—Growth curves for *Staphylococcus aureus* in raw flesh (R), cooked flesh (C) and a commercial-type crayfish product (CP) at 5, 25 and 37°C.

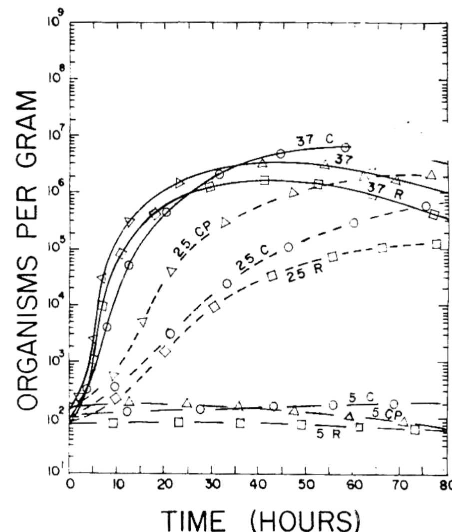

Fig. 4—Growth curves for *Salmonella typhimurium* in raw flesh (R), cooked flesh (C) and a commercial-type crayfish product (CP) at 5, 25 and 37°C.

either temperature. Apparently, some factor in the raw tissue other than the residual microflora was responsible for the retardation of growth of *E. coli* and *Streptococcus faecalis*. Pablo et al. (1966) found that "fecal enterococci" grew in competition with naturally occurring microorganisms on rehydrated chicken at a temperature of 20°C or above.

The lag time was shorter and the logarithmic growth rate was more rapid at 37°C for all of the organisms in each of the substrates. With the exception of *Staphylococcus aureus* and *Streptococcus faecalis* in the raw flesh substrate, the stationary phase of the growth curves at 37°C was reached within 10 to 20 hr, whereas, at 25°C the negative growth phase was extended and maximum cell counts were not found until 60 to 80 hr.

These data indicate that, with the exception of *Streptococcus faecalis* and possibly *E. coli* in raw flesh, these four pathogenic and fecal indicator bacteria grow well in freshwater crayfish products at temperatures of 25 and 37°C and under improper processing or handling conditions could provide a health hazard. The data also show that a storage temperature of 5°C will restrict the growth of all four of these organisms in crayfish products.

Table 2—Incidence of *C. botulinum* type E toxin production and pH change in inoculated packs of raw and cooked crayfish flesh and a commercial-type crayfish product stored at 30°C

Substrate	Hours following inoculation					
	0	24	48	72	96	120
Raw flesh						
Toxin	Neg.	Neg.	Pos.	Neg.	Neg.	Neg.
pH	6.7	7.0	7.3	8.0	8.5	8.5
Cooked flesh						
Toxin	Neg.	Neg.	Neg.	Pos.	Neg.	Neg.
pH	6.7	6.9	7.0	7.3	8.1	8.2
Commercial-type product						
Toxin	Neg.	Neg.	Pos.	Pos.	Pos.	Neg.
pH	6.8	6.7	6.4	6.3	5.9	8.5[1]

[1] The pH of the commercial product was adjusted from 5.7 to 8.5 by adding NaOH after 120 hr.

Toxin production by C. botulinum type E

Table 2 shows the results of the study of toxin production in raw flesh, cooked flesh, and a commercial product, inoculated with *C. botulinum* type E spores and incubated at 30°C. The data indicate that this strain of *C. botulinum* type E produced toxin in all three of the substrates within 72 hr at 30°C. In raw flesh, toxin production was first detected at 48 hr after inoculation; however, after 72 hr and at each subsequent analysis, the toxicity tests were negative. The cooked flesh showed similar patterns of pH and toxicity chang[illegible] as the raw flesh except at a slower ra[illegible]e. Toxin was first detected in the cooked flesh at 72 hr after inoculation and [illegible] at 96 hr when the pH had in-[illegible] 8.1.

The increase in pH in the raw flesh from 6.7 initially to 8.0 at 72 hr, which could be attributed to naturally occurring microorganisms in the nonsterile substrate, may explain the rescindance of toxicity after 72 hr. The rise in pH in the cooked (autoclaved 15 min at 121°C) flesh was not anticipated as toxic type E strains of *C. botulinum* are not considered proteolytic (Dolman, 1964 and Schmidt, private communication, 1968). The pH increase in the sterile, cooked flesh could be due to the release of volatile bases from nonprotein nitrogenous compounds, as Flick et al. (1968) found that 17.3% of the nitrogen in crayfish flesh was nonprotein.

Toxin was first found in the commercial product incubated at 30°C after 48 hr. The pH decreased to 6.4 after 48 hr and to 5.9 after 96 hr; however, toxicity persisted until the pH was raised to 8.5 by the addition of NaOH at 120 hr. The pH of one-half of this substrate was adjusted after 120 hr from 5.7 to 8.5 to confirm the supposition that the alkalinity of the medium was responsible for inactivation of the toxin of *C. botulinum* type E. The remaining half of the sample in which the pH was not changed (pH 5.7) produced toxicity in the mice.

Table 3—First detection of *C. botulinum* type E toxin in inoculated packs of raw and cooked crayfish flesh and a commercial-type crayfish product stored at 5°C and in ice-pack

Substrate	Toxin first detected	
	5°C	0°C
Raw flesh	33 days	Neg. after 56 days
Cooked flesh	33 days	Neg. after 56 days
Commercial-type product	33 days	Neg. after 56 days

At 5°C (Table 3), toxin was first found at 33 days in all three substrates. Toxin production was not found after 56 days' storage in ice-pack in any of the crayfish products.

Such a large concentration of *C. botulinum* type E spores as that used in this study would not be found in a commercial crayfish product except under extraordinary circumstances. The objective in this case, however, was to determine whether a type E strain of *C. botulinum* would produce toxin in crayfish products. The organism used in this study (Strain 8E, Continental Can Company) showed marked toxigenicity in the raw and cooked crayfish and in the commercial-type crayfish product at near its optimal growth temperature (30°C). It also produced toxin after an extended period in all three products at a temperature well within the normal range of refrigerated foods.

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OLIVE M. BATCHER
USDA, Human Nutrition Research Division, ARS, Beltsville, Maryland 20705
and A. W. BRANT and MARION SIMONE KUNZE
Department of Food Science and Technology, U. California, Davis, California 95616

Sensory Evaluation of Lamb and Yearling Mutton Flavors

SUMMARY—*Differences in flavor attributable to age of animal and to sex were detected when broth from samples of lamb and yearling mutton meat were served to panel members in triangle tests. No differences were detected in slices of roasted, broiled, or braised meat scored by the panel. Broth from the lean meat of lamb (7 to 8 months) was preferred to that prepared from lean of 15- to 16-month-old yearling mutton carcasses. Differences in flavor intensity of wether and ram meat served as patties in triangle tests were present only in patties containing 20% added fat. Full natural flavor of slices of cooked meat was not associated with either the cover fat thickness of the cut or with the fat content of the muscle.*

INTRODUCTION

FLAVOR IS frequently considered to be the most important palatability characteristic of cooked lamb meat. A consumer may accept or reject a piece of lamb meat on the basis of its flavor or odor, whereas acceptability of beef may also be based on tenderness, and pork and turkey acceptability on juiciness. The characteristic flavor of some lamb, yearling mutton, and mutton has been cited as a reason for low consumption of meat from ovine animals. In 1966, the per capita consumption of meat in the United States was 170 pounds, of which less than 2.4% was lamb and 0.2% mutton (USDA, 1967).

Many contradictory reports may be found in the literature as to the effects of such factors as age, feed, sex, and fatness of animal on the flavor of ovine meat. Although some investigators reported more intense, more pronounced and/or less desirable flavors of lean and fat with increase in age of animal (Weber et al., 1932; Paul et al., 1964), other investigators reported the reverse (Weller et al., 1962) or no association (Batcher et al., 1962). Variations in flavor among muscles from individual animals have been reported to be greater than flavor differences in meat from animals varying widely in age (Jacobson et al., 1962).

Some investigators associated increased fatness with increased flavor intensity and/or desirability (Barbella et al., 1936; Cline et al., 1937; Jones, 1952), while others reported either the reverse or no association (Hofstrand et al., 1960; Weber et al., 1931). Hornstein et al. (1963) reported that although carbonyl compounds were present in only trace amounts in the fat, the fat was responsible for the characteristic lamb aroma.

The distinction between the "characteristic" flavors of lamb and mutton meat has not been defined. People apparently differ in their concept of what constitutes mutton flavor. Mutton meat may have an entirely different flavor, or may merely represent a change in concentration. Lamb meat is usually meat from ovines less than 14 months old. Yearlings are animals 12 to 24 months old who have cut one pair of permanent incisor teeth. Mutton is from animals over 24 months who have cut two pairs of permanent incisor teeth (USDA, 1960).

The ability to distinguish between "lamb" and "mutton" flavors also varies widely among people. In preliminary studies on threshold tests, 3 out of 14 people tested were able to detect mutton flavor in ground lamb patties containing 15% mutton, 7 were able to detect the flavor in patties containing 15 to 35% mutton, but 4 other people required more than 35% mutton in the patties before the presence of mutton was detected.

The purpose of the study was to determine the effects of age, sex, and fatness on flavor of meat from ovine carcasses by evaluating the meat in different ways. Since problems arise in the marketing of yearling mutton meat when lamb meat is available, animals seven to eight months and 15 to 16 months were selected for study.

EXPERIMENTAL

LEGS, shoulders (5-rib square cut), and loins (5 lumbar vertebrae) were obtained from 80 animals of known history. The animals were wethers or rams, seven to eight months of age, fed either on pasture or on hay and grain for a minimum of five months prior to slaughter, or were 15 to 16 months of age and either fed on pasture entirely or fed half on pasture and half on hay and grain. The cuts were individually wrapped in Cryovac, quick frozen, and held at —24 to —18° C for 1 to 10 months. The frozen loins were cut into chops, placed into plastic bags and thawed at 3°C for 24 hr. The legs and shoulders were thawed at 3°C for 48 or 40 hr, respectively.

The right leg from each animal was roasted, fat side up, uncovered on racks in aluminum pans in household-type electric ovens at 163°C to an internal temperature of 80°C. Chops from the right loin of each animal were broiled in pairs on a preheated rack in a preheated pan in a 176°C oven to an internal temperature of 76°C. Each chop was turned when it reached 50°C. The right shoulder from a carcass was braised on a rack in a covered aluminum pan in a 176°C oven to an internal temperature of 80°C.

Broth was prepared from the *semimembranosus* and *adductor* muscles, which were removed from the raw, left legs and trimmed of excess fat. Equal parts of the lean muscles and water were simmered at 95°C for 2 hr. After cooling 10 min, the broth was filtered through cheesecloth into a separatory funnel to remove fat and suspended particles. The broth was served at 55°C. Broth was also prepared from the *longissimus dorsi* from the raw, left loin in the same manner.

Meat patties were prepared from the remaining muscles of the left leg, which were ground once. To part of the ground meat, 20% ground fat from that leg was added. Each part was then ground twice. A 150-g sample of ground meat, either plain or with 20% added fat, was shaped into a patty 3½-in. in diameter and 1 in. in depth. The patty was cooked in a 176°C oven for 45 min; the patty was turned once after 20 min.

At some sessions, six experienced panel members scored ¼- to ⅜-in. slices of the roasted, broiled, or braised cuts for natural flavor of lean on a 10-point semi-structured scale (as well as for tenderness and juiciness). The meat was served at normal serving temperatures on warm plates. At other sessions, samples of broth or patties were served in a triangle design, and the panel members were asked to identify the odd sample and to indicate whether the odd or duplicate sample had a more intense flavor.

The panel members were chosen from a group of 12 on the basis of their abilities to discriminate consistently between samples of meat from commercial lamb carcasses of different USDA grades.

Extraction of fat with ethyl ether was made on vacuum-dried samples of raw and cooked *semimembranosus* and *longissimus dorsi* muscles and of cooked patties.

Analysis of variance procedures included those for unequal-proportionate subclasses (Snedecor, 1946). In the patty and broth phases of the investigation, tables given by Roessler et al. (1956) were used to test for

significance of panel judgments and intensity of flavor was calculated using the method of Davis et al. (1954) in which partially correct judgments are used in the estimate of the level of significance.

RESULTS

Slices of lamb and yearling mutton meat

Differences in the flavor scores of slices of roasted, broiled, or braised meat could not be attributed to age of animal (lamb or yearling mutton), or sex (wether or ram); see Table 1. Differences in flavor scores between groups were not greater than they were within a group. Scores for flavor of meat slices from the individual lamb cuts, regardless of age or sex of animal, ranged from 4.7 to 7.8, a relatively narrow range on a 10-point scale.

Neither cover fat thickness of the cut (leg, loin, or shoulder) nor fat content of the muscle had an appreciable effect on the flavor scores of slices of cooked muscle; the correlation coefficients were both small and not significant. In fact, less than 4% of the variation in flavor scores was associated with cover fat thickness or intramuscular fat content. Neither were differences in flavor of the meat noted that could be attributed to different feeding regimes of the animals. Correlation coefficients for flavor scores with cooking losses were also small and not significant.

Broth

The broth from the lean of cuts from lambs 7 to 8 months old was more intense in flavor than comparable broth from the 15- to 16-month-old yearling mutton animals; the flavor differences were pronounced (Table 2). Differences in flavor were also detected in broths prepared from wether and ram meat. However, the panel members were not always able to indicate in triangle tests whether the broth from the wether or ram carcass had the more intense flavor. In most cases, broths from ram carcasses were cited as having the more intense flavor than the broths from wether carcasses.

Patties

Approximately half of the time panel members were able to identify correctly the odd sample (wether or ram) in triangle tests of ground meat patties prepared from leg muscles of wether or ram carcasses (Table 2). However, significant differences in flavor intensity attributable to sex (wether or ram) were observed only in meat patties with high fat contents. Although plain patties from leg muscles of wethers (15 to 16 months old) were considered to be slightly more intense in flavor than those from rams, the differences were not significant. The fat content (ether extract) of plain patties ranged from 3 to 9%. When 20% fat was added in the preparation of the patties, those prepared from wether meat had less intense flavor than those prepared from ram meat. As the fat content (ether extract) of the cooked patty increased from 13 to 33%, flavor of patties prepared from ram meat was rated more intense, but that prepared from wether meat less intense (data not shown).

DISCUSSION

IN ORGANOLEPTIC testing, the possibility is always present that responses may be dependent on the form in which samples are presented to the panel members and to the procedures used by the panel in evaluating palatability characteristics. In this investigation, samples were presented to the panel members as meat slices, as broths, and as patties with and without added fat. Flavor (as well as tenderness and juiciness) of meat slices was rated by panel members using a scoring technique. Flavor differences in broths and in patties were sought by panel members using triangle tests.

No significant differences in flavor scores of slices of roasted, broiled, or braised meat were found that could be attributed to age or sex of animal. The flavor scores may have been influenced by other more dominant palatability characteristics, such as tenderness and juiciness. Major differences in flavor of meat slices probably would have been noted by the

Table 1—Natural flavor scores[1] for cooked meat slices.

Age of animal and sex	Roasted leg (*semimembranosus*)			Broiled Loin Chops (*longissimus dorsi*)			Braised Shoulder (*triceps brachii*)		
	No.	$\bar{x}$	Range	No.	$\bar{x}$	Range	No.	$\bar{x}$	Range
Age of animal									
7 to 8 months	18	6.1	5.3–7.2	18	6.0	4.8–7.5	17	6.8	6.0–7.3
15 to 16 months	17	6.3	5.5–7.2	17	6.4	5.3–7.7	18	6.7	5.8–7.8
Sex									
7 to 8 months									
wether	9	6.1	5.5–7.0	9	6.2	4.8–7.5	9	6.7	6.2–7.0
ram	9	6.2	5.3–7.2	9	5.9	5.0–7.3	8	6.9	6.0–7.3
15 to 16 months									
wether	30	6.3	4.7–7.3	15	6.5	5.3–7.7	31	6.6	5.5–7.8
ram	31	6.1	5.2–7.2	14	6.1	5.3–7.0	31	6.4	4.7–7.7

[1] Panel members scored each muscle for natural flavor on a 10-point scale with the highest score indicating meat with very full flavor and the lowest score indicating lacking or masked flavor.

Table 2—Flavor intensities of broth and patties prepared from meat of lamb and yearling mutton wethers and rams.

	Group[1]	Age of animal	Number of observations	Number of correct identifications	Flavor intensity value[2]: Age comparison 7–8 months	Age comparison 15–16 months	Sex comparison Wether	Sex comparison Ram
			Broth					
Leg		—	108	69[3]	547	749[4]	—	—
		7–8 months	54	25[5]	—	—	363	285
	A	15–16 months	54	20	—	—	366	282
	B	15–16 months	36	19[5]	—	—	207	225
	C	15–16 months	54	31[3]	—	—	288	360
	D	15–16 months	36	24[3]	—	—	266	166[4]
Loin		—	108	60[3]	522	744[4]	—	—
		7–8 months	54	31[3]	—	—	398	250
	A	15–16 months	54	26[5]	—	—	352	296
	B	15–16 months	36	17	—	—	219	213
			Patties—leg					
Plain (no fat)		7–8 months	54	19	—	—	329	219
	B	15–16 months	36	17	—	—	197	235
	A	15–16 months	54	29[4]	—	—	309	339
	C	15–16 months	60	31[4]	—	—	351	369
	D	15–16 months	36	15	—	—	201	231
20% fat added		7–8 months	54	23	—	—	311	337
	B	15–16 months	36	23[3]	—	—	245	187
	A	15–16 months	54	28[4]	—	—	328	320
	C	15–16 months	60	27	—	—	413	307[5]
	D	15–16 months	36	19[4]	—	—	258	174[5]

[1] The groups differ in feeding regimes and sires.
[2] Lower number denotes more intense flavor. Intensity values are weighted sums of correct and partially correct identifications of samples in triangle tests.
[3] Significant at 0.001 level of significance.
[4] Significant at 0.01 level of significance.
[5] Significant at 0.05 level of significance.

panel members; subtle differences in flavor may possibly have escaped detection.

Any influence that other palatability characteristics may have had on evaluation of flavor was removed in the broths. Since the broths were homogeneous, triangle testing was possible. With the focus on just the one characteristic, flavor differences among the broths attributed to age and sex of animal were found. That the effects on flavor were subtle and perhaps attributable to small amounts of flavor components present in the fat was illustrated by the data on patties. Flavor differences were observed when patties containing sufficiently high amounts of fat were compared in triangle tests.

The conclusions reached from this study are threefold: a) Subtle flavor differences attributable to age and sex of lamb can be detected; b) trace flavor components present in the fat may account for these differences; and c) when flavor is the most important characteristic to be discerned, use of homogeneous broths or patties may be preferable to the use of heterogeneous slices of roasted, broiled, or braised meat for detecting flavor differences.

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W. MARK KLIEWER

Department of Viticulture and Enology, University of California, Davis 95616

Free Amino Acids and Other Nitrogenous Substances of Table Grape Varieties

SUMMARY—The concentration of eight amino acids, total free amino acids, total nitrogen and free amino acid fraction nitrogen in the juices of 28 table varieties of grapes was determined at both early and a late stage of fruit maturity. Arginine, proline, glutamic acid and alanine were the most prominent amino acids. The varieties were classified into four groups according to which of these acids predominated. Total free amino acids at early and late fruit maturity ranged from 1.04 to 5.53 and 1.24 to 6.45 nM/100 ml juice, respectively in the various varieties. The eight amino acids analyzed accounted for 74 to 96% of the total free amino acids. The amino acid fraction nitrogen in the juices ranged from 60 to 90% of the total nitrogen. The eight amino acids accounted for 60 to 96% of the amino acid fraction Kjeldahl nitrogen and 37 to 85% of the total Kjeldahl nitrogen. Arginine accounted for 15 to 50% of the total nitrogen in the juices of the various fruits.

INTRODUCTION

DURING RECENT years there has been renewed interest in the nitrogenous constituents of grapes, especially the free amino acids. This interest has been due largely to recent developments in chromatographic and microbiological assay methods. The results of such studies with grapes and grape products have been summarized in several reviews (Peynaud et al., 1953; Castor et al., 1956; Lafon-Lafourcade et al., 1961; Drawert, 1963, Van Wyk et al., 1965; and Tercelj, 1965). Proline, arginine, glutamic acid, α-alanine, serine, threonine, γ-aminobutyric acid, aspartic acid and valine were found in largest quantities.

The amounts of the different amino acids varied considerably, depending on variety, location, maturity, cultural conditions and method of determination. Most investigators reported an increase in concentrations of amino acids with fruit maturity. Nassar et al. (1966) and Kliewer et al. (1966) recently identified 32 free amino acids in grapevines; an additional eight unidentified amino compounds was detected. In most mature fruits, however, 90% or more of the total free amino acids were accounted for by the nine acids mentioned above.

The kinds and amounts of amino acids are not only important in such processes as growth, maturation and senescence in the grape, but also greatly influence the rate of fermentation by yeast and the formation of higher alcohols in wines (Lafon-Lafourcade et al., 1961; Webb et al., 1963).

In most studies concerned with the relative concentrations of amino acids in

Table 1—Total soluble solids, total nitrogen, amino and nonamino acid fraction nitrogen and total free amino acids of table varieties of grapes harvested at early and late stages of fruit maturity[1]

	Date Harvested		Total solids, °Brix		Total nitrogen, mg/100 ml juice		Amino acid fraction nitrogen, mg/100 ml juice		Nonamino acid fraction nitrogen, mg/100 ml juice		Total free amino acids, µmoles/100 ml (leucine equiv.)	
Variety	Early	Late	Early	Late	Early	Late	Early	Late	Early	Late	Early	Late
Barlinka	9-30	11-3	15.0	16.4	57.2	77.0	47.1	63.0	10.1	14.0	1558	2115
Beauty Seedless	8-30	9-30	17.6	18.5	161.9	198.0	128.0	140.0	33.9	58.0	5530	5400
Black Corinth	9-1	9-20	24.8	26.2	142.5	154.1	120.0	113.0	22.5	41.1	4370	4570
Black Malvoisie	8-30	9-30	19.9	24.0	99.0	140.9	88.0	107.3	11.0	33.6	4000	4850
Black Monukka	8-30	9-30	21.9	28.2	121.7	220.2	84.0	132.7	37.7	87.5	2837	5207
Black Prince	8-30	9-20	19.0	20.8	38.0	43.0	33.6	37.3	4.4	5.7	1043	1242
Blackrose	8-30	10-12	16.1	18.7	78.0	106.3	66.0	69.9	12.0	36.4	2185	3380
Canner	8-30	9-30	18.3	22.8	72.0	96.3	59.0	59.7	13.0	36.6	2175	2620
Cardinal	8-30	9-30	17.4	19.2	113.0	154.7	94.0	116.8	19.0	37.9	3392	5227
Early Muscat	8-18	9-20	20.8	26.9	96.5	126.4	72.0	81.9	24.5	44.5	2990	3588
Exotic	8-30	10-12	15.4	19.3	71.5	93.2	48.5	65.2	23.0	28.0	1861	3068
Isabella	8-30	9-30	19.4	25.2	117.5	203.2	105.3	138.2	12.2	65.0	4986	6450
Italia	8-30	10-12	17.7	20.7	92.0	103.4	56.5	64.8	35.5	38.6	1210	1991
July Muscat	8-18	9-20	20.1	26.1	89.0	97.6	55.9	59.5	33.1	38.1	2518	3038
Kishmishi	8-30	11-3	16.0	22.0	111.6	158.3	78.2	102.5	33.4	55.8	2870	3396
Loose Perlette	8-30	9-20	19.2	20.2	141.0	114.0	102.5	84.8	38.5	29.2	3233	4425
Malaga	8-30	10-12	16.4	19.8	84.0	108.3	57.5	68.5	26.5	39.8	2332	3501
Muscat of Alexandria	8-30	9-30	18.4	21.5	74.8	100.1	54.5	83.0	20.3	17.1	1730	3230
Muscat Hamburg	8-30	9-20	19.6	22.4	88.5	99.3	62.5	71.3	26.0	28.0	2325	2984
Niagara	8-30	9-20	19.4	21.9	112.5	122.0	86.5	92.4	26.0	29.6	3338	3472
Perlette	8-30	9-20	19.0	19.1	135.0	142.5	114.5	105.3	20.5	37.2	3476	4656
Queen	8-30	9-30	17.8	22.0	87.3	124.7	65.5	78.0	21.1	46.7	2377	3553
Red Malaga	8-30	10-12	17.7	22.0	69.5	111.5	57.5	98.2	12.0	13.3	2259	3958
Regina (Dattier)	9-1	10-12	16.5	20.8	97.5	98.7	61.0	69.9	36.5	28.8	2289	3215
Ribier	8-30	11-3	16.6	20.0	92.0	131.2	72.0	87.0	20.0	44.2	2738	4981
Rish Baba	8-30	10-12	15.2	19.4	85.0	162.5	63.5	119.0	21.5	43.5	2971	4163
Thompson Seedless	8-30	9-20	21.3	23.6	98.5	115.4	65.5	69.1	33.0	46.3	1827	3216
Tokay	8-30	10-12	17.4	19.6	40.5	55.4	29.5	35.2	11.0	20.2	1288	2130

[1] Figures represent the average of duplicate determinations on two samples taken at each date and for each variety.

grapes, the fruits were obtained from vines grown in various climatic regions, on different soils, under different cultural conditions and untested for the presence of viruses. The present report compares the concentrations of eight of the predominant amino acids, total nitrogen and amino acid fraction-nitrogen of fruits from 28 table varieties free from all known viruses. The vines were all grown under the same climatic, cultural and soil conditions, and the fruits were harvested at approximately the same stage of maturity.

EXPERIMENTAL

The location, sampling methods and dates and juice-extraction procedure were as described in a previous paper (Kliewer, 1967a). Each of the varieties (Table 1) was sampled in duplicate twice—once when the fruits were slightly to moderately ripe, and again when they were ripe to overripe. The samples are respectively referred to as early- and late-harvested fruits (Tables 1–3).

Preparation of amino-acid fractions

A 10-ml portion of juice from each sample was applied to a column (2 × 20 cm) of Dowex 50W-X8 (H^+ form). After thorough washing, the amino acids were eluted from the column with 150 ml of 3 *N* NH_4OH, and washed with three resin-bed volumes of distilled water. The eluate and washings were taken to dryness at 40 to 45°C under jets of filtered air. The residue was dissolved in 10 ml of 10% isopropanol.

Quantitative analysis of amino acids by paper chromatography

The amino acids were separated by two-dimensional descending paper chromatography and identified by co-chromatography with authentic compounds. The concentration of each of the free amino acids, except proline and arginine, was determined according to the procedure for paper chromatography described by Nassar et al. (1966). Samples of each variety from each sampling date were assayed in duplicate.

Determination of arginine. Arginine was determined by the Sakaguchi reaction as modified by Gilboe et al., (1956).

Determination of proline. Proline was determined by the acidic ninhydrin photometric method of Chinard (1952) as modified by Ough (1968).

Determination of total free amino acids. Total free amino acids in the amino acid fraction extract were determined according to the ninhydrin method of Jacobs (1956). The samples were corrected for the low color yield of proline, which gives only approximately 5% of the color of α-amino acids. In this report, the imino acids proline and hydroxyproline, and the amides glutamine and asparagine, are included with the amino acids.

Total nitrogen and amino acid fraction nitrogen. Total nitrogen and amino acid fraction nitrogen were determined by the Kjeldahl method on 5-ml portions of the original juice sample and the amino acid fraction extract, respectively.

Nonamino acid fraction nitrogen. Nonamino acid fraction nitrogen is the difference between total nitrogen and amino acid fraction nitrogen.

The 28 varieties studied are all used for producing table fruits. Two of the varieties, "Thompson Seedless" and "Muscat of Alexandria," are also used for raisins, but will be referred to as table varieties for simplicity.

RESULTS

In nearly every variety, there was an increase in the concentration of total nitrogen, amino acid fraction nitrogen, nonamino acid fraction nitrogen and total free amino acids with fruit maturity (Table 1). The concentrations of total nitrogen in the juice ranged from 38 to 162 mg/100 ml in the slightly to moderately ripe fruits (early harvest) to 43 to 220 mg/100 ml in the ripe to overripe fruits (late harvest). Barlinka, Black Prince, and Tokay varieties were relatively low in nitrogen (less than 80 mg N/100 ml) while Beauty Seedless, Black Monukka, Isabella and Rish Baba were high in nitrogen (greater than 160 mg N/100 ml) at the time of late harvest.

The other varieties contained intermediate amounts of nitrogen in the juice at late harvest (80 to 160 mg N/100 ml juice). The amino acid fraction nitrogen

Table 2—Concentration of several free amino acids (μmoles per 100 ml juice) of varieties of table grapes harvested at early and late stages of fruit maturity[1]

Variety	α-Alanine		γ-Amino butyric acid		Arginine		Aspartic acid		Glutamic acid		Proline		Serine		Threonine		Sum of amino acids	
	Early	Late	Early	Late	Early	Late	Early	Late	Early	Late	Early	Late	Early	Late	Early	Late	Early	Late
Barlinka	249	333	67	124	460	535	50	67	262	320	250	298	78	95	116	182	1429	1954
Beauty Seedless	700	690	105	162	1248	831	103	120	522	410	802	940	228	205	356	295	4064	4053
Black Corinth	392	344	146	201	1156	1123	73	87	370	395	851	1135	174	201	284	290	3477	3776
Black Malvoisie	555	700	146	377	922	890	65	84	322	680	905	934	184	345	150	300	3249	3410
Black Monukka	351	620	118	380	426	581	162	170	442	895	618	1060	135	164	263	300	2515	4170
Black Prince	146	167	42	43	248	315	60	55	163	168	195	228	65	59	58	68	977	1103
Blackrose	253	290	57	100	566	550	99	128	300	677	232	343	128	128	189	285	1834	2501
Canner	202	317	134	132	638	469	114	105	294	520	393	527	77	168	170	200	2022	2437
Cardinal	376	715	139	311	926	1050	87	94	503	905	702	1592	95	154	167	185	2995	5006
Early Muscat	285	350	195	363	600	534	34	62	236	294	868	1142	132	145	159	168	2509	3058
Exotic	308	366	160	200	468	455	91	109	285	912	248	598	78	115	84	104	1722	2856
Isabella	1280	1600	238	398	821	866	157	24	562	1150	248	574	190	360	278	304	3774	5276
Italia	126	131	89	106	421	549	56	65	150	460	110	396	94	81	102	120	1148	1908
July Muscat	220	284	205	268	460	436	38	32	267	290	835	1250	76	84	155	162	2256	2806
Kishmishi	152	332	87	154	938	639	64	140	263	405	786	917	67	142	180	180	2537	2909
Loose Perlette	482	600	140	186	421	587	114	135	389	416	982	1565	108	122	220	306	2856	3917
Malaga	260	448	81	151	646	567	142	140	187	656	218	633	96	103	205	260	1835	2953
Muscat of Alexandria	230	435	50	160	502	786	155	160	212	525	232	705	94	140	136	175	1611	3086
Muscat Hamburg	475	419	101	144	476	529	65	72	169	473	316	720	135	132	260	224	1997	2713
Niagara	1400	1290	254	149	466	547	85	40	298	399	214	523	204	117	160	135	3081	3200
Perlette	355	450	110	141	546	594	116	142	296	492	1080	1701	68	114	150	340	2721	3974
Queen	488	658	116	212	598	580	107	80	209	437	492	987	106	167	112	217	2228	3338
Red Malaga	275	485	54	109	600	950	74	82	237	1240	220	461	93	149	233	290	1786	3766
Regina (Dattier)	282	372	105	176	472	581	80	139	340	825	225	542	134	191	205	204	1843	3030
Ribier	359	600	125	261	519	1008	62	103	510	1017	424	980	103	184	240	271	2342	4424
Rish Baba	466	480	121	180	527	950	155	170	470	650	517	660	197	207	210	175	2643	3478
Thompson Seedless	251	360	98	110	384	546	68	109	221	395	460	859	70	131	178	215	1730	2725
Tokay	144	214	28	100	178	231	194	163	172	304	179	476	71	78	120	115	1086	1681

[1] Figures represent the average of duplicate determinations on two samples taken at each date and for each variety.

ranged from 60 to 90% of the total nitrogen (Table 1) and generally accounted for a greater percentage of the total nitrogen in the early harvest than it did in the late harvest. The opposite relationship was usually true for the nonamino acid fraction nitrogen. The concentration of total free amino acids varied greatly with variety, ranging from 1,043 to 5,530 μmole/100 ml at early harvest to 1,242 to 6,450 μmole/100 ml at late harvest (Table 1).

The level of amino acids in the different varieties usually increased with fruit ripeness (Table 2). This was especially true for glutamic acid and proline, in which the concentration in some varieties doubled or tripled between early and late harvests. There were extreme quantitative differences in the concentrations of amino acids among varieties. On the basis of the predominant amino acid present, the varieties were divided into four groups (Table 4). At the early harvest, arginine was the predominant amino acid in the greatest number of varieties; at the late harvest this was true of proline.

Alanine was the main amino acid at both harvests in only two varieties, Isabella and Niagara, both of which have American species in their parentage. Glutamic acid was predominant in six varieties. The sum of the eight amino acids determined (Table 2) accounted for 74 to 96% of the total free amino acids in the various varieties. They also accounted for 60 to 96% of the amino acid fraction nitrogen and 37 to 85% of the total N in the fruits (Table 3). Arginine contributed, by far, the most nitrogen, accounting for 23 to 63% and 25 to 65%, respectively, of the amino acid fraction nitrogen in the juice of early- and late-harvested fruits, and 17 to 50% and 15 to 48%, respectively, of the total nitrogen in the early- and late-harvested fruits.

DISCUSSION

Most of the early-harvested fruits were obtained when their total soluble solids ranged from 16 to 20°Brix. Very ripe to overripe fruits (late harvested) were obtained 30 to 60 days after the early harvest, and the °Brix of these fruits generally ranged from 19 to 27. Many varieties, however, did not increase in total soluble solids after they reached a particular concentration of sugar (Table 1). The changes in concentration of glutamic acid and of proline between the early and late harvest were most striking, increasing by as much as four-fold in some varieties (Table 2). The concentration of γ-aminobutyric acid also usually increased considerably with fruit maturity, while that of arginine increased in some varieties, decreased in others or remained about the same. These findings are in agreement with that of Lafon-Lafourcade et al. (1962) and Drawert (1963), who showed that the concentration of proline increased markedly in grapes during the latter stages of fruit maturation. The increase in concentration of free amino acids as the fruit matures may be due to a lessening in demand for these metabolites as growing processes are progressively taken over by the ripening processes.

A relationship among concentrations of arginine, proline, γ-aminobutyric acid and glutamic acid is easily seen, since the latter compound is a common intermediate in the synthesis of these other substances, and is usually formed by transamination reactions with α-ketoglutaric acid in the Krebs cycle. Arginine is the main storage form of nitrogen in the grapevine (Kliewer, 1967b), and often accumulates in seeds, fruits and other storage organs (Reuter, 1957). The synthesis of arginine also provides a mechanism for utilization of excess ammonia and may account for the decrease of this latter compound which generally accompanies fruit ripening.

The reason for the large increase in proline is not so easily understood, since this amino acid is relatively low in nitrogen. Free proline was found in very small amounts in the roots, woody tissues, bark and leaves of "Thompson Seedless"

grapevines by Nassar et al. (1966), indicating that the accumulation of this amino acid in the fruit probably results from synthesis *in situ*.

Qualitatively the general pattern of amino acid composition of the juices of the various grape varieties was similar. Quantitatively, however, there were large differences, especially in the amounts of α-alanine, arginine, glutamic acid and proline. At both harvests, arginine and proline were the predominant amino acids in the majority of the varieties, with glutamic acid and α-alanine usually the third and fourth most important quantitatively. In two varieties, Isabella and Niagara, α-alanine was the predominant amino acid. Both of these varieties have some *V. labrusca* in their parentage, and this species may carry the gene responsible for the large amount of α-alanine. The large quantitative differences in amino acid composition of the juices most probably influences their rate of fermentation by yeast. Lafon-Lafourcade et al. (1961) reported that arginine, glutamic acid and alanine are readily utilized as sources of nitrogen by yeast, while proline and threonine are difficult for yeast to assimilate. The amino acid composition of grape must may also influence the fusel oil production by yeast during fermentation (Webb et al., 1963).

The relative amounts of amino acids in mature grape berries closely resemble those in the mature orange (Clements et al., 1962). Ulrich et al. (1955) also found that proline is the predominant amino acid in pears at maturity, and Reuter (1957) reported that proline is the major ninhydrin-reacting substance in members of the *Papilionatae* family.

The amino acid fraction nitrogen includes all nitrogenous substances retained by Dowex 50 cation exchange resin. Preliminary investigations indicated that, in addition to amino acids, low molecular weight peptides such as glutathione were completely retained by the cation resin and consequently would be included as part of the amino acid fraction nitrogen. The eight amino acids in Table 2 accounted for 60 to 96% of the nitrogen in the amino acid fraction of the various varieties (Table 3). An additional 24 free amino acids plus several unidentified amino compounds have been found in fruits of grapes (Nassar et al., 1966; Kliewer et al., 1966). These, plus low molecular weight peptides, would account for most of the remaining nitrogen. Paper chromatographic analysis indicated that these undetermined amino compounds were all present in relatively low concentration.

The nonamino acid fraction nitrogen in the early- and late-harvested fruits of the different varieties ranged from 4 to 38 and 6 to 87 mg/100 ml juice, respectively, and accounted for 10 to 38% and 12 to 40% of the total nitrogen. Included in the nonamino acid fraction nitrogen were proteins, high molecular weight peptides, nucleotides and nucleosides, ammonia and

Table 3—Percentage of amino acid fraction nitrogen and total nitrogen accounted for by eight determined amino acids in juice of table grapes

Variety	Percent of amino acid fraction N accounted for by 8 determined amino acids		Percent of total N accounted for by 8 determined amino acids	
	Early	Late	Early	Late
Barlinka	83.4	79.0	68.7	64.7
Beauty Seedless	84.6	78.2	66.9	55.3
Black Corinth	81.0	88.8	68.2	65.1
Black Malvoisie	95.7	91.0	85.0	69.3
Black Monukka	63.2	62.4	43.6	37.6
Black Price	68.8	73.7	60.8	64.0
Blackrose	75.0	83.1	63.5	54.7
Canner	93.4	90.1	76.5	55.9
Cardinal	85.9	97.7	71.5	73.8
Early Muscat	83.7	79.6	62.5	51.9
Exotic	90.3	90.6	61.2	63.4
Isabella	82.9	79.7	74.3	54.2
Italia	59.8	76.8	36.7	48.2
July Muscat	91.0	96.8	57.2	59.0
Kishmishi	95.8	91.5	67.1	59.2
Loose Perlette	77.2	75.9	56.1	56.5
Malaga	91.8	95.1	62.9	60.2
Muscat of Alexandria	80.0	91.8	58.3	76.1
Muscat Hamberg	77.1	84.4	54.5	60.6
Niagara	72.5	73.4	55.7	55.6
Perlette	75.5	74.4	64.0	54.9
Queen	85.8	91.1	64.4	57.0
Red Malaga	87.3	94.3	72.2	83.0
Regina (Dattier)	74.8	95.5	46.8	67.7
Ribier	75.8	76.8	59.3	50.9
Rish Baba	91.3	74.4	68.2	54.5
Thompson Seedless	80.2	86.4	53.3	51.7
Tokay	95.9	92.3	69.9	58.7

Table 4—Classification of grape varieties according to the predominant amino acid present in the juice at early and late harvest[1]

α-alanine		Arginine		Glutamic acid		Proline	
Early	Late	Early	Late	Early	Late	Early	Late
Isabella	Isabella	Barlinka	Barlinka		Blackrose	Black Monukka	Black Corinth
Niagara	Niagara	Beauty Seedless	Beauty Seedless		Exotic	Early Muscat	Black Malvoisie
		Black Corinth	Black Prince		Malaga		Black Monukka
		Black Malvoisie	Italia		Red Malaga	July Muscat	Canner
		Black Prince	Muscat of Alexandria		Regina	Loose Perlette	Cardinal
		Blackrose	Rish Baba		Ribier	Perlette	Early Muscat
		Canner				Queen	July Muscat
		Cardinal				Thompson Seedless	Kishmishi
		Exotic				Tokay	Loose Perlette
		Italia					Muscat Hamburg
		Kishmishi					Perlette
		Malaga					Queen
		Muscat of Alexandria					Thompson Seedless
		Muscat Hamburg					Tokay
		Red Malaga					
		Ribier					
		Rish Baba					

[1] Refer to Table 1 for the °B at the early and late stages of fruit development.

nitrate nitrogen.

Bayly et al. (1967) found protein in the juice of various grape varieties ranging from 2 to 26 mg/100 ml. Amerine et al. (1967) reported that ammonia is usually present in juices of mature grapes in concentration from 1 to 10 mg/100 ml. Tercelj (1965) found that nucleosides and nucleotides accounted for about 2 mg N/100 ml juice in the variety "Sauvignon." Nitrates are present only in very small amounts in grape must (Rebelein, 1967). Peynaud et al. (1953) indicated that the more mature the grape the greater the accumulation of polypeptides; however, the exact contribution of these substances to the nitrogen in grapes is not known.

A small amount of nitrogenous substances may not have been eluted from the Dowex 50 columns with ammonia and therefore was included with the nonamino acid fraction. Burroughs (1957) noted that adenine, guanine and guanylic acid were only partly recovered by elution with ammonia from Zeokarb-225 columns.

The data reported herein are for fruits harvested in 1966 at Davis, Calif. The total number of degree days above 50°F at Davis between April 1 and Sept. 1 was 2,966, about 260 degree days above the 30-year average for this period (Winkler, 1962). However, since all data were obtained from fruits harvested from virus-free vines grown under the same climatic, cultural and soil conditions, the reported values should indicate relative differences among varieties when compared at the same stage of fruit maturity. However, the grouping of grape varieties according to the predominant amino acid should be done with caution until the influence of the region, season, rootstock and cultural conditions on the relative amounts of amino acids in grapes is known.

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C. R. REY, A. A. KRAFT, R. G. SEALS and E. W. BIRD
Department of Food Technology, Iowa State University, Ames, Iowa 50010

Influence of Temperature on Some Biochemical Characteristics of Pseudomonas Associated with Spoilage of Chicken

SUMMARY—Studies were conducted with four cultures of Pseudomonas isolated from frozen chicken. The effect of temperature on some biochemical activities of the organisms was evaluated and the individual response of the cultures to temperature was determined. Growth, survival and production of the green fluorescent pigment, pyoverdine, and extra-cellular proteinase and lipase activities were used as indices of the ability of pseudomonads to produce spoilage. The four isolates differed in their ability to perform the metabolic functions mentioned. The cultures were incubated at 15°, 5°, —18° and —29°C. Assays for proteolysis were made by means of a dye binding method; lipolysis was determined by titration of free fatty acids released from chicken fat, and a photofluorometer was used to measure fluorescent pigment. Growth was determined by colony count. At temperatures above 0°C, survival was better and growth and enzyme activity were more extensive at 5° than at 15°C. Proteinase activity increased continuously, even when viable cells were decreasing; lipase production was correlated with growth. Formation of pyoverdine declined faster than did cell numbers. Survival of the cultures was better at —18° than at —29°C. Impairment of pyoverdine secretion was observed after exposure of the organisms to freezing temperatures, but the activity of the extracellular enzymes was not affected at temperatures below 0°C. No marked differences were observed among cultures in rate of cell division, but maximum populations, survival of organisms and stability of the proteolytic, lipolytic and fluorescent activities of the isolates were inversely related to biochemical activity above 0°C.

INTRODUCTION

MODERN marketing methods use low temperatures as a common means of preserving poultry. However, at refrigeration and freezing temperatures, psychrophilic bacteria develop. Of the psychrophiles, *Pseudomonas* species have been reported as the most numerous spoilage organisms present on poultry kept at low temperatures (Ayres et al., 1950; Walker et al., 1956; Barnes et al., 1959; and Nagel et al., 1960).

Pseudomonas are usually strongly lipolytic and/or proteolytic (Witter, 1961). The action of lipases from *Pseudomonas* brings about the first steps of hydrolytic degradation of poultry fat, facilitating the subsequent destruction of the free components. Tomhnison et al. (1963) observed the accumulation of free ammonia as the catabolic product of nitrogenous compounds by pseudomonads. A water soluble fluorescent pigment is also produced by some species of *Pseudomonas* (Breed et al., 1959).

The pigment, identified as pyoverdine by Turfreijer (1941), has been related by Koepsell (1950) and Lenhoff (1963) to the functions of an accessory respiratory enzyme. Thus, growth of pseudomonads on the surface of poultry may produce degradation of nutritive factors and deleterious odors and flavors due to proteolytic and lipolytic activity. Fluorescent discoloration due to excretion of pyoverdine, may also indicate spoilage of the meat.

Witter (1961) stated that the keeping quality of food stored at low temperatures depends more on the biochemical activity of the psychrophilic contaminants than on the numbers of cells. *Pseudomonas,* being capable of good growth at temperatures just above 0°C (Ingraham, 1958), could have enzyme systems well adapted to counteract the adverse conditions that bacteria encounter under frozen and refrigerated storage.

However, individual differences existing among species of *Pseudomonas* in response to factors affecting their metabolic function have been reported by Goldman et al. (1952), Hobbs et al. (1961), and Morihara (1965).

The present study evaluates the effect of low temperatures on some of the metabolic functions associated with spoilage of chicken meat by several *Pseudomonas* cultures and investigates the relationship between the metabolic activity and the response of the individual organisms to such temperatures.

MATERIALS & METHODS

Cultures employed

The test organisms were four *Pseudomonas* cultures isolated from frozen chicken. The bacteria were Gram-negative rods, 2 to 2.5 μ in length, motile, cytochrome oxidase positive, and metabolized glucose oxidatively. The morphology of the flagella characteristic of each culture is presented in Figure 1.

Growth, lipolytic and proteolytic activities and pyoverdine production were the properties selected as indices of metabolic activity in relation to spoilage of poultry. The four isolates were classified in accordance with the rate and extent of relative metabolic activity demonstrated by each organism (Table 1).

Preparation of samples

The test organisms were inoculated into trypticase soy broth (BBL) in flasks. The flasks were shaken during incubation at 15°C for 18 to 24 hr. Subcultures were made for three consecutive days. A heavy inoculum was spread on the surface of trypticase soy agar (BBL) and incubation was performed for 18 to 24 hr. The growth was recovered from the surface of the agar and washed three times with sterile distilled water. The cells were suspended in distilled water and adjusted to give an absorbancy of 1.0 with a Spectronic 20 spectrophotometer (Bausch and Lomb Optical Company, Rochester, New York) at 600 mμ. An absorbancy of 1.0 was equivalent to a cell concentration of approximately 2.0×10^9 cells per ml as determined by prior growth studies.

For evaluating growth and survival at temperatures above freezing, dilutions of the cell suspensions were made and the inoculum was transferred to 250 ml of trypticase soy broth (BBL) in 500 ml flasks to obtain an initial concentration of 10^3 cells per ml. Incubation was 5° and 15°C for 60 days. Survival below freezing was studied by inoculating 5 ml of the cell suspension (absorbancy = 1.0) into the flasks containing 250 ml of broth. A preliminary incubation of the flasks was made for 4 days at 15°C to obtain concentrations exceeding 100 million cells per ml. The flasks were stored at —18° and —29°C for 60 days.

Experimental methods

All isolates were tested periodically during the holding period. The samples held at temperatures below freezing were thawed for sampling by immersing the flasks in a water bath at 35°C. Bacterial numbers were determined by colony count on trypticase soy agar (BBL) with plates incubated for 5 days at 15°C. Production of pyoverdine was estimated in the asparagine medium of Georgia et al. (1931). The incubataion technique and procedure of measuring pigment were described by Kraft et al. (1964).

The broth cultures were centrifuged on a Sorval Superspeed Angle centrifuge (Ivan Sorval, Inc., Norwalk, Connecticut) for 30 min at 10,300 R.C.F. $\times$ G. The supernatants were used for measuring enzyme activity.

Proteolysis was assayed spectrophotometrically by the dye binding method described by

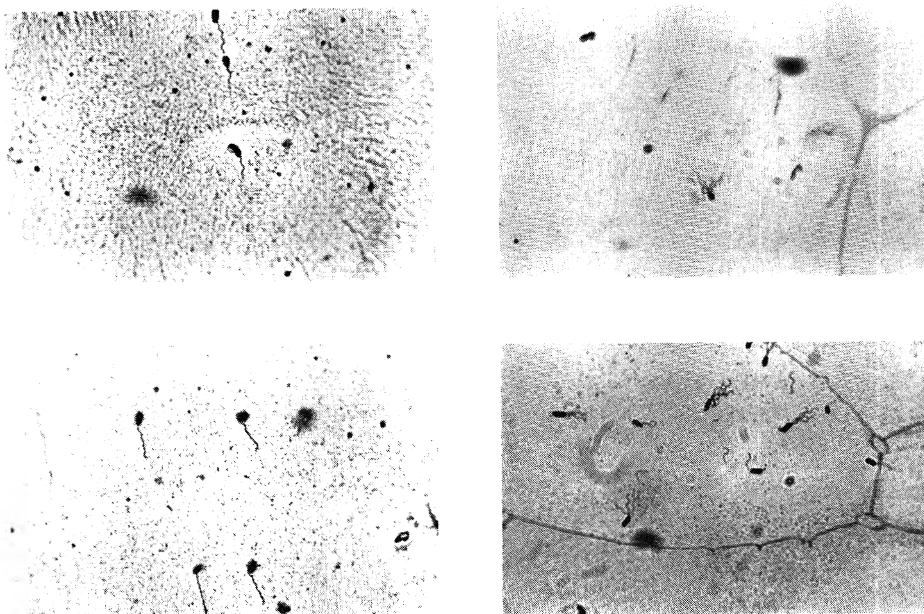


Fig. 1—Photomicrographs (500 ×) of flagella stains prepared according to the modification of Fontana's method described by Rhodes (1958). Upper left, culture F-4, single polar; upper right, culture F-14, polar multitrichous; lower left, culture F-17, single polar; lower right, culture F-21, polar multitrichous.

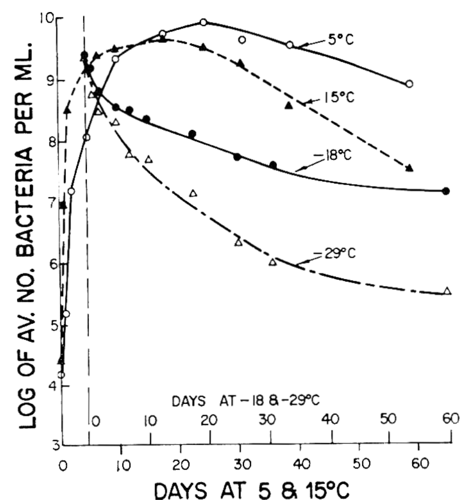


Fig. 2—Growth and survival above freezing and survival below freezing obtained by plotting average numbers of viable cells from all cultures against time of incubation at 5°, 15°, −18° and −29°C.

Hammond et al. (1966). One milliliter of the sample and 1 ml of sterile skim milk were incubated for 6 hr at 35°C. The optimum time for reaction of enzyme and substrate was determined by plotting proteolysis indices against time at intervals during 24 hr of reaction. According to this method, the proteolysis index is calculated as: $(A_S - A_C)/[(15/16)A_D - A_C]$ where: A_S = absorbancy of sample, A_C = absorbancy of unproteolyzed sample, and A_D = absorbancy of the undiluted dye solution. The index varies from 0 to 1 as the amount of dye bound decreases from that bound by intact milk to zero. A 6-hr period was chosen as the maximum time of constant rate of reaction. Controls were prepared with 1 ml of sterile trypticase soy broth (BBL) and 1 ml of skim milk.

Lipolysis was evaluated by titration of free fatty acids released from chicken fat by the method of Alford et al. (1963). Chicken fat was heated in a flask on a steam bath until liquified. To avoid separation of two liquid phases during titration, a modification of the procedure was made in accordance with the method reported by Tucker et al. (1951), as follows: 10 ml of *n*-propyl alcohol were added to serve as a binder for the titer solution in the petroleum ether; 0.01*N* methanolic potassium hydroxide was used for titration, and gum tragacanth was used as emulsifier.

For determinations of lipolytic activity, the enzyme solutions were incubated for 12 hr at 25°C. The optimum time for reaction was determined in a similar manner to that for proteolysis. Controls for initial readings were prepared using 2 ml of sterile trypticase soy broth (BBL) instead of the 2 ml of enzyme solution used for test samples. Results were reported by subtracting the amounts of methanolic potassium hydroxide used for titration of the controls from the volume used for titration of the samples.

RESULTS & DISCUSSION

Effect of temperature above freezing on growth and survival

The results of the observations obtained at each temperature of incubation for all cultures in three trials were averaged. Figure 2 presents mean values of 12 observations for bacterial growth or survival.

Optimum growth is determined by two criteria: rate of cell division or maximum population. Figure 2 shows that multiplication was faster at 15° than at 5°C, although cell yields were highest and survival was greatest at 5°C. Since higher numbers of cells and better survival were observed at 5° than at 15°C, cell production was less inhibited by low temperatures than was cell destruction. These findings are similar to those stated by Greene et al. (1954) for *Pseudomonas spp.* in milk.

Effect of temperatures above freezing on extracellular enzyme production

The production of pyoverdine (Fig. 3a), lipase (Fig. 3b), and proteinase (Fig. 3c) followed the same trend as growth in relation to temperature: the rate of production of the pigment and the enzymes was faster at 15° than at 5°C, but maximum values were observed at 5°C. The studies of Ingraham et al. (1959) indicated that psychrophilic bacteria must effect greater biochemical activity to synthesize cells and maintain old cells when grown at low temperatures than when grown at high temperatures. The observations presented in Figs. 3a, b and c seem to support their contention and suggest that an increased production of extracellular enzymes may have been induced at 5°C to compensate for the reduced rate of enzyme activity at the lowest temperature.

Table 1—Classification of the test organisms according to their metabolic activity.

Pseudomonas cultures	Growth	Production of proteinase	Production of lipase	Production of pyoverdine	General activity of the culture
F-21	Fast and extensive	Fast and extensive	Fast and extensive	Fast and extensive	Very active
F-4	Fast and extensive	Slow and limited	Slow but extensive	Fast but limited	Active
F-14	Fast and extensive	Negative	Fast and extensive	Negative	Moderately active
F-17	Slow but extensive	Negative	Negative	Slow and limited	Slowly active

Relation between growth and the metabolic functions studied

While the maximum lipolytic activity (Fig. 3b) was obtained simultaneously with the maximum number of cells at 5° and 15°C (Fig. 2), pigment production (Fig. 3a) started to decrease while bacterial numbers were still increasing. At the end of the holding period, very low levels of pyoverdine were produced even when viable cells were still present in concentrations as high as 90 million cells per ml in the samples held at 5°C.

Sinclair et al. (1963) demonstrated that oxygen is rapidly depleted in stationary cultures. Lack of oxygen causes quenching of pyoverdine production (Lenhoff, 1963). Even when pyoverdine was produced in the present investigation by transferring the cells into asparagine broth, and aeration was performed during incubation, a temporary impairment of the ability to produce pigment may have occurred. At least three sub-cultures under aeration were necessary for activation of cultures stored in broth for prolonged periods of time.

Effect of temperatures below freezing on survival and biochemical activities

The lethal effect of freezing was greater at −29° than at −18°C (Fig. 2). A rapid reduction in bacterial populations was observed during the first 48 hr below freezing. After 48 hr, the bacterial numbers decreased at a slower rate, becoming almost stationary between 30 to 60 days in frozen conditions. These data indicate that the resistance to cold temperatures varies among different cells of the same organism.

The production of pyoverdine declined faster than did cell numbers. Nonlethal metabolic injury to several species of *Pseudomonas* at sub-zero temperatures has been demonstrated by Straka et al. (1959). Apparently, freezing may affect the constitutive enzyme forming systems in some cells of the same organism, resulting in death, while other cells may be affected only in their adaptative enzyme forming systems, and remain capable of surviving frozen conditions. Pyoverdine formation is an adaptative metabolic function of *Pseudomonas* (Rhodes, 1959); hence, loss of the ability to produce pigment would not necessarily cause death of the cell.

The effect of freezing on the activity of the lipolytic and proteolytic enzymes is presented in Figures 3a and b. Freezing did not affect the activity of the extracellular proteolytic and lipolytic enzymes of these cultures of *Pseudomonas.*

Culture differences in response to temperature

The results of the observations obtained in three trials by incubation of each isolate at 5°C and 15°C for 60 days were averaged to study culture response to temperatures above 0°C; similar calculations for incubation below freezing (−18° and −29°C) were also made.

Figures 4a and b show production of pyoverdine by each fluorescent culture. The amount of pigment produced by isolate F-21, the most biochemically active of the cultures tested, was consistently high at temperatures above 0°C (Fig. 4a). However, the rate of decrease of pigment formation was the fastest for this organism. Isolate F-17, the least biochemically active of the test organisms, produced the smallest amount of pigment, but was very stable with regard to this function. It is possible that F-21, the most active of the fluorescent cultures, had to utilize the pigment as an accessory respiratory enzyme at the most rapid rate to be able to perform its active metabolic functions. Consequently, the most active of the fluorescent organisms elaborated decreasing amounts of pigment into the medium on prolonged incubation.

All cultures showed a decline in pyoverdine production after being stored at temperatures below freezing (Fig. 4b). However, isolate F-21 was the most stable in its ability to form fluorescent pigment upon immediate exposure to −18° and −29°C, and maintained this ability to a greater extent than did any of the other isolates. Culture F-17 was most affected by freezing and frozen storage.

The lipolytic activity of isolate F-21 increased at the most rapid rate at temperatures above 0°C (Fig. 5). After reaching the maximum level, the lipolytic activity also decreased at the most rapid rate for this organism. Lipolysis produced by isolate F-14 increased continuously throughout the 60 days of incubation. The rapid decrease of lipolytic activity observed with F-21 demonstrated that the lipolytic enzyme system was least stable at temperatures above 0°C for this isolate which was the most metabolically active of the three lipolytic organisms (Table 1).

The lipolytic function of isolate F-14, the least active of the three organisms, remained stable during prolonged storage. Thus, these results indicate an inverse relationship between the stability of the lipolytic functions and the overall metabolic activity of the cultures at temperatures above 0°C. At temperatures below freezing, lipolytic activity, similar to proteolytic activity (Fig. 3c), remained constant for all cultures stored at freezing temperatures. Values for lipolysis for all cultures at −18°C and −29°C were averaged, as shown in Figure 5.

Growth and survival of each culture at temperatures above 0°C are presented in Figure 6. No marked differences were observed among cultures in rate of cell division at 5° and 15°C (Fig. 6a). How-

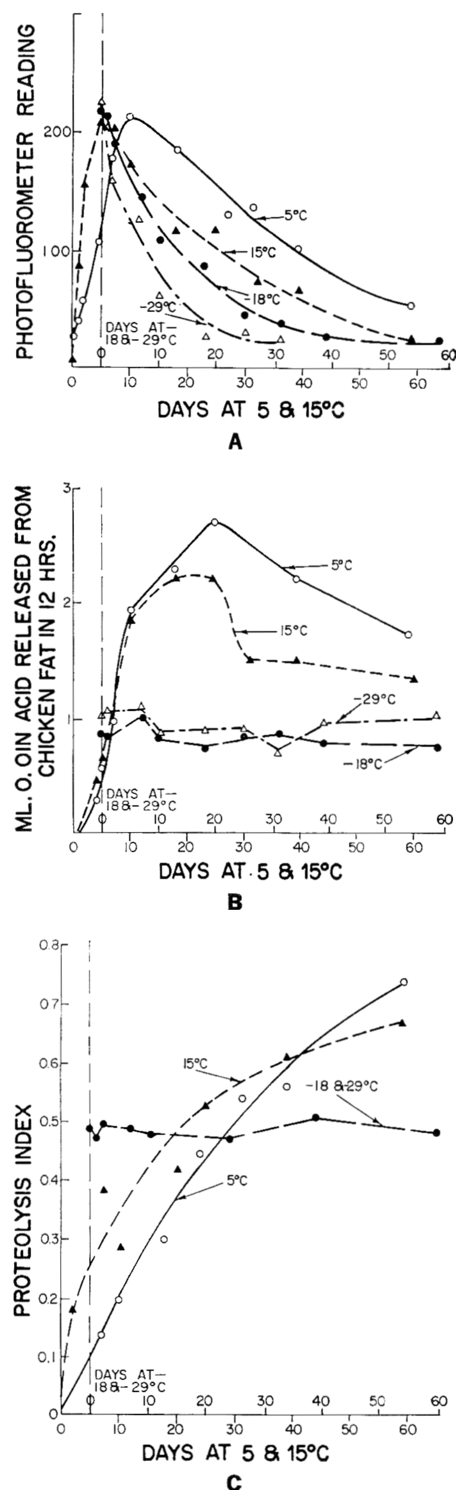
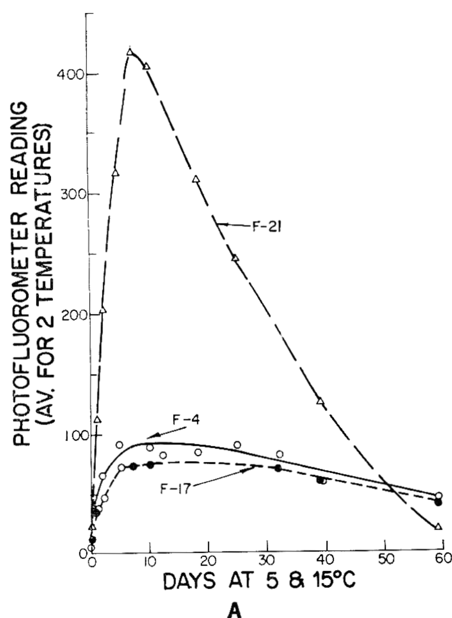


Fig. 3—Effect of temperature on proteolysis, lipolysis and pyoverdine produced by the Pseudomonas isolates with incubation at 5°, 15°, −18° and −29°C for 60 days. a. Fluorescence produced by isolates F-14, F-17 and F-21. b. Production of lipase by isolates F-4, F-14 and F-21. c. Production of proteinase by isolates F-14, and F-21.

ever, culture F-21, with the most rapid production of lipase, proteinase and pyoverdine, also had the fastest rate of death above freezing, while isolate F-17, which was the least active in its metabolic functions, maintained high numbers of viable cells over the longest period. The number of viable cells produced by each culture at the stage of maximum population was inversely related to the overall metabolic activity of the cultures, e.g., the lowest peak in growth was for isolate F-21 while the highest peak was obtained with isolate F-17. Differences among cultures seemed to be related to an inhibition of their metabolic functions proportional to the rate of formation of catabolic products and rate of utilization of substrate.

Again, contrary to the results obtained at temperatures above freezing (Fig. 6a), isolate F-21 was the most resistant to frozen storage, while F-17 was the least resistant to freezing (Fig. 6b).

Results indicated that: 1) freezing causes impairment of the ability of these cultures to produce pigment while their extracellular proteinase and lipase are not affected on prolonged storage under frozen conditions; 2) at temperatures above 0°C, maintenance of high populations, pyoverdine formation, production of extracellular lipase and proteinase were least stable for the most metabolically active cultures; 3) at temperatures below freezing, however, survival of each organism was proportional to the metabolic activity of the culture. These observations may help explain why *Pseudomonas* can survive on frozen chicken and later produce rapid spoilage after the poultry is defrosted and stored at refrigeration temperatures.



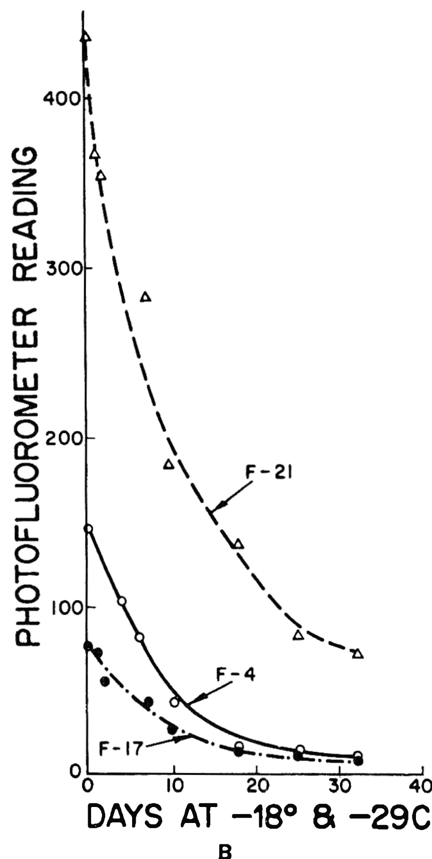


Fig. 4—Effect of temperature on production of pyoverdine by isolates F-4, F-17 and F-21. a. Average readings of fluorescence at 5° and 15°C during incubation for 60 days. b. Average readings of fluorescence at −18° and −29°C during incubation for 32 days.

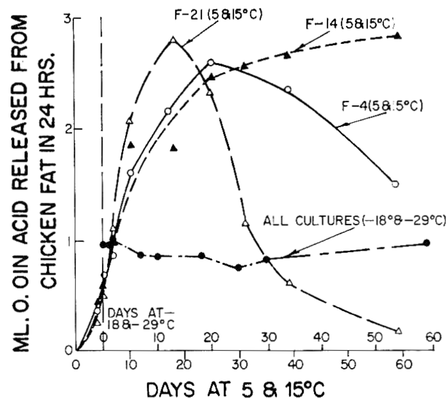
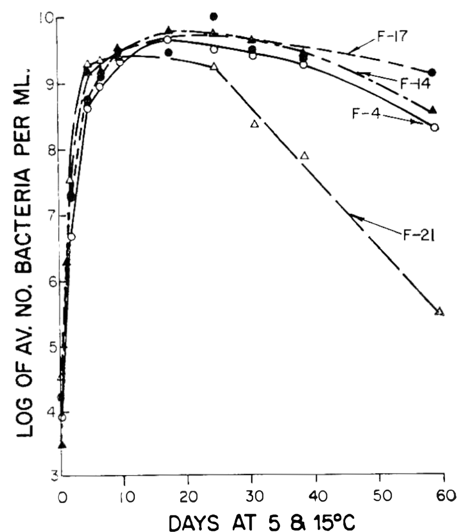


Fig. 5—Production of lipase by isolates F-4, F-14 and F-21 at temperatures above freezing, obtained by plotting average numbers of ml of 0.01 N acid released by enzymatic hydrolysis of chicken fat by each culture at 5° and 15°C against time in incubation. Lipolytic activity below freezing was obtained by plotting average lypolytic activity of the three cultures against time in incubation at −18° and −29°C.



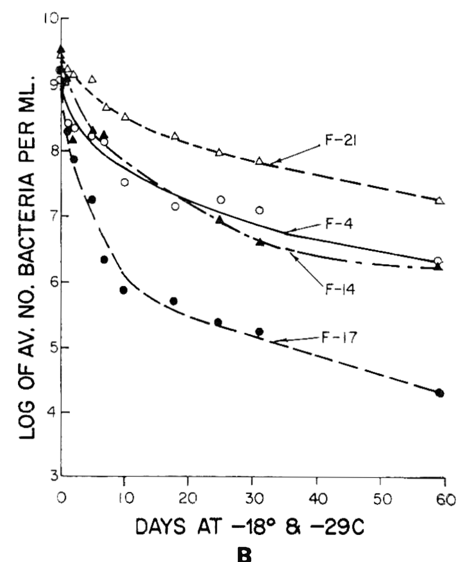


Fig. 6—Culture differences in growth or survival of Pseudomonas isolates at temperatures above and below freezing. a. Numbers of viable cells at 5° and 15°C. b. Numbers of viable cells at −18° and −29°C.

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TERYL B. PUTNAM and M. W. MONTGOMERY
Department of Food Science and Technology, Oregon State University
Corvallis, Oregon 97331

Classification of Some Esterases of the Green Bean (Phaseolus vulgaris L.)

SUMMARY—Aqueous extracts of green beans hydrolyzed the acetyl, propionyl, and n-butyryl esters of glycerol, phenol, sodium 2-naphthol-6-sulfonate and choline. Triolein and soluble long-chain esters of sodium 2-naphthol-6-sulfonate were not hydrolyzed. Optimum esterase activity occurred at pH 7.2. Three esterases were classified by studying the effects of the organophosphorus compounds, diethyl p-nitrophenyl thiophosphate (parathion), tetraethyl pyrophosphate (TEPP) and diisopropyl phosphorofluoridate (DFP), on the hydrolysis of nine substrates. One esterase, resistant to organophosphorus compounds, was classified as an arylesterase (EC 3.1.1.2). The two esterases inhibited by organophosphorus compounds were classified as carboxylesterases (EC 3.1.1.1).

INTRODUCTION

ANIMAL ESTERASES have been extensively studied and classified in recent years (Augustinsson, 1961; Anonymous, 1961), but relatively minor attention has been devoted to the esterases of plant tissues. Studies with starch-gel electrophoresis indicate that esterases of plant tissues are as complex as esterases of animal tissues (Jooste et al., 1963; Schwartz et al., 1964). Recent reports from this laboratory show that esterases of the pea (Norgaard et al., 1968) and the carrot (Carino et al., 1968) could be classified according to their substrate and inhibitor specificities.

The pea was found to possess six esterases, five of them classified as carboxylesterases (carboxylic ester hydrolases, EC 3.1.1.1) and one classified as an arylesterase (aryl ester hydrolase, EC 3.1.1.2) (Norgaard et al., 1968). Investigations with the carrot also revealed six esterases, four carboxylesterases and two arylesterases. In addition, the carrot was shown to hydrolyze certain organophosphorus compounds (Carino et al., 1968).

Substrate and inhibitor specificities of the esterases from these vegetables were quite different. Since the green bean and the pea belong to the same plant family, *Leguminosae*, an investigation of the substrate and inhibitor specificities of the green bean esterases would allow the esterases of these two closely related vegetables to be compared.

MATERIALS & METHODS

Enzyme preparation

Field grown, freshly harvested green beans (*Phaseolus vulgaris* L., variety Blue Lake) were rinsed in cold tap water, cut into 1-in. pieces, lyophilized, flushed with N_2, packed in cans in an atmosphere of N_2 and stored at −18°C. During lyophilization the temperature of the heating plates did not exceed 38°C. An aqueous extract of the dried beans was prepared and treated as described by Norgaard et al. (1968).

Substrates

Substrates used in this study are listed in Table 1. Preparation, source and concentration of the substrates were identical to that described in an earlier publication (Norgaard et al., 1968).

Inhibitors

Diethyl *p*-nitrophenyl thiophosphate (parathion) at least 95% pure, diisopropyl phosphorofluoridate (DFP) and tetraethyl pyrophosphate (TEPP) were obtained from K & K Laboratories. These inhibitors were prepared in 1% (W/V) Triton X-100 (Rohm and Hass) by the method described by Norgaard et al. (1968).

Assay procedure

The manometric assay procedure of Norgaard et al. (1968) was used with the exception that the calculated pH (Umbreit et al., 1964) of the reaction mixture was 7.2.

RESULTS & DISCUSSION

Substrate specificity

Esterase activities of the green bean extracts toward the various substrates used in this study are presented in Table 1.

Table 1—Activity of green bean extract on various substrates

Substrate	Final concentration in reaction mixture, M	Activity, units[1]
Triacetin	0.023	2.03
Tripropionin	0.10	2.98
Tri-*n*-butyrin	0.10	0.93
Triolein	0.05	0.01
Phenyl acetate	0.10	3.39
Phenyl propionate	0.10	4.91
Phenyl *n*-butyrate	0.10	4.02
Sodium 2-naphthol-6-sulfonate esters		
acetyl	0.006	0.61
propionyl	0.006	1.26
n-butyryl	0.006	1.34
n-hexyl	0.006	0.00
n-octyl	0.006	0.00
n-decyl	0.006	0.00
Acetylcholine iodide	0.10	0.17
Propionylcholine iodide	0.01	0.05
n-Butyrylcholine iodide	0.01	0.09

[1] One activity unit is one microequivalent of substrate hydrolyzed/min/ml of bean extract.

Since preliminary studies with triacetin and a titrimetric procedure indicated pH 7.7 as the optimum pH for the hydrolysis of this substrate by the bean extract, pH 7.7 was used in the substrate specificity studies. Later studies revealed the pH optimum to be closer to pH 7.2 than 7.7. Activities varied widely among the different groups of substrates and within the groups.

Acetyl, propionyl and butyryl esters of each substrate group were hydrolyzed, while triolein or the soluble long-chain esters of sodium 2-naphthol-6-sulfonate were not hydrolyzed. This suggests the absence of enzymes capable of hydrolyzing insoluble long-chain triglycerides, lipases (glycerol ester hydrolases, EC 3.1.1.3), and soluble long-chain aromatic esters in the bean extract. However, lipases have been reported in several plants (Reed, 1966). Absence of a lipase in the green bean extract suggests that the lipase was not extracted, not active under the experimental conditions used or not present in green beans.

Propionyl esters were hydrolyzed more rapidly than acetyl and butyryl esters of glycerol and phenol, whereas activity increased as the acyl chain length was increased from acetyl to butyryl esters of sodium 2-naphthol-6-sulfonate. Phenyl esters were hydrolyzed at a faster rate than the triglycerides, naphthyl and choline esters. Interestingly, as the polarity of the alcohol moiety of the substrates increased, the activity decreased. This is in agreement with Dixon et al. (1964), who postulated that van der Waals forces played a major role in enzyme-substrate binding of horse liver carboxylesterase. Similar results were reported for the pea (Norgaard et al., 1968) and the carrot (Carino et al., 1968).

Choline esters were hydrolyzed only slightly by the green bean extract. Jansen et al. (1947) reported the hydrolysis of acetylcholine by citrus acetylesterase, and choline esters were hydrolyzed by wheat germ esterase (Mounter et al., 1962). Neither of these esterases was sensitive to eserine. Acetylcholinesterase was reported in the fruit of Green Hubbard, flavedo of three citrus fruits, and orange and lemon albedo. This activity, however, was not affected by 10^{-5} *M* DFP and was slightly stimulated by 10^{-5} *M* eserine (Schwartz et al., 1964). By definition, cholinesterases are inhibited by 10^{-5} *M* of both DFP and eserine (Augustinsson, 1961). Therefore, it is questionable whether plants possess a cholinesterase, and the activity noted in Table 1 with the choline esters was probably due to esterases other than cholinesterases.

Effect of pH

Although a preliminary study was made on the effect of pH on the hydrolysis of triacetin by the green bean extract, a more detailed study was desirable, using the manometric assay procedure. Concentrations of sodium bicarbonate buffers in the reaction mixtures were varied to obtain the desired pH (Umbreit et al., 1964).

The results (Figs. 1 and 2) show the pH optimum was approximately 7.2 for the five substrates used in this study. The increased hydrolysis of triacetin at the higher pH conditions could have been due to basic hydrolysis or to an enhancement of the catalytic effect of one or more esterases by hydroxyl ions. Bergmann et al. (1958) described such a catalytic effect with the hydrolysis of *p*-nitrophenyl acetate by a C-esterase from hog kidney. The pH optimum of 7.2 for esterases of green beans is similar to that of 7.0 for the pea esterases (Norgaard et al., 1968) and 6.8 to 7.2 for carrot esterases (Carino et al., 1968).

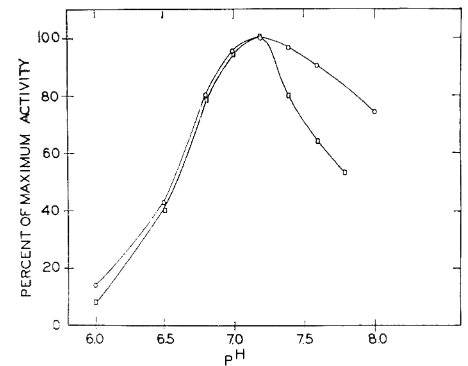

Fig. 1—Effect of pH on the hydrolysis of phenyl acetate, □——□ and phenyl propionate ○——○ by green bean extract.

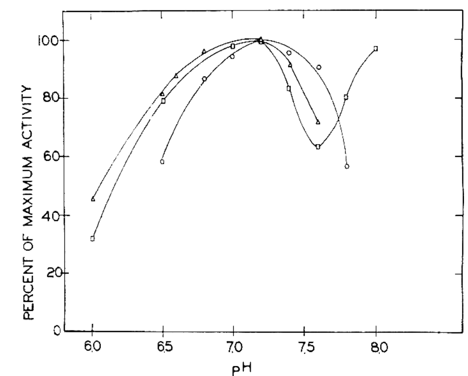

Fig. 2—Effect of pH on the hydrolysis of triacetin □——□, tripropion ○——○, and tributyrin Δ——Δ by aqueous extract of green bean.

Inhibitor specificities

Since esterases show considerable overlapping of substrate specificities, these enzymes cannot be differentiated and classified on the basis of substrate specificities alone. Sensitivity to various inhibitors has been the basis for classification of the animal esterases (Augustinsson, 1961). Aldridge (1953) distinguished two serum esterases hydrolyzing the same substrate by their sensitivity to organophosphorus inhibitors. A sigmoid curve was obtained when activity was plotted versus the negative $\log_{10}$ of the molar inhibitor concentration (pI), indicating that one esterase was inhibited. Since inhibition did not reach 100%, the serum also contained an esterase resistant to the inhibitor.

Double sigmoid curves also have been obtained (Myers et al., 1957), suggesting

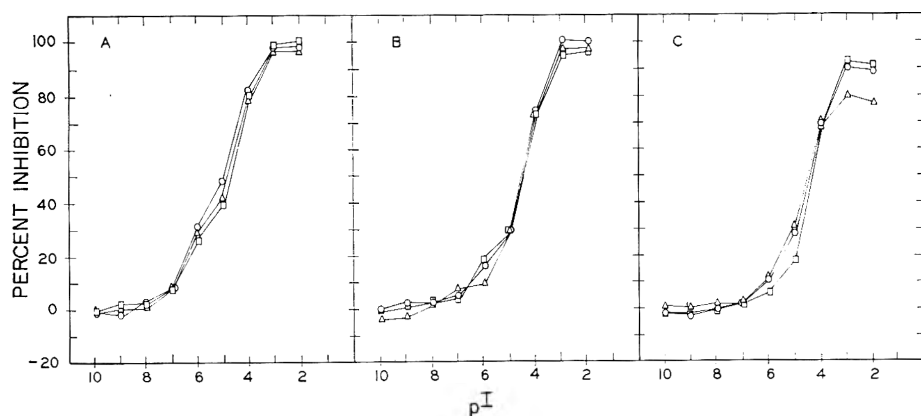

Fig. 3—Inhibition by various parathion concentrations of the hydrolysis of acetyl (Δ——Δ), propionyl (○——○), and butyryl (□——□) esters of phenol (A), glycerol (B), and 2-naphthol-6-sulfonate (C) by green bean extract. pI is the negative log_{10} of molar inhibitor concentration.

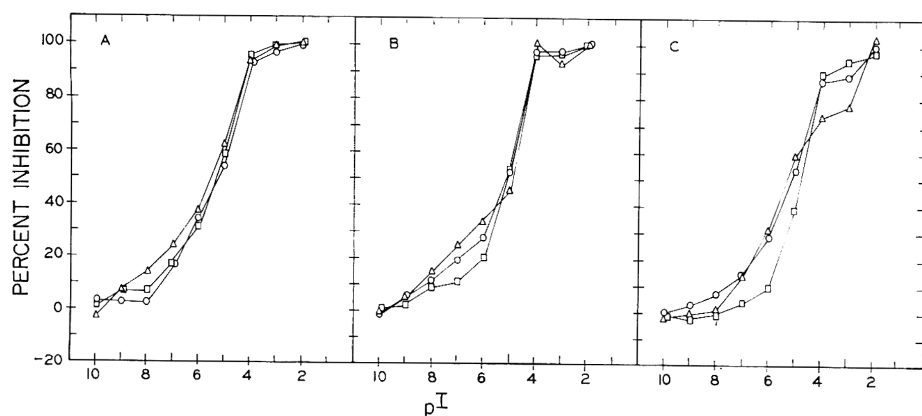

Fig. 4—Inhibition by various TEPP concentrations of the hydrolysis of acetyl (Δ——Δ), propionyl (○——○), and butyryl (□——□) esters of phenol (A), glycerol (B), and 2-naphthol-6-sulfonate (C) by green bean extract. pI is the negative log_{10} of molar inhibitor concentration.

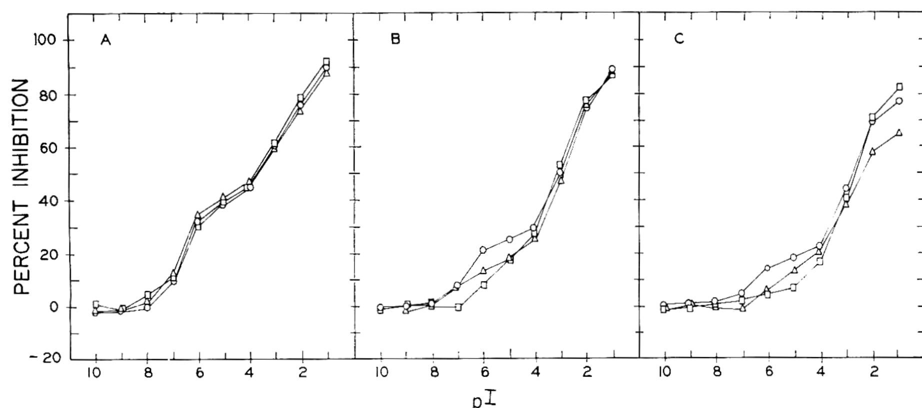

Fig. 5—Inhibition by various DFP concentrations of the hydrolysis of acetyl (Δ——Δ), propionyl (——○), and butyryl (□——□) esters of phenol (A), glycerol (B), and sodium 2-naphthol-6-sulfonate (C) by green bean extract. pI is the negative log_{10} of molar inhibitor concentration.

that two esterases with different inhibitor sensitivities hydrolyzed the same substrate. Esterases with similar substrate and inhibitor specificities would not be differentiated by this procedure.

Curves showing the inhibition of the esterase activity of the aqueous extract of green beans by parathion, TEPP and DFP are presented in Figs. 3, 4 and 5. In general, double sigmoid curves were obtained when the pI of parathion was plotted against percent inhibition of the green bean esterases (Fig. 3). This indicates the presence of at least two esterases, active toward these substrates and inhibited by parathion. The most sensitive esterase was inhibited between pI 8 and 5 of parathion. As shown by the amount of inhibition at pI 5, this esterase demonstrated a greater specificity toward the phenyl esters (Fig. 3A) than with the other substrates (Figs. 3B and 3C). The second esterase was inhibited between pI 4 and 1 of parathion and was more specific for the triglycerides.

These esterases hydrolyzed all the phenyl esters at the same ratio, as shown by the nearly parallel lines in Fig. 3A, but demonstrated various acyl chain length specificities with the triglycerides and esters of sodium 2-naphthol-6-sulfonate. The more sensitive esterase was most specific for the propionyl esters of glycerol and sodium 2-naphthol-6-sulfonate than the acetyl and butyryl esters (Figs. 3B and 3C).

Inhibition curves in Fig. 3 did not reach 100% inhibition. In Figs. 3A and 3B, the curves appear to approach 100% inhibition at pI 1 and might have reached complete inhibition if higher concentrations of parathion had been possible. In Fig. 3C the inhibition curves for the propionyl and butyryl esters of sodium 2-naphthol-6-sulfonate appear to level off at 80 to 85% inhibition, while that of the acetyl ester of sodium 2-naphthol-6-sulfonate plateaus at 70% inhibition. This suggests the pres-

ence of a third esterase in the green bean extract that was resistant to parathion and active on the naphthyl substrates, particularly the acetyl ester.

Further substantiation of the presence of an organophosphorus resistant esterase in the extract is shown in Figs. 4C and 5C. Figure 4C reveals an esterase specific for the naphthyl esters, which was not inhibited by TEPP at pI 3, but was inhibited at pI 2. Figure 5C indicates that the esterase was resistant to DFP at pI 2. Approximately 20% of the activity toward the acetyl ester, and 10% toward the propionyl and butyryl esters of sodium 2-naphthol-6-sulfonate was due to this esterase. Since 100% inhibition was obtained in Figs. 4A, 4B, 5A, and 5B, this resistant esterase did not hydrolyze the phenyl esters or the triglycerides.

Figures 4 and 5 do not show the double sigmoid curves as found in Fig. 3. This indicates that TEPP and DFP did not inhibit the organophosphorus sensitive esterases as selectively as parathion. Approximately 100% inhibition was reached with the phenyl esters and triglycerides at pI 4 of TEPP (Figs. 4A and 4B), while the same inhibition level required a 10-fold higher concentration of DFP (Figs. 5A and 5B). Therefore, the organophosphorus sensitive esterases of the bean extract appear to be more sensitive to TEPP than DFP.

Hydrolysis of the butyryl ester of sodium 2-naphthol-6-sulfonate was more resistant to lower concentrations of organophosphorus inhibitors than the acetyl and propionyl ester of this alcohol (Figs. 4C and 5C), demonstrating that the more sensitive esterase does not hydrolyze the butyryl ester as readily as the shorter acyl chain naphthyl esters.

Discussion

Three different esterases were distinguished by means of substrate and inhibitor specificity studies. Other esterases, having similar substrate and inhibitor specificities, might have existed in the bean extract and would not have been demonstrated by these type of data.

Esterases of animal tissues have been classified with regard to substrate and inhibitor specificities (Augustinsson, 1961; Anonymous, 1961). Carboxylesterases, formerly known as ali-esterases or B-esterases, are those enzymes which hydrolyze both aliphatic and aryl esters, and are inhibited by most organophosphorus inhibitors, but not physostigmine. Arylesterases, formerly known as A-esterases, are not inhibited by organophosphorus inhibitors or physostigmine, but are inhibited by *p*-hydroxymercuribenzoate. Arylesterases hydrolyze aromatic esters, but normally not aliphatic esters.

The two esterases in the green bean extract, which were inhibited by the organophosphorus inhibitors and hydrolyzed both aliphatic and aromatic esters used in this study, would be classified as carboxylesterases. The third esterase, resistant to DFP, parathion and pI 3 of TEPP, hydrolyzed the naphthyl esters, but not the phenyl esters and triglycerides. It would be classified as an arylesterase. However, the substrate specificity of this esterase would differ substantially from the animal arylesterases, which hydrolyze phenyl esters, particularly phenyl acetate (Augustinsson, 1961).

The arylesterase of the pea (Norgaard et al., 1968) had similar substrate specificity (hydrolyzing only the acetyl ester of sodium 2-naphthol-6-sulfonate) to that of the green bean, however, the pea arylesterase was inhibited by parathion and TEPP. The carrot was reported to contain two arylesterases (Carino et al., 1968). One of these arylesterases hydrolyzed the three short-chain naphthyl esters and phenyl acetate. It was also resistant to parathion, but not to DFP and TEPP. The other arylesterase hydrolyzed only the naphthyl esters, but was resistant to DFP and TEPP, and not to parathion. One of the carrot arylesterases probably was responsible for the hydrolysis of TEPP (Carino et al., 1968). Therefore, on the basis of inhibitor specificity, the arylesterase of the green bean appears to be different from the arylesterases of the pea and carrot.

Jansen et al. (1948) reported that 5×10^{-5} *M* DFP inhibited 50% of the activity of wheat and citrus arylesterase. It is interesting to note that approximately the same concentration of DFP also inhibited the hydrolysis of the triglycerides by green bean esterases (Fig. 5B). These same workers also showed that 50% of the activity of citrus acetylesterase was inhibited by 10^{-6} *M* TEPP. Since 10^{-5} *M* TEPP was required to inhibit 50% of the activity of green bean esterases toward the triglycerides (Fig. 4B), green bean esterases were more resistant to TEPP than the citrus enzymes. But this latter difference may have been due to differences in purities of the TEPP preparations. Therefore, the esterases which hydrolyze the acetate esters of glycerol in green beans are similar to those in wheat and citrus.

In an extensive study of *Solanum* tubers, Desborough et al. (1967) found 15 electrophoretically separable esterase isozymes. These authors hypothesized that these isozymes were tetramers composed of one, two or three types of monomers, and that all possible combinations of the monomers into tetramers would account for the 15 isozymes. Assuming that a similar situation exists in the green bean, the three esterases differentiated on the basis of substrate and inhibitors specificity might be the monomers, which form the tetramers. Unpublished data from this laboratory (Veerabhadrappa et al., 1968) show that green bean extract contains 12 to 13 electrophoretically separable esterase activities on polyacrylamide gel.

Two of the organophosphorus inhibitors used in this study, parathion and TEPP, are widely used insecticides. The effect of these insecticides on esterases of intact plants and plant parts used as foods is not known. This area should be the subject of further investigation.

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ROSALITA R. DE LA MAR[a] and F. J. FRANCIS
Department of Food Science and Technology, University of Massachusetts
Amherst, Massachusetts 01002

Carotenoid Degradation in Bleached Paprika

SUMMARY—Commercial samples of bleached and normal paprika were extracted with acetone, separated by countercurrent distribution fractionation, purified by column chromatography and identified by spectral and chemical methods.

The bleaching treatment of the paprika samples resulted in a loss of nearly 96% in total extractable pigment expressed as beta-carotene.

The fractionation of the carotenoid extracts revealed a great variation in the distribution patterns of the pigments. Most grades of paprika showed a predominance of diol-polyol carotenoids, but one grade had a far greater amount of hydrocarbons.

There were 54 and 37 pigments isolated from bleached and unbleached Domestic paprikas, respectively. Of the 54 and 37 pigments, only 33 and 21, respectively, were completely or tentatively identified. Sixteen of the known pigments in unbleached paprika were also observed in the bleached sample.

It is evident that the bleached paprika gave more isomers and oxidative products than did the unbleached sample. Most of the compounds appearing with the capsanthin and capsorubin fractions seemed to be isomers of more fully oxygenated derivatives of these two major pigments. Most of the more oxygenated products appeared to possess ketonic group(s).

INTRODUCTION

PAPRIKA, *Capsicum annum,* belongs to the night-shade family Solanaceae. It is manufactured from large, conical fruits and can be made sweet, semi-sweet, mildly pungent or pungent (Lieberman, 1942; Parry, 1945).

Paprika is being utilized in the United States primarily for its coloring property. It contains a fair amount of vitamin C (Jachimowicz, 1941; Suguira, 1938) and is a rich potential source of vitamin A precursors (Cholnoky, 1937; Floyd, 1939; Gomolyako, 1937). Paprika loses color on storage at a rate influenced by storage conditions, variety of pepper, harvesting conditions and drying temperatures (Lease, et al., 1956a; Van Blaricom, et al., 1951). Lease, et al. (1956b), correlated the temperature of storage and the effect of light with the color stability of peppers. They also studied the effect of drying conditions on the initial color, color retention and pungency of red peppers. Van Blaricom, et al. (1951), were able to control the loss of red color in dried and ground cayenne by the addition of antioxidants used to prevent rancidity in fats and oils.

It is the practice of some manufacturing firms to hold the dehydrated product in cold storage until the final packaging operation is completed. Some packers wish to merchandise paprika in glass containers in view of its attractive appearance, but the red color turns brown on the surface upon exposure to light. The color degradation is an important problem in the spice industry.

Extensive studies have been done on the isolation of carotenoid pigments in unbleached paprika and in fresh bell peppers (Benedek, 1957; Cholnoky, 1937, 1939, 1954, et al., 1958; Curl, 1962, 1964; Garcia, 1951; Sacetta, 1960). No known work has been done on bleached paprika.

This work was done to investigate the qualitative changes in carotenoid pigments afforded by the bleaching process.

EXPERIMENTAL

The samples used in this study were obtained through the courtesy of W. J. Stange Co., Chicago, Illinois, and Gentry Division, Division of Consolidated Foods, Glendale, California.

A severe bleaching process was used. It consisted of exposure to sunlight for three months in aluminum-lined trays covered with Pyrex glass (23 × 34 × 1 in.). The samples were then kept in airtight clear glass containers.

Pigment extraction

The extraction procedure was adapted from the method of Curl (1962). The sample, usually 250 g, was blended with 50% methanol (MeOH) in distilled water, together with magnesium carbonate and 10% Celite. After filtering, the filter cake was washed three times with 50% MeOH and then extracted with acetone until successive extracts were colorless. The combined acetone extracts were concentrated, extracted, with ethyl ether and evaporated under reduced pressure. The carotenoid preparation was then saponified with 20% KOH in MeOH and re-extracted with ether. The ethereal extracts were evaporated *in vacuo.*

Pigment fractionation

The fractionation of the carotenoid extracts was done with a counter-current distribution apparatus (CCD). Three solvent systems were used: (1) hexane–99% MeOH, 1.1:1; (2) benzene–hexane–87% MeOH, 1:1:1.15, and (3) hexane–acetone–MeOH–water, 1.25:1:1:0.65. All ratios are by volume. The concentration of the pigments in the CCD tubes was estimated with a Hitachi Perkin-Elmer 139 UV-VIS Spectrophotometer. The optical densities were measured at 478 nm where

$$E^{1\%}_{1\,cm} = 228$$

and the pigment expressed as all-trans β-carotene.

Column chromatography of fractions

Each fraction was subjected to further purification by column chromatography using one part Sea Sorb 43 (Magnesia, F. M. C. Westvaco) and one part Super Cel (John-Mansville), by weight. The pigments were separated in a column (10 × 300 nm) using a graded series of eluants for each fraction. For carotenes or hydrocarbons, 14, 20, 30 and 45% benzene in hexane was used; for diols, monoepoxide diols and diepoxide diols, 3.5, 5, 7, 10, 15, 25 and 30% EtOH in hexane; for polyols, the same eluants were used as for diols starting at 7% and going up to 50%. The eluates were evaporated under reduced pressure and then taken up with four spectra grade reagents—benzene, ethanol, hexane and carbon disulfide. The spectral characteristics were obtained using a Perkin-Elmer 450 UV-Visible-NIR Spectrophotometer and a Turner Model 210 Spectro. All absorption measurements were done in the visible region (350 to 750 nm).

Chemical tests

1. Sodium borohydride reduction of ketonic carotenoids.

The carotenoid pigment dissolved in 95% ethanol was kept overnight in the refrigerator after several crystals of $NaBH_4$ were added. It was extracted with ether, washed several times with distilled water and then evaporated under reduced pressure. Any hypsochromic shift in absorption maxima is indicative of the presence of ketone group(s), con-

[a] Present address: Department of Home Economics Research, Agricultural Experiment Station, University of Idaho, Moscow, Idaho 83843.

current with a considerable decrease in color intensity (Curl, 1962).

2. Determination of allylic hydroxyls.

The pigment was dissolved in 9 ml methanol and 1 ml of concentrated HCl. It was allowed to stand for 10 min. An excess of 20% KOH in methanol was added. It was then diluted with distilled water, extracted with ether and evaporated *in vacuo*. A bathochromic shift in spectral maxima denotes dehydration of allylic hydroxyl(s).

3. Test for 5,6-epoxides.

The carotenoid was dissolved in 5 ml absolute ethanol. After the spectral maxima were taken, a drop of concentrated HCl was added to the solution in the cuvette. The spectra were measured again from 300 to 550 nm. One epoxide group causes a hypsochromic shift of about 20 nm, two groups about 40 to 50 nm. There is a decrease of 1 to 2 nm when epoxides are absent. Another rapid qualitative test for epoxide is the HCl-ether test. This involved the treatment of an ethereal pigment solution with concentrated HCl, with the formation of a pale or light blue color. The degree of blueness indicates the number of epoxides present (Krinsky, et al., 1960; Curl, 1962).

RESULTS & DISCUSSION

Total carotenoid content

The pigment content of five different grades of paprika is shown in Table 1.

Table 1—Extractable pigment of paprika samples expressed as beta-carotene

Grade	Sample, Fresh	Bleached	Loss, %
	Total pigment (g/kg)		
Domestic	22.0	0.963	95.6
Supreme American	21.2	0.872	95.9
Regal	18.3		
Fancy	15.6		
Choice	9.6	0.424	95.6

Domestic paprika contained the greatest amount of pigment (22.0 g/kg) with *Choice* having the least (9.64 g/kg). It should be noted that the bleaching effect accounted for a loss of almost 96% in carotenoid content. The fresh samples contained 20 times more pigment than the corresponding bleached samples. Visual judgments in the degree of redness of different grades of paprika received from Gentry Division (*Supreme American, Regal, Fancy* and *Choice*) correlated very well with the total extractable color.

CCD fractionation of carotenoid pigments

The carotenoid preparations were fractionated in the CCD apparatus using Solvent System 1 (hexane–99% MeOH, 1.8:1). This system separated the pigments into fractions—diol-polyol, monol and hydrocarbons (Fig. 1). The fractionation of *Supreme American* carotenoids showed the predominance of diol-polyol

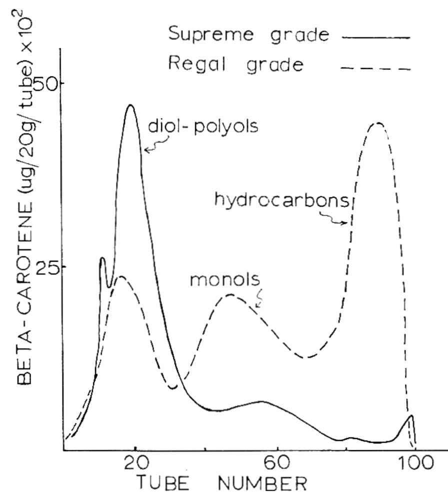

Fig. 1—Distribution of carotenoid pigments in paprika.

fraction and considerably less amounts of monols and hydrocarbons. This was also observed with *Choice* and *Fancy*. However, *Regal* showed a different distribution pattern by having a far greater amount of hydrocarbons than any other fractions.

The carotenoid extracts of *Domestic* paprika were used in the identification of pigments. The fractionation of carotenoids in bleached and unbleached *Domestic* is shown in Table 2. The unbleached paprika contained a large quantity of diepoxide diol (50.2%) and only 2.9% polyol. When the sample was bleached, a significant decrease in the amount of diepoxide diol (50.2 to 14.6%) was observed, concurrent with an increase in the amount of monepoxide diol (7.1 to 38.4%). The capsorubin fraction decreased considerably (13.9 to 7.7%) upon bleaching.

Table 2—Fractionation of paprika carotenoids with Solvent System 2 (benzene-hexane–87% MeOH, 1:1:1.15 v/v) in a 200-transfer run

Fraction	% total carotenoids[1]	N value[2]
Bleached *Domestic* paprika		
I. Polyols	4.8	5–6
II. Capsorubin (mainly capsorubin)	7.7	22–23
III. Diepoxide diols	14.6	30–31
IV. Monoepoxide diols	38.4	51–52
V. Diols	14.8	70–71
VI. Hydrocarbon-monols	19.6	99
Domestic paprika		
I. Polyols	2.9	8–9
II. Capsorubin (mainly capsorubin)	13.9	13–14
III. Diepoxide diols	50.2	24–25
IV. Monepoxide diols	7.1	44
V. Diols	14.9	61–62
VI. Hydrocarbon-monols	11.1	98

[1] Calculated as all-trans beta-carotene at 478 nm, $E^{1\%}_{1\,cm} = 228$.

[2] Tube number of maximum per 100 transfers.

Table 3—Carotenoid pigments found in both untreated and bleached Domestic paprika

Carotenoid	Spectral absorption maxima,[1] nm
I. Hydrocarbons	
1. Phytofluene	370, 349, 332
2. Beta-carotene	479, 449 (429)
3. Zeta-carotene	429, 401, 375
4. Mutatochrome-like	(450), 426–7, 402, 381–2
II. Monols	
5. Cryptoxanthin	476, 447, 428–9, 404, 374
6. Cryptoflavin	451, 427, 403 (386) (345)
7. Cryptoflavin isomer	451, 427, 407 (381) (333)
III. Diol-monoepoxide diols	
8. Zeaxanthin	485, 463 (352) (343)
9. Antheraxanthin	(490), 458–9 (430)
IV. Diepoxide diols	
10. Capsanthin	(510), 481–2, 363
11. Capsanthin isomer	(515), 478
12. Keto-capsanthin	(474), 447 (423)
V. Capsorubin fraction	
13. Capsorubin	522, 488, 460
14. Capsorubin isomer	(515), 478
15. Keto-capsorubin	(518), 450
VI. Polyols	
16. Neoxanthin-like	(478), 449–50 (422)

[1] Pigments 1 to 5 were measured in spectra grade hexane. The rest of the pigments were in benzene. The number in parentheses denotes an inflection in the spectral curve.

Identification of pigments

The pigments were identified by summarization of the countercurrent distribution behavior, chromatographic adsorption properties, spectral characteristics before and after specific chemical tests and comparison of the spectral readings with values from the literature.

There were 54 and 37 carotenoids isolated from bleached and unbleached *Domestic* paprika, respectively. Of the 54, 33 were identified completely or tentatively in the bleached samples. There were 21 known carotenoids in the unbleached samples. Sixteen known carotenoids were found in both samples (Table 3). The pigments not common to either sample are presented in Tables 4 and 5.

It is very apparent that most of the pigments in bleached paprika were oxygenated derivatives possessing hydroxyls and/or ketone groups. In the tentative identification of some oxygenated pigments, additional keto groups, in or out of the conjugated central double bond system, were noted. This condition was shown by the occurrence of several pigments characterized by having a single

Table 4—Carotenoid pigments found in bleached Domestic paprika, but not in the untreated sample

Carotenoid	Spectral absorption maxima,[1] nm
I. Hydrocarbons	
1. Alpha-carotene	474, 347 (423)
2. Zeta-carotene isomer	(425), 400–1, 377–8
3. Alpha-carotene-5,6-epoxide	472, 441 (399), 367
4. Mutatochrome	450, 426–7, 403 (381)
5. 3-keto-mutatochrome	458 (437)
6. 1–5 isomer	(460), 440 (413) (386)
II. Monols	
7. Isolutein-like	469,441 (417)
8. Isomer of 11–7	(470), 435–6
III. Diol-monoepoxide diols	
9. Capsolutein-like	484–5, 457–8, 435–6
10. Auroxanthin	(433), 410, 385
11. Isomer of 11–10	(437), 412, 385
IV. Diepoxide diols	
12. Capsanthin isomer	476, 363
13. Capsanthin-5,6-epoxide	(498), 468 (440)
V. Capsorubin fraction	
14. Capsorubin isomer	(515), 478, 453
15. Capsorubin isomer	513, 481 (454)
VI. Polyols	
16. Neoxanthin-like isomers	
a	(470), 441–2
b	(472), 446, 419

[1] Pigments 1 to 4 were measured in spectra grade hexane. The rest of the pigments were in benzene.

Table 5—Carotenoid pigments found in untreated Domestic paprika, but not in the bleached sample

Carotenoid	Spectral absorption maxima,[1] nm
Hydrocarbons	
1. Mutatochrome-like isomer	(449), 428, 404–5 (382)
Monol-diols	
2. Hydroxy-alpha-carotene	(472), 440–1, 417–8
3. Eschscholtzxanthin-like	505, 473, 444
Polyols	
4. Neochrome-like	455, 427, 404
5. Neochrome-like isomer	450, 425 (400)

[1] Pigments 1 to 3, in hexane; 4 and 5 in benzene.

maximum which is flattened, wide and broad. Another phenomenon responsible for this condition is trans-cis isomerization. In addition, there is a decrease in the extraction values.

In most instances, it is impossible to isolate all forms of the pigment. Therefore, there is every possibility that the pigment occurred as equilibrium mixture of isomers. Preliminary studies using thin-layer chromatography revealed the presence of several fluorescing compounds from a single band separated by column chromatography. Some irreversible side reactions of photoisomerization may consist of the production of fluorescent colorless cleavage compounds (Zechmeister, 1962). Morgan (1966), working on the carotenoid composition of fresh pineapples, observed considerable quantities of substances showing strong ultraviolet absorption. He inferred that these substances might have been terpenes. When resolution of each isomer is attained, its form can be detected by iodine catalysis. This reaction was not carried out in this work.

Beta-carotene was present in much lower relative concentration in bleached paprika than in untreated sample. It is presumed that most of the β-carotene had been converted into some oxidative reaction products, such as β-apo-8′-, β-apo-10′-, β-apo-12′-carotenals and some carotenones. Beta-citraurin is considered a natural degradation product of zeaxanthin (Yokoyama, et al., 1966). These pigments were not identified in either sample. However, the inability to detect these compounds did not preclude their presence in both paprika samples in very low concentration.

The diol-monoepoxide diol fraction contained antheraxanthin (Fig. 2a) and some unknown pigments possessing short chromophoric systems as predominating carotenoids. The diepoxide diol fraction consisted mainly of capsanthin (Fig. 2b) and its isomers, as well as its more oxygenated derivatives. The capsanthin and capsorubin (Fig. 2c) fractions contained the largest number of carotenoids which can be regarded as derivatives of these two major pigments. The capsanthin fraction contained six pigments whose reduced products are indicative of their close chemical relationship with reduced capsanthin, capsanthol (Fig. 2d). The compound designated keto-capsanthin may have a ketone group out of conjugation with the ethylenic bond in view of the positive ketone test and negative tests for allylic hydroxyls and epoxides. Only three capsanthin isomers, neo A, neo B and neo C, have been identified (Zechmeister, 1962). The neo A and B forms are believed to be monocis compounds and neo C to be dicis form.

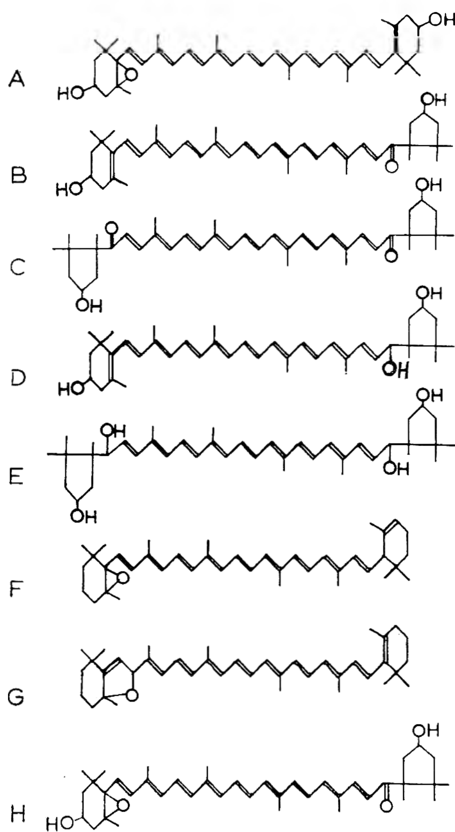

Fig. 2—Formulae of some carotenoid pigment in paprika: a, antherazanthin; b, capsanthin; c, capsorubin; d, capsanthol; e, capsorubinol; f, alpha-carotene-5,6-epoxide; g, mutatochrome; h, capsanthin-5,6-epoxide.

There were five compounds whose reduced spectra closely resembled that of the reduced capsorubin, namely capsorubinol (Fig. 2e). Only two main isomers of capsorubin were identified, the neo forms of A and B. Both forms seem to be monocis compounds. The compound designated keto-copsorubin may have a ketone group out of conjugation with the ethylenic bond, as suggested by the lack of a hypochromic shift in the reduced pigment. The single maximum at 450 nm with the lack of fine absorption peaks also suggested the presence of a ketone group out of conjugation.

Few epoxide carotenoids were isolated in both samples. Except for the presence of alpha-carotene-5,6-epoxide (Fig. 2f), mutatochrome (Fig. 2g) and mutatochrome-like pigments, auroxanthin-like carotenoid, cryptoflavin, neoxanthin, and capsanthin-5,6-epoxide (Fig. 2h), no other epoxide carotenoids were isolated. As far as the authors are aware, no capsorubin epoxide has ever been reported.

Several pigments identified by Curl (1962) in fresh red bell peppers, were not found in either sample. These are cryptocapsin, P-482 (a diol), capsolutein-5,6-epoxide and its furanoid form, mutatoxanthin, violaxanthin, luteoxanthin, P-441 (tetrahydrocapsorubin), capsochrome and hydrocapsolutein-5,6-epoxide.

The compounds in Tables 3, 4 and 5 were all tentatively identified by name or as an isomer of another pigment. There were in addition 21 compounds in the bleached paprika and 16 in the normal samples which were not identified. Data on countercurrent position, column color and position, spectra, presence of keto, allylic hydroxyl, double bonds, etc. for these compounds, as well as more detailed data for the identified compounds, are available in the original thesis. (Mar, 1967).

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K. A. CALDWELL and HANS LINEWEAVER

USDA Western Utilization R&D Div., ARS, Albany, California 94710

A Research Note

Sulfhydryl Content of Excised Chicken Breast Muscle during Postmortem Aging

INTRODUCTION

CHEMICAL changes in postmortem sekletal muscle have been described and correlated with onset of rigor mortis (Whitaker, 1964). However, the chemical reactions responsible for postmortem tenderization have not yet been elucidated.

It has been reported that the muscle of broilers is more tender if rapidly cooked within a few minutes of postmortem than if cooked at an intermediate time before aging is completed (Koonz et al., 1954 and de Fremery et al., 1963). Thus muscle that is tender initially, becomes tough, then becomes tender. The possibility that these postmortem changes in tenderness are related to the sulfhydryl-disulfide composition of muscle is attractive.

Chajuss et al. (1962) proposed that conversion of protein sulfhydryl groups to disulfide bonds may confer stained configurations on muscle proteins and stabilize the rigor state. In support of this proposal they reported observations of decreases in thiol concentration in excised chicken muscle aged in air at 25–27°C; nitroferricyanide was used to measure sulfhydryl content.

Gawronski et al. (1967) from the same laboratory utilized polarographic analyses of thiols to determine sulfhydryl and disulfide concentrations in chilled excised chicken breast muscle during aging. They reported a significant decrease in thiol concentration at 30 min postmortem only. During aging that extended to 24 hrs the sulfhydryl and disulfide concentrations remained unchanged. No other data were reported on changes which could have occurred during the first hour. Onset of rigor mortis in chicken breast muscle occurs 2 to 4 hrs postmortem (de Fremery et al., 1960) and significant changes in tenderness occur by 6 hrs postmortem (Koonz et al., 1954 and Pool et al., 1958). The time from 0 to 6 hrs postmortem encompasses the prerigor state of chicken muscle, rigor mortis, and the development of tenderness.

We wish to report results of tests designed to determine whether sulfhydryl groups are involved in these early postmortem changes.

EXPERIMENTAL

SIX COMMERCIAL broilers ranging in weight from 1.52 to 2.68 kg were electrically stunned and slaughtered by bleeding. For each bird, the outer breast muscles (*pectoralis superficialis*) were removed and cut into eight portions. The first, or zero-time sample, was taken 5 min after initiation of bleeding. The seven remaining portions were placed in a plastic bag and aged in ice for various periods. Initial and aged samples were frozen in liquid nitrogen and stored at −196°C for analysis at a later time.

Frozen tissues and dry ice were pulverized in a Waring blender. That portion which passed a twenty-mesh sieve was taken for analyses. Dry ice was sublimed at −20°C and the frozen tissue was homogenized in cold water; homogenates were maintained at 0°C. The procedure was replicated for six birds.

5,5′-Dithiobis-(2-nitrobenzoic acid) was chosen for sulfhydryl group analyses because of its high specificity (Ellman, 1959). This aromatic disulfide is reduced by sulfhydryl groups to the corresponding aromatic thiol, which is measured spectrophotometrically. The reagent was used to measure sulfhydryl content according to the modification of Jocelyn (1962), whereby total and nonprotein sulfhydryls are determined in the presence of each other by varying the pH and disulfide concentration. Nitrogen was determined by the Kjeldahl method. All tissues from one bird were analyzed on the same day. Both sulfhyryl and nitrogen analyses were carried out in duplicate.

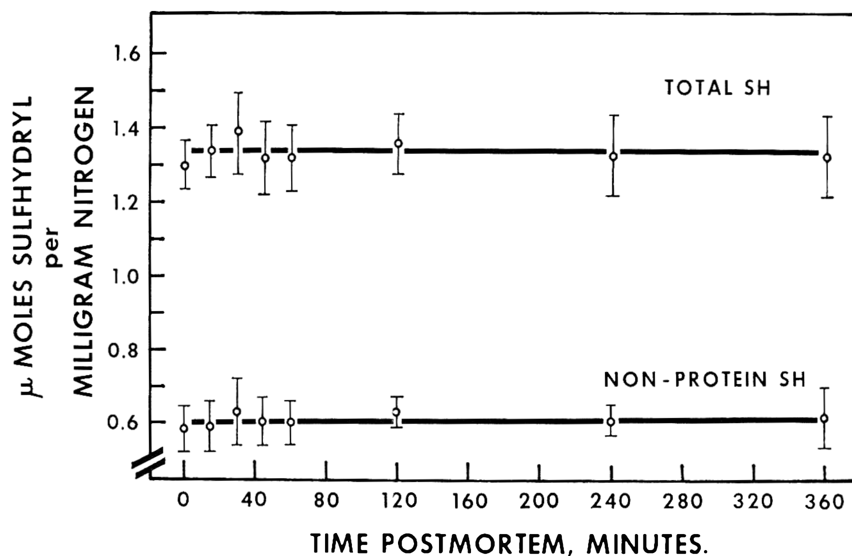

Fig. 1—Sulfhydryl concentration of excised chicken muscle as a function of time postmortem. Graph lines represent tne results of least-squares calculations; vertical lines represent two standard deviations.

RESULTS

RESULTS summarized in Figure 1 reveal no significant changes in total or nonprotein sulfhydryl concentrations during the first 6 hr postmortem. Average concentration of 1.34 and 0.61 μmoles sulfhydryl per mg nitrogen were observed for total and nonprotein sulfhydryls, respectively. Analysis of variance indicates that population means at various times postmortem do not differ significantly at the 5% level of probability. Experimental conditions were similar to those reported by Gawronski et al. (1967) except for the method of sulfhydryl analysis; the value for total sulfhydryl concentration is of the same order of magnitude as the initial or zero-time value reported by these workers. We were unable, however, to confirm their observations of decreases in sulfhydryl content. We cannot explain these differences. But the presence of other redox compounds is known to influence analysis of sulfhydryl content by either the ferricyanide or polarographic methods (Cecil et al., 1959). As both onset of rigor mortis and initiation of tenderization occur during the time period included in our experiments, data summarized in Figure 1 do not support the postulate of a correlation between sulfhydryl concentration and rigor or tenderization.

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JEAN L. KENDRICK and BETTY M. WATTS
Dept. Food and Nutrition, Florida State University, Tallahassee

Nicotinamide and Nicotinic Acid in Color Preservation of Fresh Meat

SUMMARY—Nicotinamide (NAm) protected the metmyoglobin (MetMb), reducing activity and oxygen consumption of ground beef or pork on refrigeration or freezer storage. Nicotinic acid (NA) increased MetMb in aerobically refrigerated ground meats, while NAm, particularly in combination with ascorbic acid, decreased it. In model systems and meats, hemochrome formation occurred with reduced myoglobin and either NA or NAm, but more readily with NA and at lower pH values. No hemochrome was formed in aerobically stored meats with NA or NAm, even with ascorbic acid present. Anaerobic conditions gave hemochromes, but only at the higher concentrations and lower pHs tried. The implication of these findings to color protection in fresh meats is discussed.

INTRODUCTION

NIACIN, OR nicotinic acid (NA), has been patented for use as a preservative of the red color in meats. Coleman et al. (1949, 1951) describe the formation of a red reaction product of NA and myoglobin (Mb). The amount recommended is 0.3 g/lb of meat. It was used commercially at one-tenth of this level. Side effects (general vasodilatation with flushing and itching) result from ingestion of 50 to 100 mg NA. Nicotinamide (NAm), on the other hand, does not produce such side effects (Press and Yaeger, 1962). NAm would therefore be preferable to NA for use in meats, provided it gives comparable color protection. At present, neither NA or NAm is allowed in meats subject to federal regulations (Bennett, 1968).

Coleman et al. (1951) do not identify the red reaction product. It may be presumed to be a hemochrome, possibly a mixed hemochrome as described by Lemberg and Legge (1949) with NA attached to one side of the porphyrin iron and globin to the other. NAm as well as NA forms hemochromes. Olcott and Lukton (1961), working with pure heme in model systems, reported hemochrome formation at lower concentrations of NAm than of other bases tried. Brown and Tappel (1957) ascribed pink pigments of canned tuna to hemochrome formation with NAm derived from nicotinamide adenine dinucleotide (NAD) during the heat treatment. Koizumi and Matsuma (1967) concluded that the pink color of cooked tuna could be improved by adding nitrogenous bases, e.g., NAm, and reducing agents to the meat, thus increasing ferrohemochrome formation. No direct comparisons of hemochrome formation with NAm versus NA in meat, or under conditions similar to those in meat, could be located in the literature.

The heme compound must be in the reduced form to give hemochromes with NA or NAm. Coleman et al. (1951) recommended that a reducing agent such as ascorbate be used in combination with the NA. Under anaerobic conditions, the meat itself may bring about the necessary reduction of metmyoglobin (MetMb) through NAD mediated enzyme systems (Watts et al., 1966). NAm, but not NA, protects the NAD in tissues from destruction by nucleosidase (Severin et al., 1963).

In view of these properties, NAm was felt to merit further study in meats. This paper reports enzymatic reduction of MetMb in ground meats stored with NAm, and compares hemochrome formation with the acid and the amide, both in model systems and in meat.

METHODS

Preparation of the meat, measurement of its MetMb reduction capacity and the rate of oxygen consumption in the meat slurries were described by Watts et al. (1966). The concentrations of added NA and NAm are expressed as mg %, i.e., mg per 100 g meat. The appropriate amount to be added to 50 g of meat was contained in 1 ml water. The controls had 1 ml water added. The additions of NAm for reduction protection studies were made immediately after grinding.

To study hemochrome formation in model systems, horse MetMb (Nutritional Biochemicals) in M/10 phosphate buffer was reduced with sodium hyposulfite. Hemochrome formation as a function of NAm or NA concentration, time and pH was evaluated by the ratio of absorbances at 530/572 mμ.

In storage studies with refrigerated meats, 3 mg % chlortetracycline was used as a preservative, and the meat in 50 g balls was held in air permeable bags at 4°C and analyzed for surface MetMb as described by Hutchins et al. (1967). Freezer storage was at −12°C. When it was desired to pack anaerobically, the meat was flushed with nitrogen in an airtight bag and test substances (ascorbic acid, NA or NAm) injected through the bag as described by Watts et al. (1966). Hemochrome formation in the stored meats was followed by changes in the K/S ratio at 530/572 mμ.

RESULTS

Effect of NAm on reducing activity of refrigerated and frozen meats

Stewart et al. (1965) had shown a decrease of the enzymatic reducing activity on refrigeration storage of ground meat, but had not worked with frozen meat. Greater decreases occur on freezing, e for a day (Table 1). The losses on free-

Table 1—Loss of reducing activity on freezer storage

Days frozen	% MetMb Reduced	
	[1]	[2]
Fresh	43	100
1	17	24
7	10	2

[1] Rib eye of beef, pH 5.7, 0.1% ferricyanide, 60 min.
[2] Pork ham, pH 6.1, 0.1% ferricyanide, 15 min.

ing as with refrigeration can be restored by the addition of NAD after thawing (Fig. 1).

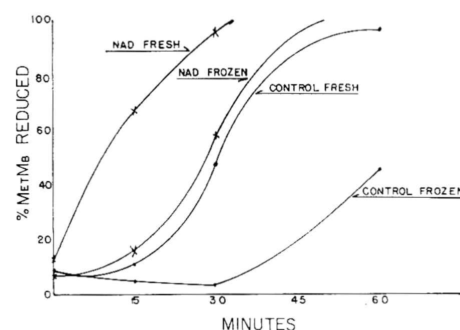

Fig. 1—Effect of NAD on reducing activity of frozen meats. Ground pork frozen 12 days, pH 6.1, 20.8 mg % NAD, 0.2 % ferricyanide.

NAm added to the ground meat before refrigeration or freezing at least partially protected the MetMb reducing activity. In 14 samples of refrigerated pork and beef and one frozen pork sample, NAm treated samples always showed greater reducing activity after storage than controls. Loss of fresh meat reducing activity averaged 68% in control samples after 4 to 6 days of refrigeration or 1 day of freezing. Table 2 gives the average losses in refrigerated ground meat treated with NAm. Concentrations as low as 6 mg % gave some protection, and there appeared to be no advantage in using concentrations greater than 60 mg %. Oxygen utilization of slurries prepared from NAm treated ground pork after refrigeration or freezing was also much higher than controls without NAm.

Hemochrome formation in model systems

The spectra obtained from reduced Mb, before and after treatment with NA and NAm, respectively, are shown in Fig. 2. The spectra of the two hemochromes are quoe similar, but it should be noted that a much higher concentration of NAm than NA was required to produce comparable hemochrome formation. With concentrations of 0.07 *M* NA and 0.28 *M* NAm, the hemochromes formed were nearly the same reddish-pink. With 0.07 *M* NAm. the color was purplish-red.

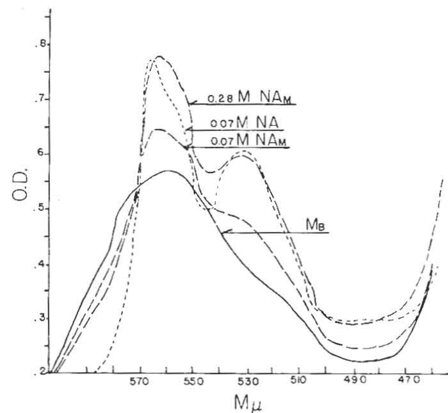

Fig. 2—Hemochrome spectra in model systems. Reduced Mb, pH 5.0 (6.7 × 10)⁻⁵M).

All three pigments are isosbestic at 572 mμ. When Mb was titrated with NA, the spectrum developed four distinct bands which on further titration reverted to the two-banded spectrum of ordinary hemochrome. Keilin (1966) noted the formation of four-banded spectra under similar conditions and ascribed them to asymmetry of the heme iron-ligand bond caused by spatial restriction of the ligand. Keilin also found that excess NA reverted the spectrum to the two bands of ordinary hemochrome, denoting displacement of globin.

Table 2—NAm protection of MetMb reducing activity in refrigerated pork

Treatment[1]	No. of samples	Average reduction loss, %	Range	Standard deviation
Control	8	68	50–85	12
6 mg % NAm	5	40	35–42	4
60 or 300 mg % NAm	5	11	0–22	11

[1] Refrigeration at 4°C for 4 to 6 days.

The two ligands differed with respect to the time required for hemochrome formation. With NA, the reaction was practically complete within a minute, whereas NAm continued to react over a period of hours and precipitation occurred before equilibrium was attained. Precipitation was especially rapid at high concentrations of ligand and low pH values, where most hemochrome formation occurred. Thus, a titration for complete hemochrome formation was not possible with NAm.

The effect of increasing concentrations of NA on the ratio of absorbances at 530/572 mμ at pH 5.6 are shown in Fig. 3. The ratio for zero hemochrome formation (100 % Mb) was 0.74. To determine the limiting ratio for complete hemochrome formation and the dissociation constant of the hemochrome formed, these data were treated according to the method of George et al. (1961). The limiting absorbance ratio for 100% hemochrome formation was found to be 1.63, and the dissociation constant at this pH was 0.072. Standard deviation of the values from the straight line was 4%.

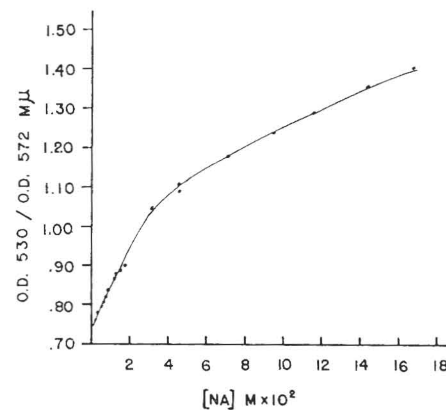

Fig. 3—Increase in the ratio of optical densities 530/572 mμ of Mb upon addition of NA at pH 5.6. Reduced Mb (3 × 10⁻⁶M). Ratios were corrected for slight volume change.

Assuming the same limiting value (1.63) for the NAm as for the NA hemochrome, the effect of pH on hemochrome formation with both NA and NAm is shown in Table 3. The dissociation constants for the NAm Mb are only an approximation, since it is questionable that the reaction had reached equilibrium at 1 hr. Keilin (1966) gave a value of 0.43 for the dissociation constant of NA Mb at a pH between 6 and 7. The pH range explored (Table 3) is that of normal meat. Obviously, pH is an important factor in hemochrome formation with either ligand. Above pH 6.0 little formation occurred.

Table 3—Variation of Hemochrome Formation with pH[1]

pH	O.D. 530 mμ[2] / O.D. 572 mμ	% Hemochrome	Dissociation constants (*M*)
NA			
5.4	1.20	52.0	0.062
5.7	1.08	38.5	0.11
5.9	1.03	33.0	0.14
6.1	0.89	17.5	0.32
6.4	0.84	11.5	0.52
NAm			
5.4	0.91	19.5	0.28
5.7	0.82	9.5	0.64
5.9	0.78	5.0	1.27
6.1	0.77	3.5	1.85
6.4	0.77	3.5	1.85

[1] Reduced Mb(2.7 × 10^{-5}*M*), NA or NAm (0.067 *M*), 1 hr.
[2] Ratio for reduced Mb, 0.74.

Analysis of surface pigments of ground meats treated with NA or NAm

Aerobic storage. No hemochrome formation occurred with either ligand, even with concentrations as high as 300 mg %, when the meat was stored aerobically, with an O_2 permeable film. MetMb increased in all samples during storage. Meat containing more than one-third MetMb was visibly inferior. Concentrations of 6 mg % of either ligand had no consistent effect on MetMb formation. Higher concentrations of NAm (60 to 300 mg %) slightly retarded MetMb

formation in most samples tested, whereas the same concentrations of NA invariably accelerated pigment oxidation.

Ascorbic acid, in the amount of 11 mg % recommended by Coleman et al. (1951), decreased MetMb in 10 different samples of meat, and combinations of ascorbic acid and NAm gave better protection than either alone. Ascorbic acid added to the freshly ground meat reduced the MetMb present and gave a bright red surface (MbO_2). Even with NA, the bright red pigment was MbO_2, not hemochrome (Table 4). The effects noted cannot be attributed to pH changes; whenever necessary, the pH of additives was adjusted to that of the meat.

Table 4—Percentage of MetMb in the surface pigment of ground meat

Sample[1] treatment	% MetMb at storage indicated		
	Fresh	1 day	6 days
I. Control[2]	17	23	47
11 mg % ascorbate	0	6	33
150 mg % NA + ascorbate	0	5	61
150 mg % NAm + ascorbate	1	0	30
			3 days
II. Control[3]	3	24	33
NAm 6 mg %	5	30	39
60 mg %	0	23	31
300 mg %	0	18	28
NA 6 mg %	2	22	48
60 mg %	0	36	65
300 mg %	6	54	70

[1] Refrigerated aerobically at 4°C.
[2] Ground beef, pH 5.7.
[3] Ground pork, pH 5.8.

Anaerobic storage. There was no significant hemochrome formation with NA below 60 mg %, or NAm below 150 mg %. At higher concentrations, partial hemochrome formation was evident with both ligands. The ratios increased with time. For a given concentration, higher ratios were always obtained with NA than with NAm, as might be expected from the results with model systems. Ascorbic acid did not increase hemochrome formation under these conditions (Table 5). Pork begins to assume a reddish-pink color and beef a deeper red with ratios above 0.80. The pH of most locally purchased meat was 5.7 or above, and at these pHs the amount of hemochrome formed with NAm was not visibly detectable. However, there was visible formation of this hemochrome in meat at pH 5.3.

Table 5—Hemochrome formation with Nam or NA and ascorbate acid in anaerobic beef[1]

Sample	K/S 530/572 mμ		
	1 day	10 days	13 days
Control	0.72	0.75	0.72
11 mg % Ascorbic acid	0.75	0.75	0.72
60 mg % NAm + Ascorbic Acid	0.76	0.75	
100 mg % NAm + Ascorbic Acid	0.78	0.77	0.75
100 mg % NA + Ascorbic Acid	0.84	0.94	0.90
100 mg % NA	0.89	0.92	0.95

[1] Refrigerated at 4°C, pH 5.7.

DISCUSSION

There seems to be no justification for the addition of NA to meats. This additive definitely accelerates MetMb formation if the meat is exposed to air. Although it forms hemochromes more readily than NAm, the concentrations required to give a noticeable reddening, even with meat of low pH, would be expected to have an adverse physiological effect.

The case for NAm is somewhat stronger. The fact that it retards loss of reducing activity in stored meats, presumably by protecting NAD, could prove useful under some conditions. Bailey et al. (1964) noted that the meat pigments of hams cured at 38°F for 6 days were more stable to light in the presence of NAm and ascorbate than were those treated either with ascorbate alone or with a mixture of ascorbate and NA. They suggested a protective effect on NAD.

The unattractive purple color of meat stored anaerobically may be improved by the addition of NAm, but only if the NAm is used in concentrations of 60 mg % or more and the pH of the meat is low. There seems to be no health hazard in the use of these rather high concentrations, but there may be some doubt that the improvement achieved is sufficient to warrant such treatment.

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J. R. GEISMAN, W. E. CAREY,[a] W. A. GOULD, and E. K. ALBAN
Department of Horticulture, Ohio Agricultural Research and Development Center, Wooster, Ohio 44691

Distribution of Arsenic Residues by Activation Analysis

SUMMARY—Arsenic bearing compounds have been used as insecticides, fungicides and herbicides. Wide-spread use of these chemicals could lead to build-up in the soil. Detection of arsenic requires special apparatus and sophisticated techniques. A method using neutron activation analysis was developed that required only drying of samples and encapsulation. Using activation analysis, it was found that arsenic was concentrated in the root of tomato plants. Data indicated a discrimination within the plant between fruit, stems and leaves, and root. Thus, it was concluded that soil concentrations of arsenic would have to be at the level that causes a reduction in fruit size and/or yield before fruit concentration of arsenic would exceed the tolerance. This situation is not likely to occur at concentrations used for defoliation of horticultural crops prior to mechanical harvest. Activation analysis could be used to detect microgram quantities of arsenic in milligram size samples. The method was precise and repeatable and did not require elaborate sample preparation techniques.

INTRODUCTION

ARSENIC COMPOUNDS are highly toxic to all forms of animal life. Recommendations for arsenicals as insecticides were published as early as 1681 (Robbins et al., 1942). Certain arsenic bearing compounds are phytocidal and have been used as nonselective herbicides (Frear, 1955). By varying concentrations of arsenic bearing compounds, these chemicals could be used as a fungicide. Lower concentrations serve to defoliate plants.

Defoliation may allow more efficient use of mechanical harvesters for several horticultural crops. However, prolonged use of arsenicals for this purpose could lead to a build-up in the soil, which could, in turn, affect yields. Arnott et al. (1967) reported a natural occurrence of arsenic concentrations in most soil of from 1 to 70 ppm.

To determine whether arsenic defoliants increased residues in plants and soils, a study was undertaken at the Ohio Agricultural Research and Development Center. As a part of this study, the methods for determining arsenic content were investigated. The Gutzeit method has been used since the early 1930s (AOAC, 1935). The colorimetric methods and the Gutzeit method (AOAC, 1965) require special glassware and apparatus, laborious and hazardous extraction procedures and sophisticated technique. Another disadvantage is the interference of certain naturally occurring compounds. It seemed feasible that activation analysis could be used for arsenic determinations in a more simplified and precise manner.

[a] Present address: Engineering Experiment Station, The Ohio State University, Columbus, Ohio 43210.

EXPERIMENTAL

Materials

Tomato plants of the Hybrid O variety were grown under greenhouse conditions in soil that had been treated for a period of at least two years with a commercially available sodium arsenite solution. The plot was divided into portions with tomato plants being grown on both the treated and drift portions. Tomato plants for untreated controls were grown in a separate area.

The plants were harvested for analysis when the fruit reached the pink stage. Soil samples were also taken at this time with the drift soil being taken from the boundary between the treated and untreated areas.

Preparation of samples

The plant and soil samples were dried in a forced draft oven at 150°C for 2 hr. The plant samples were separated into root, stems and leaves, and fruit portions. The dried samples were then weighed and encapsulated in polyethylene containers. Duplicates were made of all samples.

Preparation of standards

A series of arsenic standards were made using As_2O_5 (J. T. Baker Co., 99.2% pure). One hundred mg of the pentoxide contained 64.7 mg of As. Aqueous solutions were diluted to give standards containing 6.47×10^4, 6.47×10^3, 3.24×10^3, $\times$ 1.29×10^3, 6.47×10^2, 64.7, 6.47, 3.23 and 1.29 μg of As. After activation, standards which closely approximated the arsenic content of the plant and soil samples were used for comparisons to determine the arsenic content of the plants and soil. In addition, since sodium and potassium were present in some samples, sodium and potassium standards were prepared. Samples containing 20.8 mg of sodium and 25.8 mg potassium, respectively, were used.

Irradiation

Samples were irradiated at the Ohio State University Engineering Experiment Station's Nuclear Reactor Laboratory. Two facilities were utilized for irradiations. The "rabbit" (a fast sample insertion and removal facility) with a thermal neutron flux of approximately $2 \times 10^{10} n \cdot cm^{-1} \cdot sec^{-1}$ was used for high concentrations and the central irradiation facility with a thermal neutron flux of 2×10^{11} $n \cdot cm^{-1} \cdot sec^{-1}$ was used for low (μg) concentrations. Exposure times at a reactor power level of 10KW were 30 and 60 min, respectively, for each facility.

Determination of radioactivity

Immediately following irradiation, samples were assayed with a survey meter to determine exposure rate. The samples were then placed behind lead shielding for a period of at least 24 hr to allow decay of short-lived radioisotopes such as ^{28}Al and ^{56}Mn. (These isotopes are produced from the naturally occurring element concentrations in the plants and soils and emit gamma rays, which unnecessarily complicate the spectrum of the samples.) By waiting for the specified time period, the ^{28}Al (half-life = 2.3 min) and the ^{56}Mn (half-life = 2.6 hr) were allowed to decay to unmeasurably low levels of radioactivity. Since the half-life of ^{76}As, the isotope used in this assay, is 26.4 hr a readily measurable amount remained after the initial 24 hr waiting period.

The amount of radioactivity induced in the plant and soil samples was determined by placing the samples near a 3 $\times$ 3" NaI (Tl) scintillation crystal attached to a multichannel analyzer (Packard Model 15). The instrument was calibrated using ^{51}Cr, ^{58}Co and ^{60}Co standards. A typical calibration spectrum is shown in Figure 1. Since the energy of the gamma rays of these isotopes was known, the instrument could be adjusted to an energy calibration of approximately 20 Kev per channel (memory location). For example, a 1.6 Mev gamma ray would produce a peak at about channel 80. A typical calibration curve of gamma energy in Mev versus channel number is given in Figure 2.

The geometry used for counting both the standards and the samples was the same. A background was stored in the analyzer memory prior to counting the samples. The counting time was determined by the activity of the samples; i.e., the lower the activity the longer the count, so that a statistically significant number of total counts was recorded in each channel. In general, the counting

period did not exceed 10 min.

At the beginning of the count the sample identification, the geometry, the time, the date and counting period were recorded. These data were utilized for calculations. All counts were analyzed according to the methods described by Jarrett (1946).

Samples were assayed at approximately 24 hr intervals for periods of at least 4 days. In the case of fruit samples, where minute concentrations were involved, the assay was continued for 10 days. The purpose of continuing the assay for this period was to determine the half-life of the radioactivity being detected. These measurements were used as an additional check that indicated that ^{76}As was indeed the isotope being measured.

Calculations

Comparisons of the arsenic standards indicated that the most reliable indication of arsenic content was the sum of eight channels around the gamma peak. This was more reliable than using the count per minute (cpm) of the peak channel only, as indicated in Table 1.

Table 1—Comparison of peak channel count per minute versus eight channel sum for determining arsenic content of standards.

As content (mg)	Peak channel (cpm)	Sum of 8 channels (cpm)
64.700	49013	221,564
6.4700	4826	21,286
0.647	549	2,213

In determining the arsenic content of the soil or plant samples, counts due to sodium or potassium or both, where applicable, were subtracted graphically as in Figure 3. Background was removed from all spectra prior to graphical subtractions. The remainders were replotted for calculation of the amount of arsenic in the samples. Thus only net counts were used.

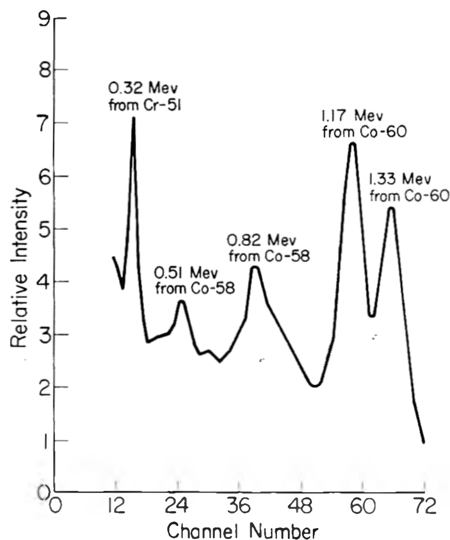

Fig. 1—Gamma-ray energy calibration spectrum.

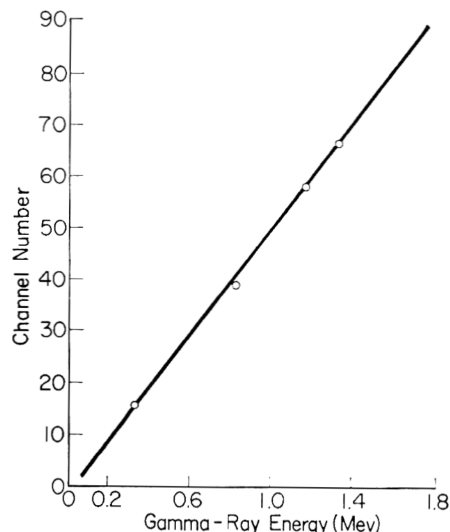

Fig. 2—Energy calibration curve.

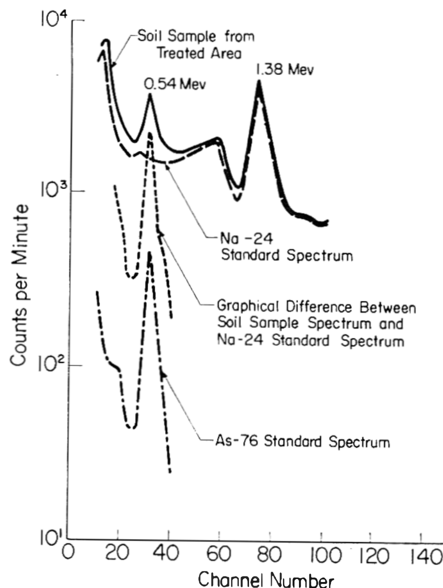

Fig. 3—Example of graphical subtraction of interfering radiations.

The peak was determined and an eight-channel sum was made as in the case of the standards. The following equation was used since the samples and standards were counted in the same geometry:

$$\Sigma \text{ cpm standard} : \Sigma \text{ cpm sample} :: \text{wt of As (std)} : \text{wt of As (sample)} \quad [1]$$

By simplification Eq. 1 becomes:

$$\text{wt As (sample)} = \frac{\Sigma \text{ cpm sample [wt of As (std)]}}{\Sigma \text{ cpm std}} \quad [2]$$

Using Eq. 2, the arsenic content of all samples was calculated. These data were converted to ppm on a dry weight basis by the following formula:

$$(\mu g) \text{ wt As sample} \div (g) \text{ wt Sample} = \text{ppm} \quad [3]$$

RESULTS

Soil

Soil samples were irradiated as previously described. After graphically subtracting sodium from the drift, treated and untreated soil scintillation spectra, it was found that a peak was obtained in the region of 0.54 Mev. This energy corresponds to the gamma ray of ^{76}AS (Heath, 1964). The half-life was also determined and found to be different for the treated and untreated samples at this gamma peak. For the treated samples, the half-life (determined graphically) was approximately 25 hr and for the untreated samples, approximately 36 hr. From these data it was concluded that the untreated soil samples contained not arsenic but some other isotope. The concentrations are presented in Table 2.

Table 2—Average concentrations of arsenic found in treated and untreated soil samples (dry weight basis).

Treatment	Ave. as concentration (ppm)
Untreated	<0.2
Drift	331.0
Treated	654.0

The data indicated that the arsenic concentrations on the treated soil samples was double the concentration of the drift area. It should be noted that the plants growing in the drift area seemed dwarfed while the plants from the treated area showed definite toxicity symptoms.

Root

After activation, spectra from the root samples were similar to those of the soil samples, in that an 0.54 Mev gamma peak was detected in the untreated samples. Again the half-life was longer than that of ^{76}As. The results are presented in Table 3.

Table 3—Average concentrations of arsenic found in treated and untreated root samples (dry weight basis).

Treatment	Ave. as concentration (ppm)
Untreated	<0.2
Drift	400.0
Treated	1707.0

The data in Table 3 indicated that arsenic accumulated in the roots in a higher concentration than the amount found in the soil. Arsenic was apparently accumulated against a concentration gradient.

Stems and leaves

The unknown element was also present in the stems and leaves of the untreated samples. The results of the arsenic concentrations are reported in Table 4.

Table 4—Average concentration of arsenic found in treated and untreated stem and leave samples (dry weight basis).

Treatment	Ave. as concentration (ppm)
Untreated	<0.2
Drift	187.0
Treated	334.0

From the data, it can be seen that the arsenic concentration of the stems and leaves was greatly reduced. This indicated that arsenic remained in the root and, as concentration in the root increased, a smaller proportion was translocated into the stems and leaves. This was probably due to the arsenic content reaching toxic level and destroying cells involved in active transport. These data demonstrate a "discrimination" within the plant.

Fruit

The arsenic concentration in the fruit was further reduced as compared to the stems, leaves and roots. These data are presented in Table 5.

Table 5—Average concentration of arsenic found in treated and untreated fruit samples (dry weight basis).

Treatment	Ave. as concentration (ppm)
Untreated	<0.2
Drift	<0.2
Treated	1.4

The data from the fruit samples indicated that there was an additional discrimination within the plant. This occurred between the stems and leaves, and the fruit. Although fruit size and yield were greatly reduced, even in the drift area, the arsenic concentration in the drift area fruit was below the limit of detectability for this method. Arsenic in the treated samples was detectable.

DISCUSSION

THE DATA indicated that although arsenic is phytotoxic, the concentration in the soil must be greater than 650 ppm to kill tomato plants. At that concentration, the plant still set fruit. These fruit were quite small in both size and number as compared to the untreated plants but the actual arsenic concentration in the fruit was 1.4 ppm.

These data also indicated a discrimination within the plant between the root, stems and leaves, and fruit. Soil concentrations of arsenic must be at a level that would reduce yields of tomatoes before the FDA tolerance of 1 ppm is reached in the fruit. Reductions of fruit size and yield by high arsenic concentrations would make the crop unprofitable and, in some cases, unmarketable. Concentrations of arsenic used as a defoliant are, at least, 100X less than were encountered in the soil samples assayed herein.

By using activation analysis, the arsenic concentration at which incipient toxicity occurs could be pinpointed more precisely. This could be accomplished with milligram size samples as demonstrated by this study.

Limits of detectability for the method

Activation analysis can be used for arsenic detection. Arsenic was identified by the characteristic gamma peak of 0.54 Mev and its half-life of 26.4 hr. The method is extremely sensitive to 0.2 ppm arsenic in less than gram quantities of material. It was also demonstrated that by increasing the neutron flux and/or the exposure time, minute quantities could be detected as in the fruit samples.

Interfering elements, sodium and potassium, could be removed through pre-irradiation chemistry. However, this is not necessary if appropriate standards are used because the spectral interference can be subtracted graphically. The simplicity involved in the preparation of samples: i.e., drying only tends to reduce errors incurred during extraction techniques. It also precludes the use of hazardous chemicals.

Identification of unknown element

The unknown element present in all the samples was identified by the following methods. The radioactive decay of an untreated fruit sample was plotted (Fig. 4) and indicated the presence of several different half-lives, one of which was about 36 hr, as indicated by the 36-hr slope, which closely approximates the slope of the decay curve through three of the data points. The presence of a longer half-life was also indicated by the data, but was not investigated further. The assumed 36-hr slope was graphically subtracted from the total decay curve and

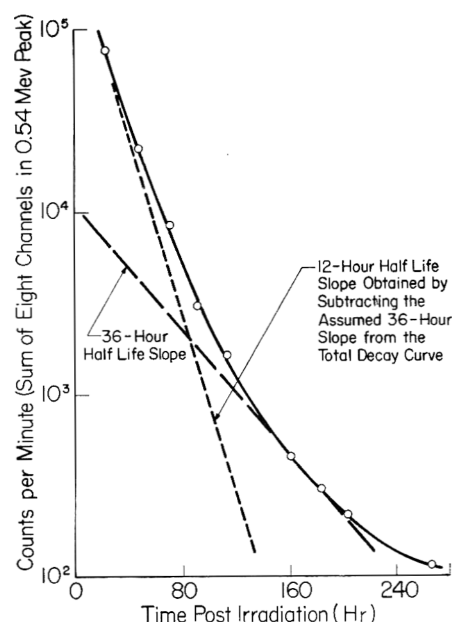

Fig. 4—Radioactive decay of an untreated fruit sample.

yielded a 12-hr half-life slope. The value of 12 hr is close to the value of 15 hr, which is given as the half-life of ^{24}Na, an isotope known to be present in all the samples from the scintillation spectra.

The energy of two gamma rays from an untreated fruit sample (Fig. 5) were identified as approximately the same energy as gamma rays emitted by ^{82}Br. Since the half-life of this isotope is accepted as

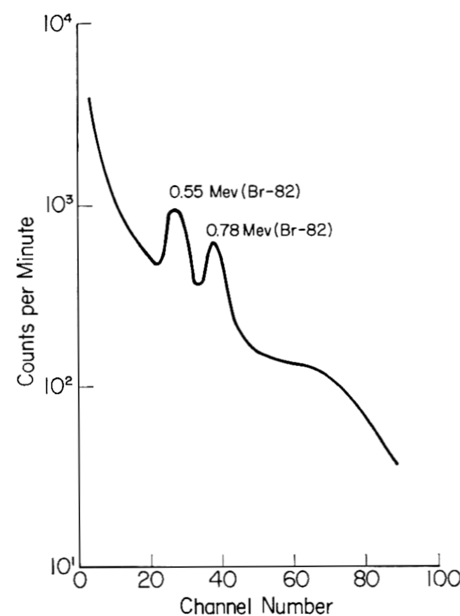

Fig. 5—Untreated fruit sample spectrum eight days after irradiation.

36 hr, an assignment of bromine was made to the previously unidentified element. No effort was made to quantify the amount present. It should be noted that the use of bromine as either a fungicide or a fumigant is a commercially accepted practice in the greenhouse culture of tomato plants. These data indicated that this element could also be detected by activation analysis to determine whether the concentration was below the federal tolerance.

CONCLUSIONS

THE PRINCIPAL conclusions drawn from this study were as follows:

1. Activation analysis could be used to detect minute quantities of arsenic in soil and tomato plant samples with precision and repeatability and without sophisticated sample preparation.
2. Soil concentrations of arsenic would have to be at the level that causes a reduction of fruit size and/or yield before fruit concentrations would be above the FDA tolerance. This is not likely to occur at the concentrations used for defoliation of certain horticultural crops prior to mechanical harvest.

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