September-October 1969
Volume 34 : Number 5

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AN OFFICIAL PUBLICATION OF THE INSTITUTE OF FOOD TECHNOLOGISTS

ห้องสมุด กรมวิทยาศาสตร์

September-October 1969
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ABSTRACTS:

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CHANGES OF SOME PROTEIN FRACTIONS OF BEEF MUSCLE POSTMORTEM. H. GUENTHER & F. TURBA. *J. Food Sci.* **34**, 469–470 (1969)—A tissue press and gel filtration were used to isolate myosin and the major degradation product from muscle postmortem. Both proteins were characterized by peptide maps. Myosin did not change in viscosity following addition of ATP and was free of actin. The degradation product exhibited reduced ATP-ase activity and SH-groups content to values approximately half those of myosin after aging 6 days. The sedimentation constant decreased from 6.2 to 4.1 and comparison of the two peptide maps showed a lack of 18 peptide spots of the original 75 myosin peptides and the appearance of 8 new peptides in the degradation product.

STUDIES ON BOVINE NATURAL ACTOMYOSIN. 1. Relationship of ATPase and contractility to tenderness of muscle. H. K. HERRING, R. G. CASSENS & E. J. BRISKEY. *J. Food Sci.* **34**, 389–391 (1969)—Actomyosin from muscle 12- and 24-hr postmortem had higher ATPase activity than that from 0 hr, 5-day aged or 12-day aged muscle. No consistent differences were found in actomyosin ATPase activity after the various periods of aging for actomyosins from tough and tender muscle. Actomyosin from 12- and 24-hr postmortem muscle superprecipitated faster than that from 0 hr muscle. However, actomyosin from 5- and 10-day aged muscles superprecipitated less rapidly than that from 12- and 24-hr postmortem muscle.

SAPID COMPONENTS IN CARROT. H. OTSUKA & T. TAKE. *J. Food Sci.* **34**, 392–394 (1969)—Relation of various substances present in carrot to its taste was studied. Relatively large amounts of amino acids and carbohydrates were detected in hot water extracts of carrot, but contents of nucleic acid derivatives and organic acids were extremely small. The taste of carrot was due mainly to the presence of glutamic acid and the buffer action of various amino acids.

FREEZER BURN OF ANIMAL TISSUE. 7. Temperature Influence on Development of Freezer Burn in Liver and Muscle Tissue. G. KAESS & J. F. WEIDEMANN, *J. Food Sci.* **34**, 394–397 (1969) Very low weight losses were necessary to initiate freezer burn in beef livers and in beef semitendinosus muscle when stored at —20°C. Fat content of livers had little influence on the onset of burn. A condensed layer was formed but no freezer burn appeared in these tissues frozen at various rates and stored at —4°C. Treatment with solutions of glycerol or sodium chloride before freezing effectively reduced formation of burn in liver and in longitudinal cuts of muscle, but was not effective in transverse cuts of muscle.

SOME SENSORY EFFECTS OF HYDROCOLLOID SOLS ON SWEETNESS. M. VAISEY, R. BRUNON & J. COOPER. *J. Food Sci.* **34**, 397–400 (1969)—Sweetness-texture interactions in cornstarch, guar and carboxymethylcellulose were assessed by a trained sensory panel. Viscosity curves over a range of sucrose levels from 2.5 to 5.5% in the three gums were determined using a Brookfield viscometer. The relationship between viscosity curves and sweetness perception determined by rates of sweetness recognition, matching of equisweetness in different gums, apparent levels of sweetness and ranking of series of gums in order of sweetness indicated that gums with less viscosity drop as shear rates increase tend to mask sweetness perception.

HYDROLYTIC ENZYMES IN BOVINE SKELETAL MUSCLE. 3. Activity of Some Catheptic Enzymes. A. J. LUTALO-BOSA & H. F. MACRAE. J. Food *Sci.* **34**, 401–404 (1969)—Maximal proteolytic activity was observed at either pH 3.8 or at pH 4.8 depending upon the enzyme studied. Activities of cathepsins B, C and D were determined in both supernatant and particulate fractions of two bovine muscles and rat liver by the use of synthetic or natural substrates. Comparison of the activities in bovine muscle with those in rat liver showed that total cathepsin B activity in skeletal muscle was approximately 52% of the activity obtained in rat liver whereas cathepsins C and D activities were approximately 2 and 25%, respectively.

FORMATION OF A GREEN PIGMENT FROM TUNA MYOGLOBINS. O. K. GROSJEAN, B. F. COBB, B. MEBINE & W. D. BROWN. *J. Food Sci.* **34**, 404–407 (1969)—A green pigment was produced when yellowfin (or other) tuna myoglobins, trimethylamine oxide (TMAO), and cysteine were heated together in 0.1 M phosphate buffer, pH 5.7. The green product was not produced with mammalian myoglobins, which contain no cysteine residue. Denaturation of myoglobin, apparently exposing a sulfhydryl group, was necessary for the greening reaction to occur. TMAO acted as a mild oxidizing agent to promote the formation of a disulfide bond between cysteine and the sulfhydryl group on the denatured myoglobins. TMAO could be replaced by oxygen, but cysteine appeared to be specific for the reaction. The green color could be reversed by sodium sulfite, but not by other reducing agents tested.

SERENDIPITY BERRIES—SOURCE OF A NEW INTENSE SWEETENER. G. E. Inglett & J. F. MAY, *J. Food Sci.* **34**, 408–411 (1969). —The fruit of *Dioscoreophyllum cumminsii*, Serendipity Berries, contain an intensely sweet principle. Chromatography of water extracts of the Berry on Sephadex G-50 and G-200 indicated that the sweetener was bound to fruit protein. Degradation of the protein fraction with a proteolytic enzyme, bromelain, gave a lower molecular weight material with intense sweetness of excellent quality.

IN THIS ISSUE

STIMULATION OF GAS PRODUCTION AND GROWTH OF *Clostridium perfringens* TYPE A (NO. 3624) BY LEGUMES. L. B. ROCKLAND, B. L. GARDINER & D. PIECZARKA *J. Food Sci.* **34,** 411–414 (1969)—Dry beans contain an unidentified factor which stimulates growth and gas production by *C. perfringens,* Type A. This factor may be related to the flatus factor in dry beans. An assay procedure was developed for estimating the response of the microorganism to various substrates. At levels of up to four times their concentrations in a synthetic basal medium, glucose, amino acids, vitamins and other nutrients had no effect. Supplements of other sugars including raffinose and stachyose produced no stimulation when added to a complete basal medium containing glucose. However, dry bean homogenates elicited prolific gas production under the same conditions.

ANTHOCYANIN PIGMENTS IN TROUSSEAU GRAPES. R. CARRENO-DIAZ & B. S. LUH. *J. Food Sci.* **34,** 415–419 (1969)—Identification of the pigments was based on R_f values in various solvents, partial acid hydrolysis, sugar moiety, alkaline degradation, fluorescence under ultraviolet radiation, color reactions, and absorption spectra in the visible region. Shown to be present in the grapes are petunidin 3-monoglucoside, cyanidin 3-monoglucoside, malvidin 3-monoglucoside and peonidin 3-monoglucoside. Based on photodensitometric measurements, malvidin 3-monoglucoside was present in largest amount (49.8%), followed by peonidin 3-monoglucoside (36.9%), cyanidin 3-monoglucoside (8.75%) and petunidin 3-monoglucoside (4.55%). The identification of all the anthocyanins as monoglucosides supports the classification of Trousseau as a cultiva of *Vitis vinifera.*

VAPOR ANALYSIS OF FERMENTED SPANISH-TYPE GREEN OLIVES BY GAS CHROMATOGRAPHY. H. P. FLEMING, J. L. ETCHELLS & T. A. BELL. *J. Food Sci.* **34,** 419–422 (1969)—Five major components were detected gas chromatographically in the headspace vapor of Spanish-type green olives fermented by pure cultures of lactic acid bacteria. Three of these compounds were identified as acetaldehyde, methyl sulfide, and ethanol. The same five compounds also were present in pasteurized, unfermented olives, but in different amounts. Olives that had undergone a natural fermentation contained the above five compounds, and, in addition, a varying number of other compounds. Methyl sulfide was found to be a major odor component of fermented as well as unfermented olives.

THE CAUSE OF DISCOLORATION OF HARD COOKED EGG ROLLS. P. G. SCHNELL, D. V. VADEHRA & R. C. BAKER. *J. Food Sci.* **34,** 423–426 (1969)—A brownish discoloration of hard cooked egg rolls was observed on storage under fluorescent lights. This discoloration was unique and caused by ultraviolet (UV) radiation. A two step mechanism was suggested: (a) the production of peroxides and/or hydroperoxides from water in coagulated albumen on exposure to UV radiation which causes the hydrolysis of the peptide chain resulting in an increase of non protein nitrogen and aromatic amino acids; (b) the oxidation of the exposed tryptophan molecule by UV radiation producing a brown color. The discoloration could be prevented by reducing agents (L-cysteine and ascorbic acid) or using a colored packaging film in the production of the roll.

MICROBIOLOGY OF FRESH APPLE AND POTATO PLUGS PRESERVED BY GAS EXCHANGE. J. G. KAFFEZAKIS, S. J. PALMER & A. KRAMER. *J. Food Sci.* **34,** 426–429 (1969)—Gas exchange as a means of preserving fresh quality of apple and potato tissue was studied. Gram negative rods in the environment of the test materials were rapidly killed when exposed either to a mixture of ethylene oxide (10%) and carbon dioxide (90%), or to pure sulfur dioxide and sulfur dioxide (50%) air mixtures. Gram positive cocci and the spores of the anaerobes showed slower rates of destruction under the same conditions. Treatment with pure carbon monoxide, nitrogen or packaging in vacuum achieved considerable freedom from color changes but had no appreciable effect on microorganisms.

UTILIZATION OF AMIDE NITROGEN BY THE YOUNG RAT. M. WOMACK & J. E. WILSON JR. *J. Food Sci.* **34,** 430–433 (1969)—Rats were unable to utilize amide nitrogen of glutamine as efficiently as an equal quantity of α-amino nitrogen from glutamic acid when the two amino acids were the sole sources of nonessential nitrogen in the diet. However, no differences in nitrogen utilization were demonstrated between two groups of rats fed different levels of amide nitrogen as wheat. It is concluded that in the usual wheat-containing diet any failure to utilize part of the amide nitrogen would not limit protein synthesis.

SENSORY DIFFERENTIATION OF BEEF TENDERNESS AND JUICINESS COMPONENTS OVER SHORT INTERVALS OF COOKING TIME. P. J. ROGERS & S. J. RITCHEY. *J. Food Sci.* **34,** 434–435 (1969)—The ability of judges to distinguish differences in juiciness and six components of tenderness of top round steaks cooked at 350°F for 20, 23, 26 and 29 min was studied by use of a paired comparison design. Concurrently, the effects of cooking time on cooking losses, percents moisture, nitrogen, and fat, shear values, "loose water" and "immobilized water" were evaluated. Judges detected differences for all factors between steaks cooked 20 and 26 min, but were unable to detect differences between steaks cooked 26 and 29 min. Differences between steaks cooked 20 and 29 min were found for all factors except fragmentation and adhesion.

ORIGIN AND NATURE OF AROMA IN FAT OF COOKED POULTRY. E. L. PIPPEN, E. P. MECCHI & M. NONAKA. *J. Food Sci.* **34,** 436–442 (1969)—Odor panel results indicate characteristic cooked poultry aroma in fat of cooked poultry is derived from the lean portions of meat. Migration of sulfur substances into the fat during cooking supports this concept. The dependence of the magnitude of the sulfur buildup in fat upon cooking conditions, the nature of aroma components found in fat of roasted turkey and the readiness with which authentic amino acids are degraded in hot fat, all suggest that protein, amino acids, sugars and other water soluble components are involved in the formation of the characteristic aroma that accumulates in fat of cooked poultry.

ABSTRACTS:

IN THIS ISSUE

HYDROGEN SULFIDE, A DIRECT AND POTENTIALLY INDIRECT CONTRIBUTOR TO COOKED CHICKEN AROMA. E. L. PIPPEN & E.P. MECCHI. *J. Food Sci.* **34,** 443–446 (1969)—The 35 parts per billion (ppb) H_2S in freshly prepared broth and the 180 to 730 ppb H_2S in meat of freshly simmered, roasted and fried chicken all substantially exceed the 10 ppb H_2S odor threshold in water. Hence, H_2S contributes directly to the aroma of these products. In a model system, H_2S was passed through molten chicken fat containing 5% acetaldehyde. After expulsion of excess H_2S and acetaldehyde the residual odorous fat exhibited a fixed sulfur content. These results suggest that a reaction between H_2S and acetaldehyde was involved and that such reactions in fat could be general. Thus H_2S may also contribute to aroma through the formation of secondary products.

TRANSAMINASES OF SKELETAL MUSCLE. 1. The Activity of Transaminases in Post-mortem Bovine and Porcine Muscles. R. HAMM, L. KORMENDY & G. GANTNER. *J. Food Sci.* **34,** 446–448 (1969)—The activity of glutamic-oxaloacetic transaminase (GOT), glutamic-pyruvic transaminase (GPT) and GOT isozymes in bovine and porcine muscles was determined. The ratio $GOT_M:GOT_S$ in the tissue was found to be about 1:1. There is only a small decrease of GOT activity during cold-storage of muscle. The small GOT_M activity in the muscle press juice does not change during storage, indicating that there is no drastic change of muscle mitochondria during aging of meat.

TRANSAMINASES OF SKELETAL MUSCLE. 2. Transaminase Activities in White and Red Muscles of Pigs and Cows. R. HAMM. *J. Food Sci.* **34,** 449–452 (1969)—The total activity of glutamic-oxaloacetic transaminase (GOT), the relative activity of the mitochondrial GOT isozyme (GOT_M) and the total activity of glutamic-pyruvic transaminase (GPT) in different kinds of bovine and porcine muscle were determined. A highly significant positive correlation was found between either GOT or GPT activity and the amount of muscle pigments. However, the relative GOT_M activity of porcine muscle was found to be almost constant in the different types of muscle.

TRANSAMINASES OF SKELETAL MUSCLE. 3. Influence of Free ing and Thawing on the Subcellular Distribution of Glutamic-Oxal acetic Transaminase in Bovine and Porcine Muscle. R. HAMM & KORMENDY *J. Food Sci.* **34,** 452–457 (1969)—Freezing and thawi of bovine and porcine muscle cause a remarkable release of the mit chondrial isozyme of glutamic-oxaloacetic transaminase (GOT_M) fro mitochondrial structure resulting in an increase of GOT_M activity in t muscle press juice. The lower the freezing temperature the stronger this effect. Repeated freezing and thawing increase the release of GOT It is considered that the level of GOT_M activity in the muscle pre juice indicates the extent of mitochondrial damage. A simple and rap routine method was developed which allows a reliable differentiation b tween nonfrozen and frozen and thawed meat.

A TEXTURE PROFILE OF FOODSTUFFS BASED UPON WEL DEFINED RHEOLOGICAL PROPERTIES. P. SHERMAN. *J. Foo Sci.* **34,** 458–462 (1969)—Many attributes contribute to texture, a panel tests should be so arranged that all these attributes are analyze The texture profile concept used by Szczesniak and her co-workers critically examined, and several modifications are proposed. The n scheme consists of primary, secondary and tertiary categories. The terms are not used in the philosophical sense proposed by Locke. P mary attributes are analytical composition, particle size and size dist bution, particle shape, air content, etc. There are only three seconda attributes viz., elasticity (E), viscosity (η) and adhesion (N). T tertiary characteristics are basically the responses most often used sensory analysis of texture. Tertiary characteristics are derived from complex blending of two or more secondary attributes.

STUDIES ON ENZYMIC ACTIVITY OF RAT LIVER SUBCELLU LAR FRACTIONS. S. SHIBKO, A.L. TAPPEL, J.P. SUSZ & R. FREEDLAND. *J. Food Sci.* **34,** 462–465 (1969)—Particulate asparta amino transferase and alanine amino transferase were shown to mitochondrial, and particulate glutathione reductase was shown to associated with both mitochondria and lysosomes. Isocitrate dehydr genase, glycerol-3-phosphate dehydrogenase, serine dehydratase, gl cose-6-phosphate dehydrogenase, xanthine oxidase, aldehyde oxida and fumarate hydratase were found not to be associated with lysosome Lysosomes were unable to incorporate labelled amino acids into pr tein but were able to incorporate acetate-^{14}C into fatty acids. Relatio ships of these findings to some properties of meats are discussed.

QUANTITATION OF FLAVORFUL FOOD COMPONENTS USIN ISOTOPE DILUTION. W. Y. COBB. *J. Food Sci.* **34,** 466–469 (196 —Flavor compounds labeled with ^{14}C are added to a food system pri to reduced-pressure distillation. The example used is benzaldehyd which in the distillate is converted to its corresponding 2,4-dinitr phenylhydrazone. Separation of the labeled hydrazone from other mat rial present is accomplished by thin-layer chromatography. Recovere material is quantitated using ultraviolet spectroscopy. Employing isoto monitoring data, the concentration of native aldehyde is calculated v isotope dilution. The method is adaptable to flavor compounds sufficient volatility to be recovered under 5 mm Hg and 65°C, ar which are stable or can be converted to a stable form for purification.

NOTICE: Page Charges for Publication of Research Papers

☐ AT ITS *March 1969 meeting, the Executive Committee of IFT voted to increase the page charge for research articles published in* Food Technology *or in the* JOURNAL OF FOOD SCIENCE. *The page charge of $50 per printed page will be effective for research manuscripts received AFTER April 1, 1969. The page charge* shall not *constitute a bar to acceptance of research manuscripts because the author is unable to pay the charge.*

H. K. HERRING,[a] R. G. CASSENS and E. J. BRISKEY
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Studies on Bovine Natural Actomyosin 1. Relationship of ATPase and Contractility to Tenderness of Muscle

SUMMARY—The effects of muscle tenderness classification and of aging muscle postmortem on ATPase activity and superprecipitation of natural actomyosin were studied. Actomyosin from muscle 12- and 24-hr postmortem had higher ATPase activity than that from 0-hr, 5-day aged or 10-day aged muscle. However, ATPase activity did not usually return to the 0-hr level. No consistent differences were found in actomyosin ATPase activity after the various periods of aging for actomyosins from tough and tender muscle. Superprecipitation of actomyosin was used as a measure of contractility. Actmyosin from 12- and 24-hr postmortem muscle superprecipitated faster than that from 0-hr muscle. However, actomyosin from 5- and 10-day aged muscle superprecipitated less rapidly than that from 12- and 24-hr postmortem muscle. Superprecipitation was more rapid in actomyosin from tough muscle than tender muscle at low KCl concentrations, but this was not true at high KCl concentrations. This observation suggested that actomyosin from tough muscle had a stronger interaction or higher amounts of some protein factor such as α-actinin than did tender muscle.

INTRODUCTION

CHANGES which accompany rigor mortis and the subsequent softening of muscle are physical and chemical in nature. The development of rigor mortis is accompanied by an increase in tension (Schmidt et al., 1968; Jungk et al., 1967), and if muscles are unrestrained, shortening can occur and the muscle toughens (Herring et al., 1967).

The formation of cross-links between actin and myosin during the onset of rigor mortis is irreversible in the absence of ATP (Bendall, 1960; Davies, 1963) or ADP (Kushmerick et al., 1969); however, if ATP is added to a myofibrillar system prepared postmortem, myofibrils can contract (Takahashi et al., 1965). Recent studies by Fujimaki et al. (1958, 1965) on natural actomyosin prepared at various times postmortem suggested that the changes in ATPase activity were possibly associated with the tenderization process in muscle. However, the basic reason for the change in ATPase activity with postmortem aging is obscure.

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The objective of the present study was to investigate the ATPase and superprecipitation of actomyosin isolated from naturally occurring tough and tender bovine muscle. In addition, the effect of aging the muscles was also studied.

EXPERIMENTAL

Muscle source and sampling procedure

Eight mature (E-maturity) bovine animals were used in these experiments. The longissimus plus all surrounding muscles, fatty tissues and bones from the fifth to the twelfth thoracic vertebrae, inclusive, were removed from the carcass and held at 4°C. Exposed muscle surfaces were covered with polyethylene. One hundred g samples were removed after aging periods of 0, 1/2, 1, 5 and 10 days.

Preparation and study of natural actomyosin

Herring et al. (1969a) describe the procedures to measure ATPase activity, degree of superprecipitation and protein concentration.

Tenderness determination and classification

Tenderness was determined on 50 g samples of the longissimus obtained adjacent to the sample used for actomyosin preparation at 1, 5 and 10 days postmortem. The muscle was cooked in boiling (100°C) water for 2 hr. Shear force was determined on six 1.25-cm cores with the Warner-Bratzler shear device. Animals classified as tough had a shear force of at least 12 kg at 24 hr and greater than 7 kg after 10 days aging. Those classified as tender had a shear force of less than 7 and 3 kg for 1 and 10 days, respectively. Five of the animals used were classified as tender and three were tough.

RESULTS & DISCUSSION

Effect of aging muscle postmortem on ATPase activity

The effect of aging muscle postmortem on actomyosin ATPase activity is summarized in Figure 1 and Table 1; the effect of KCl concentration and added Ca^{++} is indicated. The ATPase activities were lowest from the 0-hr samples and generally increased 1.1 to 1.2 times in the 12- and 24-hr aged samples. Subsequently, ATPase activity either changed little or decreased by either the 5- or 10-day aging periods.

Fujimaki et al. (1958, 1965) found ATPase activity was maximal in natural actomyosin extracted from rabbit muscle at 2 days postmortem, however maximum activity was observed after 2 and 8 days aging for veal. The onset of rigor mortis is usually complete by 6 to 9 hr in bovine muscle (Herring et al., 1964, unpublished data) and the 12- or 24-hr time periods should be reasonably close to the complete development of rigor mortis. The mechanism whereby the ATPase activity of natural actomyosin extracted from muscle 12–24 hr postmortem increased compared to that from fresh muscle could be due to a number of possibilities. Those suggested are (1) stronger binding between myosin and actin, (2) increased

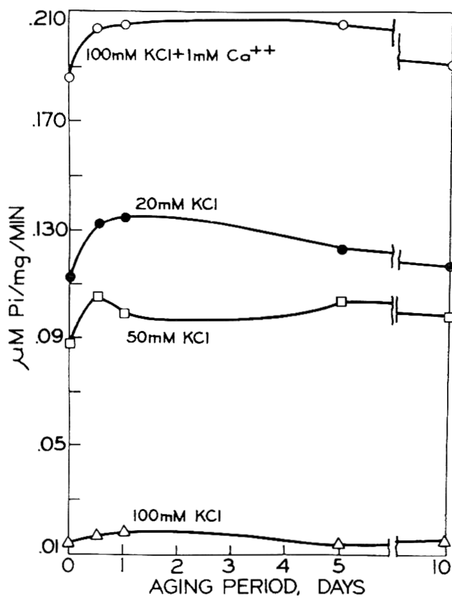

Fig. 1—Effect of aging muscle postmortem on ATPase activity of bovine natural actomyosin. Final concentrations: KCl as indicated; 1 mM ATP; 20 mM Tris-Acetate (pH 6.8); 1 mM Mg^{++} (except where 1 mM Ca^{++} indicated); 0.33 ± 0.03 mg actomyosin/ml. Temp. 25°C.

Table 1—ATPase activity[1] of natural actomyosin as influenced by muscle aging and tenderness classification.

KCl conc.	Days postmortem				
	0	1/2	1	5	10
	$\bar{x}$ ± $s_{\bar{x}}$	$\bar{x}$ ± $s_{\bar{x}}$	$\bar{x}$ ± $s_{\bar{x}}$	$\bar{x}$ ± $s_{\bar{x}}$	$\bar{x}$ ± $s_{\bar{x}}$
20 mM KCl[2]					
Tender[4]	.116 = .007	.133 ± .007	.130 ± .005	.122 ± .006	.119 ± .005
Tough[5]	.106 = .014	.130 ± .010	.142 ± .005	.126 ± .009	.116 ± .016
50 mM KCl[2]					
Tender[4]	.089 ± .005	.105 ± .006	.096 ± .011	.100 ± .006	.103 ± .007
Tough[5]	.086 ± .011	.103 ± .015	.104 ± .008	.110 ± .009	.090 ± 0.15
100 mM KCl[2]					
Tender[4]	.011 ± .001	.017 ± .003	.018 ± .003	.013 ± .001	.014 ± .003
Tough[6]	.018 ± .005	.015 ± .002	.018 ± .002	.016 ± .002	.018 ± .002
100 mM KCl+ 1 mM Ca[3]					
Tender[4]	.200 ± .010	.213 ± .016	.205 ± .012	.216 ± .012	.198 ± .016
Tough[6]	.164 ± .021	.184 ± .008	.205 ± .006	.188 ± .014	.179 ± .027

[1] ATPase activity in uM P_i/mg protein per min.
[2] Final concentrations: KCl as specified, 1 mM ATP; 20 mM Tris-Acetate (pH 6.8); 1 mM Mg^{++}; 25°C.
[3] Final concentrations: KCl and Ca^{++} as specified; 1 mM ATP; 20 mM Tris-Acetate (pH 6.8): 25°C.
[4] Five observations.
[5] Three observations.

F-actin content—F-actin-rich natural actomyosin, or (3) conformational changes in actomyosin. The first of these will be discussed later in this manuscript with respect to superprecipitation of natural actomyosin.

That natural actomyosin from 12- or 24-hr-old muscle was higher in F-actin than that from 0-hr muscle was supported by the finding that ATP sensitivity and "actin" content (polyethylene sulfonate treatment) were higher in these muscle samples (Herring et al., 1969b). Therefore the myosin ATPase could have been activated more by actin in natural actomyosin from muscle 12–24 hr postmortem than that from 0-hr muscle. The activation of myosin ATPase by actin was shown by Maruyama et al. (1962) and ATPase activity reached a plateau when a ratio of approximately 4:1 of myosin to actin by weight was reached.

Szent-Györgyi et al. (1966) reported that a conformational change in actin occurred during superprecipitation but the change was cyclical and transitory. Such a finding is applicable here only if conformational changes indeed do occur in actin during ATPase activity or superprecipitation of natural actomyosin, and the onset of rigor mortis affects such conformational changes. Levy et al. (1962) suggested a conformational change in myosin could occur during ATP hydrolysis. A conformational change in natural actomyosin was also suggested by Robson et al. (1965) and Goll et al. (1967) as being related to an increase in ATPase of actomyosin isolated from 24 hr postmortem muscle. However conformational changes are often invoked without unequivocal evidence to explain modifications in enzymatic properties (Gergely, 1966).

Relationship of ATPase activity to tenderness classification

Actomyosin ATPase activity increased as muscle aged from 0 hr to 12 or 24 hr, then decreased in the 5- and 10-day aged muscle in both tenderness groups. Although variation was evident within each tenderness group, it appeared that ATPase activity was not related to or markedly influenced by tenderness classification

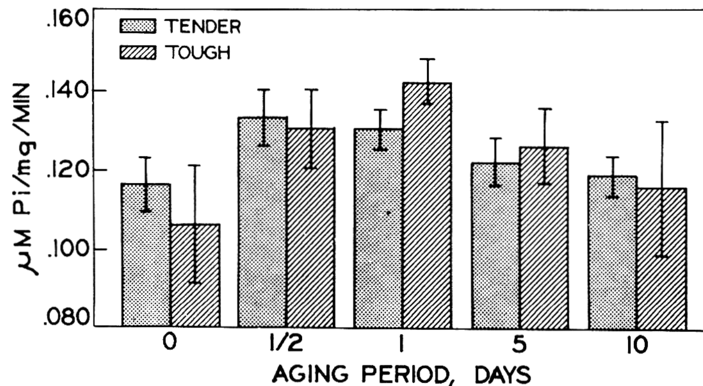

Fig. 2—ATPase of natural actomyosin of tough and tender muscle. Final concentrations: 20 mM KCl; 1 mM ATP; 20 mM Tris-Acetate (pH 6.8); 1 mM Mg^{++}; 0.33 ± 0.03 mg actomyosin/ml. Temp. 25°C.

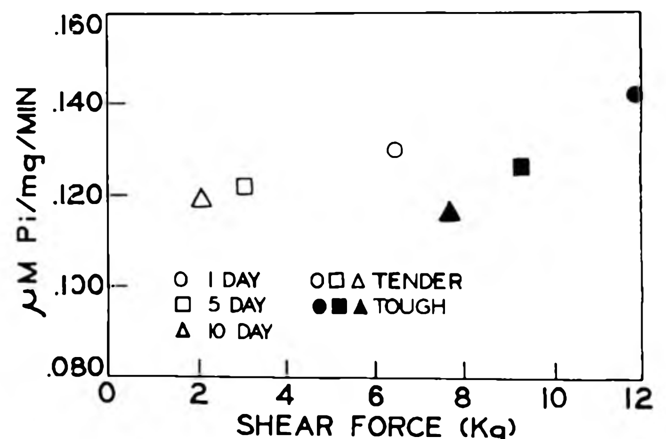

Fig. 3—Natural actomyosin ATPase vs. shear force at three aging periods. Final Concentrations: 20 mM KCl; 1 mM ATP; 20 mM Tris-Acetate (pH 6.8); 1 mM Mg^{++}; 0.33 ± 0.03 mg actomyosin/ml. Temp. 25°C.

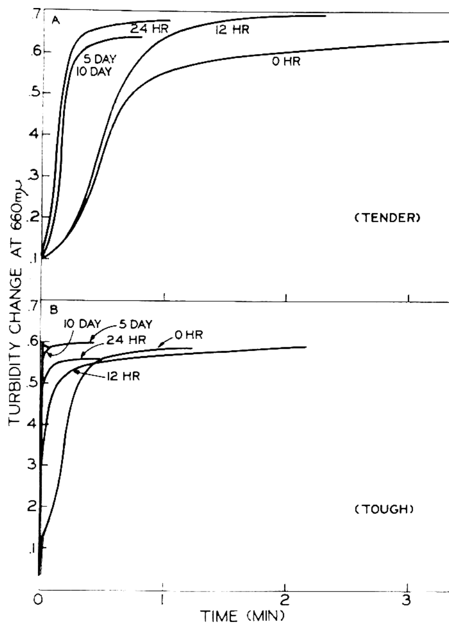

Fig. 4—Influence of aging muscle on superprecipitation of bovine natural actomyosin. Final Concentrations: 50 mM KCl; 1 mM ATP; 20 mM Tris-Acetate (pH 6.8); 1 mM Mg^{++}; 0.35 mg/ml of actomyosin. Temp. 25°C.

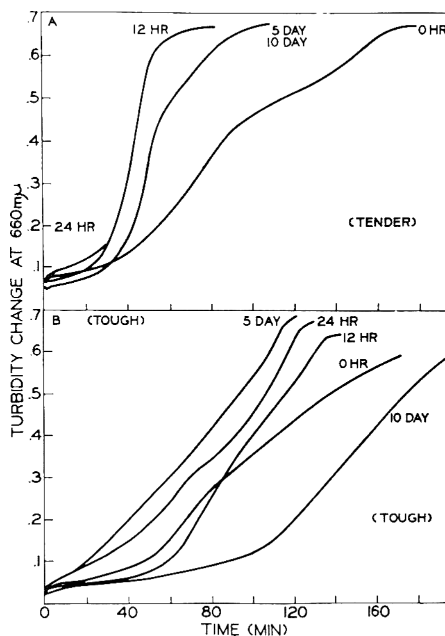

Fig. 5—Influence of aging muscle on superprecipitation of bovine natural actomyosin. Final concentrations: 100 mM KCl; 1 mM ATP; 20 mM Tris-Acetate (pH 6.8); 1 mM Mg^{++}; 0.35 mg/ml of actomyosin. Temp. 25°C.

(Fig. 2, Table 1).

These results are more clearly shown in Figure 3 where ATPase activity is plotted against shear force for the tough and tender groups at three times postmortem. With one exception, all points would fall on a line with zero slope, and therefore little relationship existed.

Effects of aging muscle postmortem and tenderness classification on superprecipitation

Figures 4 and 5 show the effects of aging postmortem and tenderness classification on contractility as measured by the turbidimetric method. Aging muscle postmortem from 0 to 12 or 24 hr resulted in an increased rate of turbidity rise in natural actomyosin from both tender and tough muscle. This suggested, perhaps, a stronger interaction of the actin and myosin moieties of actomyosin in the 12- and 24-hr samples than in 0-hr samples. It should be pointed out that results with turbidity measurements are difficult to interpret accurately (Briskey et al., 1967). It took 5 days aging in the tender group and 10 days in the tough group (Fig. 5) for the rate of super-precipitation in 100 mM KCl to decrease when compared to the 12- and 24-hr aged samples.

It appeared that actomyosin from tough muscle underwent a more rapid turbidity change than the tender muscle in 50 mM KCl (Fig. 4); however, there was a delayed rate of superprecipitation in actomyosin from the tough muscle compared to tender muscle (Fig. 5) in 100 mM KCl. This situation was puzzling but could perhaps be explained by the recently published observations of Ebashi et al. (1965) and Seraydarian et al. (1967). These workers demonstrated that a low KCl, α-actinin enhanced superprecipitation, while at high KCl α-actinin promoted clearing. Perhaps tough and tender muscle varied in character or content of this or some other regulatory protein. An alternative explanation could be that there was a stronger interaction of actin and myosin at low ionic strength for tough muscle than for tender muscle, and a weaker interaction at high ionic strength.

Furthermore, it has recently been shown by Herring et al. (1967) and Cooper et al. (1968) that contraction state of muscle postmortem was highly negatively correlated to tenderness. These results indicate that molecular phenomena are important in tenderness and that contraction state differences may reflect differences in protein interaction or composition in muscles varying in tenderness.

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Ms. received 3/5/68; revised 4/7/69; accepted 8/15/69.

Published with the approval of the Director of the Wisconsin Agricultural Experiment Station, Madison. Dept. of Meat and Animal Science manuscript No. 493. Supported in part by the Campbell Institute for Food Research. Equipment used was purchased in part through Public Health Service Grant No. UI-00266-09 from the National Center for Urban and Industrial Health.

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Sapid Components in Carrot

SUMMARY—The relationship between various substances present in carrot to its taste was studied. Nucleic acid derivatives found in hot water extracts of carrot were adenine, adenosine, inosine, hypoxanthine, 5′-AMP, 5′-UMP, UDP, but 5′-IMP and 5′-GMP were absent. The contents of these derivatives were extremely small. Silica gel chromatography showed the presence of small quantities of succinic acid, α-ketoglutaric acid, lactic acid, pyroglutamic acid, citric acid and glycolic acid.

Amino acids in hot water extracts of carrot were detected by two-dimensional thin-layer chromatography and automatic amino acid analyzer. Identified were glutamic acid, valine, leucine, aspartic acid, lysine, and serine etc. Glutamic acid content was relatively large. Sucrose, maltose and glucose were detected in carrot and these carbohydrates were responsible for the sweetness of carrot. The taste of carrot was due mainly to the presence of glutamic acid and the buffer action of various amino acids.

INTRODUCTION

MANY INVESTIGATORS, especially in Japan, have studied sapid components of various foodstuffs. Ikeda (1909, 1913) stated that monosodium glutamate was a sapid component of *Laminaria*. Kodama (1913) said that inosinic acid was a sapid component in dried bonito. Less attention has been given to the sapid components of vegetables than to those of meats and fishes.

The authors have been studying substances which are closely related to the taste of various foodstuffs. This study was made to isolate, identify and compare quantitatively various substances present in hot water extracts of carrots, and to determine the principal substances that contribute to the taste of carrot.

EXPERIMENTAL

Sample preparation

Twenty g of carrot (*Daucus Carota L.* var.) were cut finely and diluted with 100 ml of water, then heated at 100°C for 20 min. The solution was cooled and filtered through funnel cloth. The filtrate was to 100 ml with water. This solution (carrot soup) was used as a sample for analysis.

Chromatographic analysis of nucleic acid derivatives

Nucleic acid derivatives were detected by chromatography on Dowex 1 × 8 by the procedure of Nakazima et al. (1961).

Chromatographic analysis of organic acids

Organic acids were separated by silica gel column chromatography and identified by the method outlined by Ueda et al. (1959).

Analysis of amino acids

Amino acids were detected by thin-layer chromatography by the procedure of Brenner et al. (1960), and quantitative analysis was conducted by an automatic amino acid analyzer (Shibata Chem. Ins. Co. Ltd., Type AA-500).

Analysis of carotenoids

Total carotenoids were examined according to the procedure of Wall et al. (1943).

Samples for buffer action

Organic acids were removed by ether extraction. Nucleic acid derivatives were removed by the batch method of anion exchange resin (Dowex 1 × 2). Amino acids were removed by batch method of cation exchange resin (Dowex 50 × 8).

Determination of buffer action.

Buffer action of the carrot solution was examined through the pH variation caused by the varying volume of 1/10 *N*-HCl or 1/10 *N*-NaOH solution added.

Analysis of carbohydrates

The preliminary identification of carbohydrates in carrot was carried out by paper chromatography. Toyo No. 51 filter paper was used and developed with water saturated *n*-butanol. Reducing sugars was detected with ammoniacal $AgNO_3$ solution and nonreducing sugars was detected with 1% sodium metaperiodate and 1% $KMnO_4$ solution. Determination of total reducing sugars were made with Bertrand's method. Determination of glucose and maltose were made with glucose oxidase method by the procedure of Whistler et al. (1953).

Sucrose was hydrolyzed with 1/10 *N*-HCl and determined by Bertdand's method and the glucose oxidase method.

Sapidity and flavor evaluation

The method of Scalor Scoring was followed for sapidity and flavor evaluation.

The 5′-Nucleotidase was obtained through the courtesy of Dr. E. Ohara, Research Laboratories, Takeda Chemicals Industry Co. Ltd., Japan. L-Glutamate 1-Carboxylase was obtained through the courtesy of Mr. S. Yamaguchi, Kikkoman Shoyu Co. Ltd., Japan. Glucose oxidase was purchased from Nagase Industry Co Ltd., Japan.

RESULTS & DISCUSSION

THE NUCLEIC acid derivatives detected in carrot soup were adenine, adenosine, inosine, 5′-AMP, 5′-UMP, and UDP, but the present study found no traces of 5′-IMP and 5′-GMP (Fig. 1). It is widely known that nucleic acid derivatives play an important role in the taste of various foodstuffs. For instance, it is well established that 5′-IMP and 5′-GMP are sapid components in dried bonito or dried mushrooms. To ascertain the role of nucleic acid derivatives contained in carrot soup in the taste, each of them was separated by column chromatography. But it was demonstrated that the individual derivatives were irrelevant to the sapidity.

Next, all nucleic acid derivatives were removed with anion exchange resin, the taste of the resultant solution was quite similar to the original soup. The original soup treated with 5′-nucleotidase proved to be the same. From above results we may assume that nucleic acid derivatives have no relation to the sapidity of carrot soup.

The organic acids detected were α-ketoglutaric acid, succinic acid, glycolic acid, lactic acid, and citric acid, but their amount was considerably low. The removal of ether soluble organic acids and other substances from carrot soup with ether had no effect on the sapidity of carrot, although some effect was found on the flavor. In view of these results, it was ascertained that ether soluble organic acids did not affect the sapidity of carrot soup.

The amino acids detected by thin-layer chromatography were glutamic acid, aspartic acid, arginine, valine, lysine, gly-

Table 1—Content of amino acids in carrot soup.

Amino acid	mg/100 ml[1]
Aspartic acid	3.15
Threonine	75.90
Serine	140.00
Glutamic acid	60.50
Proline	1.86
Glycine	2.00
Alanine	55.40
Cystine	trace
Valine	143.60
Methionine	1.69
Isoleucine	5.28
Leucine	3.28
Tyrosine	3.74
Phenylalanine	3.53
Tryptophane	1.60
Lysine	1.22
Histidine	1.35
Arginine	2.73
Taurine	20.30

[1] 20 g of carrot in 100 ml.

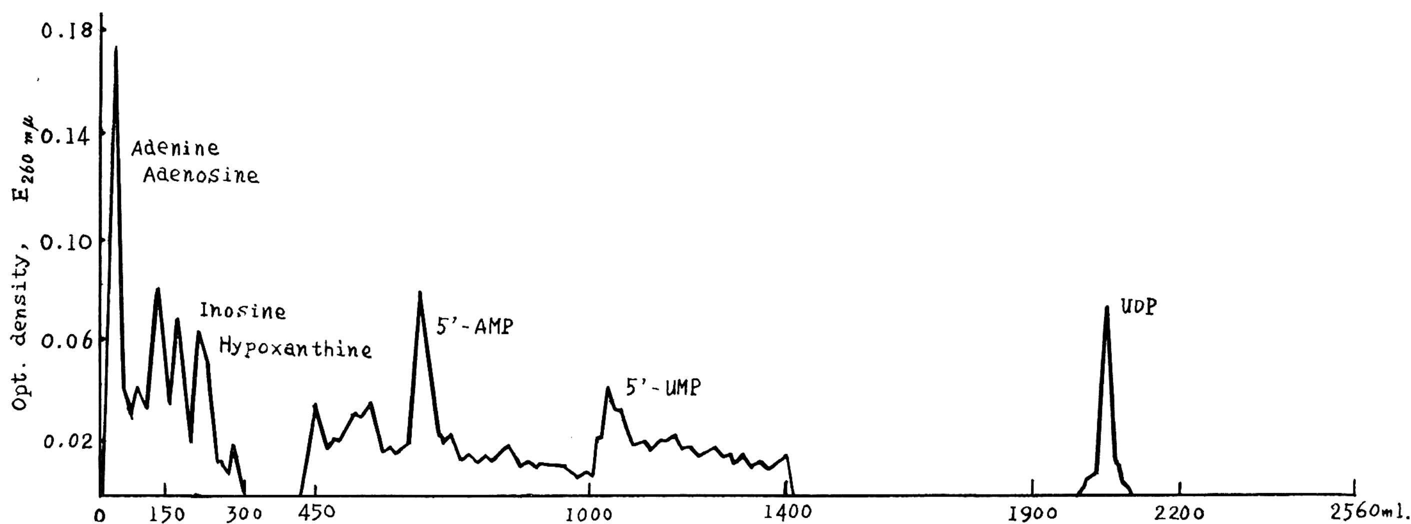

Fig. 1—Chromatograms of nucleic acid derivatives in carrot soup.

cine, and serine. Table 1 shows the content of various amino acids detected by the automatic amino acid analyzer.

Of these amino acids, glutamic acid was relatively large in content. Glutamic acid is well known as a sapid component of various foodstuffs, especially in *Laminaria.* The taste of the carrot soup from which amino acids were removed with cation exchange resin (Dowex 50 × 8) became unpalatable, and it was recognized that the sapidity of carrot soup disappeared by the treatment with L-glutamate 1-carboxylase. It was ascertained that amino acids, especially glutamic acid, were dispensable sapid components of carrot.

Table 2—Content of sugars in carrot soup.

Sugar	mg /100 ml
Glucose	75.75
Maltose	415.90
Sucrose	482.98

Table 3—Constitution of synthetic carrot soup.

Substance	mg /100 ml
Aspartic acid	3.15
Threonine	75.90
Serine	140.00
Glutamic acid	60.50
Proline	1.86
Glycine	2.00
Alanine	55.40
Cystine	trace
Valine	143.60
Methionine	1.69
Isoleucine	5.28
Leucine	3.28
Tyrosine	3.74
Phenylalanine	3.53
Tryptophane	1.60
Lysine	1.22
Histidine	1.35
Arginine	2.73
Taurine	20.30
Glucose	75.75
Maltose	415.90
Sucrose	482.98
Succinic acid	14.10

The amount of carotenoids was 5590 mg % in carrot. The carotenoids were removed by the chromatography of hyflo-supercel plus MgO mixture. Sapidity of the resultant solution was almost the same as the original carrot soup. It is inferred that carotenoids have no effect on the sapidity of carrot.

The sugars detected by paper chromatography were glucose, maltose, and sucrose, and their amounts are shown in Table 2.

It has been recognized that buffer action plays an important role in the sapidity of various foodstuffs. Figure 2 gives the buffer action of carrot soup and two foodstuff solutions used in Japan.

The buffer action of carrot solutions from which organic acids, amino acids or nucleic acid derivatives were removed, showed some decrease, especially large in the amino acids removed sample, as compared with the original carrot soup (Fig. 3).

With regard to the effects of buffer action on the sapidity of carrot soup, the results indicate that some of the influence can be attributed to amino acids whose contents are relatively large in carrot.

Next, various amino acids and carbohydrates equal in amount to those of carrot soup were dissolved in water and adjusted to pH 5.9 (pH of carrot soup) (Table 3).

The sapidity of the synthetic solution was almost similar to that of carrot soup. Buffer action of the synthetic solution was a little weaker than that of the original soup (Fig. 2). The weaker buffer action was caused by lack of undetected substances in the synthetic solution.

The sapid components of carrot were due mainly to the presence of glutamic acid, various amino acids and the buffer action of these amino acids.

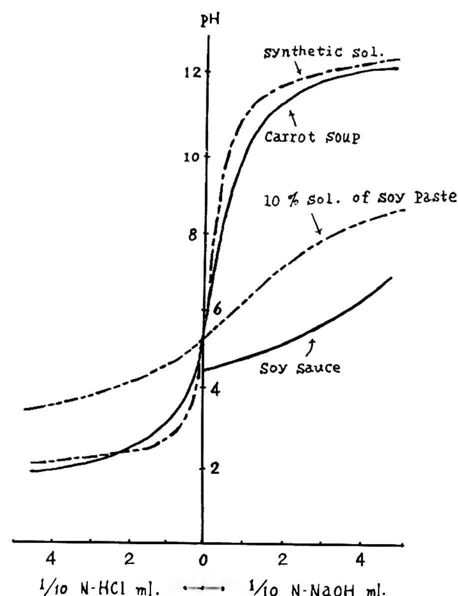

Fig. 2—Buffer action of carrot soup.

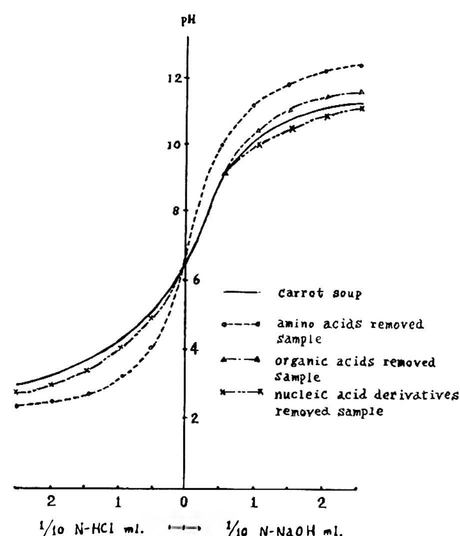

Fig. 3—Buffer action of various samples.

Detailed studies on volatile constituents and synergistic effect of the various constituents in carrot showed clarify the taste (sapidity plus flavor) of carrot.

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Ms. rec'd 4/22/68; revised 9/3/68; accepted 9/2/69

The authors thank Prof. Kinziro Tamari of the Department of Agriculture, Tohoku University, for his kind help and valuable suggestions.

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Freezer Burn of Animal Tissue. 7. Temperature Influence on Development of Freezer Burn in Liver and Muscle Tissue

SUMMARY—Various treatments found previously to affect the development of freezer burn at —10°C (Kaess et al. 1962a, 1967a,b) were applied to slices of beef liver and muscle stored at —20°C. While the general pattern of the development of freezer burn was similar to that obtained at —10°C, evaporative weight losses needed to produce a definite intensity of burn were significantly lower at —20°C. Although less freezer burn developed in low fat livers than in high fat livers at —10°C, fat content had no influence at —20°C. The layer of condensed cells at the evaporating surfaces was thinner at —20°C than at —10°C. Immersion of the tissues in solutions of glycerol or sodium chloride before freezing was effective in controlling freezer burn except with muscle slices cut across the fibers. In similar experiments carried out at —4°C desiccation of the tissue always resulted in the formation of the characteristic condensed layer at the surface but no freezer burn developed.

INTRODUCTION

EARLIER EXPERIMENTS (Kaess 1961; Kaess et al. 1962a; 1967a, b) showed that freezer burn in liver and muscle stored at −10°C was reduced when the rate of freezing was decreased, the reduction being greater in samples frozen with evaporative weight losses permitted during freezing than in samples frozen without weight loss. Increased rate of evaporation during storage also inhibited formation of burn to a small extent. Least freezer burn developed in livers of low fat content and with muscle frozen before or immediately after completion of rigor.

When weight losses were permitted during freezing and the tissue was frozen slowly, a layer of condensed cells was formed at the surface of the tissue. The depth of this layer increased during storage and development of freezer burn was delayed or prevented. When freezer burn cavities were present they occurred below the condensed layer. Tissue frozen rapidly without weight loss was highly susceptible to burn, and it was shown with this method of freezing that the positions of the freezer burn cavities and the condensed layer, which form during storage, are reversed. It was further shown that a condensed layer, similar to that seen in tissue slowly frozen with weight loss, developed in rapidly frozen tissue during storage when the tissue was dipped in a solution of glycerol, sorbitol, hexose, urea or sodium chloride before freezing (Kaess et al. 1962b, 1967a).

It seems likely that the development of this layer depends largely on the displacement of tissue into the spaces earlier occupied by ice crystals. If this assumption is correct, rising temperatures would facilitate the formation of the condensed layer, while with decreasing temperatures and increasing tissue viscosity its development would become less probable and freezer burn cavities would form. Because of the increasing emphasis on lower storage temperatures in cold stores, the previous experiments at −10°C were repeated at −20°C to provide further information on the effect of temperature on the development of the condensed layer and freezer burn. Some experiments were also carried out at −4°C.

EXPERIMENTAL

THE METHODS used in the preparation, freezing and storage of samples, estimation of weight loss and fat content, and treatment with dipping solutions, were essentially as reported before (Kaess 1961; Kaess et al. 1962a, b; 1967a, b). Samples of liver or muscle were frozen at two or three different rates (with and without evaporative weight loss) and stored at −4°C and −20°C in atmospheres of two different humidities.

Rates of freezing (the times to lower the temperature from 0°C to the storage temperatures −4°C and −20°C, respectively) were varied by changing the rate of conduction of heat from the trays holding the samples. To obtain a final temperature of −4°C the samples were frozen in the same freezing room used for earlier experiments (Kaess et al. 1962a). For rapidly lowering the temperature from 0°C to −20°C, the trays containing the samples were floated on mercury cooled to −30°C with dry ice, and placed in a freezing room with an air temperature of −25°C and an RH of 62%. The lower rates of freezing at −20°C were also obtained in this room. Samples were frozen either with weight loss permitted, or covered with plastic film impermeable to water vapor. Before storage the plastic film was removed.

The relative humidity in the experimental drums was controlled either with saturated salt solutions (with excess of salt) or a sulfuric acid solution (Kaess 1961). RH values of 89% and 65% (referred to ice) were obtained for saturated calcium nitrate solutions at −20°C and −4°C, respectively. The concentrations of sulphuric acid used to establish the RH of 97% (referred to ice) were 6.4 *N* at −20°C and 3.1 *N* at −4°C.

The dipping solutions used contained glycerol or sodium chloride at concentrations (30% and 20% w/w respectively) known to control freezer burn at a temperature of −10°C or at a higher concentration. Solutions containing both substances were also used.

For histological examination, samples from fresh tissue and from stored frozen and thawed tissue were fixed at room temperature in 10% buffered formalin (pH 6.5). This solution had a freezing point of approximately −4°C and was also used for freeze-fixing of samples from frozen slices and from slices with freezer burn at this freezing point. For freeze-fixation at −20°C the formalin concentration was increased to 66.5% v/v. Paraffin sections of the fixed material were cut at a thickness of 7μ (liver) and 10μ (muscle) and stained with hematoxylin-eosin.

RESULTS

Effects of temperature

Experiments at −20°C. Total evaporative weight losses for the initiation of freezer burn of intensities I_1 (just detectable colorless pin-points), I_2 (distinctive white dots or patches), and I_3 (continuous greyish-white areas of blemish) at −20°C and 89% RH were higher when samples were allowed to lose weight during freezing than when they were frozen while covered with a plastic film impermeable to water vapor. Figures 1 and 2 show total weight losses necessary for the initiation of freezer burn of intensities I_1 and I_3 in liver and muscle tissue.

The weight loss needed to produce a definite intensity of burn increased with freezing time. This increase was roughly the same for the lower intensities, but smaller for intensity I_3. The fat content of livers influenced the onset and development of burn only to a small degree. The weight losses needed to produce the three levels of intensities of burn in prerigor muscle were higher than those obtained for postrigor muscle. Storage of liver or muscle at the RH of 97% had no influence on the weight losses at which a definite intensity of freezer burn occurred.

Weight losses necessary for the onset of freezer burn were lower at −20°C than at −10°C, but this difference tended to disappear with samples frozen at a high rate. A statistical analysis was made of the influence of storage temperature (−20°C, −10°C), freezing time (3 levels), fat content (low and high) and type of freezing (with and without weight loss) on the development of freezer burn of intensity I_1. A complete set of data was available for 96 liver samples. The analysis confirmed that the weight losses necessary for the initiation of intensity I_1, were affected by storage temperature ($P < 1\%$), weight loss during freezing ($<1\%$), rate of freezing ($<1\%$) and fat content ($<5\%$), when pooled second and third order interactions were used as error term.

Significant first-order interactions were obtained between the effects of storage temperature, rate of freezing and type of freezing, due to the increase with the rate of freezing of total weight losses necessary

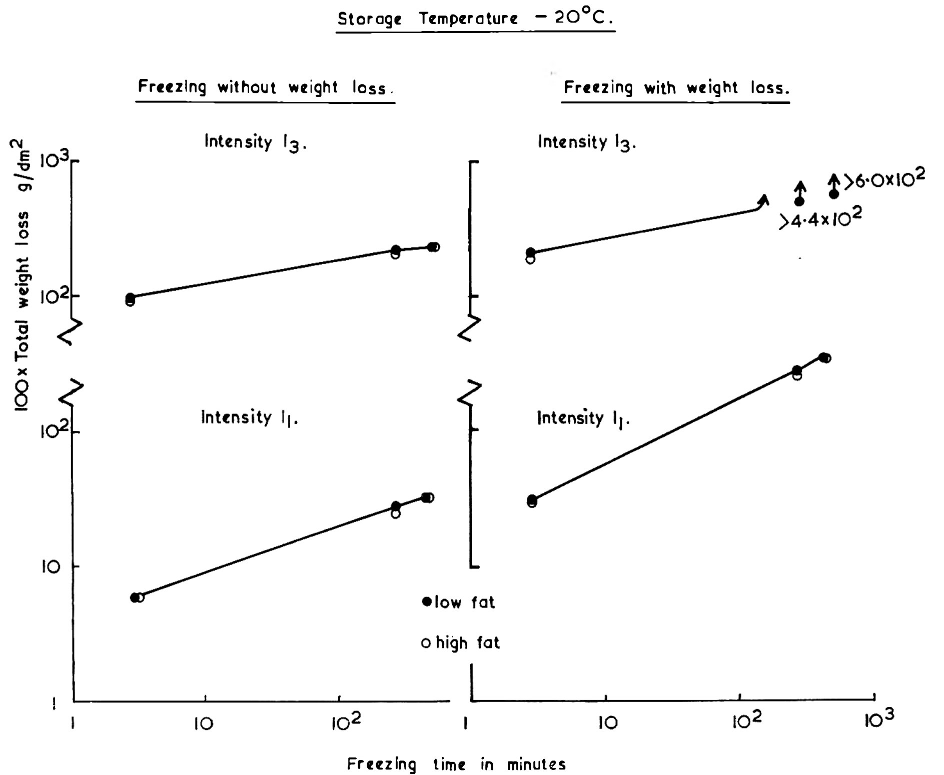

Fig. 1—Relation between total weight losses (freezing and storage) at which freezer burn of intensities I_1 and I_3 was first apparent, and freezing times of samples of low and high fat livers from adult cows, stored at −20°C and 89% R.H. after freezing. Fat contents were low fat 4·4–9.1%, high fat 19.1–33.1%.

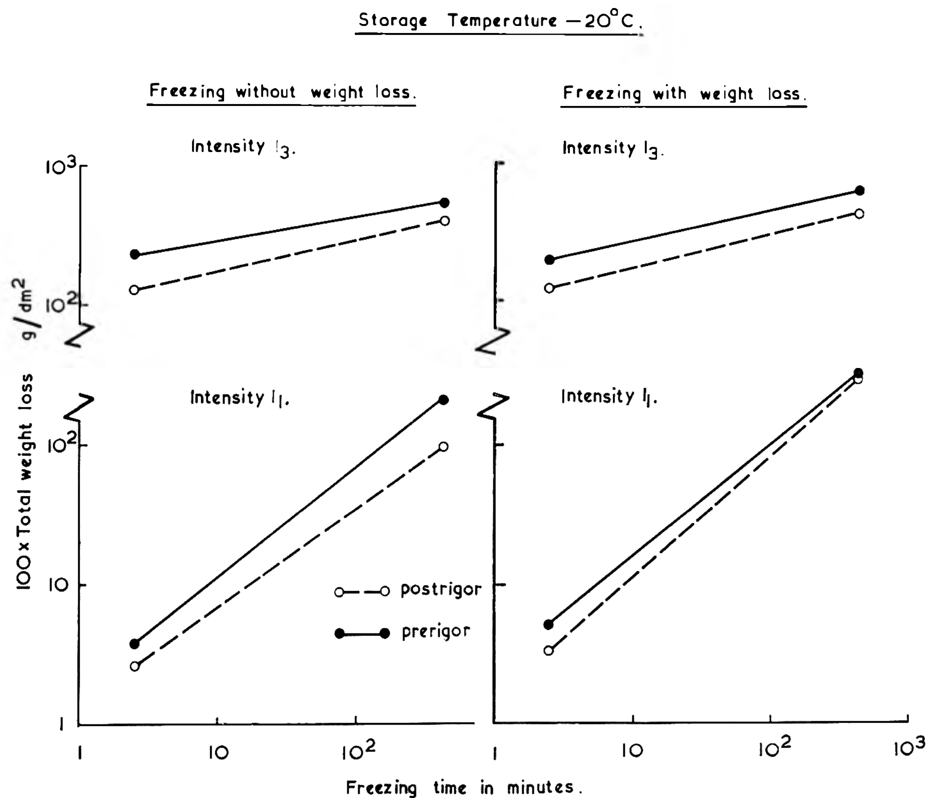

Fig. 2—Relation between total weight losses (freezing and storage) at which freezer burn of intensities I_1 and I_3 was first apparent and freezing times of steer semitendinosus muscle initially held at 0°C, then frozen prerigor or postrigor and subsequently stored at −20°C.

to introduce intensity I_1. This increase varied with storage temperature and type of freezing.

A similar analysis of variance carried out with the data for total weight losses needed to induce intensities I_1 and I_2 in prerigor and postrigor muscle samples, frozen at two rates (rapid and slow), with or without weight loss, and stored at $-20°C$ or $-10°C$, confirmed that at the lower temperature a definite intensity of burn was initiated at significantly lower weight loss ($P < 1\%$).

Using the term for experimental error which was comparable with high order interactions, all other main order effects were also significant ($P < 1\%$). Variation of rates of diffusion due to difference in composition between and within muscles, variation of rheological properties of the tissue surrounding ice crystals and changes of such conditions introduced by the various treatments and the course of rigor, were probable causes for several significant first order and two second order interactions.

Experiments at −4°C. No traces of freezer burn developed at −4°C although total weight loss of liver reached 18.6 g/dm² and muscle 21.0 g/dm² during storage. This temperature effect was independent of the rates of freezing (with or without evaporative weight loss), the fat content of the livers and the physiological condition of muscles (prerigor and postrigor).

Dipping treatments

Experiments with liver. The onset of freezer burn in liver stored at −20°C was considerably retarded when samples were dipped for 10 min in glycerol or sodium chloride solutions and then frozen in 2.7 min without weight loss. At a weight loss of 3.5 to 4 g/dm² samples showed a definite darkening of color in comparison with fresh frozen controls kept separately in air-tight containers. After a treatment with a 30% (w/w) glycerol solution or a 20% (w/w) sodium chloride solution, the onset of burn occurred at higher weight losses than the onset of dark discoloration.

Experiments with muscle. Although the direction of fibers on the muscle surface did not influence the onset of freezer burn in untreated samples, it influenced the protective effect of dipping solutions. A glycerol concentration of 50% gave sufficient protection for longitudinally cut slices, but with slices cut transversely the burn set in before dark discoloration, even when 100% glycerol was used. A sodium chloride solution of 30% was less effective in inhibiting burn formation than a glycerol solution of 50%. The application of a mixture of saturated glycerol (75%) and saturated sodium chloride solution (25%) did not increase the protective effects.

Histological observations

Observations with liver. A condensed layer did not develop in tissue frozen rapidly without evaporative weight loss and stored at −20°C. This is in contrast to the results obtained at −10°C (Kaess et al. 1962a; 1967a) when a thin layer of condensed cells was formed. In tissue frozen with weight loss after prolonged storage at −20°C, the condensed layer extended hardly beyond the width of the surface membrane. In liver frozen slowly with or without weight loss a condensed layer was formed on the exposed surfaces. It was however thinner and seemed less compact than in tissue frozen and stored

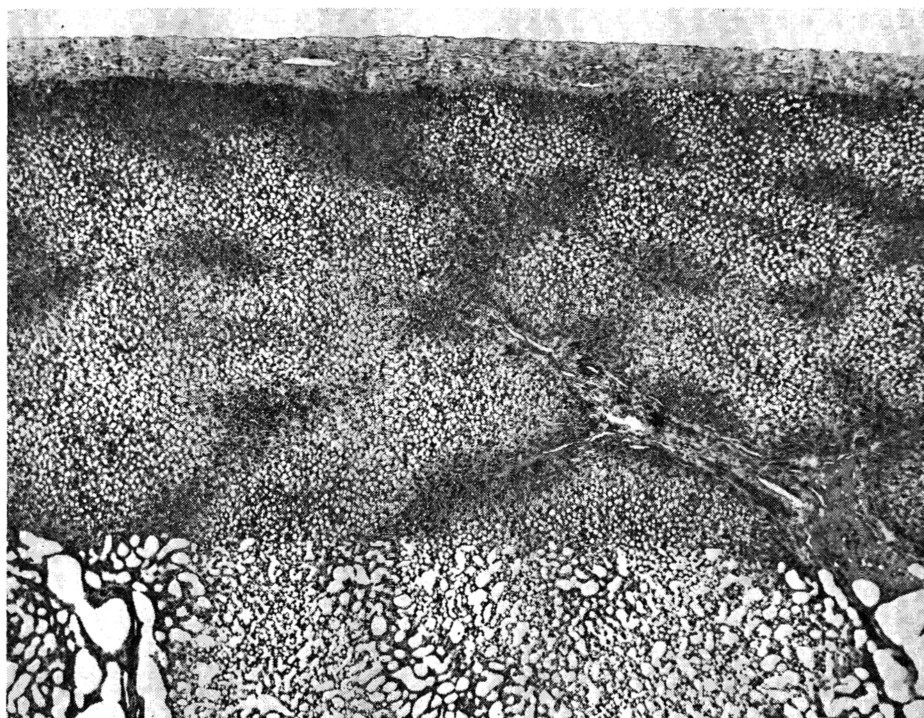

Fig. 3—Section from liver (26.9% fat), frozen in 2.3 min. without weight loss and stored at −4°C. Weight loss in store 11.3 g/sq.dm. Sample freeze-fixed at −4°. Hematoxylin-eosin stain. Magnification × 50.5.

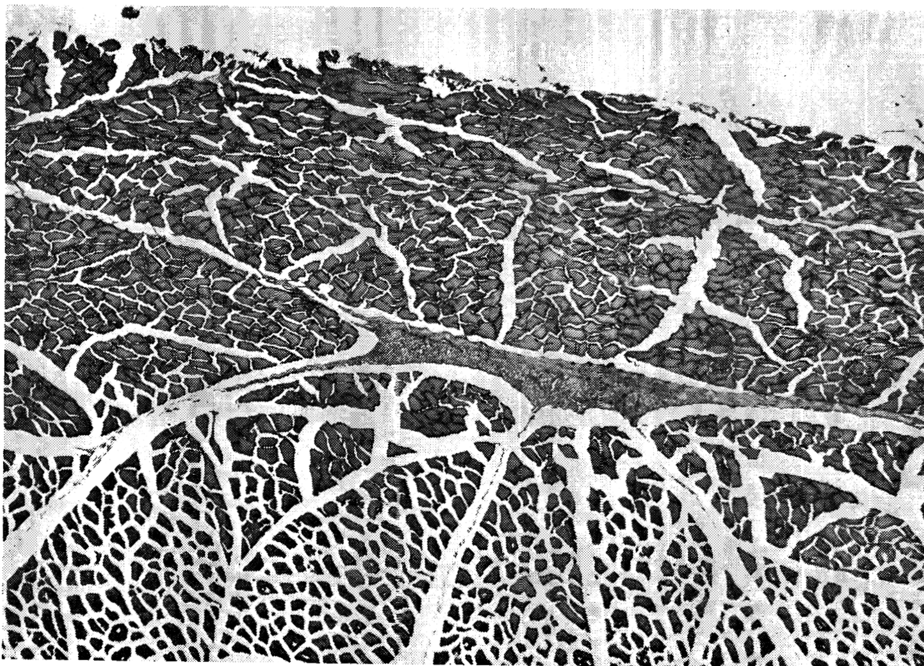

Fig. 4—Micrograph of semitendinosus muscle frozen postrigor in 2.3 min. without weight loss. Storage temperature −4°C. Weight loss in store 10.1 g/sq.dm. Fixation in 10% formalin at 23°C. Hematoxylin-eosin stain. Magnification × 50.5.

under comparable conditions at −10°C. Freezer burn cavities developed directly at the surface of liver frozen rapidly without weight loss but below the condensed layer in slowly frozen livers.

In liver stored at −4°C no freezer burn cavities could be detected and only a condensed layer formed (Fig. 3), an effect which was independent on fat content, method of freezing, or the RH of the storage atmosphere.

Observations with muscle. The development of the condensed layer and of the freezer burn cavities, in muscle tissue stored at −20°C, generally followed a pattern similar to that seen in liver tissue. However, in muscle frozen before completion of rigor, freezer burn cavities were frequently initiated intracellularly, while in muscle frozen postrigor, cavities formed extracellularly.

As with liver, no freezer burn cavities developed at a storage temperature of −4°C (Fig. 4), even in rapidly frozen postrigor muscle which was most susceptible to freezer burn at lower temperatures.

Effects of dipping treatments. The penetration of dipping solutions into the surface of liver or muscle tissue (Kaess et al. 1962b) promoted the formation of considerably thicker condensed layers than were seen in untreated controls.

Cavities were not seen in muscle slices which were cut longitudinally to the fiber direction, treated 10 min with 65% glycerol before freezing and stored at −20°C till evaporative weight loss reached 9.8 g/dm^2. However, sections from slices cut transversely to the fiber direction and dipped in 100% glycerol, or from longitudinal slices dipped in 20% solutions of sodium chloride before freezing and storage, always contained freezer burn cavities. Solutions of glycerol (30%) and sodium chloride (20%) were effective in suppressing cavity formation in liver tissue.

DISCUSSION

RESULTS have shown some marked differences in the development of freezer burn and the condensed layer at different storage temperatures.

Freezer burn cavities probably represent the spaces, initially occupied by ice crystals, which continue to exist after the sublimation of the ice, due to greatly reduced flow properties of the tissue at low temperature (−20°C). There is no equivalent at −20°C of cavities forming within the condensed layer as seen at −10°C in tissue frozen rapidly without weight loss. At −10°C tissue displacement seems less restricted and cavities initiated in the condensed layer grow by shrinkage of the tissue along channels formerly occupied by ice crystals.

Figure 3 suggests that the formation of a condensed layer is caused by movement of tissue into the spaces earlier occupied by ice crystals, after the sublimation of the ice. The flow properties of the tissue at the freezing point seem to allow the tissue to move into the space provided by subliming ice during the process of freezing with evaporative weight loss. In the frozen state, tissue free of ice is compressed between ice crystals which have a larger volume than the water from which they originated. The release of this tension, as the ice sublimes, may assist movement of tissue and the consequent formation of a condensed layer at low temperatures.

The shrinkage of the condensed layer near the surface, due to dehydration during storage, also would introduce tension that would tend to move tissue into cavities from which ice had sublimed. The development of the condensed layer was favored by the presence of large ice crystals (slow freezing) and was predominant during storage at a relatively high temperature (−4°C).

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Ms. received 8/1/68; accepted 9/2/68.

Dr. A. Howard's programming and processing of statistical data with the G.E.225 Computer at the University of Queensland's Computer Centre is gratefully acknowledged. Mrs. M. Shevill and Mr. R. Turner skillfully assisted in the experimental work.

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Some Sensory Effects of Hydrocolloid Sols on Sweetness

SUMMARY—Sweetness-texture interactions in cornstarch, guar and carboxymethylcellulose were assessed by a trained sensory panel. Viscosity curves over a range of sucrose levels from 2.5 to 5.5% in the three gums were determined using a Brookfield viscometer. The relationship between viscosity curves and sweetness perception determined by rates of sweetness recognition, matching of equisweetness in different gums, apparent levels of sweetness and ranking of series of gums in order of sweetness indicated that gums with less viscosity drop as shear rates increase tend to mask sweetness perception.

INTRODUCTION

INFORMATION about the sensory properties of hydrocolloid sols becomes more important as their use in foods for bodying, thickening, bulking, masking aftertastes, flavor-blending, controlling freezing and melting, and improving carbonation retention (Glicksman et al., 1966) increases. Mouthfeel of hydrocolloid sols is complicated by the interrelationships of their molecular weights, molecular structure and bonding, degree of particle dispersion and other chemical characteristics. The addition of electrolytes (Zabik et al., 1967; Masson et al., 1955), other foreign chemical radicals, pH (Pederson, 1966) and the temperature of the sol also influence mouthfeel.

The mouthfeel of many gums has been correlated with the rate of viscosity drop as shear rate increased. Gums were classified into three sliminess groups, those showing most shear thinning being the least slimy (Szczesniak et al., 1962).

Work on the interactions of hydrocolloid sol textures with the basic tastes is limited. Mackey et al. (1956) reported

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that the 4 basic tastes were easier to detect in liquids than in gels. Further studies by Mackey (1958) showed that caffeine, quinine and saccharin were slightly less discernible in water with methylcellulose than in plain water. These reports suggest that systems showing more shear thinning in the mouth would allow easier perception of the basic tastes.

Stone et al. (1966) studied the effects of certain gums on sweetness and reported contradictory results. While sensitivity to threshold levels of sucrose decreased with increased gum sliminess, indicating the more slimy gums masked sweetness, the rankings of relative sweetness at sucrose levels of 1, 2, 5 and 10% tended to be greater with more slimy gums.

The present investigation used other sensory methods to further examine the effects of cornstarch, guar and carboxymethylcellulose (CMC), three hydrocolloids varying in shear thinning performance, on sweetness perception.

MATERIALS & METHODS

Materials

Three gums, representing the three groups of sliminess as determined by Szczesniak et al. (1962) were obtained commercially. These gums were cornstarch (The Canada Starch Co. Ltd.), guar (Stein-Hall, Jaguar A-20-D) and CMC (sodium carboxymethylcellulose, high viscosity, British Drug Houses Ltd.). The gum concentrations were selected to give a viscosity of 5000 centipoises at a shear rate of 0.5 rpm at 23°C.

Cornstarch, 2.7%, was blended into distilled water and heated with stirring to 90°C. Guar, 0.81%, was dispersed in distilled water at 25°C. The CMC, 1.55%, was blended into distilled water, placed over high heat and beaten at a high speed with an electric beater for 5 min for each 1000 ml water. Each sol was portioned for the addition of sucrose. The rates and times of mixing and beating were standardized for each hydrocolloid.

Samples of each sol at each of seven levels of sucrose concentration were set aside for viscosity tests and the remaining portions were served into ¾ oz containers which were capped and brought to 24°C for sensory testing.

Viscosity measurements

The sols were allowed to stand for at least 3 hr. Viscosity measurements were made on each sol with no sucrose added and at each sucrose concentration at 23°C using a Brookfield viscometer, model RVT, on a helipath stand with spindle T-A at speeds of 0.5, 1.0, 2.5, 5.0, 10.0, 20.0, 50.0 and 100.0 rpm. Three replicates were performed.

Panel selection

From an original group of 11 people, seven panel members were selected on their ability to rank two sets of cornstarch sols of the following sucrose concentrations:

a) 2, 4, 6, 8 and 10%
b) 2, 3, 4, 5 and 6%

Table 1—Hydrocolloid combinations for one replicate of sensory evaluation.

Test	Hydrocolloid X	Series
3.5% Sucrose		
1.	Cornstarch	Guar
2.	Cornstarch	CMC
3.	Guar	Cornstarch
4.	Guar	CMC
5.	CMC	Cornstarch
6.	CMC	Guar
4.5% Sucrose		
7.	Cornstarch	Guar
8.	Cornstarch	CMC
9.	Guar	Cornstarch
10.	Guar	CMC
11.	CMC	Cornstarch
12.	CMC	Guar

The results were scored according to the method of Kramer et al. (1966).

Panel training

During training, the seven panelists were asked to match a hydrocolloid sol of unknown sucrose concentration to one in a series in which the concentrations varied by 0.5% sucrose over a range of 2.5–5.0%. During this period sols used for the matching were in various combinations as follows: cornstarch to cornstarch, guar to guar, CMC to CMC and cornstarch to CMC. Because the panelists reported the 3.5 and 4.5% sucrose solutions in cornstarch to be even sweeter than the 5.0% sucrose in CMC, the range of sucrose concentrations in the CMC for the actual test period was raised to 3.0–5.5% sucrose. The panelists rapidly achieved a high degree of accuracy during the training period.

Sensory evaluations

These tests were conducted in fluorescent-lit, individual booths in a quiet, air-conditioned room. Panelists were provided with distilled water for rinsing their mouths.

For each test, each panelist was presented with one hydrocolloid sol sample, X, with an unknown sucrose concentration of 3.5 or 4.5%, and a series of six samples of a single hydrocolloid other than X. The series ranged in sucrose concentration from 2.5–5.0% in cornstarch and guar, and from 3.0–5.5% in CMC. The series was coded and presented in a randomized order to the panelist.

Panelists were asked a) to record the time, using a stop watch, required to recognize the sweetness taste in X (this was repeated three times in each test and an average taken), b) to arrange or rank the series in order of sweetness; c) to match X to one sample in the series.

There were four replications of the experiment (Table 1), thus timing and matching were carried out in 48 tests. Complete sets of rankings were obtained in 39 of these tests—13 tests for each gum. The test order was randomized within replications and two to three tests were performed at one sitting.

Analysis of data

Viscosity. Viscosity data from readings at eight shear rates and six sucrose levels of 0, 3.0, 3.5, 4.0, 4.5 and 5.0%, for the three gums were analyzed as an $8 \times 6 \times 3$ factorial design (Cochran et al. 1957). The 2.5 and 5.5% sucrose levels were omitted to confine the analysis to sucrose levels used with all gums. To compare means of sucrose levels at specific shear rates, sub-unit analyses of variance were performed.

Rate of sweetness recognization. Analysis of variance according to the split-plot design (Cochran et al., 1957) was applied to the time in seconds required by each panelist to recognize sweetness in each hydrocolloid at the 3.5% and 4.5% sucrose levels. There were eight replications and "t" values were calculated to determine significant differences between rates of sweetness recognition between gums.

Ability to match equisweetness in different gums. To estimate numerically the effect of textural differences between the gums when matched for equisweetness, a scoring method was devised which indicates matching accuracy. For example: a panelist was asked to match a cornstarch sol, X, of 3.5% sucrose to one of the samples in a series of six guar sols ranging in concentration from 2.5–5.0% sucrose by increments of 0.5%. If X was matched to the 3.5 sample in the series, a score of 0 was assigned; if matched to 3.0 or 4.0 a score of 1 was given; if matched to 2.5 or 4.5 a score of 2 was given; if matched to 5.0 a score of 3 was given. Thus the more accurate the matching, the lower the score. Analysis of variance was applied to these scores.

Effect of different gums on apparent level of sweetness. Further analysis of matching data was performed to determine whether or not certain hydrocolloids affected the apparent sweetness of the samples. The levels of sweetness assigned to the X sample by the panelists are referred to as the *apparent* sweetness of X. The *actual* level of sweetness of X was either 3.5 or 4.5% sucrose. Confidence intervals of the apparent sweetness were determined for each of the six combinations of gums. The two levels of sucrose, 3.5 and 4.5%, were analyzed separately. Thus, there were 12 sets of confidence intervals. If the actual sucrose level lay outside these intervals, then the effect of the hydrocolloid of sweetness was considered to be significant.

Ranking Ranking refers to the ability to arrange the six levels of sucrose concentration differing by 0.5% sucrose occurring in the same hydrocolloid sol in order of sweetness. Analysis of variance was applied to inversion values from rank scores calculated as described by Amerine et al. (1965).

RESULTS & DISCUSSION

Viscosity measurements

Factorial analysis of the viscosities of the three gums as shear rates and sucrose levels increased showed significant differences in all main effects (Table 2). The significant first order interactions show that the gums behaved differently in response to changes in both shear rate and sucrose level.

Increasing shear rates had a greater thinning effect on cornstarch than on either guar or CMC (Fig. 1). Guar thinned slightly more than CMC but the general viscosity patterns of guar and CMC did not differ as markedly as expected from

Table 2—Analysis of variance of viscosity changes in 3 hydrocolloids over 8 rates of shear and 6 sucrose levels[1] (3 × 8 × 6 factorial).

Source	Degrees of freedom	Sum of squares	Mean square	F Calculated	F Tabulated (P = 0.01)
Total	431	12490210			
Reps	2	44760	22380		
Sugar levels	5	81429	16286	6.49	3.02
Rates of shear	7	9264796	1323542	528.00	2.80
Hydrocolloids	2	1827150	913575	364.55	4.60
Sugar × Rates	35	104281	2979	1.19	1.70
Sugar × Hydrocolloid	10	75642	7564	3.02	2.32
Rates × Hydrocolloid	14	258662	18476	7.37	2.18
Sugar × Rates × Hydroc.	70	116546	1665		
Error	286	716944	2506		

[1] (0, 3, 3.5, 4, 4.5, and 5%).

Table 3—Rates of sweetness recognition in hydrocolloid sols.

% Sucrose in hydrocolloid	Hydrocolloid	Mean time to recognize sweetness (sec)
3.5	Cornstarch	1.4[a]
	Guar	1.8[b]
	CMC	1.8[b]
4.5	Cornstarch	1.1[a]
	Guar	1.3[a]
	CMC	1.8[b]

[ab] Values bearing different superscripts are significantly different at P = 0.05 by "t" tests where error variance = 0.32 with 35 df.

earlier reports (Szczesniak et al., 1962).

Increasing the sucrose level from 0 through 5% had no distinct effect on the viscosity of either guar or CMC. Carlson et al. (1965) have reported a distinct viscosity reduction in guar sols with sugar increments of 10, 20, 30, 40, 45, 50 and 65%. In the present work, cornstarch sols thinned more at slower shear rates as sucrose level increased. This thinning effect of sucrose on cornstarch was significant at shear rates up to 20 rpm (P = 0.01 where F = 5.75 at 6/12 df) but not at 50 or 100 rpm (Fig. 1). The shear rate exercised by the tongue in the mouth is believed to be about 30 to 50 rpm (Szczesniak et al., 1962).

The fact that the cornstarch sols were thinner in the presence of sucrose, while guar and CMC were not, suggests that guar and CMC compete more effectively than cornstarch for water.

Sensory evaluations

Rates of sweetness recognition. Times required to recognize a sweet sensation varied with the shear-thinning tendency of the gums (Table 3). At both sucrose concentrations perception of sweetness was delayed in CMC when compared to cornstarch. The rate of sweetness detection in guar was not consistent between sucrose concentrations. Sweetness recognition times for guar and CMC were closer than those for guar and cornstarch, which would be expected if sweetness perception is related to the viscosity curves (Fig. 1).

There was a marked difference between panelists in rates of sweetness recognition (F = 14.3 at 6/249 df), confirming other reports of wide individual variation in the detection of primary tastes (Stone et al., 1966; Schutz et al., 1957).

Also, a significant interaction between gums and panelists (F = 3.2 at 30/249 df) indicates that mouthfeel of gums affected the rate of sweetness recognition to a greater degree in some panelists than in others. One panelist reversed the order between guar and CMC.

If certain hydrocolloids delay the rate of sweetness recognition, they may also delay recognition of other flavor notes. Among the generally desirable characteristics of a food product are rapid development in the mouth of full flavor and perception of the character notes in close succession (Cairncross et al., 1953). Some hydrocolloids may adversely affect these characteristics.

Matching equisweetness in different gums. Judgments of equisweetness between sweetened hydrocolloid sols, assessed by assigning scores for accuracy of matching, showed differences in matching precision between different comparisons (Table 4). However, calculated "t" values showed no significant differences between mean scores. These scores did show a tendency for greater accuracy in matching at the higher sucrose concentration. More accurate matching was consistently evident when guar and CMC were compared than when either was matched with cornstarch. That is, sweetness matching appeared to be easier between gums showing similar viscosity curves.

Effect of different gums on the apparent levels of sweetness. The confidence inter-

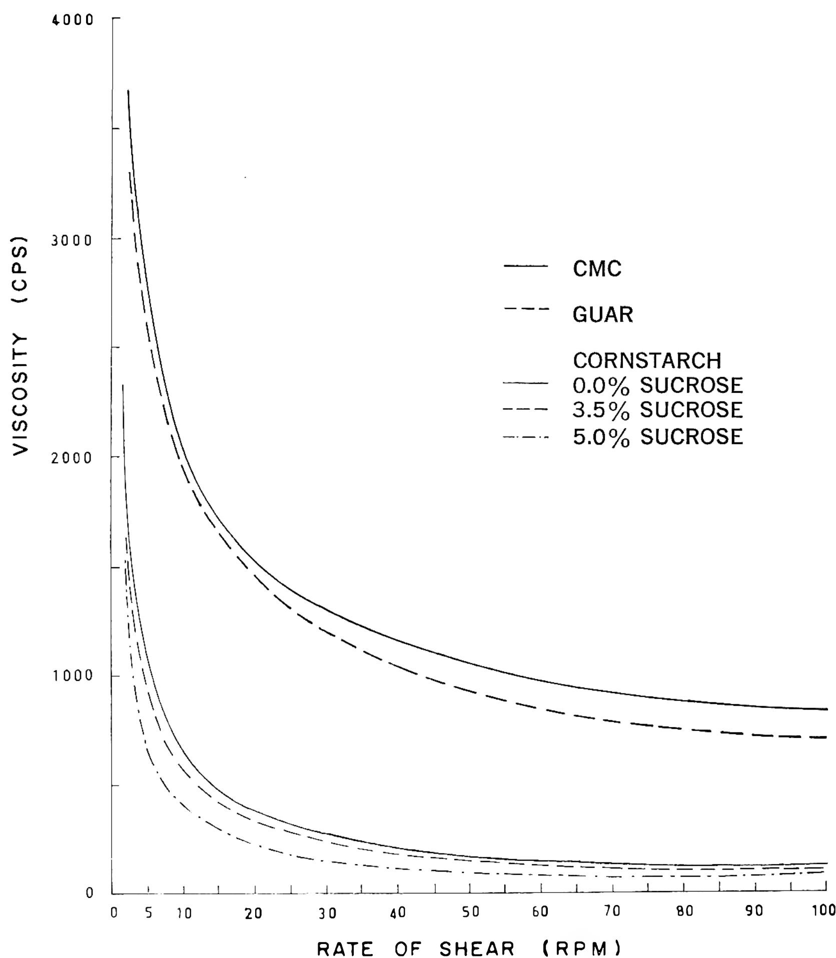

Fig. 1—Viscosities of sweetened hydrocolloid sols with increasing rates of shear. Data used for guar and CMC curves were means of all sucrose levels.

Table 4—Precision in matching equisweetness of different gums.

Gums compared[1]	Mean scores[2] 3.5% Sucrose	4.5% Sucrose
Cornstarch to CMC	1.77	1.20
Cornstarch to guar	1.30	1.00
Guar to CMC	1.00	0.89

[1] Difference between comparisons significant at P = 0.05 with 5/35 df.
[2] Zero indicates totally correct matching.

Table 5—Confidence intervals of apparent sweetness of gums.

Actual sucrose level	Hydrocolloid Series	X	Confidence intervals	Interpretation
3.5%	Cornstarch	Guar	3.05, 3.35	Cornstarch seems sweeter than guar
		CMC	3.57, 4.15	Cornstarch seems less sweet than CMC[1]
	Guar	Cornstarch	4.22, 4.60	Guar seems less sweet than cornstarch
		CMC	3.81, 4.15	Guar seems less sweet than CMC[1]
	CMC	Cornstarch	4.26, 4.84	CMC seems less sweet than cornstarch
		Guar	3.43, 3.89	CMC seems equal to guar
4.5%	Cornstarch	Guar	3.79, 4.17	Cornstarch seems sweeter than guar
		CMC	3.88, 4.36	Cornstarch seems sweeter than CMC
	Guar	Cornstarch	4.69, 4.91	Guar seems less sweet than cornstarch
		CMC	4.60, 4.90	Guar seems less sweet than CMC[1]
	CMC	Cornstarch	4.46, 5.00	CMC seems equal to cornstarch
		Guar	4.02, 4.56	CMC seems equal to guar

[1] These relationships are exceptions to the general trend.

vals calculated from the sweetness levels provided a means for determining the effect of texture in enhancing or depressing sweetness (Table 5). Where the confidence interval of the apparent sweetness is lower than the actual sucrose level of X, then the hydrocolloid in X depressed its sweetness and thus seemed less sweet than the hydrocolloid in the series to which it was compared. With three exceptions in the 12 comparisons shown, the gums exhibiting less thinning with increasing shear rates either depressed perception of sweetness or had no effect.

From Table 4, it can be seen that cornstarch was always sweeter than guar and tended to be sweeter than CMC. Guar was judged less sweet or equally as sweet as CMC. This lack of any distinct effect between guar and CMC might be accounted for by the similarity in viscosity curves.

Ranking of hydrocolloid series in order of sweetness. Sucrose levels could be ranked more accurately in cornstarch than in CMC (Table 6). The incidence of ranking error for guar was intermediate between cornstarch and CMC suggesting that accuracy in ranking sweetness becomes greater in gums exhibiting greater thinning as shear rates increase. This again suggests the thesis that gums with less drop in viscosity as shear rates increase tend to mask sweetness perception.

The analysis of variance of the inversion values showed no difference in replications (F = 0.6 at 12/24 df). This indicates the panelists' ranking ability was constant throughout the test period. The significant difference between panelists (P = 0.01 where F = 9.76 at 6/213 df) verifies the investigators' observation that although four of the panelists attained remarkable accuracy in ranking before the actual test period began, the remaining three maintained a lower level of accuracy throughout the test period.

Table 6—Comparison of mean inversion values in ranking.

Hydrocolloid	Mean inversion values[1]
Cornstarch	0.46[a]
Guar	0.57[ab]
CMC	0.89[b]

[1] Zero indicates totally correct ranking.
[ab] Values bearing different superscripts are significantly different at P = 0.05 by "t" tests, where error variance = 1.25 with 24 df.

The trends in all the sensory tests, rates of sweetness recognition, matching of equisweetness in different gums, apparent levels of sweetness in these gums and ability to rank series of gums in order of sweetness seem to indicate the validity of the thesis that hydrocolloid sols that thin less rapidly as shear rates increase mask perception of sweetness.

More conclusive results might be obtained by more closely simulating mouth conditions by a) adjusting the concentration of the hydrocolloids being studied so that their viscosities are similar at the shear rates of between 30 and 50 rpm that occur in the mouth, and b) taking viscosity readings at mouth temperature of 37°C.

In the present investigation, the panelists became highly sensitive to sweetness so that any effects that the hydrocolloids may have had on sweetness perception were likely minimized. Several of the panelists reported that they increased the shear rate in their mouths to increase their accuracy in the tests. Obviously, when this occurs, a normal eating situation no longer exists and the results of such a test cannot be directly interpreted for application in the development of food products.

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Ms. received 6/18/68; revised 9/9/68; accepted 8/20/69.

This study was supported by a research grant from the University of Manitoba Graduate Research Fund.

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Hydrolytic Enzymes in Bovine Skeletal Muscle. 3. Activity of Some Catheptic Enzymes

SUMMARY—The present study was undertaken to examine the effects of pH, substrate concentration, time of incubation and temperature on proteolytic activity in the skeletal muscle of the bovine and to determine in this tissue enzymatic activities resembling those of cathepsins B, C and D. Maximal proteolytic activity was observed at either pH 3.8 or at pH 4.8 depending upon the enzyme studied. Activities of cathepsins B, C and D were determined in both supernatant and particulate fractions of two bovine muscles and rat liver by the use of synthetic or natural substrates. Comparison of the activities in bovine muscle with those in rat liver showed that total cathepsin B activity in skeletal muscle was approximately 52% of the activity obtained in rat liver whereas cathepsins C and D activities were approximately 2 and 25%, respectively.

INTRODUCTION

STUDIES BASED on histochemical and biochemical methods have indicated that intracellular proteolytic enzymes (cathepsins) are present in skeletal muscle (Gutmann et al., 1948; Koszalka et al., 1960a, 1960b; Snoke et al., 1950; Weber, 1963). Studies on the role of the cathepsins during the aging (storage) of beef have been hampered by observations which suggest that proteolytic activity in bovine skeletal muscle is low in comparison with the activity of similar enzymes in organ tissues such as liver, spleen, lung and kidney (Landmann, 1963). However, limited studies indicate that proteases of relatively low activity are present in bovine skeletal muscle, that they are active in the pH ranges 4.0 to 6.5 and 8.5 to 9.5, and that they are possibly identical to the cathepsins isolated from spleen and other organ tissues (Fruton et al., 1941).

Bodwell et al. (1964) were unable to attribute the proteolytic activity in extracts of bovine skeletal muscle to either cathepsin B or C. They concluded that the enzyme fraction obtained by them displayed endopeptidase activity similar to that of cathepsin A. Bodwell et al. (1964) and Sharp (1963) concluded that the sarcoplasmic proteins were the major substrates for the natural proteolytic enzymes. Randall et al. (1967) examined the proteolytic activity of the water-soluble proteins of bovine skeletal muscle separated by starch gel electrophoresis and presumed the presence of proteolytic activity resembling that of cathepsins B and C.

The present paper describes some properties of proteolytic activity in bovine skeletal muscle. Activities of cathepsins B, C and D were determined in both supernatant and particulate fractions of two bovine muscles and rat liver. Since the cathepsins are classified on their ability to hydrolyze certain substrates (Tallan et al., 1952), cathepsin B activity was determined by the use of benzoyl-L-argininamide, cathepsin C by the use of glycyl-L-tyrosinamide and cathepsin D by the use of denatured hemoglobin as the substrates. The activity of aryl sulfatase in these tissues was also determined since the enzyme has been used as a lysosomal "marker."

EXPERIMENTAL

PRELIMINARY investigations were conducted on the proteolytic activity of bovine skeletal muscle using a soluble fraction from muscle as the enzyme source and endogeneous muscle proteins as the substrate.

Samples of skeletal muscle, namely *obliquus abdominus externus* (flank) or *psoas major* (loin) were obtained from beef carcasses 1 to 2 hr post-slaughter. The samples were placed in polythene bags, chilled on ice and taken promptly to the laboratory.

Preparation of enzyme source

The method used was a modification of the method described by Berlinguet et al. (1966). Excess fat and connective tissue were dissected away from the muscle and the tissue was minced in an ordinary food chopper. Ten grams of minced muscle tissue was homogenized with 40 ml of 2% KCl in a Waring Blendor for 1/2 min. The homogenate was filtered through four layers of cheesecloth to remove any remaining connective tissue, etc. The filtrate was dialyzed for 18 hr at 2 to 4°C with four changes of 2% KCl solution. The dialyzed homogenate was centrifuged for 2 hr at 36,900 × G.

The supernatant was decanted and the residue was washed twice with 20 ml of 2% KCl solution and centrifuged at 36,900 × G for 2 hr after each wash. The washings and the original supernatant were combined and lyophilized. The material thus obtained constituted the enzyme source. The enzyme solution was prepared by dissolving 15.0 mg of the lyophilized enzyme source in 30 ml buffer solution of the desired pH.

Preparation of endogenous substrate

The residue obtained as described above was blotted between several layers of filter paper to remove excess moisture, was ground in a mortar and used as the source of substrate. One g of the substrate material was dispersed in 20 ml buffer solution of the desired pH.

Determination of proteolytic activity

Proteolytic activity of the enzymes was determined by measuring at 280 mμ the release of tyrosine and tryptophan from the endogenous muscle proteins used as substrate according to the method described by Koszalka et al. (1961). The reaction mixture contained 2.0 ml of buffer solution of the desired pH, 0.5 ml of the enzyme solution and 1.0 ml of the substrate suspension. The mixture was incubated at 37°C for 2 hr. In each case, the enzyme source in buffer solution was pre-incubated at 37°C for 5 min to attain a constant temperature before the substrate was added.

The reaction was stopped by the addition of 3.5 ml of cold 10% tricholoacetic acid (TCA) and the tubes were left to stand overnight at 4°C. The reaction mixture was centrifuged at 12,800 × G for 10 min in a refrigerated centrifuge. Proteolytic activity was expressed as the absorbance at 280 mμ of the reaction mixture incubated at 37°C for 2 hr and read against a control at zero time of incubation. The analysis was carried out in duplicate.

Total nitrogen was determined on each lyophilized fraction of muscle tissue (5 mg) according to the method described by Lang (1958).

Determining cathepsins B, C, D and aryl sulfatase activities

Enzyme activities were carried out on supernatant and particulate fractions of the two bovine skeletal muscles (loin and flank). Concurrently, assays were carried out for comparative purposes on similar fractions prepared from rat liver since the activities of these enzymes in rat liver have been reported by others (Bouma et al., 1964).

Sprague-Dawley male rats 3 months of age were killed by decapitation, the liver removed and weighed in 20 ml of cold 0.15*M* KCl. The volume of KCl solution was adjusted to give a tissue to extractant ratio of 1:4. The tissue was minced with scissors and homogenized in a Potter-Elvehjem homogenizer for six strokes. The homogenate was centrifuged at 39,600 × G for 40 min. The supernatant was decanted and the residue was washed twice with 15 ml of 0.15*M* KCl.

The washings and original supernatant were combined and lyophilized and will be referred to as the supernatant fraction. The residue was also lyophilized and will be referred to as the particulate fraction. The two bovine muscle tissues were processed in a similar manner except that these tissues in KCl solution were subjected to the action of a Waring Blendor for 1 min prior to homogenization.

Assay methods for cathepsins B, C and D were modifications of the methods described by Bowers et al. (1967).

Cathepsin B. Five milligrams of the lyophilized supernatant fraction were dissolved in 1 ml of 0.1*M* citrate buffer (pH 4.8) containing 0.04*M* cysteine and placed in the outer well of a Conway microdiffusion cell (Obrink type—Fisher Scientific). The substrate (0.5 ml of 10 mM benzoyl-L-argininamide—Mann Research Laboratories Inc., N.Y.) was added and incubation was carried out for 1 hr at 37°C. The incubation period was terminated by the addition of 1.0 ml of 5% TCA and 1.0 ml of buffered K_2CO_3 (45%, pH 11.0) was added to release the NH_3 which was absorbed in 1.0 ml of 0.015 N H_2SO_4 in the center well of the cell.

The diffusion time was 50 min. The blank determination was carried out exactly as described for the sample except that the substrate solution was replaced by 0.5 ml citrate buffer. The activity of the particulate fraction was determined exactly as described above except that one drop of Triton X-100 was added to the incubation mixture.

The acid solution containing the NH_3 was transferred from the center well to a graduated tube (15 ml) and diluted to 3.0 ml with glass-distilled water. The color was developed as described by Russell (1944) and read in a spectrophotometer at 525 mμ.

Cathepsin C. The procedure was the same as that described for Cathepsin B, except that 0.5 ml of 10 mM glycyl-L-tyrosinamide (Mann Research Laboratories Inc., N.Y.) was used as the substrate.

Cathepsin D. Five milligrams of the lyophilized supernatant fraction were dissolved in 3.0 ml of 0.1*M* lactate buffer (pH 3.8) and 0.5 ml of a 2% solution of denatured hemoglobin was added as substrate. The mixture was incubated in a water bath at 37°C for 1 hr and the reaction was stopped by the addition of 2 ml of cold 5% TCA. The mixture was allowed to stand for 30 min at 4°C and then centrifuged. The color was developed by the addition of 5.0 ml of 0.5*N* NaOH to 2 ml of the clear supernatant followed by the addition of 2.0 ml of Folin-Ciocalteau reagent. The color was read at 660 mμ. The blank was prepared exactly as described for the sample except that the TCA solution was added immediately after addition of the substrate.

Aryl sulfatase

Five milligrams of the lyophilized supernatant or particulate fraction were dissolved in 3.0 ml of 0.05*M* acetate buffer (pH 4.8) containing 0.2*M* KCl. The substrate (0.5 ml of a 20mM solution of nitrocatechol sulfate) was added and the mixture was incubated for 1 hr at 37°C. The reaction was stopped by the addition of 2.0 ml of cold 5% TCA and centrifuged at 12,800 × G for 30 min. Alkaline quinol reagent (3.0 ml) was added to 2 ml of clear supernatant and the color read at 540 mμ (Roy, 1958). The blank determination was carried out as described above except that the TCA solution was added immediately after the addition of the substrate.

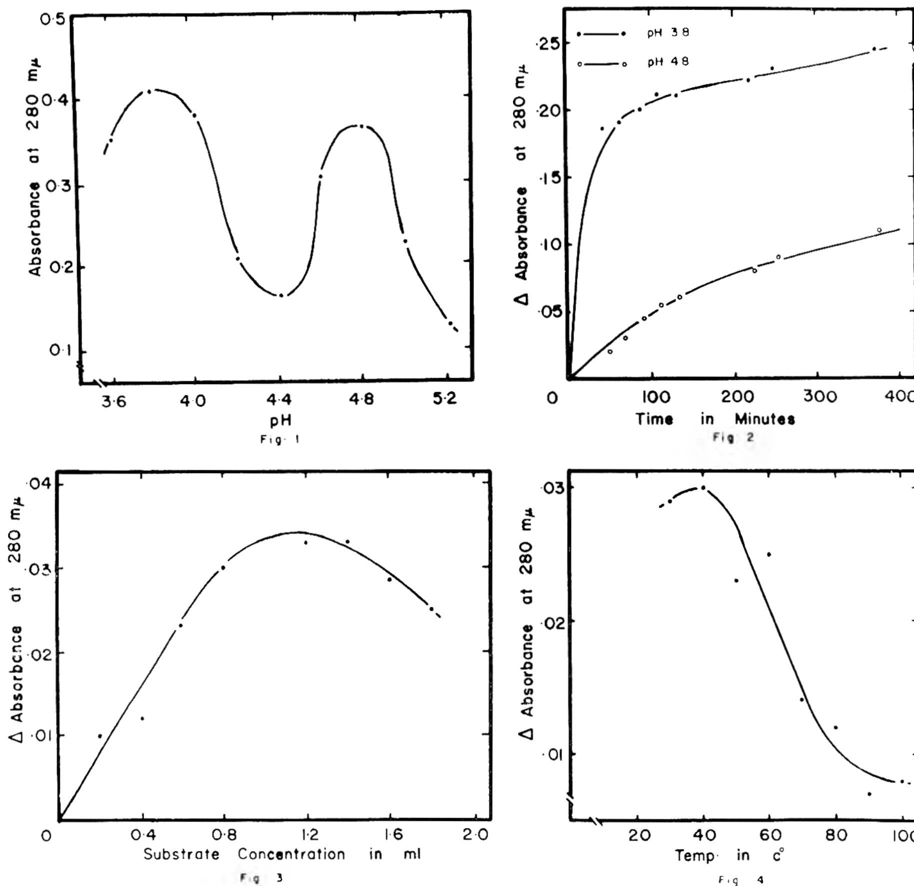

Fig. 1—Activity optima of proteolytic enzymes in pH range 3.6–5.2.

Fig. 2—Activity of proteolytic enzymes on endogenous muscle proteins as a function of incubation time at pH 3.8 and 4.8.

Fig. 3—Effect of substrate concentration on proteolytic activity using endogenous muscle protein as substrate.

Fig. 4—Effect of temperature on activity of proteolytic enzymes using endogenous muscle proteins as substrate at pH 3.8.

RESULTS & DISCUSSION

PRELIMINARY studies on the proteolytic activity of a soluble fraction from skeletal muscle, prepared as described above, were carried out with endogenous muscle protein as the substrate. Effect of pH, time, substrate concentration and temperature were studied.

Effect of pH on proteolytic activity

The proteolytic activity of the enzymes toward endogenous muscle protein was examined at intervals of 0.5 pH units between pH 4.0 and 9.0. Since the present study was concerned principally with those enzymes which exhibit optimum activity in the acid range, the activity was re-examined at intervals of 0.2 pH units between pH 3.6 to pH 5.2. The results obtained are presented in Figure 1. The activity observed at pH 3.8 was higher than that at pH 4.8.

Effect of time on activity

The reaction was linear for approximately the first $^1/_2$ hr at a pH of 3.8 and at a pH of 4.8. Subsequently, the rate of the reaction tended to level off. The results are presented in Figure 2.

Effect of substrate concentration

The substrate concentration was expressed in milliliters of a preparation containing 1.0 g of endogenous protein in 20 ml of acetate buffer (pH 3.8). The results are presented in Figure 3. The reaction was linear at low concentrations and attained a maximum at a substrate concentration of 1.2 ml. Above this concentration there was a decrease in activity which could be attributed to "product inhibition" of the enzymes (Walter et al., 1963).

Effect of temperature on activity

Enzyme solutions were maintained at

Table 1—Distribution of enzyme activities in particulate and supernatant fractions of bovine skeletal muscle and rat liver.

	Activities[1]											
	Cathepsin B			Cathepsin C			Cathepsin D			Aryl Sulfatase		
	Rat liver	Bovine loin	Bovine flank	Rat liver	Bovine loin	Bovine flank	Rat liver	Bovine loin	Bovine flank	Rat liver	Bovine loin	Bovine flank
Supernatant[2]	0.29	0.28	0.24	1.02	0.17	0.13	0.30	0.40	0.32	0.72	0.02	0.02
Fraction	±0.06	±0.01	±0.02	±0.01	±0.03	±0.04	±0.02	±0.04	±0.05	±0.07	±0.01	±0.01
Particulate[3]	0.42	0.13	0.11	7.58	0.08	0.07	1.71	0.14	0.14	3.21	0.01	0.02
Fraction	±0.03	±0.02	±0.01	±0.72	±0.05	±0.05	±0.10	±0.04	±0.01	±0.45	±0.00	±0.00
Total	0.71	0.41	0.35	8.60	0.25	0.20	2.01	0.54	0.46	3.93	0.03	0.04
	±0.08	±0.06	±0.03	±0.68	±0.04	±0.05	±0.10	±0.06	±0.05	±0.50	±0.03	±0.02

[1] Activities of Cathepsins B and C are expressed as the difference in absorbance/mg N between the sample and the substrate-free blank at 1 hr of incubation. Activities of Cathepsin D and Aryl Sulfatase are expressed as absorbance/mg N of the sample at 1 hr of incubation and the blank at zero incubation time. Each value represents the average for six animals, ± S.E.

[2] Fraction soluble in 0.15 *M* KCl.

[3] Fraction insoluble in 0.15 *M* KCl.

different temperatures in a water bath for 20 min before addition to the reaction mixture. The activity was expressed as the difference in absorbance of a reaction mixture incubated for 2 hr at 37°C and that of its control at zero time of incubation. The results are shown in Figure 4. Maximal activity was obtained with an enzyme solution maintained at 40°C for 20 min. Above this temperature, there was a rapid decrease in activity. The activity observed above 60°C was possibly due to an enzyme with properties similar to that of cathepsin C since the latter enzyme has been shown to be heat stable at 65°C (Fruton, 1960), whereas the other known cathepsins are labile above this temperature. The residual activity at 100°C is undoubtedly due to nonenzyamtic hydrolysis at a high temperature and at pH 3.8.

Activities of cathepsins B, C, D and aryl sulfatase

The results obtained for cathepsins B, C, D and aryl sulfatase activities in both particulate and supernatant fractions of two bovine muscles and rat liver are presented in Table 1. Each assay was determined in triplicate and each value represents the average for six animals.

The order of activities in bovine skeletal muscle was found to be cathepsin D > B > C whereas in rat liver the order was cathepsin C > D > B. The latter order of activities in rat liver and the high activity of cathepsin C are in agreement with the results reported by Bouma et al. (1964) for the same tissue.

The distribution of activities in supernatant and particulate fractions (Table 1) was of interest because such a distribution was considered to be a measure of "soluble" and "bound" enzyme. Activities for all enzymes studied in rat liver were higher in the particulate fraction than in the supernatant fraction whereas the activities in fractions of the bovine muscle were in the reverse order. The values obtained for aryl sulfatase activity in muscle tissue were extremely low. These results could be interpreted either as a simple lack of aryl sulfatase or as an indication that the lysosomes had not been ruptured sufficiently to release aryl sulfatase.

The reverse pattern of enzyme distribution as between rat liver and skeletal muscle was considered due a) to the circumstance that the activities in rat liver were determined immediately post-slaughter whereas the activities in skeletal muscle were determined several hours post-slaughter and b) skeletal muscle and not rat liver was subjected to the action of a Waring Blendor.

In this connection, studies currently in progress indicate that the activities of these enzymes in rat liver increase in the supernatant fraction relative to the activities in the particulate fraction as a result either of aging of the tissue or by the use of more drastic homogenization techniques than those employed in the present study.

Figure 5 shows graphically the total activities of these enzymes in skeletal muscle relative to the activities in rat liver. The combined activities for cathepsin B in the two skeletal muscles was approximately 52% of that in rat liver whereas cathepsins C and D activities were approximately 2 and 25% respectively. The activity of aryl sulfatase in skeletal muscle was less than 1% of the activity in rat liver.

A search of the literature has not revealed any study which involved the determination of the total activity of cathepsins B, C and D in bovine skeletal muscle as reported here although there have been reports on the activity of cathepsin D in chicken muscle (Berman, 1967; Martins et al., 1968). Bouma et al. (1964) observed that the activity of cathepsin B in rat skeletal muscle was approximately 25% of the activity in rat liver whereas cathepsins C and D were approximately 2 and 7%, respectively. However, the

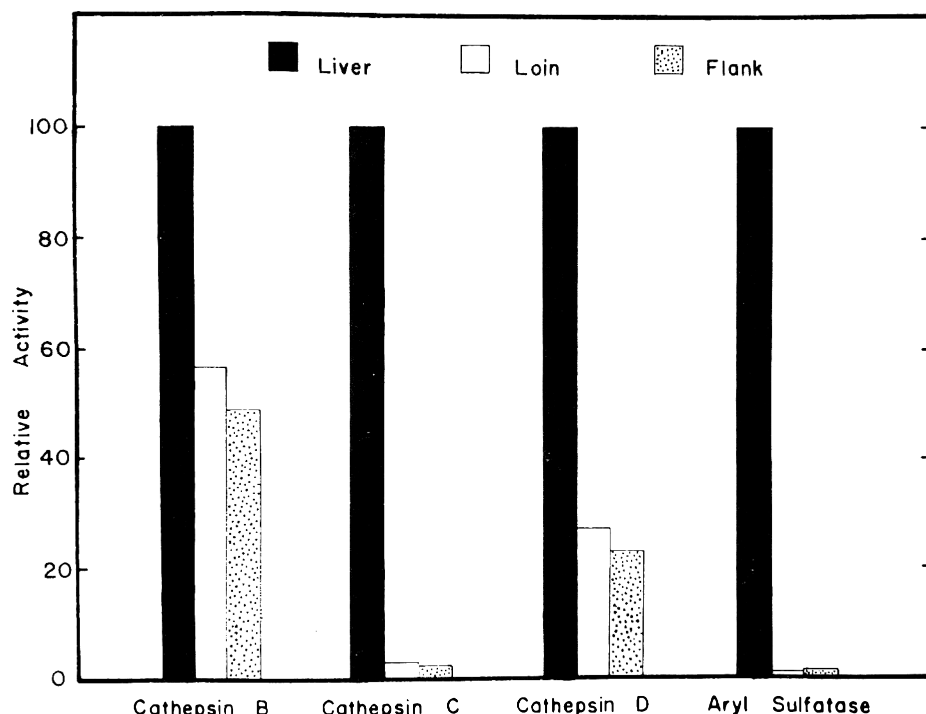

Fig. 5—Activities of enzymes in bovine skeletal muscle relative to the activities in rat liver.

latter workers determined the enzyme activity in extracts of skeletal muscle and not in homogenates.

Finally, the present study suggests that the activities of cathepsins B and D are higher in bovine skeletal muscle than previously indicated and points to the importance of optimum conditions for homogenization, extraction and assay of these enzymes in skeletal muscle.

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Ms. received 5/20/68; revised 9/16/68; accepted 8/20/69.

The authors are indebted to the National Research Council of Canada for financial support and to Steinberg's Ltd., Montreal, for the provision of suitable muscle samples.

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Formation of a Green Pigment from Tuna Myoglobins

SUMMARY—A green pigment was produced when yellowfin (and other) tuna myoglobins, trimethylamine oxide (TMAO), and cysteine were heated together in 0.1 M phosphate buffer pH 5.7. The green product could not be produced with mammalian myoglobins, which contain no cysteine residues. The roles of myoglobin, cysteine, and TMAO in the production of off-color (green) cooked tuna were investigated. Denaturation of myoglobin, apparently exposing a sulfhydryl group, was necessary for the greening reaction to occur. TMAO acted as a mild oxidizing agent to promote the formation of a disulfide bond between cysteine and the sulfhydryl group on the denatured myoglobin. TMAO could be replaced by oxygen (air), but cysteine appeared to be specific for the reaction. The green color could be reversed by sodium sulfite, but not by other reducing agents tested.

INTRODUCTION

GREENING, the industrial name given an off-color reaction in cooked tuna, has been encountered in albacore (*Germo alalunga*), big-eye (*Thunnus obesus*), bluefin (*Thunnus orientalis*), yellowfin (*Thunnus albacares*), and skipjack (*Katsuwonus pelamis*) tuna (Tomlinson, 1966). It is not clear that the greening phenomenon is due to a single type of pigment formation.

Brown et al. (1958) suggested that one off-color is due to oxidation to the ferri- state of the desirable ferrohemochromes, the pigments responsible for normal tuna color. More recent work shows species differences in susceptibility to off-color (Barrett et al., 1965). Brown et al. (1967) related the length of tuna to color, longer fish having poorer color.

Apart from these considerations, which are believed to be related to the ferrihemochrome type of off-color, there have been efforts to establish a possible relationship of trimethylamine oxide (TMAO) and/or trimethylamine (TMA) to, perhaps, a different type of off-color.

Sasano et al. (1961; 1962) found that an unknown substance contained in an alcohol extract of tuna meat was possibly involved in the greening reaction. The substance gave a yellow color on treatment with ninhydrin and was tentatively identified as a peptide. When this compound was mixed with raw tuna meat of good quality, it produced greening when the meat was subsequently cooked. It was later reported that the alcohol-soluble substance that produced a yellow color with ninhydrin was TMAO, and that the addition of TMAO to raw meat induces greening on subsequent cooking of yellowfin and albacore tuna (Koizumi et al., 1965a; 1965b).

These studies also showed that heat degradation products of TMAO such as dimethylamine, methylamine, and formaldehyde did not produce greening. At least part of the color changes brought about in tuna meat precooked with added TMAO were not reversible by treatment

with a reducing agent. However, a suitable reducing agent added in addition to the TMAO prior to cooking prevented the development of off-color. They concluded that this was not due to reduction of some heme derivative, but rather to the breakdown of TMAO to substances that did not participate in the greening phenomenon.

A reasonable correlation was found between TMAO concentration in raw fish and the subsequent extent of greening in cooked fish (Koizumi et al., 1967a). Nagaoka et al. (1964) also reported finding a positive correlation between the TMA content of cooked tuna, the TMAO content of raw tuna, and greening. They suggested that the green color is derived from a combination of metmyoglobin and some substance produced from TMAO.

The purpose of this investigation was to explore further the roles of myoglobin, TMAO, amino acids, mercapto compounds, and oxidizing and reducing agents in the greening reaction. While the present work was in progress, Koizumi et al. (1967b; 1968) have reported on possible relationships among cysteine, myoglobin, and TMAO to produce greening. Their work paralleled the present investigation in some aspects.

EXPERIMENTAL

Materials

Yellowfin tuna myoglobin was purified by the method of Brown (1961). The myoglobin was in the met (ferri-) state after the isolation procedure. Sperm whale myoglobin and equine myoglobin were obtained from Calbiochem, Los Angeles, California.

Trimethylamine (TMA) and trimethylamine-n-oxide dihydrate (TMAO) were obtained from Eastman Organic Chemicals, Rochester, New York. Cysteine hydrochloride was obtained from Nutritional Biochemical Corporation, Cleveland, Ohio. Cysteine sulfinic acid, cysteic acid, and cystine were obtained from Sigma Chemical Company, St. Louis, Missouri. Glutathione was obtained from Schwarz Bioresearch, Inc., Mount Vernon, New York.

All chemicals were reagent grade or better. Glass distilled water was used.

Spectral measurement

Absorption spectra were recorded with a Cary Model 11 spectrophotometer. Reflectance spectra were obtained with a Beckman DK-2 spectrophotometer equipped with a reflectance attachment.

Preparation of oxymyoglobin (MbO_2)

Three mg of sodium dithionite were placed in a 50 ml erlenmeyer flask. After the flask was cooled in an ice bath, 2 ml of a yellowfin metmyoglobin (metMb) solution (60 mg) were added to the flask and it was swirled gently. The solution was deionized by running it through a precooled Bio-Rad AG501-X8 mixed bed resin column (2 cm × 12 cm), at a flow rate of 1 drop per sec.

Sulfhydryl group analysis

Sulfhydryl groups were determined by the nitroprusside test for free SH group described by Toennies et al. (1951).

Model systems for study of pigment formation

(1) To 4 ml of 0.1 M phosphate buffer, pH 5.7, 0.5 ml (15 mg) of yellowfin metmyoglobin, 0.5 ml of 1.0 M TMAO, and 5 mg cysteine were added. The mixture was heat denatured by holding in a water bath at 70°C for 15 min to produce greening. A control contained no cysteine.

(2) Other chemicals (5 mg per tube) were substituted for cysteine or TMAO in the system described above. A control containing cysteine was also used in order to compare green color development.

(3) To reduce disulfide bonds, sodium sulfite was added to the greening system and additional heating was used. The procedure of Pechere et al. (1958) was also employed for this purpose.

(4) In order to maintain the denatured myoglobin in solution, 4 ml of 10 M urea, pH 5.6, was substituted for the buffer, and the solution was then heated.

(5) In order to approximate the dilution of metmyoglobin by other proteins in the tuna muscle, 1 ml of human albumin (50 mg) dissolved in 0.1 M phosphate buffer, pH 5.7, was added both to the greening system and to a control.

(6) Heme groups were extracted by the method of Koizumi et al. (1968).

RESULTS & DISCUSSION

The role of metmyoglobin, TMAO, and cysteine in greening

Experiments were conducted to find out whether TMA or TMAO will react directly with myoglobin to produce any green derivatives. TMAO or TMA was added to purified metmyoglobin and oxymyoglobin solutions over a pH range of 5.0 to 9.0 (0.1 M phosphate buffer). No visual or spectral changes could be detected either immediately or after standing.

Myoglobin was then heat denatured in order to make the experimental conditions more closely approximate those in tuna muscle during cooking. Table 1 summarizes the effect on the final color when cysteine, TMA, and TMAO were added to oxymyoglobin and metmyoglobin solutions subjected to urea and heat denaturation. When metmyoglobin was heat denatured in the presence of TMA or TMAO, only brown precipitates were obtained. However, when cysteine was added with the TMAO, a dull light olive green precipitate was obtained. The green color was enhanced in the presence of albumin. A green precipitate was not obtained with TMA.

Table 1—The color of yellowfin tuna MbO_2 and metMb denatured in the presence of cysteine, TMA, and TMAO.

	Color in presence of indicated additives			
	TMAO	Cysteine	TMAO + Cysteine	TMA + Cysteine
YF-metMb (urea denatured)	Brown	Reddish brown	Green	Brown
YF-met Mb (heat denatured)	Brown	Reddish brown	Green[1]	Brown
YF-metMb + Albumin (heat denatured)	Brown	Reddish brown	Green[1]	Brown
YF-MbO_2 (heat denatured)	Pinkish brown	Greenish brown	Green[2]	—

[1] After 10 min.
[2] After 30 min.

When purified oxymyoglobin was treated with TMAO and cysteine and then heat denatured, a green precipitate was obtained, but it took longer to form than when metmyoglobin was used. The delayed time in producing greening with oxymyoglobin suggested that metmyoglobin is involved in greening and that oxymyoglobin must be converted into metmyoglobin before the reaction proceeds. The results obtained when metmyoglobin was denatured by urea were similar to those obtained when heat was the denaturing agent.

Reflectance spectra of the different precipitates were obtained, as were absorption spectra of similar mixtures denatured in urea. The reflectance spectra, particularly, were quite non-descript. Absorption spectra were better, but still poor. In the green systems, there was diminished absorption in the 500 and 630 mμ areas associated with metmyoglobin, and an enhanced absorption over the 550 to 600 mμ range.

Table I clearly shows that both TMAO and cysteine are involved in the reaction with denatured myoglobin, forming a product having a green color. Since some free cysteine likely occurs in tuna muscle, it is possible for this reaction to occur in tuna flesh. The following experiments were designed to determine whether TMAO and cysteine were reacting with

Table 2—Pigments from yellowfin tuna metMb or yellowfin tuna metMb plus TMAO with various oxidative products of cysteine and glutathione.

Additive	Color of heat denatured pigment	
	YF-metMb	YF-metMb + TMAO
Cysteine	Brown	Green
Cysteine sulfinic acid	Brown	Brown
Cysteic acid	Brown	Brown
Cystine	Brown	Brown
Glutathione-SH	Brown	Brown

Table 3—The effect of reducing agents on yellowfin tuna metMb, TMAO system.

Additive	Color of heat denatured pigment YF-metMb + TMAO
Sodium sulfite	Reddish brown
Formamidine sulfinic acid	Reddish brown
Hydrindantin	Brown
Ascorbic acid	Brown
Thioglycol	Brown

Table 4—The effect of the order of denaturation or additive incorporation on color change.

	Color produced under conditions shown					
			Reagents added prior to reheating for 15 min			
	Heat 70°, 15 min	24 hr sit.	metMb	TMAO	cysteine	cysteine + TMAO
YF-metMb + cysteine	Brown	Greenish brown		Green	—	—
YF-metMb + TMAO	Pink	Reddish brown		—	Green	—
TMAO + cysteine	Clear solution	Clear solution	Reddish brown	—	—	—
YF-metMb	Pink	Reddish brown		—	—	Green

Table 5—The participation of different myoglobins in greening reaction.

Mb	Color produced on heat denaturation of different Mbs		
	Buffer	TMAO	Cysteine & TMAO
YF tuna	Pinkish brown	Pinkish brown	Green
Sperm whale	Pinkish brown	Pinkish brown	Brown
Equine	Brown	Brown	Brown
Albacore tuna	Brown	Pinkish brown	Green
Skipjack tuna	Brown	Reddish brown	Green
Bluefin tuna	Brown	Reddish brown	Green

each other or whether both TMAO and cysteine were reacting with the denatured myoglobin.

The nature of involvement of cysteine

In order to determine if oxidative products of cysteine are involved in the greening reaction, they were tested in the model system. As shown in Table 2, none of these oxidative products of cysteine and glutathione produced greening.

In Table 3 are shown the results of replacing cysteine with various reducing agents. It is evident from the results that cysteine is not acting simply as a reducing agent in the greening reaction. In fact, greening does not occur when reducing agents are added along with metmyoglobin, cysteine, and TMAO, indicating that reducing agents may prevent greening. If cysteine is replaced with amino acids such as aspartic acid, arginine, glutamic acid, methionine, histidine, tryptophan, and tyrosine, the green pigment is not produced.

When metmyoglobin was added to a solution of cysteine and TMAO that had previously been heated, greening did not occur. Under this condition, SH groups were not detectable. Presumably, the cystine formed will not replace cysteine in the reaction. When metmyoglobin was first heat denatured, and TMAO and cysteine then added, greening was produced (Table 4).

From these results, it is clear that cysteine cannot be replaced in the greening reaction by its own oxidative products, other reducing agents, or other amino acids. Free cysteine must be present for greening to occur.

Involvement of cysteine residue in myoglobin

Brown et al. (1961; 1962) found that tuna myoglobins contain the amino acid cysteine. In contrast, myoglobins from whale and other mammals do not contain this amino acid.

Table 5 indicates that the greening reaction involves the -SH group of the cysteine residue in the tuna myoglobin. No greening was produced when myoglobins such as sperm whale myoglobin and equine myoglobin, which do not contain a -SH group, were heat denatured in the presence of cysteine and TMAO. Therefore, it appears that the greening reaction involves the -SH group of both free cysteine and the cysteine residue in the tuna myoglobin. To confirm the participation of -SH groups in the greening reaction, -SH blocking agents such as iodoacetamide (Mayaudon et al. 1957) and formamidine disulfide (Sullivan et al. 1966) were added with myoglobin, TMAO and cysteine in the model system. Only brown precipitates were obtained. Thus these -SH blocking agents prevented greening from occurring in the model system.

The nature of involvement of TMAO

A variety of oxidizing agents were used in efforts to produce greening with metmyoglobin and cysteine. Results are shown in Table 6. TMAO is not as specific to the reaction as cysteine since TMAO can be replaced by air. However, the reaction occurs much more slowly with air alone than it does with TMAO. In contrast to the results of Koizumi et al. (1967b) the reaction did not occur when O_2 was replaced by N_2. There is no explanation for this discrepancy since in many aspects the findings are virtually identical.

Both hydrogen peroxide and potassium persulfate produced greening with metmyoglobin alone. This color formation may have resulted from a different mechanism than that when cysteine is involved. Table 6 clearly demonstrates that TMAO functions as a mild oxidizing agent in the reaction of the cysteine -SH with the protein -SH to form a disulfide bond.

Hird et al. (1961) studied the oxidation of cysteine, glutathione and thioglycollate by bromate, iodate, persulfate and air at 28°C and near neutral pH values. They found that oxidation of these sulfhydryl compounds was rapid with iodate, slow with bromate and persulfate and slower still with air. Their results showed that the major oxidation product from cysteine is cystine. With iodate and bromate there was evidence of the production of higher oxidation products, namely cysteine sulphinic acid and cysteine sulphonic acid.

To determine if a disulfide bond was formed in the greening system, sodium sulfite was reacted with experimentally produced green myoglobin. The ability of sulfite to cleave disulfide bonds has been demonstrated (Cecil et al. 1959). Sodium sulfite reacted with the green pigment and reversed the color to pink. Other reducing agents, namely, formamidine sulfinic acid and hydrindantin had no effect on the green color. A small amount of the green color was not reversed until the precipitate was dissolved in 10 M urea and treated with sulfite and ammoniacal copper at pH 10.2.

Table 6—The effect of different oxidizing agents and cysteine on off colors produced by heat denaturation of yellowfin tuna MbO_2 and metMb.

	Color of heat denatured pigment				
	TMAO	[1]H_2O_2	[2]$K_2S_2O_8$	O_2 (air)	N_2
YF-metMb	Brown	Green	Green	Brown	—
YF-metMb + cysteine	Green (10 min)	Green	Green	Green (18 hr)	Reddish bown (18 hr)
YF-MbO_2 + cysteine	Green (30 min)	—	—	Green (18 hr)	—

[1] 0.05 ml 0.3%.
[2] 5 mg.

To determine if cysteine were reacting with metmyoglobin before denaturation, the following experiment was carried out: Solutions of (1) metMb, (2) metMb + cysteine, (3) metMb + TMAO, and (4) metMb + TMAO + cysteine were placed in four test tubes. The pH values were measured and the absorption spectra were determined. Then the test tubes were incubated at 30°C for 23 hr and the absorption spectra were measured again.

In the tubes containing cysteine and metmyoglobin a yellowish-brown product was formed. The color of the product was not affected by sodium sulfite as determined by spectral measurements. These results suggest that the product formed under these conditions is markedly different from that formed when the metmyoglobin is denatured before reacting with the cysteine.

These results clearly indicate that myoglobin must be denatured for greening to occur, and that the formation of a disulfide bond is involved in the greening reaction.

Green pigment

The substitution of various mercapto compounds for cysteine resulted in the production of green pigment with mercaptoacetic acid, mercaptopropionic acid, mercaptobutyric acid, mercaptoethanol, and homocysteine but not with thiodiglycol. The green color changed with the chain length of the mercapto compound—the mercaptoacetic acid product was the deepest green while that of the homocysteine and mercaptobutyric acid products was almost brown.

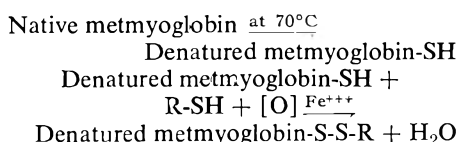
The mercaptoacid and mercaptoethanol myoglobin products appeared to be very resistant to the action of sulfite, suggesting that these products may differ from those formed with cysteine and homocysteine. In support of this conclusion, the color of the mercaptoethanol product can be reversed with sodium hydrosulfite. This reagent will not cleave disulfide bonds. This possible alternate mechanism for formation of green pigments will be the subject of future study.

An attempt was made to extract the heme group from the control and green myoglobin. Although the heme group of heat denatured myoglobin could be readily extracted, that of the green pigment formed with cysteine could not. Reversal with sulfite, however, increased the amount of pigment which could be extracted.

Possible mechanism

Vaisey (1956) found that TMAO at temperatures between 22–24°C is readily reduced by cysteine in the presence of iron or hemoglobin as a catalyst. The major product of this reduction was TMA (and, presumably, cystine). This observation agreed with that of Nagaoka et al. (1962; 1964), who found a positive correlation between the TMA content of cooked tuna, the TMAO content of raw tuna, and greening. Vaisey also found that glutathione could not replace cysteine as a reducing agent.

The greening reaction appears to involve the reaction of free cysteine with the -SH group of heat denatured tuna myoglobin (under mild oxidizing conditions provided either by TMAO or air) to produce a disulfide bond and TMA, with perhaps a catalytic influence by the iron (Fe^{+++}) of metmyoglobin. The overall mechanism, therefore, for the greening caused by the reaction of denatured myoglobin with cysteine and TMAO may be summarized by the equations below:



$$\text{Native metmyoglobin} \xrightarrow{\text{at } 70^\circ C} \text{Denatured metmyoglobin-SH}$$

$$\text{Denatured metmyoglobin-SH} + \text{R-SH} + [O] \xrightarrow{Fe^{+++}} \text{Denatured metmyoglobin-S-S-R} + H_2O$$

Apparently the cysteine molecule which is attached via the disulfide bond is in close proximity to the porphyrin ring on the metmyoglobin and interacts in some manner to produce a change in the light absorbing properties of the porphyrin group.

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Ms. received 7/17/68; revised 11/1/68; accepted 9/10/69.

This work was supported in part by a contract with the U.S. Bureau of Commercial Fisheries Technological Laboratory, Terminal Island, California. We thank members of the Star-Kist Laboratories for supplying tuna used for myoglobin preparations.

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Serendipity Berries—Source of a New Intense Sweetener

SUMMARY—The fruit of Dioscoreophyllum cumminsii, *Serendipity Berries, contains an intensely sweet principle. Chromatography of water extracts of the berry on G-50 and G-200 Sephadex indicated that the sweetener was bound to fruit protein. Degradation of the fruit extract with bromelain, a proteolytic enzyme, yielded a lower molecular weight material with intense sweetness of excellent quality. Functional group tests indicated that this material was not proteinaceous, but rather a carbohydrate type substance. Threshold taste response gave a sweetness value of 1500 times sweeter than sucrose.*

INTRODUCTION

THE RECENT application of synthetic sweeteners to new diet foods has revealed that there are several limiting taste qualities to commercially available sweeteners (Rader et al., 1967; Bottle, 1964). A synthetic sweetener with superior taste qualities could expand the present sweetener market. Several research approaches were used to find a superior synthetic sweetener. Organic synthesis of analogs of the dihydrochalcone sweeteners, naringin dihydrochalcone and neohesperidin dihydrochalcone, gave compounds that show considerable commercial promise (Krbechek et al., 1968).

Another approach used was screening tropical plant materials for intense sweetness. Plant materials studied included many perishable rare tropical fruits (Inglett et al., 1968). An early tropical fruit to receive research attention was Miracle Fruit (*Synsepalum dulcificum*) (Inglett et al., 1965). This fruit has the remarkable taste-modifying property of causing sour materials to taste sweet after the mouth has been exposed to the fruit's mucilaginous pulp.

The Serendipity Berries were originally received from Nigeria as an unidentified tropical fruit. The unexpected properties of intense sweetness and the water solubility of the sweetener were discovered the same day that research was discontinued on the Miracle Fruit. Hence, its name "Serendipity" was very appropriate. It was some time later that the berry was botanically identified as *Dioscoreophyllum cumminsii* (Inglett *et al.*, 1967). A picture of these berries is shown in Figure 1. The Serendipity Berries are indigenous to tropical West Africa.

The *Dioscoreophyllum cumminsii* plant grows from Guinea to the Cameroons and is also found in Gaboon, the Congo, the Sudan, and Southern Rhodesia. It grows in thick forest areas during the rainy season from approximately July to October. The Serendipity Berries are borne by hairy climbing vines which are sometimes 15 feet long and $^1/_8$ to $^3/_{16}$ in. in diameter. These vines are usually found supported by other vines; the leaves, which are heart-shaped with ragged edges and measure about 3 in. from the tip to the stem, are found attached at 6-in. intervals on the vine. The berries are red, approximately $^1/_2$-in. in diameter, and grow in grapelike clusters with approximately 50 to 100 berries in each bunch.

The tough outer skin of the berry encloses a white, semisolid, mucilaginous material surrounding a friable thorny seed. The fruit is not commonly cultivated or used by the natives of Nigeria because of its intense sweetness. The tubers of the plant are reported to be eaten in some parts of Africa and used medicinally. The fruit has remarkable stability properties keeping for several weeks at room temperature.

EXPERIMENTAL

General procedures

The berries used in these studies were obtained fresh by air transport from Nigeria. The berries were immediately washed, frozen, and stored until used.

Preliminary extraction of the sweet principle was carried out as follows: the whole berries were homogenized in deionized water using a Waring blendor equipped with a polyethylene blade. Thus, pulp and skins were separated from the seeds without cutting into the seeds which are extremely bitter. This preliminary extraction procedure was facilitated by the addition of pectinase to the mixture of whole berries and water to a ratio of 1 g of pectinase to 250 g whole berries. After the homogenization, the enzyme was allowed to incubate at 25°C with the mixture for 24 hr. This procedure greatly reduced the viscosity of the homogenate, enabling the ensuing filtration to proceed at a much greater rate.

After incubation, the mixture of pulp, skin, and seeds was filtered through a large Buchner funnel to eliminate the seeds from the rest of the homogenate. The filtrate was then dialyzed against deionized water for 24 hr at 4°C. The water soluble materials in the dialysate were devoid of the sweetener principle, but the non-dialyzable material was intensely sweet. This material was lyophilized and stored at 4°C for further purification.

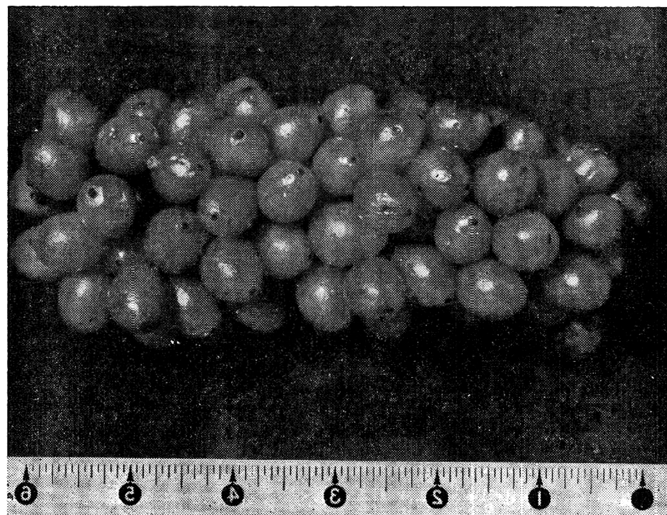

Fig. 1—Serendipity Berries (Dioscoreophyllum cumminsii).

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The active principle of *Dioscoreophyllum cumminsii* can be most readily detected by the sense of taste. However, another parameter, the ultraviolet absorption (optical density) at 280 mμ, is an equally valid test of the presence of the active principle. An ultraviolet spectrum showed a peak in absorbance to occur at 270–275 mμ. However, 280 mμ proved to be sufficient for the detection of the active principle.

Molecular sieve chromatography

G-50 Sephadex chromatography. The crude berry extract was carried through a preliminary purification by means of G-50 Sephadex chromatography. (All molecular sieve gels used were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden.) The column employed was 100 cm. × 2.5 cm.

Several samples of crude berry extract (ranging in weight from 200 to 500 mg each) were dissolved in 10.0 ml of acetate buffer (pH 4.6) to help reduce the viscosity and surface tension of the extract solution. The sample was introduced onto the top of the column and was eluted with deionized water for 18 hr. The flow rate was 30.0 ml/hour, and the eluate was collected in 10 ml fractions by a Gilson Medical Electronics fraction collector.

In 10 separate trials, the sweetener was found to be eluted in fractions 13–24 (average values) immediately following the forerun. (The forerun was determined to be approximately 110 ml by chromatographing a high molecular weight dye, Dextran 2000.) The ultraviolet absorbance at 280 mμ was measured for each eluted fraction on a Beckman DU Spectrophotometer.

In every trial, the first peak in the optical density coincided with the elution of the sweetener, and the second peak marked the position of the elution of the brown pigments. The fractions containing the active principle were lyophilized to a white or light tan powder with an average yield of about 50% by weight of the material placed on the column.

In the descriptive literature published by Pharmacia on Sephadex, it is stated that the approximate molecular weight limit for complete exclusion by G-50 Sephadex is 10,000. The sweet principle of *Dioscoreophyllum cumminsii* was eluted immediately following the forerun in G-50 Sephadex chromatography, indicating that it is, in this crude state, of molecular weight 10,000 or greater.

G-200 Sephadex chromatography. The Serendipity sweetener, which had been preliminarily purified by fractionation on a G-50 Sephadex column, was chromatographed on a G-200 Sephadex column. Two trials were run using the upward flow technique and a third using the normal downward flow method. The chromatography was monitored by measuring the ultraviolet absorbance at 280 mμ of the eluate. In trial I, the sweetener was eluted in 10 ml fractions 31–40 and 51. The dry weight of the two sweetener peaks were 18% and 1.3%, respectively, of the total material placed on the column.

The dry weight of the remaining unsweet fractions was 61% of the total; hence, 80% of the material placed on the column was recovered. The indication is that the substance eluted in fraction 51 is of a considerably lower molecular weight and thus of a different structure than that eluted in fractions 31–40. A cursory study into the nature of these two substances was carried out by means of disc-gel electrophoresis to be discussed at a later point.

In the second G-200 Sephadex upward flow chromatography experiment, the sweetener was eluted in fractions 21–45. The difference in the results of these two trials was not accounted for.

A third G-200 Sephadex column was run using a downward flow technique. The preliminarily purified sample was introduced onto the top of the column; and in this trial, the sweetener was eluted in fractions 22–35 (dry weight, 24% of the total amount placed on the column). In all three cases, the sweet fractions coincided with the peaks in the ultraviolet absorbance pattern.

The molecular weight exclusion limit for G-200 Sephadex is listed as 200,000. Since the sweetener was somewhat retarded by the molecular sieve gel and in all three cases was eluted several fractions following the forerun, it can be assumed that the substance and/or substances have a molecular weight lower than 200,000.

Sweetness evaluation

Lyophilized sweet extract recovered from the downward flow G-200 Sephadex column was evaluated for sweetness by a panel of five members. Values were first obtained by comparing the threshold sweetness of the Serendipity Berry material to that of sucrose. (Threshold sweetness is defined as that concentration at which one first perceives a sweet taste.) Results obtained from this method ranged from 1000–2000X sweeter than sucrose with a mean value of 1500X sweeter than sugar.

The second method of sweetness evaluation was carried out by matching a solution of known concentration of the Serendipity Berry sweetener to a sucrose solution of equivalent sweetness level. Values obtained in this manner ranged from 500–1250X sweeter than sucrose with a mean value of 800X sweeter than sugar. The limitations of these evaluations are the subjectivity and variability of the individual tasters. The results must be treated as only a crude approximation of the sweetness value of the material.

Enzymatic studies

The effect of three different enzymes—papain, bromelain, and trypsin—on Serendipity Berry sweetener was studied. All enzymes used were obtained from Nutritional Biochemicals Co. Dilute solutions (0.05%) of papain and bromelain were prepared in sodium acetate-acetic acid buffer (pH 4.5) and activated with cysteine hydrochloride. A dilute solution of trypsin (0.05%) was prepared in phosphate buffer (pH 7.0). 1 ml of each enzyme solution was added to a 10.0 ml aliquot of 0.02% sweetener solution. (The sweetener used was extract which had been preliminarily purified by G-50 Sephadex chromatography.) Each sample was tested for sweetness against a control of 0.02% sweetener at the outset of the experiment and after time lapses of 30 min, 1 hr, and 24 hr.

Table 1—Protein bands obtained by disc gel electrophoresis of sweetener fractions.

Description of sample	Number and nature of bands
726 μg G-50 fractionated sweetener	3 strong, 1 weak
Fraction 51 (sweet) from first G-200 upward flow column, 215 μg	0
200 μg G-50 fractionated sweetener incubated 24 hours with 13.7 μg papain	2 weak
200 μg G-50 fractionated sweetener incubated 24 hours with 8 μg bromelain	2 weak
200 μg G-50 fractionated sweetener incubated 24 hours with 8 μg trypsin in phosphate buffer	1–2 diffuse, weak
200 μg G-50 fractionated sweetener in phosphate buffer	2 weak

The samples were incubated at 25°C. No reduction in sweetness value was observed in the samples containing the bromelain and the papain. However, in the trypsin-phosphate buffer solution, the sweetness was reduced almost immediately, and after 24 hr, almost none remained. The phosphate buffer, not the trypsin, was responsible for the loss of sweetness. The cause of this unusual effect was not ascertained, but chromatographic studies showed that the sweetness lost could be regenerated by G-50 Sephadex chromatography. This suggests that the loss of sweetness may have been due to a masking effect by the buffer rather than to a chemical change.

Sephadex G-50 chromatography of the enzyme treated sweeteners showed that, in each case, the sweetener was fractionated into two optically dense peaks. In the cases of trypsin, papain, and the phosphate buffer, both optically dense peaks contained sweetener material. In the bromelain hydrolysis, however, only the second peak contained the sweetener (Fig. 2).

In ensuing bromelain hydrolysis experiments, the sweetener was sometimes eluted in the first peak also. Nevertheless, the indication is that the bromelain effects a more complete cleavage of the sweetener moiety than the other three preparations. Thus, bromelain was selected for use in further enzymatic degradation studies.

The second peak eluted in the previous chromatography experiments is necessarily of smaller molecular size (according to the theory of molecular sieve chromatography) and was therefore selected for further analysis.

The two optically dense peaks fractionated by G-50 Sephadex will henceforth be referred to as Peak I (higher molecular weight) and Peak II (lower molecular weight). Several trials were carried out in which preliminarily purified sweetener was hydrolyzed by bromelain and then chromatographed on G-50 Sephadex. The ratio of yields of Peak I to Peak II were approximately 10 to 1 by weight.

The action of enzymes and phosphate buffer on the Serendipity Berry sweetener was studied by means of disc gel electrophoresis (see Table 1). The procedures fol-

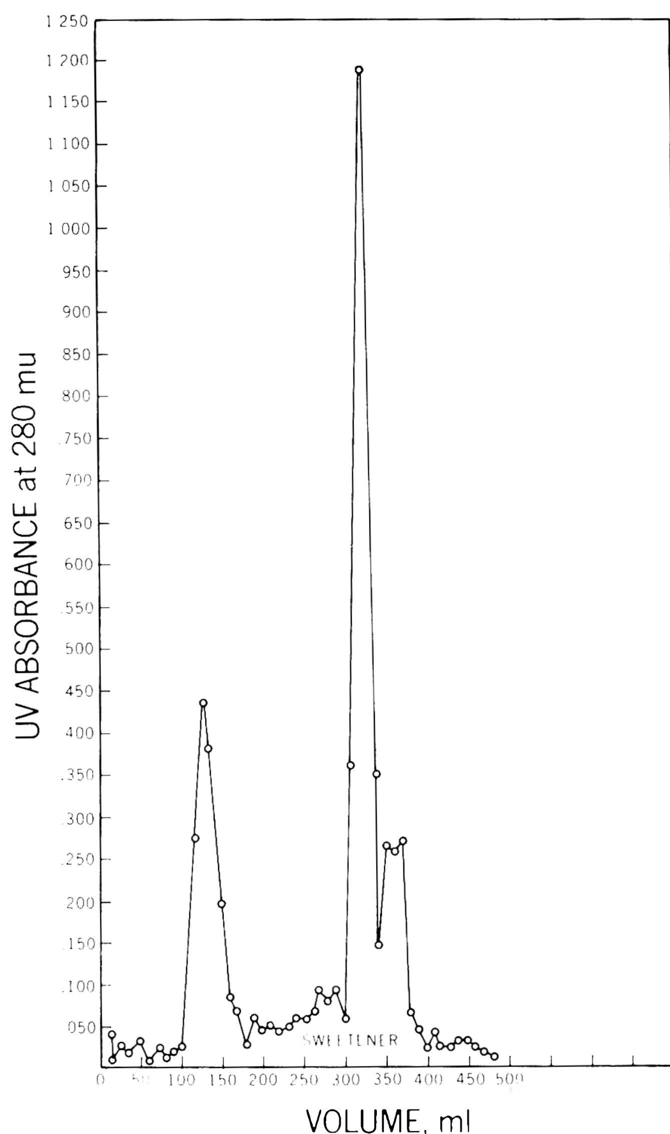

Fig. 2—Natural sweetener (bromelain treated) fractionated on Sephadex G-50.

lowed and the preparations used were those found in the literature published by Canalco Corporation. The samples were placed in columns containing two different layers of polyacrylamide gel.

After the electrophoresis, the columns were placed in a solution of trichloroacetic acid (TCA) for a preliminary protein analysis. No precipitation occurred. The columns were then stained with Amido Schwartz dye which is 10 times more sensitive than TCA and can detect the presence of as little as 100 μg of protein. Destaining was effected by rimming the gels from the column and washing them in 10% acetic acid. All but one sample showed one or more protein bands, and the results are tabulated in Table 1.

All bands in the gel were due to protein in the sweetener material, because the concentration of the enzyme was not sufficient for their detection. The results indicate that there is more than one protein in almost every sample. The strongest bands occurred in the sample containing only sweetener material. This is an expected result, because much of the protein in the other samples was probably degraded by the enzymes. However, the sample considered to be the most pure, Fraction 51 (sample 2), contained no protein bands. These and following data suggest that the Serendipity Berry sweetener is not a protein.

Analysis of Peak II sweetener

Infrared spectra (KBr pellets) were obtained for (1) the G-50 Sephadex preliminarily purified sweetener, and for (2) Peak I and (3) Peak II sweeteners obtained by bromelain hydrolysis and ensuing G-50 Sephadex chromatography of (1). Results showed that (1) and (2) are the same substance whereas (3) is definitely of a different character. It appears, therefore, that Peak I sweetener is merely unhydrolyzed starting material. Thus it was decided to concentrate the ensuing studies on the lower molecular weight and possibly more pure Peak II sweetener.

n-Butanol-acetic acid-water (4:1:5), using ascending chromatography on Whatman No. 1 paper, resolved this purified sample into three spots, two of which were ultraviolet light fluorescent ($R_f = 0.00$ and $R_f = 0.31$) and one which gave rise to a pink color upon spraying with analine phthalate indicator ($R_f = 0.11$). After the chromatography, the spots of the three different R_f's were eluted in 0.5 ml of water. The eluents were tasted, and it was determined that the sweetener had remained at the origin.

The eluted sweetener was then hydrolyzed in 6*N* HCl (reflux, 2 hr). A ninhydrin test on the hydrolysate was negative, indicating the absence of any amino acids. Presence of carbohydrate positive substances in the Peak II sweetener was confirmed by the use of the anthrone reagent.

DISCUSSION

THE FRUIT of *Dioscoreophyllum cumminsii* contains an intensely sweet, water-soluble principle. Mild extractive procedures were used in the isolation of this sweet principle from the fruit pulp. The techniques used consisted mainly of various types of chromatography in conjunction with enzymatic and acid hydrolysis procedures.

Classification of the sweetener substance was not completely established. However, several statements concerning the character of the sweetener can be made. Enzymatic, electrophoretic, and ninhydrin studies showed that although the sweetener is not a protein or polypeptide, it is associated with the fruit's protein fraction during early stages of isolation. Molecular weight estimation by means of Sephadex chromatography substantiates this assumption.

The position of the elution of the Peak II sweetener from a G-50 Sephadex column (320–360 ml, average values) indicates that its molecular weight is considerably lower than the 10,000 value estimated for the crude extract. The fact that the Peak II sweetener retains its optical density at 280 mμ following enzymatic degradation sheds some light on the nature of its structure. The substance is either aromatic or contains some other form of unsaturated bonds.

Paper chromatography showed that Peak II contained at least three different substances. Although it was possible to identify and isolate the sweetener material following the paper chromatography, all ensuing experiments on this substance were inconclusive. Hence, final classification of this material was not achieved. Although it was necessary to stop work on the Serendipity Berries short of the point where meaningful interpretation of the data could be achieved, any future purification and characterization work may be based on the possible presence of an aglycone glycoside.

Lyophilized berry extract recovered from a Sephadex G-200 column was evaluated for sweetness by a panel of five

members. Results were that the Serendipity Berry extract was 800-1500X sweeter than sucrose, depending upon the method of evaluation.

Stevioside has been regarded as the sweetest naturally occurring compound known, being 300 times sweeter than sucrose (Bottle, 1964). Another well-known naturally occurring compound is glycyrrhizin. It is a triterpenoid glycoside from licorice root and appears to be 50 to 100 times sweeter than sucrose. The new sweetening agent from the fruit of *Dioscoreophyllum cumminsii* now appears to be the sweetest naturally occurring substance known.

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Ms. received 4/1/68; revised 11/15/68; accepted 12/12/68.

The authors are grateful to Dr. J.F. Kincaid, Dr. James H. Ware, and Dr. F.A. Hoglan for helpful interest in this work, and to Mrs. Nonja Bisgard for some preliminary laboratory assistance.

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Stimulation of Gas Production and Growth of Clostridium perfringens Type A (No. 3624) by Legumes

SUMMARY—Dry beans and other legumes contain an unidentified factor which stimulates rapid growth and gas production by Clostridium perfringens, *Type A. This factor may be related to the flatus-inducing properties of dry beans. It is suggested that flatus gases are the product of accelerated gas production by the intestinal anaerobe. Gas production and growth of* C. perfringens *were inhibited by some of the same antibiotics that are known to block flatulence in higher animals. Hydrogen and carbon dioxide, the major constituents in flatus gases, were also found to be the primary gases collected over cultures of the anaerobe grown in a synthetic medium. Bland foods, such as rice and barley, evoked minimal responses. Pure carbohydrates including lactose, raffinose, stachyose and starch had no effect on gas production when the organism was grown in a complete basal medium containing glucose. An assay procedure has been developed for measuring the response of the microorganism to various substrates. This procedure should facilitate isolation, purification and characterization of the unknown factor. If a direct relationship can be established between this factor and the flatulence factor in dry beans, the assay procedure should find applications in establishing a flatus index for foods and aid in the development of nonflatulent food products.*

INTRODUCTION

IT IS generally acknowledged that ingestion of cooked dry beans causes human flatulence (Hedin et al., 1962). This characteristic discourages broader use of these low cost, high protein foods. The factor responsible for the flatus-inducing properties of dry beans has not been established. Evidence reviewed below suggests that *Clostridium perfringens*, normally present in the gastrointestinal tract, may be a dominant factor in the production of flatus gases and that the legume factor stimulates gas production by the anaerobe.

Flatus gases contain major proportions of carbon dioxide and hydrogen, (Kirk, 1949; and Askevold, 1956). Present knowledge of intermediary metabolism precludes the production of hydrogen by higher animals. The production of these gases by anaerobes including many Clostridial species is well documented (Gray et al., 1965). The primary intestinal flora appears to be anaerobic (Weiss et al., 1937) with the population increasing toward the distal end of the intestine (Richards et al., 1966). Nelson (1933) reported that flatus was proportional to the *C. perfringens* population.

The antibiotic, Neomycin, reduced flatus volume and percentages of hydrogen and carbon dioxide in rat flatus (Hedin, 1962). These decreases corresponded to a reduction in the population of all intestinal microorganisms except mold. Mexiform and Vioform inhibited flatus in dogs (Richards et al., 1966), and destroyed anaerobic bacteria in the intestinal tract while the aerobic and coliform organisms increased in total numbers (Eisman et al., 1961). Therefore, gas production resulting from ingestion of bean homogenate must have been due to the anaerobic flora.

In both rats (Hedin et al., 1962) and humans (Murphy, 1964) an induction period of about 4 hr is required before flatus gases are passed. Since gases introduced directly into the digestive tract are generally passed within 30 min (Danhof, 1953), flatus gases must be formed no earlier than 3 hr after ingestion of the legume substrate. This delay corresponds to the time required for the ingested food to reach the region of the intestine (Goldman, 1924) dominated by the anaerobes (Weiss et al., 1937). Isolation and identification of the flatus factor have been hampered by the unavailability of a satisfactory method for estimating flatulence. Richards et al. (1965, 1966) and Rackis et al. (1966) reported that cultures of *C. perfringens* have been used in studies directed toward the fractionation and isolation of the flatus factor in soybeans. However, details of the procedures have not been described completely.

This study was undertaken to develop a reliable method for estimating the stimulation of gas production and growth of *C. perfringens* by legume homogenates,

and to provide a convenient assay procedure to use as a guide to the isolation of sufficient amounts of pure active factor to permit its chemical characterization and testing of its physiological effects.

MATERIALS & METHODS

Culture

Clostridium perfringens, Type A, No. 3624 was obtained from the American Type Culture Collection as a lyophilized culture. Stock subcultures were transferred monthly into Bacto Egg Meat Medium. They were incubated at 45°C for 4 to 5 hr and held in the dark at 20°C. Sixteen hrs prior to the preparation of the inoculum, 2 ml of the stock culture were transferred to 750 ml of Bacto Brain Heart Infusion in a 1 L screw-capped, Erlenmeyer flask. It was incubated at 45°C with the cap partly unscrewed. The seed culture was centrifuged at 4080 × g for 5 min in 250-ml centrifuge bottles and the supernatant liquid decanted.

The pellets were thoroughly dispersed in sterile distilled water, combined and recentrifuged. The supernatant wash water was decanted and the pellet was redispersed in sterile distilled water and adjusted to a standard optical density by a Bausch and Lomb Spectronic 20 spectrophotometer. The supectrophotometer was standardized by use of a Coleman-Nephlos 22 standard adjusted to an absorbance value of 0.1 An absorbance reading of 0.3 at 535 mμ corresponded to an inoculum containing 10^6 microorganisms per ml. Each sample tube was inoculated with 1.5 ml of the standardized inoculum.

Basal medium

A completely defined synthetic basal medium facilitates standardization of an assay procedure and permits non-equivocal recognition of stimulatory or inhibitory substrates. Several synthetic media have been developed for *C. perfringens*. After a systematic study of the nutritional requirements of *C. perfringens* BP6K, Boyd et al. (1948) developed a defined medium for the microbiological assay of essential nutrients. Fuchs et al. (1957) and Murata et al. (1964) modified the synthetic medium to optimize toxin production. However, since maximum toxin production is not related directly to maximum growth (Mueller et al., 1948), this study employed the medium of Boyd et al. (1948) without significant modification.

Each constituent of this synthetic medium was tested at levels up to four times the standard level to determine if supplementary amounts of any of the constituents, which may be introduced with legume substrates, would produce either stimulation or inhibition. No changes in growth rate or gas production were observed at the higher levels of each component. Reduction of several constituent levels to half or less of the standard medium decreased growth rates indicating their essentiality as well as their near optimum levels in the standard medium.

The basal medium was prepared from a dry, ball-milled mixture of pure amino acids (Rockland et al., 1946) and solutions of vitamins, minerals and other supplements described by Boyd et al. (1948). Ten ml of double strength basal medium were placed in 20 × 150 mm borosilicate glass screw-capped test tubes. Aliquots of substrate, representing 4 concentration levels of finely dispersed bean slurries were added to each tube. Sterile distilled water was added to the control and sample tubes so that each tube contained 17.5 ml of solution. Screw caps were fitted loosely and the tubes were autoclaved for 15 min at 121°C. One ml of a sterile, 20% glucose solution and 1.5 ml of standardized washed inoculum were added bringing the final volume of each tube to 20 ml. The caps were tightened and the contents mixed thoroughly.

A sterile, inverted 10 × 75 mm borosilicate glass test tube was placed aseptically in each tube and the screw caps refitted. The tubes were inverted to fill the inner tube and reinverted so that the completely filled inner-tube rested at the bottom of the larger tube. Screw caps were loosened to allow pressure equilibration during incubation in a circulating-water bath at 45°C. Generally two replicate tubes were employed at each of four substrate levels. The height of medium displaced from the inner tube and exact incubation time were recorded for each tube periodically during active gas evolution. The effect of incubation time and substrate level on the response of *C. perfringens* to Lima beam homogenate is shown in Table 1.

Table 1—Effect of incubation time on gas production by C. perfringens grown in a synthetic medium containing rehydrated whole dry Lima bean homogenate.

Dry Lima bean (mg/tube)	Gas produced (mm)[1] after incubation time (min) of: 95	165	240
0	1	4	9
100	1.5	10	26
200	2	12	34
300	4	17	39

[1] Height of medium displaced in duplicate inverted 10 × 75 mm test tubes.

At highest substrate levels, maximum gas displacement (75 mm) was obtained within about 6 hr. Suspended solids in tubes containing beam homogenates did not allow a direct comparison between gas production and growth estimated turbidimetrically. Therefore, proteose peptone, which gave a clear solution, was employed as substrate to determine the relationship between growth and gas production. During incubation at 45°C over a period of 6 hr, a linear relationship was observed between growth and gas production (Fig. 1). Microscopic examina-

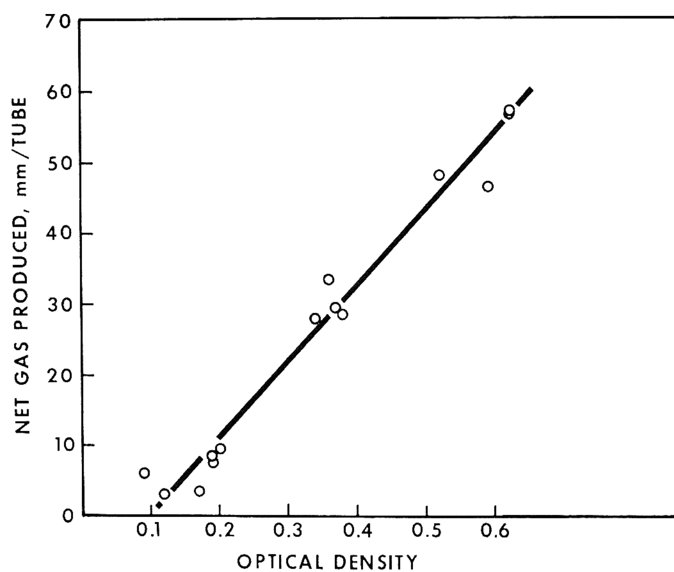

Fig. 1—Relationship between gas evolution and turbidimetrically estimated growth compared in a proteose peptone supplemented synthetic basal medium incubated at 45°C for up to 6 hr.

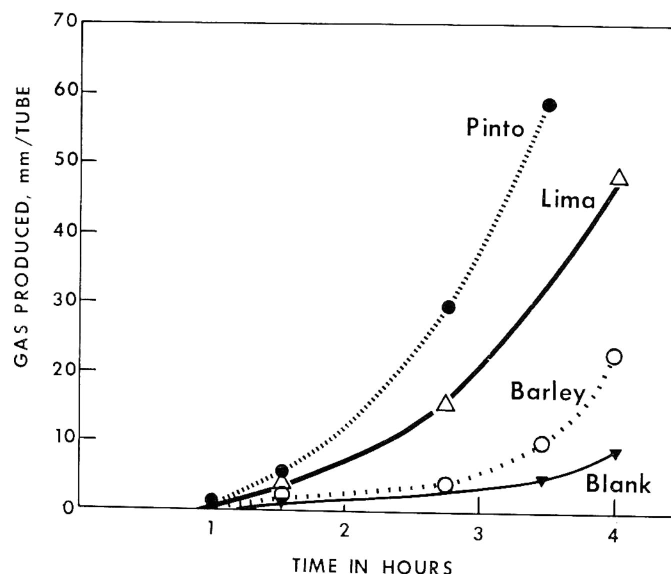

Fig. 2—Effects of barley, pinto and Lima beans on the rate of gas production by C. perfringens. Substrate level 270 mg/tube.

tion of cultures after incubation with bean homogenates indicated that accelerated gas production was associated with prolific growth.

Activity of each test substance was estimated in the following manner. Gas production was measured periodically at each substrate level during a 6-hr incubation period. At each substrate level gas production was plotted against incubation time as illustrated in Figure 2. Substrate activity was estimated at an incubation time chosen so that tubes containing the lowest substrate level displaced less than 30 mm of medium from the inverted inner tube and tubes containing highest substrate level displaced more than 30 mm of medium. A gas production vs substrate concentration curve was prepared for each sample (Fig. 3) and the amount of substrate required to produce a unit, generally 30 mm, gas displacement was estimated. Gas displacements as low as 20 mm were employed when materials such as rice or barley elicited minimal response during the usual range of incubation periods.

Since activities were measured over the near linear portion of the curve, activities calculated within the range 20 to 30 mm gas displacement were not appreciably different. Incubation periods ranged from 3.5 to 5 hr, depending upon the specific activities of substrates. The relative activity of each substrate was calculated as the ratio of the weight of Lima beans (standard) to the weight of test substance. Since all of the samples contained about the same moisture content under the same ambient conditions, it was not considered necessary to correct relative activity values for moisture content of the dry samples. An estimate of the precision of an assay is presented in Table 2. It is presumed that the lower values obtained for single bean samples were due to mechanical losses involved in the preparation of single bean homogenates.

Table 2—Precision of the response of C. perfringens to Lima bean homogenates.

Sample homogenate	Replicate assays	Average ($\bar{x}$)	Activity[1] Range	Standard deviation (σ)	Probable error of mean (γ)	$\sigma/\bar{x}$
Composite[2]	5	1.01	0.97 to 1.08	0.088	0.024	0.088
Single beans	15	0.79	0.66 to 1.02	0.048	0.022	0.061

[1] See text.
[2] Composite of 20 beans.

Gas chromatography

Fixed gases were estimated using an Aerograph A-100 gas chromatograph equipped with a thermal conductivity detector operated at 185 mv. Separation of hydrogen, oxygen, nitrogen, methane and carbon dioxide was accomplished at 25°C on a single coiled 20 ft × ⅛ in. (0.101 in. ID) Type 304, Weldrawn stainless steel column packed with Barneby-Cheney chromatographic grade activated carbon type SA 1850. Helium was employed as carrier gas at a flow rate of 30 ml/min. Approximately 1 to 2 ml of gas sample were injected through a silicone rubber septum using a gas-tight 2 ml syringe. Standard gas mixtures were prepared volumetrically by displacement of a vertical column of mercury in a gas buret. The relative retentions of the gases were: H_2, 0.33; O_2, 0.93; N_2, 1.00; CH_4, 3.7; and CO_2, 11.9. The sensitivity of the detector for each gas was: H_2, 1.0; O_2, 31; N_2, 29; CH_4, 1.6; and CO_2, 2.3.

RESULTS

ACTIVITY VALUES for homogenates of rice, barley and various dry beans are presented in Table 3. Rice and barley, both of which have a high starch content and are not considered flatulent, had less than 30% of the activity of Lima beans. Several other legumes appeared more active than Lima beans. Interestingly, a mixture of equal weights of rice and highly active soybeans had less than half of the sum of the activities of these products evaluated individually.

Legumes contain significant amounts of the two unique sugars stachyose and

Table 3—Relative activities of dry beans and other products for stimulation of gas production by C. perfringens.

Product	Activity Exp 1	Exp 2	Average
Pearl barley	0.16	0.23	0.20
Pearl rice	0.34	0.22	0.28
Blackeye beans	0.73	—	—
Lima beans, large	1.0	1.0	1.0
Garbanzo beans	1.5	1.6	1.6
Lima beans, baby	1.4	1.7	1.6
Whole green peas	1.9	1.6	1.8
Great Northern beans	1.8	—	—
California small white beans	1.9	—	—
Pinto beans	1.9	—	—
Black beans	2.4	2.4	2.4
Pink beans	2.4	2.7	2.6
Soybeans	4.4	3.9	4.2
Pearl rice plus soybeans	—	1.3	—

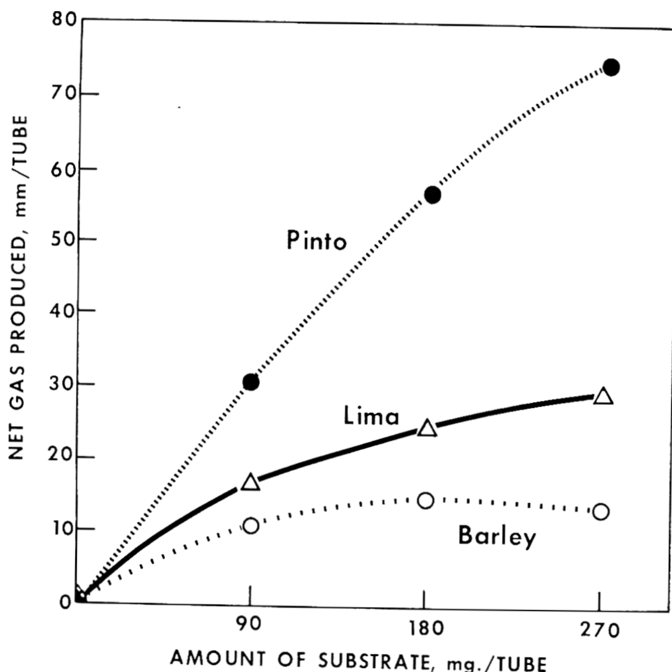

Fig. 3—Effects of substrate levels on gas production by C. perfringens grown for 4 hr in a synthetic medium containing supplements of barley, pinto and Lima bean homogenates.

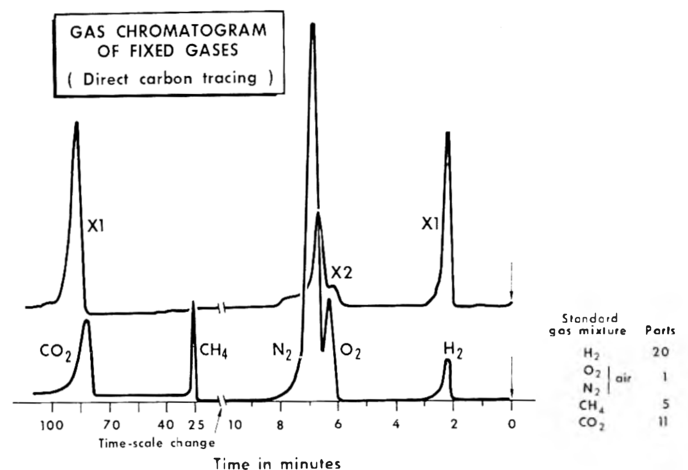

Fig. 4.—Qualitative analysis by gas chromatography of gases generated over cultures of C. perfringens incubated with a homogenate of Lima beans.

raffinose. It has been suggested that these compounds may be involved in flatulence resulting from the ingestion of dry beans (Murphy, 1964; and Steggerda et al., 1967). Neither of these sugars or starch stimulated gas production by *C. perfringens* when added to a synthetic basal medium containing 0.5% glucose (Table 4). Similarly, no stimulation of gas production was obtained when the more easily fermentable lactose was employed as a supplement (Table 5). On the other hand, addition of Lima bean homogenate with or without lactose produced a marked acceleration of gas production. These experiments demonstrate conclusively that the primary stimulant in dry beans is something other than one of these simple sugars.

Table 4—Effects of sugars on gas production by C. perfringens.[1]

Sugar	Relative volume of gas produced (%)
Maltose	101
Glucose	100
Fructose	55
Sucrose	45
Galactose	35
Arabinose	10
Raffinose	10
Stachyose	0

[1] Incubated 210 min at 45°C. Basal medium contained 0.5% sugar. Glucose arbitrarily assigned a value of 100%.

Table 5—Effects of sugars and a homogenate of rehydrated large dry Lima beans on gas production by C. perfringens.

Medium supplement	Gas production (mm)[1] at Carbohydrate level (mg/tube) of			
	200	400	600	800
Glucose	12	12	12	9
Glucose + 200 mg dry Lima bean	33	36	36	36
Lactose + 200 mg glucose	12	13	11	—
Lactose + 200 mg glucose + 200 mg dry Lima bean	34	37	33	—

[1] Height of medium displaced in inverted 10 × 75 mm test tubes. Average of duplicate tubes incubated 330 min at 45°C.

The assay was developed primarily to aid in the isolation of the factor in dry beans which stimulates gas production by *C. perfringens*. However, it was of interest to compare the results obtained using the microbiological assay procedure with the only quantitative data available from assays of similar products with human subjects (Table 6). The similarity in the relative activity values observed for Lima, kidney and small white beans is striking, although the materials studied were obtained from different sources.

A variety of substances, including several antibiotics, inhibited growth and gas production at low substrate levels (Table 7). These effects may be related to the inhibition of flatus by addition of antibiotics to flatus-inducing foods (Kakade et al., 1967). Gas chromatography was employed for the qualitative analysis of the gases evolved during incubation of *C. perfringens* with Lima bean slurries (Fig. 4). About equal amounts of H_2 and CO_2 were evolved after incubation of *C. perfringens* ATCC No. 3624 with a Lima bean homogenate. These observations are consistent with the premise that *C. perfringens* fermentation may be the direct source of flatus gases and that the pure culture might be employed as an assay organism for estimating the flatulence factor in food products.

Table 6—Comparison of the results of human flatus assays of dry beans and the relative activities of similar products for the production of gas by C. perfringens.

Dry bean	Human flatus assay[1]		Activity for *C. perfringens*	
	Total flatus (ml/3 hrs)	Relative gas volume	Relative gas[2] volume	Relative activity
Lima, large	525	1.0	1.0	1.0
Red kidney	1120	2.1	1.9	2.2
California small white	1050	1.9	1.6	1.9

[1] Murphy, 1964.
[2] Incubated with 50 mg/tube of homogenate for 90 min at 45°C.

Table 7—Substances inhibiting growth and gas production by C. perfringens.

Substance	Levels tested (mg/100 ml)	Inhibitory level	
		50% inhibition	100% inhibition[1]
Penicillin G, potassium	0.10 to 100	0.10	100
p-Aminobenzene sulfonamide	0.10 to 100	1.0	—
Sodium sulfide	0.05 to 500	5	400
Azosulfamide	0.10 to 100	10	—
Ethionine	0.10 to 250	250	—
Sodium thioglycolate	0.50 to 1500	500	—
Picolinic acid	5 to 500	—	500

[1] No growth or gas formed within 24 hr at 45°C.

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Ms. received 6/14/68; revised 8/26/68; accepted 9/5/69.

The Fruit and Vegetable Chemistry Laboratory is a laboratory of the Western Utilization Research and Development Division of the Agricultural Research Service.

This work was supported in part by the California Lima Bean Advisory Board. The mention of specific instruments or materials does not imply endorsement of such instruments or material by the U.S. Department of Agriculture over others of a similar nature.

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Anthocyanin Pigments in Trousseau Grapes

SUMMARY—The anthocyanin pigments in Trousseau grapes were extracted with 0.1% HCl in methanol, purified with Dowex 50 WX 4 cation exchange resin in the hydrogen form, and further purified by paper chromatography. Identification of the pigments was based on R_f values in various solvents, partial acid hydrolysis, sugar moiety, alkaline degradation, fluorescence under ultraviolet radiation, color reactions, and absorption spectra in the visible region. Shown to be present in the grapes are petunidin 3-monoglucoside, cyanidin 3-monoglucoside, malvidin 3-monoglucoside, and peonidin 3-monoglucoside. Based on photodensitometric measurement, malvidin 3-monoglucoside was present in largest amount (49.8%), followed by peonidin 3-monoglucoside (36.9%), cyanidin 3-monoglucoside (8.75%), and petunidin 3-monoglucoside (4.55%). The identification of all the anthocyanins as monoglucosides supports the classification of Trousseau as a cultiva of Vitis vinifera.

INTRODUCTION

ANTHOCYANINS are the pigments responsible for the red color in grapes. Anderson et al. (1926) studied the pigments in Isabella grape, a hybrid of *V. vinifera* and *V. labrusca*. They established the structure of the anthocyanidin obtained from acid hydrolysis of oenin which is presently called malvidin. Rankine et al. (1958) surveyed the pigments of 55 vinifera samples from 42 known varieties. They found the same pigment in greatest concentration in all varieties. Akiyoshi et al. (1963) studied the major anthocyanins of *V. vinifera* table-grape varieties Flame Tokay, Emperor, and Red Malaga. They reported that the principal skin pigment was cyanidin 3-monoglucoside.

Somaatmadga et al. (1963) isolated six anthocyanins from Cabernet Sauvignon grapes. The major pigments were malvidin 3-monoglucoside, delphinidin 3-monoglucoside, petunidin 3-monoglucoside, and malvidin 3-monoglucoside acylated with chlorogenic acid. Ribéreau-Gayon et al. (1955, 1958) showed a genetic relationship among *V. vinifera* varieties with regard to pigmentation. They studied about 80 hybrids and 30 varieties of *V. vinifera* and concluded that *V. vinifera* contained monoglucosides, but no diglucosides. This is a genetic characteristic of this variety. Reuther (1961) confirmed the findings of Ribéreau-Gayon and postulated the mode of inheritance of the diglycosides of malvidin, petunidin, and delphinidin. He also discussed the importance of genetic markers in breeding grapes.

Albach et al. (1963) reported the presence of peonidin 3-monoglucoside in *V. vinifera* grapes. Albach et al. (1965) studied the structures of acylated anthocyanin pigments in *Vitis vinifera* variety Tinta pinheira. They identified the pigments as 3-monoglucosides of malvidin, peonidin, delphinidin and petunidin, each acylated with p-coumaric acid. They also found the same anthocyanins, each acylated with caffeic acid. Anthocyanins in Rubired grapes have been identified by Smith et al. (1965). Chen et al. (1967) used Dowex-50 W X 4 cation exchange resin and two-dimensional paper chromatography for separation and identification of anthocyanins in Royalty grapes. Koeppen et al. (1966) reported the anthocyanins in Barlinka grapes. The pigments were identified as oenin (malvidin 3-glucoside), mono-p-coumaroyl oenin and the 3-glucosides of peonidin, petunidin and delphinidin. Oenin was by far the major pigment of Barlinka grape skins.

The Trousseau grape, originally from Portugal, is a variety long known in the San Joaquin Valley for its vigor and productivity of early-ripening fruit that makes excellent dessert wines (Olmo et al., 1962). The chemistry of the anthocyanins in this variety is not known. The present work covers the isolation, purification, and identification of the anthocyanins pigments in Trousseau grape.

MATERIALS & METHODS

Grapes

Ripe Trousseau grapes were supplied by Dr. H. P. Olmo and Mr. A. Koyama of the Department of Viticulture and Enology. The berries were harvested from 10-year-old vines from the University vineyard at Davis, washed with tap water, stemmed, sealed in cellophane bags, and stored at 0°F.

The Trousseau grapes had the following characteristics:

Soluble solids at 20°C	21.5 Brix
Total acidity	0.76% (as tartaric acid)
Total pigment	0.085 g/100 g

Approximately 3 lb of grapes were thawed at room temperature. The skin portion was blended under a nitrogen gas atmosphere in a Waring Blendor with 300 ml of 0.1% HCl in absolute methanol for 5 min. The resulting mixture was filtered through Whatman No. 1 paper under vacuum. The residue was extracted two more times with the same solvent. The combined extracts were mixed with a sufficient amount of Dowex 50 W X 4 cation exchange resin in the hydrogen form with occasional stirring (Chen et al., 1967).

After setting for a short time, the resin was thoroughly washed with distilled water to remove free sugars and pure methanol to remove organic compounds. The pigments were eluted from the resin by successive extractions with 1 L of 0.1, 0.2, 0.3, 0.4 and 0.5% methanolic HCl (v/v). The combined extract was concentrated in a flask evaporator under vacuum almost to dryness and then redissolved in a small amount of 0.01% HCl in methanol. The pigment mixture was stored at 0°C in the dark under a nitrogen atmosphere.

Paper chromatography

The various solvent systems for paper chromatography of the components have been described previously (Chen et al., 1967).

Whatman No. 3 MM papers (67 × 46.5 cm) were used for purification and photodensitometric measurement of pigments. Whatman No. 1 papers (67 × 46.5 cm) were used for identification of anthocyanins, aglycones, sugars and alkaline degradation products.

Bar technique. For partial acid hydrolysis and alkaline degradation studies, a small amount of pure pigment was obtained by the bar technique. (Smith et al., 1965).

Development of the chromatograms was carried out by the descending flow of a BAW system for 24 hr. After air drying, 4 well-defined bands, numbered from 1 to 4 in increasing distance from the origin, were cut from the papers. Corresponding bands from 60 papers were combined and cut in small pieces and eluted with 0.1% methanolic HCl immediately after the papers just became dry. Eluate from each band was concentrated to a small volume and then restreaked on

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Whatman No. 3 MM papers for descending development in AWH for 12 hr.

Each of the four bands did not separate into additional bands. The papers containing the pigment were cut again, eluted with the same solvent, concentrated and dissolved in a small volume of 0.01% methanolic HCl. The purified pigments were kept at 0°C in the dark under a nitrogen atmosphere.

Two dimensional paper chromatography

For absorption spectra measurement and for R_f determination, a small amount of pure pigment was obtained by two dimensional paper chromatography on several Whatman No. 3 MM papers. The paper was irrigated with BAW as the first solvent in the long direction and AWH as the second in the short direction. Like spots from the papers were cut and combined. The pigments were eluted several times at room temperature with 0.1% methanolic HCl. The corresponding eluates were combined, concentrated to smaller volume in a flask evaporator and stored under nitrogen atmosphere at 0°C.

Sugar moiety. One ml of concentrated pigment solution was hydrolyzed by refluxing with 2 ml 1 *N* HCl in a micro-flask for 60 min in a boiling brine bath. Dowex 50 W X 4 cation exchanger in the hydrogen form and Dowex 1 X 8 anion exchanger in the acetate form. Forty µl of the clear supernatant was spotted on Whatman No. 1 chromatography papers. Separate spots of 8 µl each of 0.5% solution of glucose, galactose, rhamnose, arabinose and xylose were used as references. The chromatograms were developed separately with BAW for 18 hr, Et-A-W for 9 hr, and Bu-Py-W for 18 hr. They were allowed to run off the paper for R_g value determination.

The sugar spots were visualized by spraying with the aniline hydrogen phthalate reagent (Partridge, 1949; Chandler et al., 1961; Lynn et al., 1964).

Studies of aglycone. The aglycone from acid hydrolysis of the anthocyanin in HCl was absorbed on Dowex 50 X 4 cation exchange resin as described above. The resin was washed with distilled water, and the anthocyanidin was eluted with 0.1% methanolic HCl. The eluate was evaporated to a small volume in a flask evaporator. The resulting concentrate was kept at −16°F in the dark for aglycone studies.

R_f values of the aglycones. The aglycones were chromatographed on acid-washed Whatman No. 1 papers with BAW for 18 hr, Forestal for 15 hr and For-HCl-W for 6 hr. R_f values were measured shortly after the papers dried.

Alkaline degradation of aglycones. The remaining aglycone concentrate was combined and refluxed with 6 ml of 15% $Ba(OH)_2$ for 30 min in a microflask with a continuous flow of nitrogen gas as described previously (Luh et al., 1965).

Partial acid hydrolysis. Partial acid hydrolysis of purified pigments was done following the method described by Abe et al. (1956).

Properties of anthocyanins

R_f values. The individual purified pigments were spotted on Whatman No. 1 papers. The chromatograms were developed with BAW for 18 hr, AWH solvent for 6 hr, and 1% HCl for 4 hr. The papers were air-dried and the R_f values were measured. The pigments on the freshly dried chromatograms were examined for fluorescent behavior with an ultraviolet light lamp of 3,600 Å unit output (Model 1910, Burton Medi-Quip. Co., Van Nuys, Calif.).

Color reaction with aluminum chloride. The dried two-dimensional chromatograms were sprayed with 5% aluminum chloride in 95% ethanol. This reagent distinguishes between anthocyanins having two hydroxyl groups in the ortho position in the B-ring such as cyanidin, petunidin and delphinidin, which give a positive color change, and pelargonidin, peonidin and malvidin, which give no color change.

Absorption spectra in the visible region. The absorption spectra of the pure pigments in the visible region were recorded with a Beckman DB recording spectrophotometer. The purified pigment was dissolved in methanol containing 0.01% conc. HCl. The solvent was used as a blank. The shift in the absorption peak after adding 3 drops of 5% aluminum chloride in 95% ethanol was recorded.

Photodensitometric measurement. Photodensitometric measurement of all pigments on Whatman No. 3 paper strips were recorded with a recording Photovolt Densitometer, using a green filter having a maximum transmission at 525 mµ. A fresh methanolic pigment extract was chromatographed in two dimensions on Whatman No. 3 sheets. Each pigment was isolated on air-dried strips, 4 cm wide from the same chromatogram and passed through the densitometer standardized against a strip from the blank portion of the chromatogram. The density of each pigment was recorded and the area under each peak measured with a planimeter (Gelman Instrument Co., Germany).

Table 1—Comparison of R_f and R_g values of sugar moiety of Trousseau grape anthocyanins with those of authentic sugar samples by paper chromatography.

	BAW R_f		BAW R_g		Bu-Py-W R_g		Et-A-W R_g		Identification
Pigment									
1	0.17		1.00		0.99		0.99		glucose
2	0.16		0.98		0.97		0.99		glucose
3	0.17		0.99		1.00		1.00		glucose
4	0.18		1.00		0.98		1.00		glucose
Total Extract	0.18		0.98		0.99		0.98		glucose
Authentic Sugars									
	Found	Ref[1]	Found	Ref[2]	Found	Ref[3]	Found	Ref[4]	
Glucose	0.17	0.18	1.00	1.00	1.00	1.00	1.00	1.00	
Galactose	0.15	0.16	0.88	0.90	0.79	0.81	0.88	0.87	
Rhamnose	0.38	0.37	1.61	1.61	2.33	2.34	2.15	1.96	
Arabinose	0.21	0.21	1.18	1.17	1.28	1.36	1.45	1.43	
Xylose	0.26	0.28	1.30	1.27	1.60	1.62	1.62	1.77	

[1] Partridge, 1948.
[2] Lynn et al., 1964.
[3] Albach et al., 1963.
[4] Jermyn et al., 1949.

RESULTS

Paper chromatography of anthocyanins

The anthocyanins were separated both by two-dimensional paper chromatography and the bar technique. Four pigments were found. The two-dimensional technique was done using BAW solvent in the first direction and AWH solvent in the second direction. Best results were obtained when the solvent was allowed to run off the paper.

Chromatography of the extract by the bar technique with BAW as solvent gave four distinct bands. They were designated as bands 1, 2, 3, and 4. Since each band contained only one pigment, the same number for the band was assigned to that for the pigment. The results are in agreement with those obtained by Albach et al. (1963), who found that the best general solvent is the organic phase of BAW for separation of grape anthocyanins on paper. However, AWH was run as a second solvent in order to detect possible presence of minor pigments.

Sugar moieties

Table 1 shows the R_f and R_g values of the sugar moieties obtained from acid hydrolysis of the pigments and those of authentic sugar samples with BAW, Bu-Py-W, and Et-A-W as irrigating solvents. Results show that R_f and R_g values of the sugar moieties from all pigments were close to that of glucose. The same result was obtained when the total extract of the pigments was hydrolyzed. Thus, glucose was the only sugar moiety present in the anthocyanins of Trousseau grapes. The result is in agreement with the results of Ribéreau-Gayon et al. (1958), Akiyoski et al. (1963), Reuther (1961), and Albach et al. (1963, 1965) who reported that glucose was the only sugar moiety in the anthocyanins of several varieties of grapes.

R_f values of the aglycones

The agylcones of the pigments were determined by paper chromatography. Table 2 shows the R_f values of the aglycone moieties of the anthocyanins and those of anthocyanins reported in the literature with BAW, Forestal, and For-HCl-W as solvent systems. R_f values of

Table 2—R_f values of the anthocyanidins derived from Trousseau grape anthocyanins.

	R_f values of anthocyanidins			
	BAW	Forestal	For-HCl-W	Identification
Pigment				
1	0.50	0.47	0.23	Petunidin
2	0.67	0.52	0.20	Cyanidin
3	0.58	0.61	0.33	Malvidin
4	0.70	0.67	0.35	Peonidin
Reported				
By Harborne (1958a)				
Petunidin	0.52	0.46	0.20	
Cyanidin	0.68	0.49	0.22	
Malvidin	0.58	0.60	0.27	
Peonidin	0.71	0.63	0.30	
By Albach (1963)				
Petunidin	0.47	0.54	0.23	
Cyanidin	0.67	0.58	0.25	
Malvidin	0.56	0.68	0.26	
Peonidin	0.71	0.75	0.34	

Table 3—Chromatographic comparison of alkaline degradation products of Trousseau grape anthocyanins with phenolic compounds.

Pigment	R_f values		Color[1]			Identification
	BAW	2% HOAc	UV + NH_3	DPNA[2]	DPNA + NH_3	
1	0.76	0.50	—	Op	RP	3-o-Methylgallic acid
	0.70	0.62	BF	O	DO	Phloroglucinol
2	0.81	0.55	—	T	dP	Protocatechuic acid
	0.70	0.62	BF	O	DO	Phloroglucinol
3	0.83	0.52	—	O	NB	Syringic acid
	0.70	0.62	BF	O	DO	Phloroglucinol
4	0.86	0.57	—	Y	DP	Vanillic acid
	0.70	0.62	BF	O	DO	Phloroglucinol
Total	0.76	0.50	—	Op	RP	3-o-Methylgallic acid
Extract	0.81	0.55	—	T	dP	Protocatechuic acid
	0.83	0.52	—	O	NB	Syringic acid
	0.86	0.57	—	Y	DP	Vanillic acid
	0.70	0.62	BF	O	DO	Phloroglucinol
Authentic phenolic compounds						
Vanillic acid	0.86	0.57	—	Y	DP	
Syringic acid	0.83	0.52	—	O	NB	
3-o-Methylgallic acid	0.76	0.50	—	Op	RP	
Protocatechuic acid	0.81	0.55	—	T	dP	
Phloroglucinol	0.70	0.62	BF	O	DO	

[1] (B) blue, (F) fluorescent, (O) orange, (D) dark, (R) red, (P) purple, (p) pink, (T) tan, (d) dull, (Y) yellow, (N) navy.
[2] Diazotized P-nitroaniline spray.

anthocyanidins are quite close to those reported in the literature. It appears that the aglycone moiety of pigment 1 is petunidin; pigment 2, cyanidin; pigment 3, malvidin; and pigment 4 peonidin, respectively.

The stability of anthocyanidins is markedly dependent on pH. They are less stable at higher pH values and tend to fade when chromatographed in solvents containing no mineral acids, such as BAW. However, this solvent can be used in paper chromatography of anthocyanidins, when the paper sheets are previously washed with dilute hydrochloric acid.

Bate-Smith (1950) suggested that when using solvents containing no mineral acid, it is important to have sufficient HCl present in the original extract or on the paper to keep the anthocyanidins in the chloride form. Thus the chromatograms using BAW as solvent were developed on acid-washed papers. The Forestal and For-HCl-W solvents gave good results.

Alkaline degradation of the aglycones

The aglycones are hydrolytically cleaved by alkali degradation at C_2-C_3 and C_4-A ring bonds to yield phloroglucinol (derived from ring A) and benzoic acid derivatives. The identification of the benzoic acid derivatives plays a very important role in proving the structure of the pigments, because each anthocyanidin yields a distinct benzoic acid derivative which can be identified by its R_f value and color characteristics, using phenolic compounds as references (Smith et al., 1965). The results are shown in Table 3. The benzoic acid derivatives and phloroglucinol were examined under ultraviolet radiation in the presence and absence of ammonia vapor. Also, the distinctive color of each spot was observed after spraying with diazotized P-nitroaniline (DPNA) and the color change when exposed to ammonia vapor.

The results contain further evidence that the aglycone of pigment 1 was petunidin; pigment 2, cyanidin; pigment 3, malvidin; and pigment 4 peonidin, respectively.

Partial acid hydrolysis

In order to determine the position and number of sugar residues in the anthocyanins, the partial acid hydrolysis method described by Abe et al. (1956) was used. In anthocyanins, sugar molecules are generally attached to the 3-position or to the 3- and 5-positions. During acid hydrolysis, 3 monoglucoside and 3,5-diglucoside may be distinguished by the fact that the former yields only one simpler product, while the latter yielded two. The 3,5- and 5-glycosides appear as intensely fluorescent under ultraviolet radiation, while 3-glycosides are not fluorescent (Harborne 1958a, 1958b). Partial acid hydrolysis was done only on pigments 3 and 4, because they were the only ones available in sufficient quantity. They gave only one hydrolysis product each, the respective aglycone. Since these pigments did not fluoresce under ultraviolet radiation, it was concluded that they were 3-monoglucosides.

R_f values and aluminum chloride reaction

Table 4 lists the R_f values of the anthocyanins from Trousseau grapes purified by two-dimensional chromatography as compared with those reported in the literature. The relationship between R_f values and structure of the anthocyanins has been noted by Bate-Smith et al. (1950), Abe et al. (1956) and Harborne (1958a). They found that (a) the greater the number of hydroxyl groups present in the anthocyanidin molecule, the lower is its R_f value in both alcoholic and aqueous solvents; (b) methylation reverses the effect of hydroxylation, i.e., the greater the number of methoxyl groups, the higher is the R_f value in both alcoholic and aque-

Table 4—R_f values of the anthocyanins in Trousseau grapes.

	Identification	R_f values of anthocyanins		
		BAW	1% HCl	AWH
Pigment				
1	Petunidin 3 G	0.36	0.04	0.23
2	Cyanidin 3 G	0.40	0.08	0.26
3	Malvidin 3 G	0.38	0.06	0.28
4	Peonidin 3 G	0.42	0.09	0.31
Reported[1]				
Petunidin 3 G		0.35	0.04	0.22
Cyanidin 3 G		0.38	0.07	0.26
Malvidin 3 G		0.38	0.06	0.29
Peonidin 3 G		0.41	0.09	0.33

[1] Harborne, 1958a.

Table 5—Color characteristics and relative amounts of anthocyanins in Trousseau grapes.

Pigment	Identification	Color	Color under ultraviolet radiation	Color change with $AlCl_3$	Relative Amts. %
1	Petunidin-3-monoglucoside	Light purple	Dull purple	+	4.55
2	Cyanidin-3-monoglucoside	Magenta	Dull magenta	+	8.75
3	Malvidin-3-monoglucoside	Magenta	Dull mauve	–	49.80
4	Peonidin-3-monoglucoside	Orange pink	Dull pink	–	36.90

Table 6—The spectral absorption characteristics of the anthocyanins in Trousseau grapes.

	Identification	λmax (mμ)[1]	E_{440}/E_{max} (%)	$AlCl_3$ shift[2] (mμ)
Pigment				
1	Petunidin 3-monoglucoside	536	20	−38
2	Cyanidin 3-monoglucoside	525	19	−18
3	Malvidin 3-monoglucoside	535	18	0
4	Peonidin 3-monoglucoside	523	25	0
Reported[3]				
Malvidin 3-monoglucoside		535	18	0
Peonidin 3-monoglucoside		523	26	0
Cyanidin 3-monoglucoside		525	22	+18
Petunidin 3-monoglucoside		535	18	+24

[1] Measured in 0.01% methanolic HCl.
[2] On addition of 5% ethanolic aluminum chloride.
[3] Harborne, 1958b.

ous solvents. The increase in R_f value brought about by methylation is rather less than the decrease caused by hydroxylation; (c) glycosidation increases the R_f value in aqueous solvents, but decreases it in alcoholic solvents; and (d) acylation causes an increase in R_f value in alcoholic solvents, but lowers the R_f value in aqueous solvents.

On paper chromatograms, the anthocyanins exhibit different colors by reflected light and under ultraviolet radiation. Some anthocyanins on the chromatograms, when sprayed with a 5% ethanolic solution of aluminum chloride, show a positive color change from purple to blue. The results are listed in Table 5. Pigments 1 and 2 changed to a blue color when sprayed with $AlCl_3$ reagent, indicating the presence of two hydroxyl groups in the ortho position. This further supports the previous finding that the aglycone of pigment 1 was petunidin, and that of pigment 2 was cyanidin. Pigments 3 and 4 did not show chelation reaction with $AlCl_3$, supporting the previous finding that the aglycone of pigment 3 was malvidin, and that of pigment 4 was peonidin.

Photodensitometric measurements

Table 5 also shows the relative amounts of the anthocyanins in Trousseau grapes as measured from the two-dimensional chromatogram with the photodensitometer. Pigments 3 (malvidin 3-glucoside) and 4 (peonidin 3-glucoside) were about 87%, and pigments 1 (petunidin 3-glucoside) and 2 (cyanidin 3-glucoside) were about 13% of the total anthocyanins. The results are in agreement with those of Rankine et al. (1958) who stated that in the majority of *V. vinifera* varieties malvidin 3-monoglucoside and peonidin 3-monoglucoside are present in the largest quantity.

Absorption characteristics in the visible region

Table 6 lists the spectral absorption characteristics, the ratios of the optical density (E_{440}/E_{max}) as percentage, and the shift in absorption peak after adding $AlCl_3$. Harborne (1958b) found that the ratio of O.D. at 440 mμ to O.D at maximum peak of 5-free hydroxyl anthocyanin was twice that of the corresponding pigment which has its 5-position substituted. This is a very useful method to differentiate 3-glycosides from 3,5-diglucosides.

From the absorption maxima and the bathochromic shifts with aluminum chloride it was concluded that pigment 1 was petunidin 3-monoglucoside, pigment 2 was cyanidin 3-monoglucoside, pigment 3 was malvidin 3-monoglucoside and pigment 4 was peonidin 3-monoglucoside.

DISCUSSION

THE EXTRACTION of anthocyanins from grapes was easily carried out with 0.1% methanolic HCl under an atmosphere of nitrogen gas to avoid oxidation reactions. Adsorption of the anthocyanins on Dowex 50 W X 4 cation exchange resin eliminates many impurities which would otherwise interfere with the movements of the pigments on paper chromatograms. It is important to point out that the ion-exchange resin should be washed several times, first with 2 *N* HCl, then with distilled water and finally with methanol to remove impurities. It is desirable to allow the pigments to be adsorbed on the resin for a shorter time, preferably less than an hour. This greatly facilitates the elution of the pigments from the resin with acidified methanol.

Arabinose appears as a contaminant in the anthocyanins isolated from Trousseau grapes. Harborne et al. (1957) found that arabinose was produced as an artifact in consequence of the action of HCl on the paper during the purification procedure. To determine whether arabinose was present as a sugar moiety or as an artifact, hydrolysis of the total extract, which had not been chromatographed, was done. No arabinose was found in the hydrolysate. Thus glucose was the only sugar moiety present in the anthocyanins of Trousseau grapes.

The genetic relationship among *Vitis vinifera* grapes with regard to pigmentation was discussed by Ribéreau-Gayon et al. (1955; 1958). They concluded that *V. vinifera* does not contain diglucoside, and that diglycosides were characteristics of *V. riparia* and *V. rupestrics*. The diglycosides are dominant genetically. Reuther (1961) also states that the anthocyanins could be divided into the monoglucoside as a genetic marker in hybrid for *V. vinifera*, and diglycosides as genetic markers for *V. riparia*. The identification of all the anthocyanins as monoglycosides supports the classification of Trousseau as a cultivar of *Vitis vinifera*.

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Ms. received 3/28/68; revised 5/29/68; accepted 9/5/69.

Paper presented at the 28th Annual Meeting of the Institute of Food Technologists in Philadelphia.
This investigation was supported by Public Health Grant EF-00157 from the Division of Environmental Engineering and Food Protection. We thank Professor Harold P. Olmo and Mr. A. Koyama, of the Department of Viticulture and Enology for supplying the grapes.

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Vapor Analysis of Fermented Spanish-type Green Olives by Gas Chromatography

SUMMARY—Five major components were detected gas chromatographically in the head-space vapor (HSV) of Spanish-type green olives fermented by pure cultures of Lactobacillus plantarum, Pediococcus cerevisiae *and* Leuconostoc mesenteroides. *Three of these compounds were identified as acetaldehyde, methyl sulfide, and ethanol. The same compounds were present in unfermented olives but in different amounts. Olives that had undergone a natural fermentation contained the above five compounds, and, in addition, a varying number of other compounds. These results indicated that HSV analysis may be a rapid method for detecting volatile end products resulting from the metabolism of various microorganisms. A high ethanol content was found in olive brines that contained a predominance of yeasts. Abnormal fermentations gave unique HSV profiles, one of which indicated a high level of 2-butanol. Methyl sulfide was found to be a major odor component of fermented as well as unfermented olives. Acetaldehyde and ethanol contributed secondarily to the odor. Primary contributions of fermentation by the above lactic acid bacteria to the flavor of olives were: (1) production of a desirable level of acidity, and (2) utilization of fermentable sugars to the exclusion of microorganisms which produce metabolic end products with undesirable flavor characteristics.*

INTRODUCTION

THE PRESERVATION of fruits and vegetables by brining has been practiced for centuries, with comparatively few technological advances. Organoleptic qualities of products preserved in this manner, such as cucumbers, olives and cabbage, are greatly influenced by the variable nature of microbial activity. Recent efforts have been made to control more closely the quality of such products. This is done by regulating the microbial flora with the addition of pure cultures of lactic acid bacteria to heat-shocked cucumbers and olives (Etchells et al., 1964, 1966).

The concentration of lactic acid, measured as titratable acidity, indicates the extent to which lactic acid bacteria were active in the fermentation of olives. It is an important criterion used to determine the success of olive fermentations. Other end products of microbial metabolism, whether present in large or trace amounts, may be of great significance with respect to organoleptic properties of olives, especially flavor.

Alcohols, esters, aldehydes, ketones, etc., as well as acids, are among end products known to be formed by microorganisms which are competitive with lactic acid bacteria in brined olives. Clostridia, coliforms, yeasts, and molds have been associated with spoilage problems of fermented olives (Vaughn, 1954; Vaughn et al., 1943) and may produce many of the previously mentioned end products.

Gas chromatographic analysis of head-space vapors has been successfully employed in determining volatile constituents of many foods including vegetables (Buttery et al., 1961), milk (Bassette et al., 1963), and alcoholic beverages (Kepner et al., 1964). This work was undertaken to establish the value of the head-space vapor method for detecting volatile compounds as an indication of the types of microorganisms active in the fermentation of olives. It was further desired to ascribe relative significance of the components detectable by the method to the odor of fermented olives.

EXPERIMENTAL

Fermented olives

The pure-culture fermented Spanish-type green olives (Manzanillo variety) examined in this study were samples from a previous study (Etchells et al., 1966). The processing of these samples involved lye treatment to remove bitterness, removal of the lye by leaching in water, heat-shocking the olives at 74°C for 3 min in a water bath, followed by packing into 0.5-gal glass jars with a pasteurized and cooled 40° salometer brine (10.6% NaCl). The addition of 2.5 ml of 85% lactic acid to each jar provided an initial pH of 7.0–7.4.

The jars scheduled for inoculation received 8 ml of a 30 hr culture of the designated species of lactic acid bacterium which had been grown at 32°C in Trypticase Sugar Broth (BBL). After incubating the samples at 21–24°C for 7.5 months, the olives were evaluated as to flavor and other organoleptic

and physical qualities as described by Etchells et al. (1966). Assays of the brines for titratable acidity, pH, and transmittance, and microscopic examination of brines were determined according to Etchells et al. (1964).

A portion of the olives was repacked into quart jars, with the addition of one ml of a one percent aqueous Merthiolate solution to preclude further microbial activity. These jars were then held at 5°C until being assayed by gas chromatography.

Head-space vapor analysis

Olives were removed from refrigeration and immediately pitted with a hand-operated mechanical olive pitter. Ten grams of finely diced olive tissue, 20 ml brine, 40 ml distilled and cooled (5°C) water, 30 g sodium sulfate and a 1 in. Teflon-coated magnetic bar were added to a 125 ml erlenmeyer flask. Two ml of 0.01% (v/v) 2-butanol in water were added to serve as an internal standard.

A rubber septum was fitted onto the flask and, while the contents of the flask were still cool, 30 ml of vapor were removed through the septum with a 50-ml syringe to provide sufficient vacuum to prevent excessive pressure inside the flask upon heating. The flask was submerged up to the neck in a water bath held at 60°C on a hot plate magnetic stirrer.

After holding the flask in the bath for 15 min, with the magnetic bar slowly stirring, 5 ml of vapor were removed with a 6-ml disposable syringe (Monoject 506 S). The syringe, fitted with a 25-gauge needle, was filled and emptied back into the flask four times. The fifth filling was injected into a Barber-Colman Model 10 gas chromatograph equipped with a hydrogen flame detector. A stream splitter allowed 50% of the injected sample to enter the detector and 50% to emerge through the collector port for odor monitoring. The column consisted of a 9-ft U-shaped glass tubing, $^1/_4$-in. i.d., packed with 5% Carbowax 20 M on 60/80 mesh GC-22 Firebrick. The operating conditions were: column, 75°C; detector, 190°C; flash heater, 170°C; nitrogen carrier gas, 23 ml/min.

Relative peak heights of chromatograms were expressed as the peak heights for unknown components divided by the peak height for 2-butanol which was added to each sample. Adjustment in attenuation was necessary to record all of the peaks for a sample and calculations included multiplication by the appropriate factor. The attenuation was the same, however, for respective peaks obtained from different samples.

Assays were performed in duplicate. Vapor components arising from the sampling syringe, flask, reagents, etc. involved in the assay were negligible and did not significantly influence relative peak heights of the major components reported herein.

Identification of head-space vapor compounds was made by comparing their retention times with known compounds on three columns. These columns and the conditions were: (1) Carbowax 20 M, as described above; (2) Diisodecyl phthalate, 5%, on 60/80 mesh Firebrick; 9-ft U-shaped, $^1/_4$ in. i.d. glass column; 75°C; N_2 carrier gas, 23 ml/min; (3) Polyethylene glycol 600, 15%, on 60/80 mesh Firebrick; 6-ft U-shaped, $^1/_4$-in. i.d. glass column; 75°C; N_2 carrier gas, 23 ml/min.

The syringe reaction technique of Hoff et al. (1964) was employed to establish which peaks represented aldehydes or ketones. This procedure involved coating the inside of 5-ml ground-glass hypodermic syringes used to sample the head-space vapor with a solution of either potassium permanganate or hydroxylamine. The absence of a peak after this treatment indicated that the compound was either an aldehyde or a ketone.

Vacuum distillation

Fermented olives were pitted and 200 g of tissue along with 100 ml of cover brine from the olives and 200 ml of distilled water were placed in a 5-L flask. This flask was connected to a high vacuum system containing three traps in series. These traps, in order, were: (1) ice-salt, (2) dry ice-acetone and (3) liquid nitrogen. The assembly was similar to that described by Aurand et al. (1965); but the sample was not swept with nitrogen.

The sample was distilled under a pressure of 35μ of mercury for 3 hr. with the distillation flask being maintained at about 25°C. Approximately 40% of the sample volume was distilled by this procedure.

Mass spectrometry

Mass spectrometry was performed with a coupled Barber-Colman Model 5000 gas chromatograph and a Bendix Model *12*-107 Time-of-Flight Mass Spectrometer. The system was similar to that described by Teranishi et al. (1963). The GLC column was a 6-ft, $^1/_4$-in. stainless steel tubing packed with 5% Carbowax 20 M on 60/80 mesh Chromosorb G. Components eluted from the GLC column were observed as the mass spectral output on an oscilloscope and the mass spectral pattern recorded by rapid scan at appropriate times.

Prior to the run, contents of the liquid nitrogen trap from vacuum distillation were trapped in a U-shaped, 6-in. long, $^1/_4$-in. diameter, stainless steel pre-column of 5% Carbowax 20 M on 60/80 mesh Chromosorb G. This transfer was effected by holding the pre-column in liquid nitrogen while sweeping in the contents of the trap from vacuum distillation with nitrogen gas. The pre-column was then removed from liquid nitrogen and coupled with the injection port of the gas chromatograph. The system was swept with helium as the carrier gas. The temperature of the GLC column was 50°C initially and was programmed at 3°C per min up to a final temperature of 150°C.

RESULTS & DISCUSSION

Pure-culture fermented olives

Gas chromatography of the head-space vapor of pure-culture fermented olives on Carbowax 20 M resulted in chromatogram profiles possessing 5 major peaks. A typical chromatogram obtained from a fermentation by *L. plantarum* is shown in Figure 1A. Peak No. 6 represents 2-butanol which was added as an internal standard. Profiles from olives pure-culture fermented by *P. cerevisiae* and *L. mesenteroides*, as well as those from unfermented (Fig. 1B) and natural fermented (Fig. 1C) olives, indicated the presence of these same 5 components on the basis of retention times.

Compounds represented as peak No. 1, 2 and 5 (Fig. 1) were identified as acetaldehyde, methyl sulfide and ethanol, respectively, by comparison of retention times with known compounds on three different columns (Experimental). The

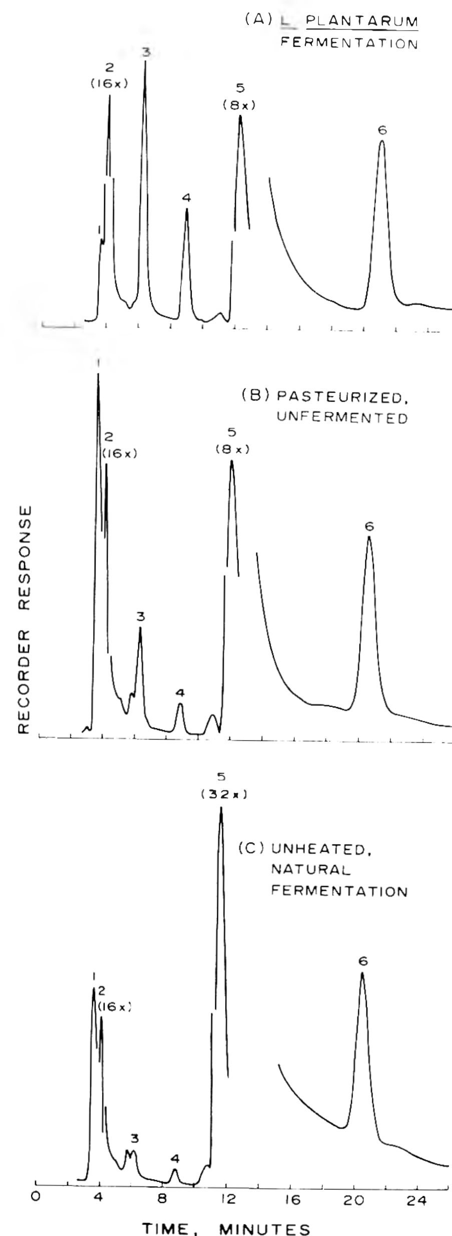

Fig. 1—Head-space vapor GLC profiles of (A) pure-culture fermented; (B) pasteurized, unfermented; and, (C) naturally fermented olives. Numbers above peaks were identified as (1) acetaldehyde, (2) methyl sulfide, (3) unknown, (4) unknown, (5) ethanol, and (6) 2-butanol added as an internal standard. The attenuation was 2 X except as noted in parentheses above peaks. The column was Carbowax 20 M and was operated at 75°C.

Table 1—Vapor and brine analyses of fermented olives.

Fermentation Type	Vapor analysis[1] Acetal-dehyde	Methyl sulfide	Peak No. 3 unknown	Peak No. 4 unknown	Ethanol	Brine analysis pH	Acidity as lactic	Trans-mittance	Microscopic examination
							%	%	
Lactobacillus plantarum	0.48	9.82	1.43	0.64	4.60	3.8	1.10	7	Abundant short rods only
Pediococcus cerevisiae	0.63	8.41	0.58	0.27	4.85	4.6	0.47	25	Abundant pediococci only
Lactobacillus plantarum + *Pediococcus cerevisiae*	0.58	11.30	1.61	0.78	6.05	3.8	1.08	7	Abundant short rods, few pediococci
Leuconostoc mesenteroides	0.72	9.43	0.63	0.70	18.87	5.1	0.34	35	Leuconostoc, in chains, not abundant
Leuconostoc mesenteroides[2]	1.64	10.77	0.19	0.04	7.89	6.7	0.08	75	Few yeasts and rods, not established
Pasteurized, uninoculated control	1.99	11.28	0.56	0.17	6.53	6.2	0.13	77	No microbes seen
Unheated, natural fermentation	1.16	8.44	0.16	0.09	36.31	5.8	0.13	67	Yeasts only
Av. % difference from mean of duplicate assays for 24 samples	4.6	8.4	2.9	3.2	6.0				

[1] Relative peak heights from head-space vapor analysis; average of duplicate assays.
[2] This sample of olives differed from the others in that it did not receive 2.5 ml of 85% lactic acid per 0.5-gal jar as described in the Experimental section.

identification of peak No. 1 as acetaldehyde was further verified by the syringe reaction methods of Hoff et al. (1964) for the removal of aldehydes.

The presence of methyl sulfide was confirmed by vacuum distillation, trapping with liquid nitrogen, and subsequent mass spectrometry. Concentrations of the other volatiles from this distillation were insufficient for identification by mass spectrometry.

While HSV profiles from the various olive samples indicated qualitative similarity, quantitative differences in the 5 major vapor components among fermentations existed, as evidenced by peak heights (Fig. 1). Relative peak heights of HSV profiles were determined to serve as an index of these quantitative differences (Table 1). The reproducibility of relative peak heights, as determined by duplicate assays, is indicated in Table 1, and was considered sufficient for detecting major quantitative variations in vapor composition of olive samples.

Brine analyses which are performed routinely to indicate the success of olive fermentations (Vaughn et al., 1943; 1954) are reported in Table 1 for comparison with the HSV data.

Olive samples inoculated with *L. Plantarum* and *P. cerevisiae* resulted in successful fermentations as determined by the abundant growth of these microorganisms with resulting pH and acidity values typical of a desirable product (Table 1). *L. mesenteroides* was less dependable as the fermenting bacterium as was reported by Etchells et al. (1966).

One sample which fermented to some extent and another which failed to ferment are reported in Table 1. The sample which failed to ferment did not receive 2.5 ml of lactic acid prior to inoculation as did the other samples reported in Table 1. Thus, fermentation failure was probably related to high initial pH. No apparent fermentation occurred in the pasteurized (i.e., heat-shocked at 74°C for 3 min prior to packing) olives. The unheated, natural fermentation resulted in a predominance of yeasts with the development of little acidity.

Several correlations existed between relative peak heights and microbial activity among the olive samples reported in Table 1. Acetaldehyde content apparently was reduced as a consequence of fermentation by lactic acid bacteria, based on comparison with the pasteurized, unfermented sample. Metabolism of this compound in milk by certain cultures of *Leuconostoc citrovorum* has been reported (Lindsay et al., 1965) and may explain its reduction in the present instance.

Ethanol was present in all products; but its increased concentration in brines with high populations of yeasts was apparent. Fermentation by *L. mesenteroides* also resulted in a high level of ethanol, which is consistent with the end products expected for this heterofermentative bacterium. Fermentations by the homofermentative bacteria *L. plantarum* and *P. cerevisiae* resulted in comparatively low levels of ethanol.

Methyl sulfide content in the vapor did not vary greatly among the various fermentation types. Similar levels were present in the vapor of the unfermented and the fermented olives.

Relative peak heights for the unidentified vapor components, indicated as peak No. 3 and 4, also served to characterize the fermentation type (Fig. 1, Table 1). Peak heights for component No. 3 were characteristically high when *L. plantarum* was the fermenting bacterium. The most striking observation concerning component No. 4 was its relatively low concentration in the vapor of unfermented olives and those that underwent natural fermentation with a predominance of yeast activity.

Commercially brined olives

Samples of commercially brined olives that had undergone a natural fermentation gave HSV profiles that indicated variations in qualitative as well as quantitative composition of volatile compounds (Fig. 2). Most commercial brines gave a HSV profile similar to that shown in Figure 2A. The same five major peaks were present in the chromatograms of these brines as were found with the pure culture fermented olives. In addition, several other peaks were evident.

The ethanol peak varied greatly among the commercial brines, presumably reflecting varying degrees of activity by yeasts and heterofermentative lactic acid bacteria. The very low level of acetaldehyde in the commercial brines may have been due to dissipation of this highly volatile compound during handling of the olives by the packer.

Two samples of commercially brined olives considered to be atypical are represented by the HSV profiles in Figure 2B and 2C. In several samples there was a very high level of 2-butanol, ironically the compound used earlier as an internal standard (Fig. 2B). Identity of 2-butanol was made on the basis of retention time on the three GLC columns as described in the Experimental section.

The apparently high concentration of

2-butanol in the brine strongly indicates that appreciable microbial activity in addition to or to the exclusion of lactic acid bacteria occurred in this lot of olives. The odor of these olives, although acceptable, was considered "unclean" in comparison with the pure culture fermented olives.

Another sample of commercial olives had a repulsively strong butyric acid odor and was designated as "malodorous" on the basis of the description by Vaughn (1954). These olives also were soft, and definitely unacceptable for consumer use. The characteristic volatiles of fermented olives were greatly reduced or absent from this sample (Fig. 2C). It seems likely that these olives had been washed several times in an effort to remove the unpleasant odor, but without success as the odor of butyric acid is detectable at very low levels.

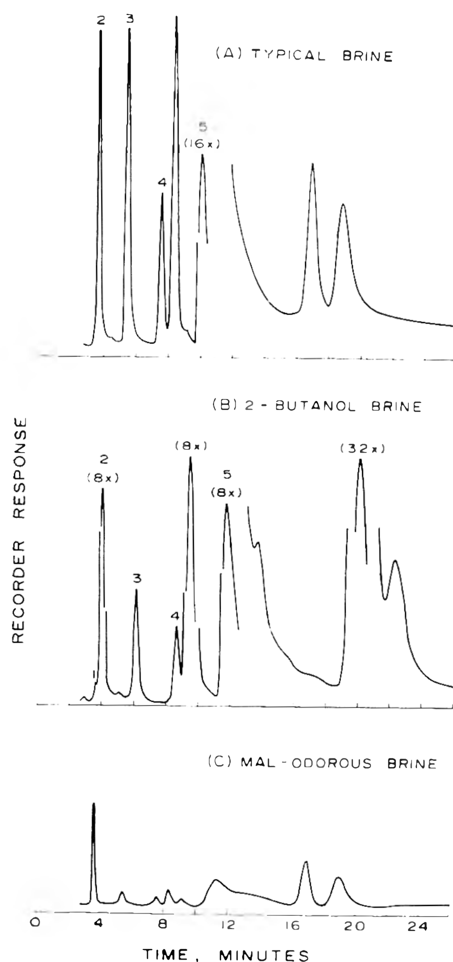

Fig. 2—Head-space vapor GLC profiles of naturally fermented olives from commercial sources showing variations that existed in volatile compounds. Sample (A) was a typical brine with a pleasant odor; (B) a brine that contained a high level of 2-butanol (peak attenuated 32 X); and, (C) a brine that possessed a strong odor of butyric acid. The attenuation was 2 X except as noted. The column was Carbowax 20 M and was operated at 75°C.

Table 2—Fractionation of fermented olive volatiles by vacuum distillation.

Trap	Odor description
Ice-salt	Weakly alcoholic
Dry ice-acetone	Strongly alcoholic
Liquid nitrogen	Strong fermented olive, methyl sulfide-like
Residue	Weak fermented olive, nearly bland

Major odor components

The volatile compounds of olives fermented by *L. plantarum* were fractionated into two groups by vacuum distillation (Table 2). The ice-salt and dry ice-acetone traps contained alcoholic odors. The liquid nitrogen trap contained what was considered the primary odor of fermented olives.

Odor-monitoring of GLC effluents from chromatographing samples from the liquid nitrogen trap revealed only two components that could be detected by smelling. These components were methyl sulfide and acetaldehyde as indicated by retention times.

Methyl sulfide odor could be detected when chromatographing on any of the three stationary phases used in this study. The odor of acetaldehyde could be detected only when chromatographing on diisodecylphthalate, as the other two columns did not adequately resolve this component from methyl sulfide. When the two components emerged in mixtures, only the odor of methyl sulfide was detected.

Odor-monitoring of GLC effluents from head-space vapor samples revealed only one component in effluents, methyl sulfide, that could be detected by smelling. This compound was concluded to be a major odor component of brined olives as the compound alone in certain concentrations gave an odor very similar to that of the olives. Thus, brined olives are among the increasing number of foods which have been reported to contain flavor threshold levels of methyl sulfide. Other such foods include tomatoes (Miers, 1966), potatoes (Gumbmann et al., 1964) and Swiss cheese (Langler et al., 1967).

Similar concentrations of methyl sulfide in the head-space vapor of brined olives, whether fermented or unfermented, indicates that it is derived from the olive under normal brining conditions.

Acetaldehyde, and to a lesser extent ethanol, were considered to contribute secondarily to the odor of brined olives. Unidentified components indicated as peak no. 3 and 4, and other compounds may also modify the basic odor ascribed to methyl sulfide.

Samples of olives were subjected to informal taste panel evaluation. Pure-culture fermented olives were described as having a "clean" fermented olive flavor. The term "clean" was defined as the absence of off-flavors. The flavor of the unfermented product was rated as being more bland and lacking sharpness, probably due to the lack of acidity. The odors of the fermented and unfermented products were similar.

From the above observations it was concluded that the primary contributions of fermentation by lactic acid bacteria to the flavor of olives include: (1) the production of a desirable level of acidity; and (2) utilization of fermentable sugars to the exclusion of microorganisms which produce metabolic end products with undesirable flavor characteristics.

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Ms. received 9/18/68; accepted 9/8/69.

Paper number 2696 of the Journal Series of the North Carolina State University Agricultural Experiment Station, Raleigh, North Carolina.

The authors wish to thank the following individuals of the Department of Food Science, North Carolina State University: J.A. Singleton and R.J. Bingham for mass spectral analysis, W.Y. Cobb for helpful advice, and R.M. Swindell for technical assistance.

Mention of firms or products is not intended to imply endorsement.

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The Cause of Discoloration of Hard Cooked Egg Rolls

SUMMARY—The brownish discoloration of coagulated albumen in hard cooked egg rolls by ultraviolet (UV) radiation involves the splitting and conformation changes of the peptide chain as suggested by an increase in the amount of nonprotein nitrogen and aromatic amino acids. The presence of water was essential in the development of discoloration, indicating that hydroperoxides and/or peroxides produced from water by the action of UV radiation could cause oxidation of tryptophan. The oxidative type of reaction was confirmed by the development of a similar brown color when coagulated albumen was heated in the presence of oxidizing agents. The discoloration could be prevented by reducing agents, however, once the color has been developed the agents become ineffective. Therefore, a two step mechanism was suggested for the UV discoloration of coagulated albumen: first, the peptide chain is hydrolyzed resulting in an increase in the amount of tryptophan available; second, the tryptophan molecule is oxidized producing a brown color.

INTRODUCTION

IN 1964 Cornell University developed a new egg product known as prepackaged Hard Cooked Eggs (Egg Rolls). During the eighth week of market-testing a brownish discoloration appeared on the surface of the egg roll. Areas directly exposed to the fluorescent light in the market display case developed the brownish color. This study was undertaken to determine the cause of the discoloration and to find methods for its prevention.

Joly (1965) reviewed the effect of ultraviolet (UV) radiation on denaturation of albumen. Ultraviolet denaturation of albumen is a unimolecular reaction and is not affected by pH and temperature. Alexander (1959) and Woodward (1933) reported that UV radiation of albumen caused emission of electrons from albumen which resulted in an increased number of disulfide bonds. Proteolysis is associated with the UV denaturation of ovalbumen and appeared after exposure to UV radiation for 1 hr. The effectiveness of the UV radiation is dependent on the product of intensity and time of exposure (Joly 1965). Mitchell et al (1938) suggested that the photoreaction effects the peptide linkage adjacent to an aromatic amino acid causing oxidative photolysis.

The denaturation of native albumen with UV radiation has been studied by several workers, however, no work has been reported on the effect of UV radiation on heat coagulated albumen.

MATERIALS & METHODS

Preparation and exposure to UV radiation of egg rolls

Four, one day old eggs of White Leghorn hens were broken and their contents were placed in a polypropylene tube and sealed. The rolls were heated in a 93°C water bath for 20 min, then cooled in a refrigerator (1–4°C). The egg rolls were exposed to UV radiation by placing them 25 cm from a RE lamp with two black ray bulbs (40 rapid start, UV Products, Inc., San Gabriel, Calif.) in a 7–14°C refrigerator.

Removal of sugar and water from albumen

One day old eggs of White Leghorn hens were desugared by an enzymatic reaction involving glucose oxidase. The desugared albumen was then coagulated and exposed to UV radiation. The presence of glucose was tested with Benedicts test according to Clark (1964).

Water was removed from coagulated albumen by freeze-drying in a Stokes Freeze Dryer. The dried albumen was then exposed to UV radiation for a maximum of 20 days.

Effect of oxidizing and reducing agents on albumen

Oxidizing agents. Ammonium persulfate was added to albumen to a final concentration of 5% and was heated in a 93°C water bath for 20 min. 5% hydrogen peroxide was also used.

The concentration effect of oxidizing agents was studied by adding 5, 4, 3, 2, 1, 0.5, 0.25 and 0.125% ammonium persulfate to albumen. The solutions were heated at 93°C for 20 min. The color produced in the albumen was compared with an egg roll exposed to UV radiation for 10 days to determine the time of reaction.

Reducing agents. A 5% solution of L-cysteine in albumen was heated at 93°C for 20 min. Samples were then exposed to UV radiation for a maximum of 20 days. Ascorbic acid and 2-mercaptoethanol were also used.

The effect of reducing agents on preventing discoloration in albumen was investigated by preparing 1% solutions of ammonium persulfate in albumen (base oxidizing solution). Varying quantities of L-cysteine was added to a base oxidizing solution to give final concentrations of 0.5, 0.2, 0.1, 0.05 and 0.025 M. The solutions were then heated at 93°C for 20 min.

Effect of minerals

1 ml of a 1% solution of ferric chloride was added to (a) 99 ml of albumen, and (b) 99 ml of base oxidizing solution. These were heated at 93°C for 20 min. The color produced was measured using a Gardner Color Difference Meter. Ferrous chloride, cobalt chloride, magnesium chloride and manganese chloride were also used in place of ferric chloride.

Determination of nonprotein nitrogen

10 g of brown UV treated egg roll was added to 50 ml of 12% trichloroacetic acid (TCA) and blended in a Waring Blendor for 30 sec. The mixture was then centrifuged for 30 min at 48,000 × G (Sorvall RC2-B centrifuge). The supernatant was neutralized and tested for nitrogen using the micro-kjeldahl, aromatic amino acids using the Lowry modification of the Folin test, and tyrosine and tryptophan using the 295:280 ratio as reported by Colowick et al. (1964). The central portion of the egg roll which was not brown was tested for NPN in a similar manner and served as the control.

Visible effect of UV radiation on amino acids

1% solution of tryptophan was subjected to UV radiation for 5 days at 7–14°C and observed for discoloration. Tyrosine, phenylalanine, serine, histidine and threonine were also used. The effect of concentration on discoloration was determined using 0.1, 0.3 and 0.5% tryptophan solutions. The intensity of discoloration was tested by optical density (OD) at 500 mμ from 0 to 45 hr. Water was used as a reference.

Effect of reducing agents on discoloration of tryptophan

Solutions containing 0.5, 0.25, 0.20, 0.10 and 0.05 *M* 2-mercaptoethanol in 1% tryptophan were exposed to UV radiation for 48 hr at 7–14°C and OD measured at 500 mμ. L-cysteine and ascorbic acid were also used.

RESULTS & DISCUSSION

Effect of sugar and water

The nature of discoloration of egg rolls was determined by removing the glucose and water from egg albumen. It was found that the desugared albumen on exposure to UV radiation developed a brownish discoloration. These data (Table 1) indicated that glucose was not involved and the discoloration was not a carbonyl-amine type of browning. Enzymatic browning and bacterial pigmentation were eliminated due to the heat treatment involved in the production of rolls (93°C

for 20 min). Water was found to be an essential part of the discoloration reaction as the freeze dried coagulated albumen did not brown under UV radiation. The prevention of UV browning by removal of water indicates that water is probably acting as a substrate for the production of peroxides and hydroperoxides by UV radiation and in turn may cause the hydrolysis of the peptide chain.

Oxidizing and reducing agents

The effects of oxidizing and reducing agents on discoloration of albumen are shown in Table 2. The discoloration of coagulated albumen by UV radiation was similar to that produced by oxidizing agents and could be prevented by reducing agents, suggesting that color development involves an oxidative process. However, once the brown color has been produced it cannot be changed or removed by reducing agents. It appears that hydroperoxides by virtue of their ability to degrade products could make this an irreversible reaction.

The time for and extent of brown discoloration produced by ammonium persulfate in coagulated albumen was found to depend on persulfate concentration. A minimum concentration of 1% ammonium persulfate was required to obtain the same degree of browning as produced by UV radiation. However, as the concentration of ammonium persulfate was increased, the time required to achieve the same amount of discoloration decreased (Fig. 1).

The effect of L-cysteine and cystine in preventing the brown color produced by albumen in the presence of ammonium persulfate is shown in Figure 2. L-cysteine at a concentration of 0.2 M completely prevented the development of such a color. However, cystine did not influence the brown color at any concentrations tested, indicating the importance of reduc-

Table 1—Effect of water and sugar on discoloration of coagulated albumen by ultraviolet radiation.

Treatment	Development of color (days)
Coagulated albumen	5
Freeze dried coagulated albumen	>20
Desugared coagulated albumen	5
Native albumen (uncoagulated)	>20
Freeze dried native albumen	>20

Table 2—Effect of oxidizing and reducing agents on discoloration of coagulated albumen.

Treatment	Color developed	
	Non UV treated	UV treated
Ammonium persulfate	Brown	[1]
Hydrogen peroxide	Brown	
L-cysteine	No color	No color
2-Mercaptoethanol	No color	No color
Ascorbic acid	No color	No color
Untreated	No color	Brown

[1] Already brown.

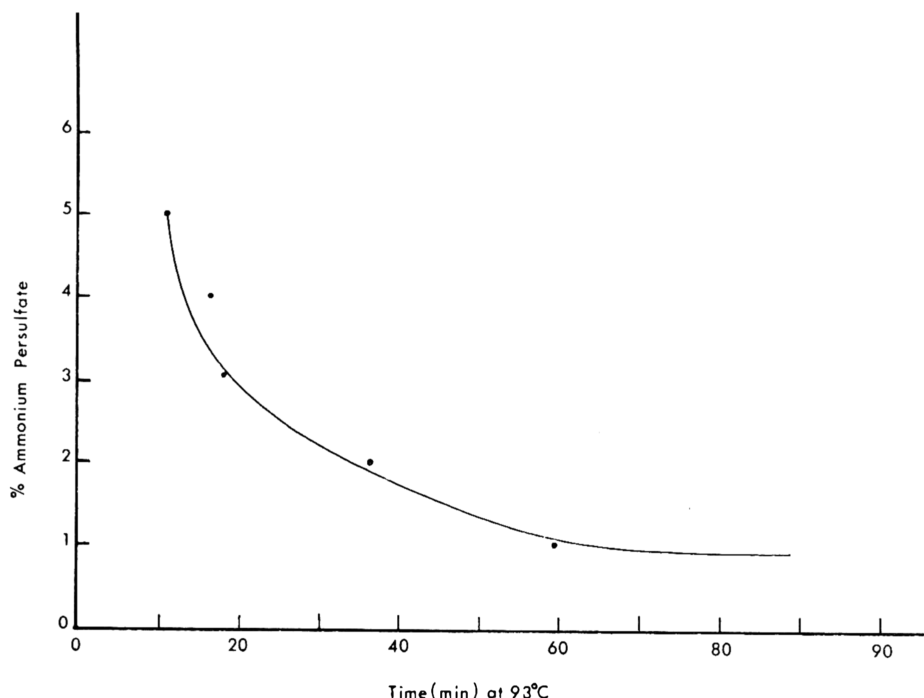

Fig. 1—Time required for browning of egg albumen with various amounts of ammonium persulfate at 93°C.

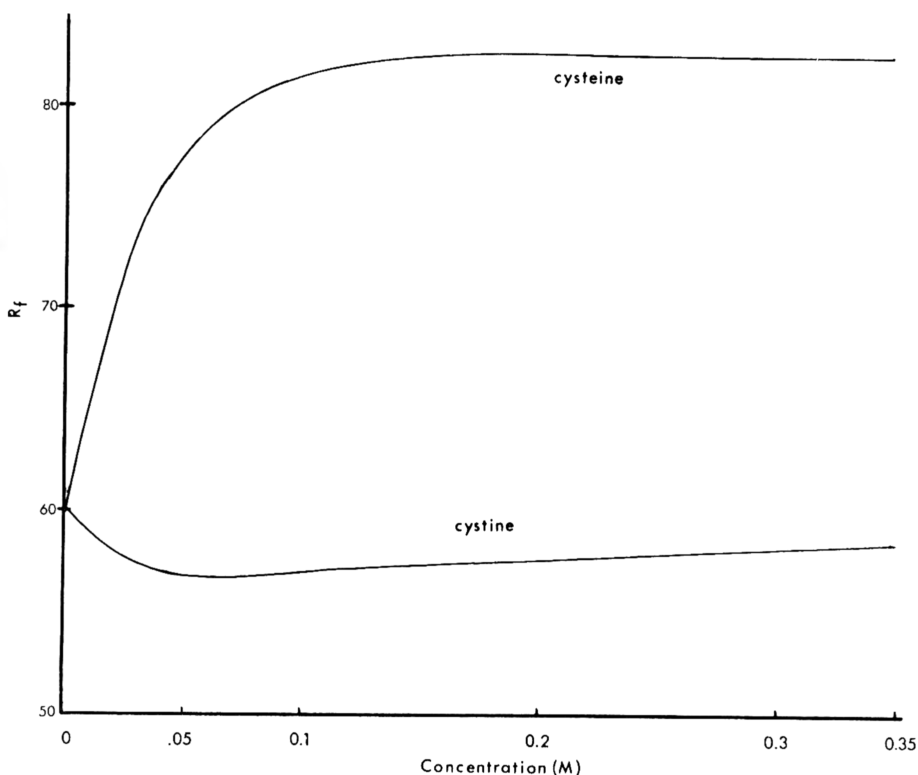

Fig. 2—Effect of varying concentrations of cysteine and cystine on the prevention of browning in base oxidizing solution.

ing properties of sulfhydryl groups of L-cysteine.

Effect of cations

Cations commonly associated with known color reactions had no significant effect on the brownish discoloration of coagulated albumen (Table 3) when measured on a Gardner Color Difference Meter. This indicates that metal ions may not be involved in the brown discoloration of coagulated albumen.

Nonprotein nitrogen

The amount of nonprotein nitrogen (NPN) in a 10% TCA supernatant from control and UV treated coagulated albumen was determined and these data are shown in Table 4.

The microkjeldahl data show that there is approximately $1^1/_2$ times more nitrogen in the TCA supernatant of the UV treated albumen than the control (non UV treated). The Folin procedure also showed a similar increase in the amount of aromatic amino acid when compared to control. The 295:280 ratio which is specific for tyrosine and tryptophan showed 1.7 and 1.3 times more tyrosine and tryptophan in UV treated albumen than the control.

These data indicate an increase in aromatic amino acids which is primarily caused by the hydrolytic (proteolytic) activity of UV radiation (Mitchell et al., 1938). This suggests that amino acids which were shielded prior to the hydrolysis and conformational change have become available for reaction in the degradation by UV radiation (Joly, 1965).

Visible effects of UV radiation

The discoloration of UV radiation on several amino acids was investigated and the data (Table 5) show that tryptophan was the only amino acid that produced a brown color. The concentration depen-

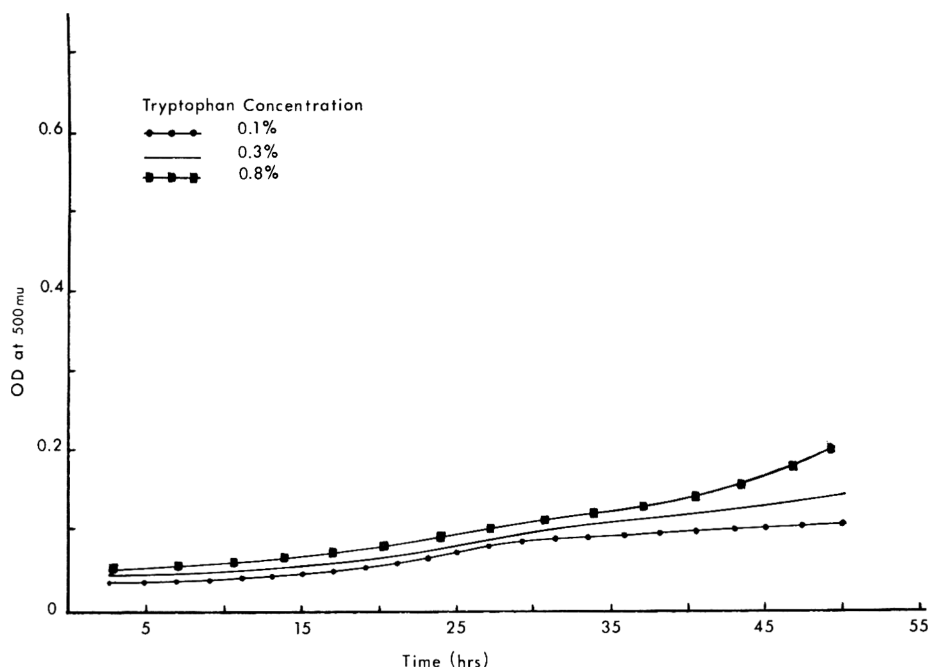

Fig. 3—Effect of tryptophan concentration on the intensity of brown color on exposure to UV radiation at different time intervals.

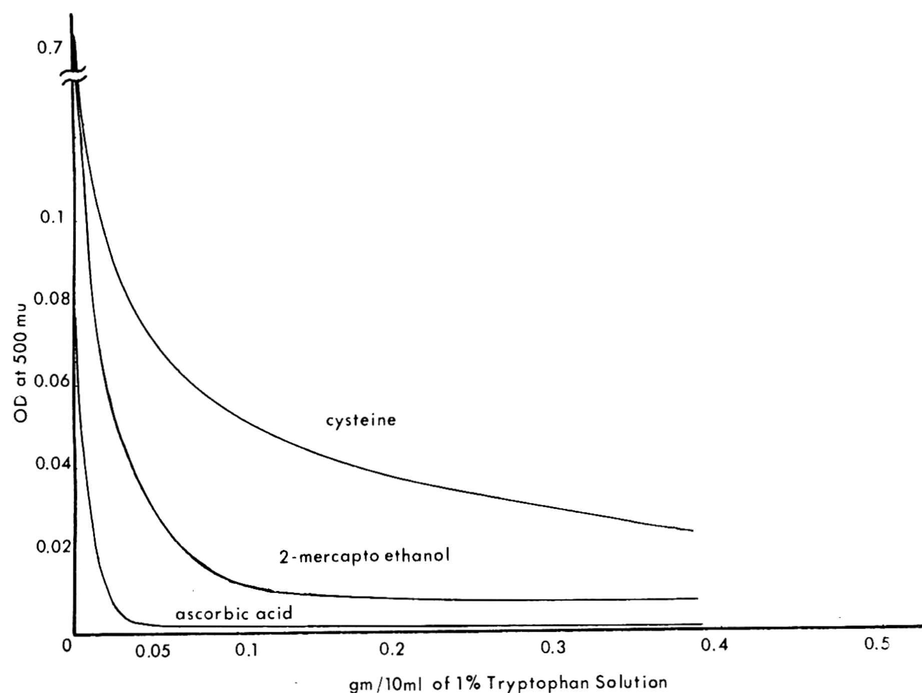

Fig. 4—Effect of varying concentration of reducing agents on the prevention of browning of tryptophan when exposed to UV radiation.

Table 3—Effect of various cations on discoloration as measured with a Gardner Color Difference Meter.

Cation	R_f[1] Cation + albumen	Cation + albumen + 1% ammonium persulfate
Fe++	71.5[2]	65.6
Fe+++	70.6[2]	63.3
Co++	78.8	63.8
Mg++	78.8	66.6
Mn++	77.5	66.4
Control	79.3	70.5

[1] R_f is the white to black scale.
[2] Samples were black.

Table 4—Quantities of NPN in 10% TCA supernatant from UV- and non-UV-treated coagulated albumen.

Method	Treatment	Amount of nonprotein nitrogen	
		mg of nitrogen/g of sample	
Micro Kejldahl	Normal	0.273	
	UV	0.427	
		mg NPN as tyrosine	
Folin	Normal	4.44	
	UV	7.58	
		mg mmole/g of protein tyrosine	tryptophan
295:280 ratio	Normal	48.4	34.8
	UV	81.3	45.0

Table 5—Effect of UV radiation on discoloration of some amino acids.

Amino acid	Brown discoloration development in 5 days
Tryptophan	+
Tyrosine	−
Phenylalanine	−
Serine	−
Lysine	−
Threonine	−
Cysteine	−
Cystine	−
Methionine	−

dence of tryptophan to discoloration was investigated and the results are shown in Figure 3. Investigations as reviewed by Joly (1965) have shown that there are molecular changes in tryptophan when exposed to UV radiation, but any color change in tryptophan solution when treated with UV has not been reported previously. This could be due in part to the dilute solutions used for UV analysis. These data indicate that a 0.1% tryptophan solution gives a visible brownish color after 24 hr of exposure to UV radiation. Lower concentrations of tryptophan solution take a longer time to develop and the intensity of the color is low.

The effect of reducing agents on prevention of browning of tryptophan was investigated (Fig. 3) and it was observed that the brownish color was retarded by the reducing agents tested. However, the concentration necessary for prevention of brown color varied with the reagent used (Fig. 4). The necessity for an aqueous solution was confirmed when no color was developed on exposure of dry tryptophan to UV radiation.

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Ms. received 7/30/68; revised 10/25/68; accepted 9/5/69

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Microbiology of Fresh Apple and Potato Plugs Preserved by Gas Exchange

SUMMARY—Gas exchange as a means of preservation of fresh disk-shaped plugs of apples and potatoes at ambient temperatures was studied. Death kinetics of the microorganisms E. coli, St. aureus and Cl. botulinum were determined. The concurrent effects of the gas on the browning enzyme systems of the products was followed. Gram negative rods in the environment of the test materials were rapidly killed when exposed either to a mixture of ethylene oxide (10%) and carbon dioxide (90%), or to pure sulfur dioxide and sulfur dioxide (50%) air mixtures. Gram positive cocci and the spores of the anaerobes showed somewhat slower rates of destruction under the same conditions. Treatment with pure carbon monoxide, nitrogen or packaging in vacuum achieved considerable freedom from color changes but had no appreciable effect on microorganisms.

INTRODUCTION

GASEOUS sterilization, a specialized type of chemical sterilization where the chemicals used are gases, has found application in the treatment of a variety of nonfeed materials (Kaye, 1949; Phillips, 1957), such as plastics, leather, certain biological products and delicate laboratory instruments.

The intriguing advantages of such a system are: simplicity in operation since a gas distributes itself freely throughout an enclosed space, its relatively high power of penetration, avoidance of a water soaked appearance, damage to heat sensitive materials and relatively easy removal of excess disinfectant by airing or evacuation. With this technique, sterilization at ambient temperatures can be achieved (Kramer et al., 1968). Cotton et al. (1928) were the first to report the use of ethylene oxide as a fumigant. Practical sterility of dry foodstuffs, i.e., spices, grains and the like, has been obtained by the use of ethylene oxide (Heath, 1964). This gas is truly bactericidal in its action through alkylation of one or more of the nitrogen atoms in the DNA bases of the microorganisms, subsequently causing anomalies in the base pairing in the DNA helix (Rose, 1965). It penetrates readily plant tissues and has a broad spectrum of activity.

Sulfur dioxide inhibits enzyme catalyzed oxidative discoloration of fruits and vegetables (Joslyn et al., 1954). It has been widely used for the treatment of fruits and vegetables before dehydration in order to preserve their color. It has also been employed in order to extend the storage life of fresh grapes by its effect on *Botryis* (Pryor, 1950) and to prevent the growth of undesirable microorganisms during wine making (Amerine et al., 1951). It is a strong reducing agent and it acts both through enzyme poisoning and as an antioxidant.

The effect of carbon monoxide as a preservative has received little attention. It displays very high toxicity (Rose, 1965) as an enzyme inhibitor by combining with an iron-containing prosthetic group or coenzyme that is required for the activity of certain enzymes.

Nitrogen atmospheres have been successfully employed (Bayes, 1950; Kramer et al., 1966) for retarding oxidative deterioration of perishable food products.

A number of other gases such as ozone, carbon dioxide, nitrogen trichloride, formaldehyde, nitrous oxide and *b*-propiolac-

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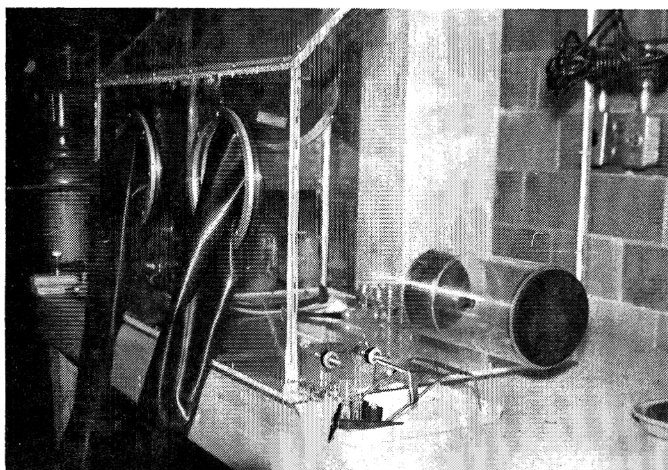

Fig. 1—Product preparation chamber.

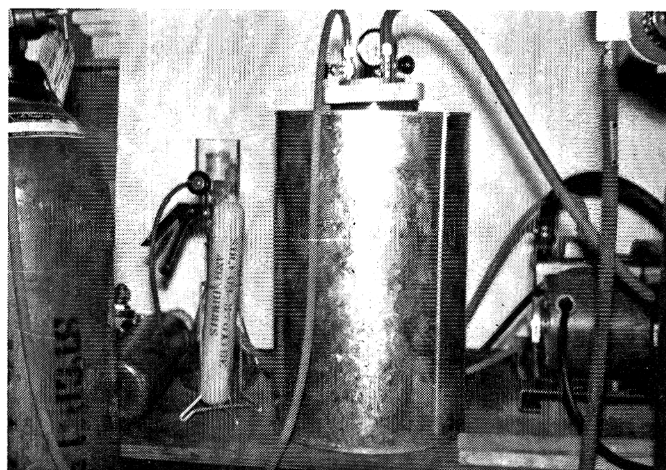

Fig. 2—Gas exposure chamber.

tone have attracted attention as potential preservatives and disinfectants with varying degrees of success (Dunn, 1957; Phillips et al., 1958).

The lack of current information on the effect of a number of gases on spoilage and pathogenic microorganisms in the environment of fresh plant food products and the removal of residual gases and their effect on the browning enzyme system indicated the need for further exploration in this area.

MATERIALS & METHODS

Test organisms

Pure cultures of typical food spoilage and food poisoning microorganisms were selected. They were *Escherichia coli* ATCC II229, a standardized suspension of spores of *Clostridium botulinum* 62-A and *Staphylococcus aureus* ATCC 6538. The test bacteria were grown in Brain Heart Infusion broth (Difco) at 37°C for 18 hr. The bacterial cells were harvested by centrifugation, washed twice with sterile distilled water and suspended in buffered sterile distilled water. The inoculum was adjusted to a population of approximately 10^6 cells per gram of product. The standardized spore suspension of the *Cl. botulinum* was diluted to an equivalent of 10^6 spores per gram of product. Both the cell and the spore inocula are considered high levels of initial contamination for food products.

Preparation of plant materials

Apples and potatoes were selected since they represent important crops, as well as affording a wide range of pH within which most plant food materials fall. The products were washed and placed in a preparation chamber made of heavy plastic film (Fig. 1).

The cutting tools, bottles and stoppers were presterilized. The chamber was evacuated repeatedly and broken with nitrogen which was then maintained at a slight pressure. The samples, therefore, were prepared in an atmosphere approaching 100% nitrogen in order that oxidative enzymatic browning would not be initiated prior to the final treatment with the appropriate gas.

The products were sliced by a slicer and cylindrical plugs weighing about 1 g were cut using a cork borer. Ten plugs were placed in each of 50 ml capacity rubber stoppered glass containers. A number of bottles in each batch were inoculated with the test microorganisms. Appropriate controls were included.

Exposure procedure

A modified case anaerobic jar was found to be a suitable exposure chamber for the treatment of the samples with the different gases (Fig. 2). The capacity of the jar is 16 L and it is equipped with a vacuum-pressure gage which can conveniently indicate the degree of evacuation and the degree of replacement with the desired gas. Gas (or gas mixture) at the rate of about 1 g per L of space was used; and the chamber was operated at room temperature which was found to be effective by a number of researchers (Kaye, 1949; Pappas et al., 1952).

The bottles containing the samples were placed inside the chamber, the stoppers were partially undone and a vacuum of 30 in. was drawn and maintained for 10 min. The desired gas was then connected and allowed to fill the chamber until the gage returned to the zero position. The gas remained in contact with the product for the determined length of time. The chamber was subsequently evacuated for 15 min and following this the product was "washed" with sterile air for an additional 10 min. A bacteriological glass filter was used in filtering the air. The chamber was then opened and the stoppers were securely adjusted in place to avoid recontamination.

Gas analysis of the headspace of the bottled samples by the use of the Fisher-Hamilton-Partitioner failed to register the presence of the tested gas. In the case of sulfur dioxide, the residual sulfur dioxide content was further determined by titration as suggested by Ruck (1963). Gases used in this experiment were: Anhydrous sulfur dioxide, C.P. grade; carbon monoxide, C.P. grade; and Carboxide—a 10% ethylene oxide and 90% carbon dioxide mixture which is considered nonflammable.

Microbiological examination.

The 10 g sample was transferred aseptically from the treatment bottle to a sterile Osterizer jar. 90 ml of phosphate buffered sterile distilled water was added and the sample was blended for 2 min. This 1:10 dilution was used in preparing a series of decimal dilutions from 10^{-2} to 10^{-6}. Microbiological techniques described by a number of workers were employed (Hartman, 1960; APHA, 1965; Angelotti et al., 1966). *E. coli* were enumerated by plating in duplicate on Violet-red bile agar (Difco) and incubating at 37°C for 24 hr. *St. aureus* were surface plated on Mannitol salt agar (Difco) and the plates were incubated at 35°C for 48 hr. Bright yellow colonies developed in this medium. Reinforced clostridial agar (Oxide, Consolidated Laboratories) was used for counting the *Cl. botulinum*. Thick plates were poured and incubated in a case anaero jar in an atmosphere of 90% nitrogen and 10% carbon dioxide at 30°C for 72 hr.

Determination of enzyme activity

The method described by Summer et al. (1953) was used, by which polyphenoloxidase activity can be determined colorimetrically by measuring the ability of this enzyme to catalyze the oxidation of catechol with the formation of quinones. The optical density of the solution was recorded at 405 mμ in a Spectronic '20.'

RESULTS & DISCUSSION

PRELIMINARY work on improved methods of preserving fresh foods had included the total withdrawal of oxygen from plant materials followed in some cases by packaging under nitrogen (Kramer et al., 1966). It was observed that over a period of several weeks, color changes were arrested while changes in texture were attenuated. In later studies in which air withdrawal was followed with flushing with certain gases, color, texture and flavor changes were arrested (Kramer et al., 1968).

In this study inoculated samples of the plant products were treated with the ex-

Fig. 3—Survivor curves for microorganisms exposed to carbon monoxide (——), ethylene oxide (— —), or sulfur dioxide (---) gas. a. E. coli *on apple plugs; b.* St. aureus *on apple plugs; c.* Cl. botulinum *on apple plugs; d.* E. coli *on potato plugs; e.* St. aureus *on potato plugs; f.* Cl. botulinum *on potato plugs.*

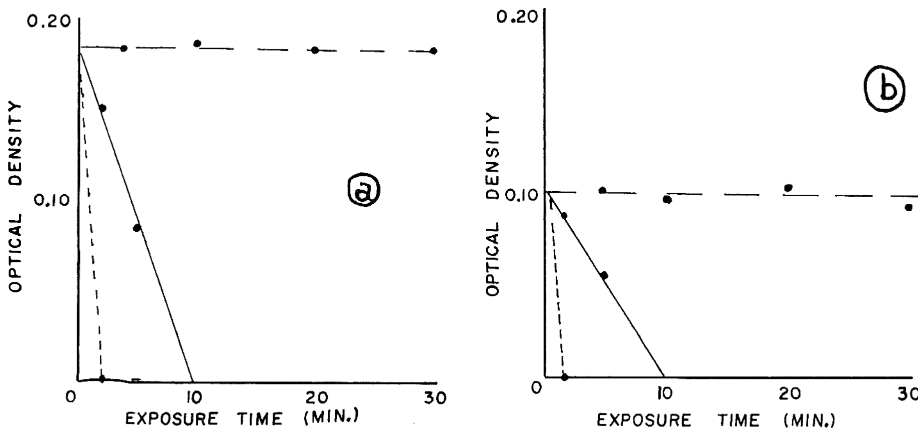

Fig. 4—Polyphenoloxidase activity following exposure to carbon monoxide (——), ethylene oxide (— —), or sulfur dioxide (- - - -) gas. a. Potato plugs; b. Apple plugs.

perimental gases. Survivor curves for *E. coli, Cl. botulinum* and *St. aureus* in inoculated sample disks are presented in Figure 3.

The effect of the experimental gases on polyphenoloxidase is shown in Figure 4.

Carbon monoxide, although preserving substantially the color of the plant materials by inactivating the polyphenoloxidase, has little effect on the bacteria. Exposure of the apple plugs to the ethylene oxide mixture proved satisfactory as far as the destruction of microorganisms is concerned but it did not inactivate the browning enzyme system. Since plant products are generally high in moisture, the difficulties encountered in the sterilization of dried materials or materials of low water content with this gas, as reported by Kaye et al. (1949), were not evident in this study.

The gram negative rods used in this experiment were more vulnerable to the

gas than the cocci. The clumping effect and the general hardiness of the cocci has been postulated by Rose (1965) as favoring their survival, as well as the fact that all the microorganisms in a clump need to be killed before the loss of one colony forming unit is observed. The spores of *Cl. botulinum* displayed the same pattern of destruction as the cocci. The differential rate of destruction within a microbial population was apparent by the presence of the characteristic "shoulder" often observed in certain lethality curves.

All test microorganisms were quickly destroyed after a 5 min exposure to pure sulfur dioxide or 50% sulfur dioxide-air mixture. Lower sulfur dioxide-air mixtures required a proportionately longer exposure time to achieve complete inactivation of the bacteria. This gas effectively destroyed the polyphenoloxidase. A possible synergistic effect of low pH and an antimicrobial agent was demonstrated in the case of apple plugs, while the potato plugs were less easily sterilized, and their polyphenol oxidase activity decreased at a lesser rate.

In all cases no evidence of the studied gas was detected in the headspace of the sample bottles by the Fisher-Hamilton Gas Partitioner, following 15 min evacuation and 10 min washing with sterile air.

In the case of sulfur dioxide treated samples, the plant tissues were further tested for the presence of sulfur dioxide, in view of the ease with which this dissolves in water. A number of evacuation times were employed and the sulfur dioxide residual in the tissues was determined by titration. The results are summarized in Figure 5.

A statistically significant interaction between products and evacuation time ($P = 0.01$) was noted; the more compact cellular structure of the potato plugs retaining more residual gas than the apple tissues. At the end of a 60 min evacuation, the SO_2 residue in the tissues of both potatoes and apples was reduced to below 100 ppm and could not be readily detected organoleptically.

It would appear that in the future, should all process development and all nutritional and toxicological tests be completed successfully, gaseous sterilization could be applied to the preservation of a number of fresh food products.

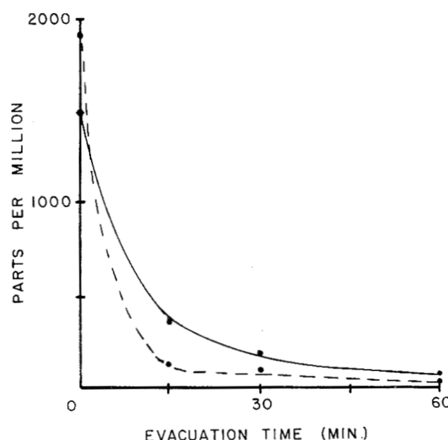

Fig. 5—Sulfur dioxide residue in potato (——) and apple (- - - -) plugs treated with SO_2 for 5 min and subsequently evacuated.

CONCLUSIONS

CONSIDERABLE work remains to be done to determine the nutritional and toxicological effects of such gaseous treatments. Further studies may reveal, for example, possible toxicity of ethylene oxide on hydrated products, and if that should be the case the use of another epoxide such as propylene oxide may be necessary although its bactericidal activity is lower (Whelton et al., 1948). Undoubtedly additional more rigorous microbiological and enzymological studies need to be performed over an extended storage time, particularly with such organisms as *Cl. botulinum* on low acid foods before a gas exchange procedure can be approved.

From the results obtained thus far, however, it would appear that there is a possibility that such a gas exchange procedure would be applied not only to extend shelf life by a drastic reduction in bacterial population and enzymatic activity, but by a total destruction of bacteria and enzyme activity, to preserve a number of fresh food products without altering their fresh-like sensory quality.

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Ms. received 4/22/68; revised 10/14/68; accepted 9/5/69.

Scientific paper No. A1437, Contribution No. 4063 of the Maryland Agricultural Experiment Station (Department of Horticulture).

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Utilization of Amide Nitrogen by the Young Rat

SUMMARY—Effects on body nitrogen gains of supplying nonessential nitrogen as glutamic or aspartic acids, as glutamine or asparagine or as wheat were investigated. It was found that nitrogen gains of rats fed diets containing only purified amino acids as the nitrogen source and relatively high levels of amide nitrogen were significantly lower than those of rats fed the same amounts of total nitrogen, all as α-amino nitrogen. However, they were significanty higher than those of rats fed the same amounts of α-amino nitrogen, but no additional amide nitrogen, indicating some effect of amide nitrogen in body nitrogen storage. Rats fed ad libitum consumed more of the glutamine-containing diet than of one containing isonitrogenous amounts of glutamic acid; analysis of covariance indicated that ad libitum-fed rats also utilized the nitrogen of glutamic acid more efficiently than that of glutamine. Addition of enough sodium bicarbonate to neutralize the hydrochlorides of dietary amino acids had no effect on utilization of amide nitrogen. No differences in nitrogen utilization were found between two groups of rats fed different levels of amide nitrogen as wheat.

INTRODUCTION

ABOUT 20% of the total nitrogen in wheat is in the amide form but the extent to which amide nitrogen in the diet can contribute to nitrogen stores in the body has not been extensively investigated. Barry (1956) showed that in the casein of milk of the lactating goat glutamine residues came from free blood glutamine and glutamic acid from free blood glutamic acid. Studies using animal cell cultures (Levintow et al., 1957) indicated that the amide nitrogen of glutamine is utilized for the synthesis of purines and pyrimidines but was not a precursor of α-amino nitrogen.

Studies from this laboratory (Womack et al., 1964) indicated that a low level of wheat gluten adequate for maintenance of tissue protein in the young adult rat was inadequate for older animals. Data were interpreted to indicate that the requirement for at least one amino acid is higher for the older than for the young adult animal. The possibility exists, however, that the differences observed may have been due to a difference in utilization of amide or amino acid nitrogen, or both, by animals of the two age groups. A study was therefore undertaken of the utilization of α-amino acid versus amide nitrogen by animals of different ages. Studies with the young rat are reported here. It was found that amide nitrogen was able to contribute to body nitrogen stores but that it was less efficiently used than an equal quantity of α-amino nitrogen.

EXPERIMENTAL

Experimental plan

The experimental plan is shown in Table 1. In preliminary studies it was found that 7 g food was near the upper limit that all rats receiving high levels of glutamic acid would consume per day. Levels of essential amino acids were used which would allow for growth when suitably supplemented with nonessential nitrogen without furnishing excesses which might be utilized for formation of nonessential amino acids. Also, it was necessary to determine the amounts of total nitrogen to be fed to produce readily detectable effects on nitrogen gains. Then the utilization of α-amino nitrogen versus amide nitrogen in various feeding patterns and dietary combinations was studied. Details of the experiments accompany discussion of results.

Procedures

Weanling rats obtained from the supplier were housed individually in a temperature-humidity controlled room and fed a stock diet for 3–5 days until they reached the desired weight. After being fed a nitrogen-free diet for 4 days (experiments 1–4) or fasted for 24 hr (experiments 5 and 6), they were divided into groups and fed the experimental diets or killed for initial controls.

Feces were collected daily from the rats of experiment 4, frozen until the end of the feeding trials, dried, ground, and analyzed for nitrogen by the macro-Kjeldahl method. At the end of the feeding period, all rats were killed with chloroform, contents of the gastrointestinal tract were removed, and carcasses autoclaved for 15 min at 15 lb pressure. They were homogenized in a blender with a known amount of water and analyzed for nitrogen. Initial carcass nitrogen of the experimental animals was determined from the regression lines calculated for control groups.

The amino acids were either N.R.C. grade or products analyzed for purity for another study (Ahrens et al., 1966). The amino acid mixture (Table 2) was patterned after the amino acid requirements of the growing rat (Rama Rao et al., 1959; Ranhotra et al., 1965). The wheat, lye-peeled bleached bulgar (WURLD wheat) was ground before use. Amino acid content of the product was determined using a Technicon Amino Acid Analyzer. For some amino acids (cystine, histidine, isoleucine, tryptophan, and valine) the microbiological procedures used previously (Ahrens et al., 1966) were also employed because values from the analyzer were not in the same range as values for other wheat products.

To minimize scattering, water (0.5 ml per

Table 1—Plan of experiments.

Experiment no.	Preexperimental treatment	Feeding pattern	Total N intake mg/day	EAAN[1] intake mg/day	Nitrogen source	Length of experiment days
1	Nitrogen-free diet 4 days	7-g food per day	160	76, 67, 54, 48	PAA[2]	21
2	Nitrogen-free diet 4 days	7-g food per day	60, 80, 100, 120, 140	60	PAA	21
3	Nitrogen-free diet 4 days	7-g food per day	90, 120	60	PAA	21
4	Nitrogen-free diet 4 days	Ad libitum	Not controlled	Not controlled	PAA	21
5	Starved 24 hr	2 1-hr feeding periods daily	[3]	[3]	PAA	24
6	Starved 24 hr	2 1-hr feeding periods daily	[3]	[3]	PAA and wheat	24

[1] Essential amino acid nitrogen.
[2] Purified amino acids.
[3] Rats were allowed only the amount of food that all would consume in two 1-hr feeding periods daily. They were given 1 g the first day and amounts were increased gradually to 3.5 g per feeding. Total food intakes were the same as in experiments 1–3.

Table 2—Amino acid mixture.

	g
L-arginine HCl	0.50
L-cystine	0.34
L-histidine HCl·H_2O	0.34
L-isoleucine	0.55
L-leucine	0.70
L-lysine HCl	1.12
L-methionine	0.16
L-phenylalanine	0.42
L-threonine	0.50
L-tryptophan	0.11
L-tyrosine	0.30
L-valine	0.55
	5.59

gram food when raw cornstarch was the carbohydrate, and 2.0 ml when the diets contained wheat or pregelatinized wheat starch) was mixed into all diets just before they were given to the rats. For the ad libitum fed rats (experiment 4) uneaten food was removed daily, dried, allowed to equilibrate to room moisture, and weighed. Scattered food was treated in a similar manner.

RESULTS

Experiments 1 and 2

The aim of the first two experiments was to arrive at levels of essential and nonessential amino acid nitrogen such that a small change in the kind or amount of dietary nitrogen might be expected to produce readily detectable effects on growth or nitrogen retention. In experiment 1, rats were fed 7 g food per day in diets providing 160 mg total nitrogen and the proportion of EAAN and glutamic acid was varied. A change from 67 to 57 mg EAAN per day decreased growth from 36.6 to 28.8 g (Table 3). An amount of amino acid mixture which would furnish 60 mg EAAN per 7 g food was used in other diets.

In the second experiment, all rats received 60 mg per day of EAAN and varied amounts of glutamic acid. Nitrogen gains (Table 4) increased as nitrogen intake increased up to 120 mg total nitrogen per day, with no further increase in gains when intake was increased to 140 mg.

Experiment 3

The question of the utilization of amide versus α-amino nitrogen was then studied under conditions in which limited amounts of food, EAAN, and total nitrogen were supplied. Groups of animals were fed, in addition to 60 mg EAAN per day, amounts of aspartic (ASP) or glutamic (GLU) acid to furnish either 30 (90 ASP and 90 GLU) or 60 (120 ASP and 120 GLU) mg α-amino nitrogen per day, or amounts of asparagine (ASN) and glutamine (GLN) (120 ASN and 120 GLN) to furnish 30 mg α-amino nitrogen and 30 mg amide nitrogen (Table 5). When total nitrogen intakes were equal, rats fed aspartic or glutamic acid stored more nitrogen than those fed asparagine or glutamine (1.36 vs 1.31 and 1.38 vs 1.30 mg). On the other hand, when α-amino nitrogen intakes were equal, rats fed asparagine and glutamine stored more nitrogen than those without the extra (amide) nitrogen (1.31 vs 1.26 and 1.30 vs 1.23 mg). Thus, amide nitrogen was able to contribute to body nitrogen stores, but not to the same extent that an equal quantity of α-amino nitrogen did.

Due to the small increases involved, a

Table 3—(Experiment 1) Diet composition and average weight gains of rats fed 7 g food per day for 21 days.

Group	76 N	67 N	57 N	48 N
	%	%	%	%
Diets[1]				
Amino acid mixture	8.00	7.00	6.00	5.00
L-glutamic acid	12.60	14.00	15.40	16.90
Cornstarch	65.35	64.95	64.55	64.05
Constant ingredients[2]	14.05	14.05	14.05	14.05
No. rats per group	7	7	6	6
Weight gains, g	36.9	36.6	28.8	26.8

[1] Seven g food supplied 160 mg total nitrogen and 76, 67, 57 or 48 mg essential amino acid nitrogen.

[2] Constant ingredients were Jones and Foster salt mixture (1942) 4.00; celluflour (Chicago Dietetic Supply House, Chicago), 4.00; vitamin A and D concentrate (Percomorph oil, Mead Johnson Co., Evansville, Indiana), 0.05; corn oil (Mazola), 5.00; and vitamin mixture, 1.00. The vitamin mixture was formulated so that each kg of diet contained thiamine.HCl, 5 mg; pyridoxine-HCl, 5 mg; niacin, 5 mg; riboflavin, 10 mg; Ca D-pantothenate, 25 mg; *p*-aminobenzoic acid, 300 mg; α-tocopheryl acetate, 25 mg; 2-methyl-1,4-naphthoquinone, 2 mg; folic acid, 2 mg; biotin, 100 μg; vitamin B_{12}, 30 μg; choline chloride, 2 g; and inositol, 1 g.

Table 4—(Experiment 2) Diet composition, weight and nitrogen gains and nitrogen intakes of young rats fed the same amounts of food and essential amino acids but with total nitrogen varied by the addition of glutamic acid.

Group[1]	60 N	80 N	100 N	120 N	140 N
	%	%	%	%	%
Diets					
Amino acid mixture	6.30	6.30	6.30	6.30	6.30
Glutamic acid	—	3.00	6.01	9.00	12.01
Cornstarch	79.65	76.65	73.64	70.65	67.75
Constant ingredients[2]	14.05	14.05	14.05	14.05	14.05
No. rats per group	12	11	11	11	10
Weight gain, g[3]	24	30	36	41	43
Nitrogen gain and standard error of the mean, g	0.65± 0.023	0.97± 0.025	1.23± 0.025	1.40± 0.043	1.40± 0.037

[1] 60, 80, 100, 120, and 140 refer to daily nitrogen intakes (calculated); actual intakes were 62, 80, 101, 121, and 141 mg.

[2] See Table 3.

[3] Average initial weights of the groups were 70–75 g before and 60–64 g after 4 days on nitrogen-free diet.

Table 5—(Experiment 3) Diet composition, nitrogen intakes and nitrogen gains of rats fed constant amounts of food and essential amino acids with aspartic acid, asparagine, glutamic acid, or glutamine added as the sole source of NEN.[1]

Group	90 ASP[1]	120 ASP[1]	120 ASN[1]	90 GLU[1]	120 GLU[1]	120 GLN[1]	120 GLN[1] + $NaHCO_3$
	%	%	%	%	%	%	%
Diets							
Amino acid mixture	6.30	6.30	6.30	6.30	6.30	6.30	6.30
L-aspartic acid	4.07	8.15					
L-asparagine			4.04				
L-glutamic acid				4.50	9.00		
L-glutamine						4.47	4.47
Cornstarch	75.58	71.50	75.61	75.15	70.65	75.18	74.22
Constant ingredients[2]	14.05	14.05	14.05	14.05	14.05	14.05	14.05
Sodium bicarbonate							0.96
No. rats per group	10	9	10	20	21	21	14
Total N intake, g	1.92	2.54	2.54	1.92	2.54	2.55	2.49
Amide N intake, g			0.63			0.64	0.62
Nitrogen gain and standard error of the mean, g	1.26± 0.027	1.36± 0.044	1.31± 0.038	1.23± 0.020	1.38± 0.026	1.30± 0.028	1.27± 0.025

[1] NEN = nonessential nitrogen; ASP = L-aspartic acid; ASN = L-asparagine; GLU = L-glutamic acid; GLN = L-glutamine; 90 and 120 indicate mg nitrogen intake per day. Average initial weights were 65–69 g before and 55–60 g after 4 days on the nitrogen-free diet.

[2] See Table 3.

Table 6—(Experiment 4) Food intakes and weight and nitrogen gains of rats fed diets containing L-glutamic acid or L-glutamine ad libitum.

Group[1]	Intake		Nitrogen gain	
	Food g	Nitrogen g	Observed g	Corrected[2] g
120 GLU[3]	193	3.38	1.92 ± 0.100[4]	2.02
120 GLN[3]	217	3.62	1.99 ± 0.088[4]	1.90

[1] Average initial weights of the groups were 65 g before and 56 g after 4 days on the nitrogen-free diet. There were 15 animals per group.
[2] Corrected by analysis of covariance to the same intake of apparently absorbed nitrogen. The values are significantly different ($P < 0.05$).
[3] GLU = L-glutamic acid; GLN = L-glutamine. 120 has no significance in this experiment but is retained in the group designation to indicate that the diets were the same as those fed in experiment 3, Table 5.
[4] Standard error of the mean.

Table 7—(Experiment 5) Average nitrogen gains of rats fed the same amounts of food and of total and essential amino acid nitrogen, with isonitrogenous amounts of glutamic acid or glutamine added, and restricted to two 1-hr feeding periods per day.[1]

NEN[2] source	No. rats per group	Intake[3]			Nitrogen gains g
		Total N g	α-NH_2 N g	Amide N g	
Glutamic acid	12	2.49	1.25	—	1.39 ± 0.027[4]
Glutamine	14	2.48	0.62	0.62	1.15 ± 0.023

[1] The diets used were those designated 120 GLU and 120 GLN in Tables 5 and 6.
[2] Nonessential nitrogen.
[3] Totals for 24 days.
[4] Standard error of the mean.

Table 8—(Experiment 6) Composition of diets containing two levels of wheat, supplemented to contain the same levels of essential amino acids and nitrogen, but with different levels of amide nitrogen, and nitrogen intakes and gains of rats fed the diets.[1]

Group[2]	34 W	67 W	PAA
	%	%	%
Diets			
L-alanine			0.281
L-arginine HCl	0.295	0.040	0.559
L-asparagine			0.335
L-cystine	0.298	0.216	0.383
L-glutamic acid	4.357		
L-glutamine			2.565
Glycine			0.429
L-histidine HCl·H_2O	0.247	0.119	0.381
L-isoleucine	0.440	0.265	0.620
L-leucine	0.476	0.173	0.789
L-lysine HCl	1.127	0.991	1.267
L-methionine	0.119	0.059	0.180
L-phenylalanine	0.221		0.473
L-proline			0.864
L-serine			0.395
L-threonine	0.430	0.302	0.563
L-tryptophan	0.076	0.030	0.124
L-tyrosine	0.178		0.338
L-valine	0.419	0.225	0.620
Wheat	34.000	67.000	
Wheat starch	42.532	16.010	73.825
Constant ingredients[3]	14.050	14.050	14.050
Sodium bicarbonate	0.735	0.520	0.959
No. rats per group	17	18	16
Nitrogen intake, g	2.41	2.48	2.42
Amide N intake, g	0.20	0.41	0.40
Nitrogen gain and standard error, g	1.46± 0.019	1.48± 0.022	1.37± 0.031

[1] Each diet contained the same amounts of essential amino acids (EAA) used in experiments 2–5. The amounts listed for 34 W and 67 W were those added to the wheat; total EAA was the same as is listed for diet PAA. Diets 67 W and PAA contained the same quantities of nonessential amino acids, all supplied by wheat in 67 W and by purified amino acids in PAA. Glutamic acid was added to 34 W to compensate for the smaller amounts of nonessential amino acids in that diet.
[2] Average initial weights of the animals were 68–69 g before and 57–58 g after they were starved for 24 hr.
[3] See Table 3.

large number of animals was required to establish the statistical validity of these findings. Only the differences between the glutamic acid-glutamine-fed groups (20–21 rats each) is statistically significant ($P < 0.05$).

The addition of sodium bicarbonate to the glutamine-containing diet to neutralize the hydrochlorides of arginine, histidine, and lysine had no effect on nitrogen gains.

Glutamine nitrogen has been shown to be the precursor of a large percentage of the urinary ammonia in acidotic dogs (Pitts et al., 1965). However, in this study, results were the same whether or not the hydrochlorides in the diets were neutralized.

Experiment 4

The effects of feeding ad libitum the amide and α-amino acid nitrogen-containing diets was next studied (Table 6). Under these conditions rats showed a preference for the glutamine (120 GLN) over the glutamic acid-containing diet (120 GLU). Average weight (66 vs 68 g) and nitrogen gains, however, were not significantly different between the two groups. Feces were collected from these animals because it was anticipated that the higher food intakes expected of the glutamine-fed rats would result in higher fecal nitrogen excretion by this group. Differences were small but significant ($P < 0.01$), 0.17 vs 0.19 g.

Nitrogen gains were plotted against absorbed nitrogen and the regressions calculated. The slopes of the lines were not significantly different. Nitrogen gains of glutamic acid-fed rats were significantly higher than of glutamine-fed rats when adjusted to equal nitrogen intakes by analysis of covariance. Thus, more efficient utilization of glutamic acid than of glutamine nitrogen was again shown.

Experiment 5

Because glutamine-fed rats tended to eat their food much more rapidly than glutamic acid-fed rats the effect of feeding pattern on nitrogen utilization was then studied. Cohn et al., (1964) showed that rats force-fed their rations in one or two daily feedings wasted more nitrogen than those allowed to eat over a longer period of time. There was also a chance that enzyme systems involved in amide nitrogen utilization had been depleted by the initial feeding of a nitrogen-free diet for four days, and that the low intake of food, nitrogen and EAAN were starved for 24 hr and then given twice daily only the amount of food which would be consumed by the slowest eaters in one hr. The diets were the same as those fed to groups 120 GLU and 120 GLN, Table 5. The results show (Table 7) that under these ex-

perimental conditions, differences in utilization of α- and amide nitrogen were even greater than in the previous studies.

Experiment 6

In the final study (Table 8) three diets were used to study the utilization of amide and α-amino acid nitrogen when additional nonessential amino acids were supplied to the animals and when part of the amino acids were furnished by wheat. Two of the diets contained either 34 or 67% wheat plus some purified amino acids and a third (designated PAA) contained purified amino acids but no wheat. 67 W and PAA had exactly the same composition except that in 67 W, wheat plus purified amino acids was the nitrogen source.

All three diets contained the same quantities of essential amino acids. Purified essential amino acids were added to the two wheat-containing diets to bring the amounts to the levels used in the earlier experiments.

The level of 67% wheat was selected because it was the highest amount which could be incorporated into the diet without furnishing more essential amino acids than were supplied by the earlier diets; the level of 34% wheat was selected arbitrarily. Glutamic acid was added to the diet containing the low level of wheat in an amount calculated to equalize nitrogen content. No other nonessential amino acids were added.

The results shown in Table 8 do not give an unequivocal answer to the question of the utilization of the amide nitrogen of wheat. Small unplanned differences in nitrogen content of the two wheat-containing diets (1.64 vs 1.69%) resulted in differences in nitrogen intake (100 vs 103 mg per day). However, nitrogen gains as percent of nitrogen intakes were not significantly different (60.8 ± 0.81 and 59.7 ± 0.89 g). Thus, the two groups of rats fed diets containing wheat and two different levels of amide nitrogen appeared to utilize their ingested nitrogen in a similar manner.

Ahrens et al. (1966) reported that when calorie intakes were the same, nitrogen gains of young rats receiving casein were higher than those of rats receiving an amino acid mixture simulating casein. In those studies the amino acid diets contained asparagine but no glutamine. The present study in which both asparagine and glutamine were fed demonstrates again that better nitrogen utilization occurs when the diet contains some intact protein.

The question arises as to the effect of varying proportions of intact dietary protein on nitrogen storage and there are no data available to resolve the point. Rats receiving no intact protein stored 1.37 g nitrogen (diet PAA, Table 8) and those receiving 81% of their dietary nitrogen as wheat (diet 67W) stored 1.48 g. If the effect of intact protein on nitrogen utilization is linear, rats receiving 43% of their nitrogen as intact protein (diet 34W) should have stored 1.43 g. The slightly higher storage found (1.46 g), while not statistically significant, may be considered an indication of better utilization of the nitrogen of the diet with lower amide nitrogen content.

Rigorous control of dietary essential amino acids and nitrogen, and of ratios of essential to nonessential nitrogen was required to demonstrate differences in utilization of the nitrogen compounds by the young rat. The conclusion seems warranted that any wheat-containing diet suitably supplemented by other foods to contain adequate amounts of all essential amino acids would have sufficient amounts of α-amino acid nitrogen either as excesses of certain of the essential amino acids or as other nonessential amino acids so that any failure to utilize part of the amide nitrogen would not limit protein synthesis.

The finding that glutamine nitrogen is less efficiently utilized for body nitrogen gain by the young rat than an equal quantity of glutamic acid nitrogen when they are the sole sources of nonessential nitrogen in the diet may be linked to blood levels and ammonia formation. According to Meister (1965), in general, the concentration of glutamine is higher in the body fluids than that of glutamic acid. The reverse situation is true in the tissues.

Ammonia is formed in the kidney from glutamine and almost none appears to be formed from glutamic acid (Kamin et al., 1951). Thus glutamic acid would be taken up by other tissues while the kidney absorbed glutamine from the blood and converted part of the amide nitrogen to ammonia and excreted it.

That losses through the kidney may be responsible for the differences observed is suggested by the finding of even greater differences in nitrogen gains of rats fed glutamic acid- and glutamine-containing diets for two 1-hr periods daily (Table 7) than for those allowed to eat for longer periods of time (Table 5). Presumably blood glutamine levels would be higher in the animals consuming their food rapidly, and thus more glutamine nitrogen might be converted to ammonia.

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Ms. received 7/29/68; revised 9/4/69; accepted 9/10/69.

The authors gratefully acknowledge the technical assistance of William Bagley and Mrs. Ethel Hall.

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Sensory Differentiation of Beef Tenderness and Juiciness Components Over Short Intervals of Cooking Time

SUMMARY—The ability of judges to distinguish differences in juiciness and six components of tenderness of top round steaks cooked at 350°F for 20, 23, 26, and 29 min was studied by use of a paired comparison design. Concurrently, the effects of cooking time on cooking losses, percents moisture, nitrogen, and fat, shear values, "loose water" and "immobilized water" were evaluated. Judges detected differences for all factors between steaks cooked 20 and 26 min, but were unable to detect differences between steaks cooked 26 and 29 min. Differences between steaks cooked 20 and 29 min were found for all factors except fragmentation and adhesion.

INTRODUCTION

TENDERNESS is recognized as a primary factor influencing the eating quality of meat and juiciness tends to be related to tenderness. Subjective scores for the eating quality of beef have been divided into juiciness and six components of tenderness by Cover et al. (1962a; b; c). In these studies, the steaks were cooked to internal temperatures of 61, 80 and 100°C. Ritchey et al. (1965) studied the effect of temperature changes over a range of 6–7°C on steaks from the Longissimus dorsi and Biceps femoris muscles and observed that the judges could detect differences in eating quality.

However, certain components of beef tenderness seem to be altered as the meat passes a certain end point temperature during the heating process. The ability of judges to detect changes resulting from small differences in cooking times coupled with the possibility of relating the changes detected by sensory analysis to alterations in the biochemical components of the meat could lead to a better understanding of beef quality. This study was designed to determine if judges could detect differences in steaks cooked over small differences in time increments. Physical and chemical measurements including cooking losses, fat, nitrogen, shear force values, and total "loose" and "immobilized" water were obtained for each length of cooking time.

MATERIALS & METHODS

MEAT was obtained from 7 steers similar in age and carcass grade from the Virginia Polytechnic Institute, Animal Science Department. After the carcasses were aged for 4 days the top round muscles from each animal were removed, wrapped, and frozen at −10°F for 3 days. Each top round was then cut into steaks 7 × 1½ × 1 in., vacuum packaged in individual pliofilm bags, and held in a household freezer from 1 to 2 months until used.

A paired comparison design was used with 2 steaks from adjacent positions of the same side or 2 steaks from corresponding positions on the left and right sides being cooked on the same day. Each thawed steak was placed on a wire rack in the center of the oven and baked at 350°F in an electric oven for a period of 20, 23, 26, or 29 min. Each possible combination of two cooking times occurred at 3 different cooking periods, including the combination of each cooking time with itself.

The cooked steaks were rare to medium in doneness. Time, rather than temperature, was used as the criterion of doneness. It was felt it would be as accurate, and use of time avoided the difficulty in positioning of thermocouples in the steaks, avoidance of fat and connective tissue, and variations in the reading of the thermocouples.

Immediately following cooking of each steak, total, volatile, and drip losses were determined. Each steak was evaluated for its sensory qualities as well as additional physical and chemical properties. Sensory analysis for each steak was performed using the quality factors defined by Cover et al. (1962a). Thus, 2 steaks representing either the same or different cooking times were compared by judges for amount of juiciness, softness to tooth pressure, softness to tongue and cheek, fragmentation and adhesion, mealiness, softness of connective tissue, and amount of connective tissue.

A score of 1 was given to the sample which was most juicy, lowest in fragmentation and adhesion, softest to tooth pressure, softest to tongue and cheek, least amount of connective tissue, least mealy, and softest connective tissue. A score of 2 was given to the other sample. Tie scores indicating no difference in evaluated components were permitted.

Nine judges scored the 2 samples for all factors Monday through Friday for 30 sessions. One-half in. cube samples were taken from ½ in. strips removed from the center of each steak. Each judge evaluated samples from the same relative position within each steak. No attempt was made to keep the samples warm as this would tend to cook the sample more and dry out the meat cubes. Judges were selected from within faculty and graduate students.

Preliminary work was done over a 2 month period with evaluations being made twice a week. For the first 4 and last 4 sessions the score card and meanings of terms were discussed. Results were discussed at the end of each preliminary session. Pairs of samples were given that were either cooked for the same length of time or represented various degrees of doneness to enable the judges to determine a frame of reference.

For sensory analysis of the data, it was assumed that for each factor the cooking times have true ratings $\pi_1, \ldots, \pi_i, \ldots \pi_n$. Every $\pi_i \geq 0$ and $\Sigma\pi_i = 1$. When cooking time i appears with cooking time j in a block, the probability that treatment i obtains top rating or a score of 1 is $\pi_i/(\pi_i + \pi_j)$. These ratings were estimated by the method of Bradley et al. (1952). Duncan's multiple range test was used to determine if differences between lengths of cooking times for the quality factors were statistically significant.

Shear values were determined for ½ in. cores, taken from the center and corners of each steak. All 5 values were averaged and the mean value reported.

The remaining meat was ground in an electric grinder and used as sample for the other physical and chemical measurements. The methods of AOAC (1960) were used for determination of total moisture, fat and nitrogen. "Loose water" and "immobilized water" were determined by the method of Ritchey et al. (1964a) except that a pressure of 5,000 lb was used instead of 12,500 lb. The method of calculation was as follows:

$$\text{"Loose water" (\%)} = \text{total moisture (\%)} - \text{"immobilized water" (\%)}$$

$$\text{"Immobilized water" (\%)} = \frac{\text{wt pressed sample (g)} - \text{wt dried sample (g)}}{\text{wt original sample (g)}} \times 100.$$

A correlation coefficient was determined for each pair of physical and chemical measurements.

RESULTS & DISCUSSION

ESTIMATES of the true ratings of cooking times with respect to each quality factor are presented in Table 1. Judges detected differences ($P \leq 0.05$) in all 6

Table 1—Estimates of true ratings for juiciness and tenderness components of top round steaks cooked for 20, 23, 26, and 29 min using Duncan's multiple range test.[1]

Components of Eating Quality	Cooking time, min			
	20	23	26	29
Juiciness	0.5100	0.3154	0.1208	0.0784
Softness to tongue and cheek	0.4637	0.2629	0.1774	0.1074
Softness to tooth pressure	0.3769	0.2443	0.1904	0.1918
Fragmentation and adhesion	0.1620	0.2443	0.3138	0.2810
Mealiness	0.3454	0.3056	0.1782	0.1722
Softness of connective tissue	0.1301	0.2186	0.3092	0.3425
Amount of connective tissue	0.1268	0.2184	0.3487	0.3073

[1] Differences between values underlined by a line on the same level are not statistically significant ($P \leq 0.05$).

Table 2—Mean objective values of top round steaks cooked for four different lengths of cooking time.

Factor	Cooking time, min			
	20	23	26	29
Fat, %	4.39 ± 1.91[1]	4.35 ± 1.56	4.42 ± 1.21	4.74 ± 1.97
Total moisture, %	70.49 ± 1.72	70.33 ± 1.96	69.15 ± 1.46	68.14 ± 1.78
Nitrogen, %	3.76 ± 0.16	3.86 ± 0.16	3.93 ± 0.19	4.01 ± 0.29
Loose water, %	40.10 ± 2.48	40.10 ± 4.03	38.68 ± 2.64	35.91 ± 3.69
Immobilized water, %	30.40 ± 2.19	30.24 ± 2.97	30.57 ± 1.75	32.23 ± 2.45
Shear values, lb.	5.46 ± 0.56	5.15 ± 0.79	5.49 ± 0.63	5.98 ± 0.84
Volatile loss, %	11.51 ± 1.45	14.12 ± 1.20	16.02 ± 1.59	18.63 ± 1.53
Drip loss, %	1.47 ± 0.55	1.99 ± 0.75	2.32 ± 0.91	2.61 ± 0.77
Total losses, %	13.70 ± 1.87	17.04 ± 1.60	19.03 ± 2.22	23.02 ± 2.36
Cooking time, min/lb	29.13 ± 0.83	33.98 ± 1.41	38.35 ± 1.51	42.13 ± 1.19
End point temperature, °C	68.00 ± 4.45	73.80 ± 5.40	77.05 ± 4.10	78.30 ± 4.20

[1] Mean ± SD.

sensory factors between steaks cooked 20 and 26 min but were unable to detect any statistically significant differences between steaks cooked 26 and 29 min. Differences between steaks cooked 20 and 29 min were significant for all factors except fragmentation and adhesion. This may be attributed to the fact that the average differences between end point temperatures of steaks cooked 26 and 29 min was 1.2°C, whereas for steaks cooked 20 and 29 min the difference was 10.3°C (Table 2).

Juiciness, softness to tongue and cheek, softness to tooth pressure, fragmentation and adhesion, and amount of connective tissue decreased as the cooking time increased. For the latter two, values were slightly higher for the 26 min steak than the 29 min steak. Fragmentation and adhesion values tended to toughen with heat. Softness of the connective tissue and mealiness of the sample increased as the cooking time increased. Similar results have been reported by Cover et al. (1962a) and Ritchey et al. (1964b; 1965).

Physical and chemical data (Table 2) revealed little change in percent fat, percent nitrogen, or shear values as the cooking time increased. "Immobilized water" showed little change except between 26 and 29 min. Percent total moisture, and "loose water" decreased, whereas cooking losses (total, volatile and drip), cooking time in min per lb, and end point temperature increased as the cooking time increased.

"Loose water" was positively correlated with "immobilized water" at each length of cooking time. Total moisture and "loose water" were linearly and positively related at all lengths of cooking time except 20 min and this was significant at the 10% level. Percent nitrogen and "loose water" were positively correlated at all lengths of cooking time except 26 min. Volatile losses and total cooking losses; drip losses and total cooking losses were positively correlated for all cooking times.

It appears that rapid changes occur within the meat between 20 and 26 min of cooking and perhaps a slowing down of changes in juiciness and tenderness of the meat occurs around 26 min of cooking. This probably represents a change in the rate of heat penetration, and thus a decreased rate of change in chemical components of meat.

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Ms. received 3/22/68; revised 8/22/68; accepted 9/10/69.

The authors wish to express their appreciation to Mrs. Patricia V. Brooks and Miss Lucy Towe for technical assistance.

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Origin and Nature of Aroma in Fat of Cooked Poultry

SUMMARY—Fat of raw poultry, separated from solid tissue and washed with water at temperatures not exceeding 40°C, does not contain cooked poultry aroma and does not develop it when heated. Hence, cooked poultry aroma cannot be derived from the fat alone. Washed and filtered fat from cooked poultry contains characteristic cooked poultry aroma dissolved in it. The aroma of this fat is caused by compounds which dissolve in it during cooking and which apparently stem from nonfat or lean portions of the meat. The ability of fat to dissolve or acquire substances during cooking was demonstrated by showing that fat of cooked poultry contains more sulfur than does fat of raw poultry. Less than 2% of this sulfur build-up occurs as hydrogen sulfide. The magnitude of the sulfur build-up was 8 to 14 times greater in the fat of roasted poultry than it was in the fat of simmered chicken. Authentic amino acids in contact with poultry fat at a typical roasting temperature readily underwent Strecker type degradation. Furthermore, aroma components representing typical amino acid degradation products were found in fat from roasted turkey. These analytical results indicate protein, amino acids and probably also sugars and other water soluble components are invoved in aroma formation. Hence fat contributes to cooked poultry aroma indirectly and passively through its ability to dissolve and retain aroma components formed during cooking. Consequently, the characteristic cooked poultry aroma in fat of cooked poultry is not derived from the fat itself but comes from and is thus dependent on the "lean."

INTRODUCTION

THE ORIGIN of the characteristic cooked meat aroma which occurs in fat of cooked meat is controversial. It does not stem from oxidative fat deterioration for this leads instead to rancidity. The contribution of other compounds derivable from fat such as free fatty acids, methyl ketones, lactones, etc. (Forss, 1967) may be of some importance. However, their contribution to cooked meat aroma must be minor because they are derivable from and occur in fats which neither possess cooked meat aroma nor come from animal sources. Hence, from our knowledge of triglycerides, it is difficult to see how characteristic cooked meat aroma can be derived from the fat itself. Nevertheless the view is held that the characteristic flavor of meat is derived from the fat (Hornstein et al., 1960).

On the other hand, the ability of fat to dissolve and retain aroma that originates outside the fat is well known and has been demonstrated and practiced for many generations in the ancient process of *enfleurage* in the perfume industry (Guenther, 1948) and in the smoking of bacon in the meat industry. Viewed in this way, it is easy to see how the fat could acquire its cooked meat aroma from the lean indirectly by dissolving and retaining aroma components formed and released from the lean during cooking. This explanation, offered by Bouthilet (1951) and supported later by Pippen et al. (1954), can also be deduced from the results of Hofstrand et al. (1960) on lamb and mutton flavor and is also supported by the recent results of Klose (1967).

The diverse conclusions arrived at by these two points of view illustrate the need for additional studies that will lead to definite conclusions regarding the origin and nature of the components responsible for the flavor and aroma that occurs in the fat of cooked meat. This study was carried out to determine whether the characteristic cooked poultry aroma in fat of cooked poultry is derived from fat itself or from lean portions of the meat and also to inquire into the nature of components responsible for this aroma.

Aroma and total sulfur values of fat from raw and cooked poultry were compared. Fat from raw poultry was defined as the clear oil obtained from crude raw fatty tissue following pressing, water washing and filtration at temperatures not exceeding 40°C. Similarly, fat from cooked poultry was defined as the clear oil obtained following washing and filtering of the fat that rendered out during cooking. Hence the "lean" consists of protein, water soluble components and other meat components not dissolved in this washed and filtered fat.

Volatiles were isolated from roasted turkey drip oil and separated by gas chromatography. Odors of the fractions were observed and particular attention was given to detection of odor and compound types which would logically originate in the lean. Finally, the possibility that amino acids in contact with hot fat during the roasting of poultry would be degraded to volatile compounds was investigated.

MATERIALS & METHODS

Poultry raw material

All raw poultry was obtained in ready-to-cook form from commercial sources. Chilled but unfrozen roasting chickens were used within 24 hr of purchase time. Ice packed unfrozen stewing fowl, obtained within 24 hr of processing, were bagged in polyethylene, frozen in a blast freezer at −34°C and held at −23°C until used. Tom turkeys (24–26 lb oven-ready weight) were obtained packaged and frozen and were stored at −23°C until used. Cut-up tray-packed frying chickens were held at −23°C for a few weeks before thawing, dredging in flour and salt and frying.

Cooking methods

Chicken was simmered in an equal weight of water for 2 hr at 95–100°C in a lidded stainless steel vessel. Roasting was carried out in a rotary electric oven. Three chickens (total weight 17 lb) were simultaneously roasted at 218°C for about 2 hr to an internal temperature of 91°C. Turkeys were roasted in batches of one or two birds per batch. Typical conditions for roasting a pair of turkeys (total weight 50 lb) was 7.5 hr at 191°C. Chicken was deep fat fried in peanut oil at 141°C to an internal temperature of 85°C. Approximately 24 chickens were fried in the 12 in. × 18 in. × 10 in. fryer before a sample of frying oil was taken for analysis.

Preparation of fat samples

a. Fat from raw fowl for aroma evaluation and for total sulfur analysis. Three ready-to-cook fowl were removed from frozen storage and band sawed into right and left halves. Right halves were returned to frozen storage. Left halves, while thawing, were skinned. Fatty tissue cut from the visceral cavity, skin and muscle was combined. The fatty tissue was extruded at room temperature from a stainless steel cylinder-and-piston press through a plate having 0.04-in. holes.

The extruded fatty tissue was parcelled into 250 ml glass centrifuge tubes so that each was about 40% full. Warm (50°C) distilled water about equal to the volume of fatty tissue was added to each tube, and tubes were capped and vigorously shaken. Tube contents were adjusted to 40°C by gentle stirring while immersed in a warm water bath. Tubes were then promptly centrifuged for 10 min at 1500–2000 rpm on a Model BE International Centrifuge. The lower water and fibrous tissue phase was frozen by immersing the tube in a dry-ice-alcohol bath to a depth

sufficient to freeze the lower phase while leaving the upper fat phase in a semi-solid state.

At this point, the upper fat phase was transferred with a spatula to a clean centrifuge tube. It was subjected to the process of warm water washing, centrifuging, freezing out of lower water phase and transferring of the upper fat phase to a clean centrifuge tube two more times. The fat, warmed gently until melted, was filtered by suction through a carefully cleaned, dry, medium porosity sintered glass filter. The resulting clear oil was free of visible solid particles and seldom contained any visible water.

b. Fat from simmered fowl for aroma evaluation and for total sulfur analysis. The three right halves mentioned above were removed from frozen storage, thawed and simmered. At the end of the simmering period, the hot broth with its melted fat was decanted from the meat through cheese cloth into a separatory funnel where the aqueous phase was withdrawn. The fat was then distributed into 250 ml centrifuge tubes, each filled to about 40% capacity. Fat in each tube was washed with warm water and centrifuged as described above.

After centrifuging, the aqueous phase and interfacial material were withdrawn with a large-bore pipette and discarded. The process of warm water washing, centrifuging and phase separation was repeated two more times. The warm melted fat was filtered by suction through a clean, dry, medium porosity sintered glass filter. The clear oils obtained contained no visible solid particles and usually contained no visible water.

Starting with another batch of 3 fowls, steps (a) and (b) were repeated as needed to prepare additional pairs of fat for aroma and total sulfur analyses.

c. Fat from raw chicken roasters. Fatty tissue was removed principally from the visceral cavities of the carcasses just before they were roasted. The fat was rendered from this tissue with the pressing, washing, centrifuging and filtering steps described in detail above.

d. Fat from roasted chicken. Drippings were collected and kept during roasting in an aluminum tray placed in the oven under the roasting chicken. After roasting, the drippings were transferred to a screw cap bottle and stored at −34°C. The thawed and warmed (40°C) drip stock was mixed and a portion removed, homogenized in a glass homogenizer and stored at −34°C for analysis. The remaining drip stock, warmed to 40°C, was centrifuged until it separated into oil and sediment phases. The oil was carefully withdrawn and portions of it were filtered or water washed, centrifuged and filtered as described in detail in part (b) above. See also Figure 1.

e. Fat from roasted turkey. Washed and filtered fat from raw turkey was obtained and worked up as described above for roast chicken, part (c). Drippings from roasted turkey were also worked up into oil and sediment fractions, a filtered oil fraction and a water washed and filtered oil fraction as described in parts (b) and (d) above (Fig. 1).

f. Peanut oil. A sample of fresh unused peanut oil was taken from the same lot of oil used for the deep fat frying of chicken. Another sample of peanut oil was taken from the deep fat fryer after it had been extensively used to fry chicken. Both the fresh peanut oil and the frying oil were water washed, filtered, etc. as described in part (b) before sulfur analyses were carried out.

g. Fat from simmered fowl for H_2S determinations. Two batches (3 fowl/batch) were simmered as described above. Hot broth was transferred to a separatory funnel where the aqueous phase was withdrawn. In the first batch, the hot fat was filtered only through a milk filter (a coarse cheese cloth and paper filter), cooled to 65°C and immediately transferred and sealed in a one liter round bottomed flask for H_2S analysis. (Sample I, Table 1.) In the second batch, the hot fat, separated from the aqueous broth, was filtered through a milk filter and separated into two equal portions. One portion was suction filtered through a medium porosity sintered glass filter to give Sample II, Table 1. The second portion was washed 3 times with water and filtered through the glass filter as described in detail under section (b) to give Sample III, Table 1.

Table 1—Quantity of hydrogen sulfide in fat of simmered fowl.

Sample no.	Description	Quantity of fat analyzed (g)	Total H_2S found (μg)	Sulfur as H_2S ($\mu g/g$ fat)
I	Hot fat from simmered fowl, filtered through milk filter, cooled to 65°C, sealed in analytical flask, and nitrogen purged 1/2 hr at 30°C	243	2.13	0.008
	Same fat nitrogen purged additional 1/2 hr at 95°C	243	1.92	0.007
II	Hot fat from simmered fowl, filtered through milk filter and then through medium porosity sintered glass filter, nitrogen purged 1 hr at 30°C	150	0.00	0.00
	Same fat nitrogen purged additional hr at 100°C	150	0.53	0.003
III	Same fat as Sample II, but washed 3 times with water before final filtering and then nitrogen purged 1 hr at 30°C	124	0.00	0.00
	Same fat nitrogen purged additional hr at 100°C	124	0.1	0.0008

Table 2—Odor panel evaluation of washed and filtered chicken fat from simmered and from raw fowl.

	Judgments		
Pairs compared	Showing discrimination between pairs (Correct/total)	Indicating little or no aroma in fat from raw (% of total)	Indicating chickeny aroma in fat from simmered (% of total)
I Simmered vs raw	15/16	81	69
II Simmered vs raw heated dry 2 hr at 100°C	8/8	87	87
III Simmered vs raw heated in water 2 hr at 100°C	6/7	100	100

Aroma panel

Fat samples were presented to experienced trained judges as warm (40°C) clear oils in clear glass screw cap bottles. There were no visible differences between samples. Each judge was first given a coded sample identical to one of the two samples being compared. After smelling this control sample, the judge was presented with the two coded comparison samples and a score card (Fig. 2), which he filled out after smelling each of the two samples. There were two replicates of each of the three comparisons (Table 2). All of the fat samples evaluated came from raw or simmered fowl and all were freed of solid material, water washed, filtered, etc. as described above.

Determination of total sulfur in samples

A method was developed for completely

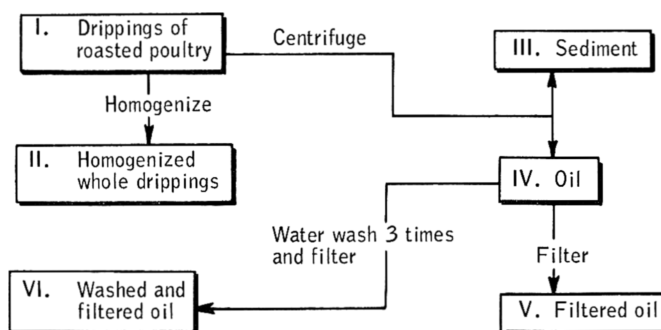

Fig. 1—Fractions of roasted poultry drippings.

Sample No.	Check sample that smells most like control	Aroma characteristics	
		Little or no aroma	If aroma is detected, please describe

Fig. 2—Form used by judges for evaluating odor of chicken fat samples.

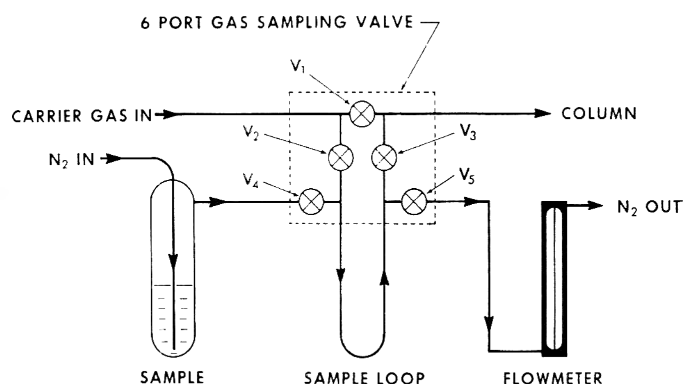

Fig. 3—Schematic representation of the apparatus used for isolating and injecting volatile components into the gas chromatograph.

burning the sample in a quartz tube filled with alumina granules carrying a vanadium pentoxide catalyst. The combustion products were trapped in chilled hydrogen peroxide solution where SO_2 formed during combustion was oxidized to SO_4^{-2}. The SO_4^{-2} was concentrated and reduced to H_2S which was quantitatively determined as methylene blue. The method was tested by analyzing poultry fat samples containing known amounts of added sulfur. It was thus shown to be highly suitable for the quantitative determination of trace quantities of sulfur in fat and also easily capable of distinguishing between fat samples that differed by as little as 0.5 μg S/g fat. A manuscript describing the method in detail is being prepared for publication elsewhere.

Determination of hydrogen sulfide in fat

The melted (30°C) fat, with sufficient triple distilled water to make a total volume of 500 ml, was added to a 1-liter round bottomed flask. High purity nitrogen was then passed through the mixture, and H_2S entrained in the nitrogen stream was absorbed in zinc acetate solution and determined as methylene blue as described by Mecchi et al. (1964). A sample was first analyzed by nitrogen-purging the fat-water mixture, kept at 30°C, for at least $^1/_2$ hr. Then, with a fresh zinc acetate trap in place, the same fat-water sample, warmed to and kept at 95–100°C, was nitrogen purged for at least an additional $^1/_2$ hr to determine whether it would yield more H_2S.

Procedure for isolating volatiles

The hardware and gas flow arrangements are shown schematically in Figure 3. The five valves shown as part of the gas sampling valve (V1-V5, Fig. 3) were all an integral part of the heated 6-port stainless steel gas sampling valve (Varian Aerograph No. 57-000036-00) which was attached directly to the injector of the Varian Aerograph Model 1525-B gas chromatograph. The valve was equipped with Viton Quad rings and 40 volts a.c. were applied to its 50 watt cartridge heater. In one of two possible positions of the valve plunger, V1, V4 and V5 were open and V2 and V3 were closed. In this position (the "sample collect" position) carrier gas passed directly into the injector and column while nitrogen could be passed through the sample, sample loop and out through a flow meter. Thus, by heating the sample while cooling the sample loop, volatiles were transferred from the sample to the sample loop.

In the other valve plunger position (the "inject" position), V1, V4 and V5 were closed and V2 and V3 were open thus sending all the carrier gas first through the sample loop and then into the column. Thus, by heating the sample loop immediately after placing the plunger in the "inject" position, the trapped sample was vaporized and swept into the column.

The approximately 12 ml glass sample container was connected to the nitrogen supply and to the gas sampling valve with glass to stainless steel ball and socket joints. The stainless steel tube (8 in. long × $^1/_8$ in. OD × $^1/_{16}$ in. ID) connecting sample flask to gas sampling valve was heated electrically to minimize condensation. The sample loop was made from stainless steel tubing ($8^1/_2$ in. long × $^1/_8$ in. OD × 0.093 in. ID) packed with 45/50 mesh acid washed dimethyldichlorosilane treated chromosorb G, plugged at each end with glass wool, bent into a "U" shape and fastened to the gas sampling valve with Swagelok fittings.

Standard conditions for isolating volatiles were as follows. With the gas sampling valve plunger in the "sample collect" position, the sample flask containing 8 ml (7.2 g) of melted, filtered roast turkey drip oil (Fraction V, Fig. 1) was connected to the high purity nitrogen source and to the gas sampling valve. With a stream of nitrogen established and maintained at a 10 ml/min rate through the sample and through the sample loop, the latter was cooled in liquid nitrogen and the sample was heated in a silicone oil bath at 175°C. These conditions were usually maintained for 30 min, when heat was removed from the sample and nitrogen flow through the sample was stopped. Injection of trapped volatiles was accomplished by removing the liquid nitrogen from the sample loop, moving the valve plunger to the "inject" position and immediately applying a hot oil bath (230°C) to the sample loop.

Isolation and gas chromatography of volatile decomposition products

The amino acids, all commercially available dl-forms, were used as received. After charging the sample flask (Fig. 3) with 8 ml of warmed (40°C) melted roast turkey drip oil (Fraction V, Fig. 1), about 0.5 mg of each amino acid being used was added to the oil. The sample flask was then attached to the gas sampling valve and heated and purged with nitrogen. Volatiles from it were trapped and injected into the gas chromatograph using the standard conditions described in detail in the preceding section.

Gas chromatography

The general conditions used for gas chromatography are shown in Table 3. Details not supplied in this table, such as column length, column packing, and column temperatures will be found in the legends for figures. Modifications to the instrument included fitting it with a 6-port gas sampling valve (as described above) and with a 50–50 column effluent stream splitter. Half of the column effluent was connected to the hydrogen flame detector while the other half led out of the detector oven to a heated smelling tip thus permitting simultaneous smelling and recording of fractions.

The chromatograph was also equipped, as needed, with a laboratory designed and constructed device for injecting authentic samples into the sample loop. This injection device was constructed of stainless steel and was provided with a rubber septum for sample introduction. Its outlet was connected directly to the sample loop input fitting on the 6-port gas sampling valve and its inlet was connected to a regulated source of high purity nitrogen. Thus, with nitrogen flowing through the injector and out through the sample loop a sample injected through the septum into the nitrogen stream was carried into the sample loop where it could be condensed and held at liquid nitrogen temperature until injected into the column by actuating the valve plunger and heating the sample loop. Authentic samples generally consisted of 5 to 30 μl of vapor taken from the headspace over the authentic sample.

Table 3—General gas chromatography conditions.

Instrument:	Varian-Aerograph Model 1525-B
Columns:	$^1/_8$-in. OD × 0.093-in. ID stainless steel
Sample loop heating bath:	230°C
Injector temp:	200°C
Detector temp:	215°C
Detector type:	Hydrogen flame, electron capture, olfactory
Carrier gas:	Nitrogen
Carrier flow rate:	25 ml/min
Recorder:	Leeds and Northrup Speedomax W

RESULTS & DISCUSSION

DURING PREPARATION it was apparent that fat from simmered fowl had a distinct "chickeny" aroma whereas fat from the raw fowl had relatively little odor. This observation was confirmed by the odor panel (Table 2, Pair I).

Since all samples in Table 2 had been water washed and filtered before their aromas were evaluated, the water soluble components and the solid tissue that often accompany crude or poorly defined animal fat samples could not be direct contributors to the aroma of any of these fat samples. Therefore the aroma of these fat samples, and hence the aroma differences observed, must be caused by substances in them that are truly fat soluble. Heating the fat from raw fowl whether dry (Table 2, Pair II) or with water (Table 2, Pair III) not only failed to generate a chickeny aroma but also left them relatively odorless.

Evidently the chickeny odor that develops in fat of simmered fowl depends to a great extent upon its ability to dissolve aroma components released or formed during cooking. Futhermore, these results strongly indicate that the substances responsible for the chickeny odor of fat from simmered fowl originate partly, if not entirely, in the lean, proteinaceous or water soluble portions of the meat.

Consequently we should expect to find a predominantly lean meat element at a higher level in fat from cooked poultry than in fat from raw poultry. Sulfur is such an element because nearly all the sulfur in chicken meat occurs in muscle protein (Beach et al., 1943) with some also occurring to a lesser extent in other compounds such as glutathione, thiamine and the free amino acids methionine and cysteine. Futhermore, sulfur compounds in chicken meat are known to decompose significantly during cooking (Mecchi et al., 1964; Ballance, 1961; Libbey, private communication). We should not be surprised to find that sulfur compounds released and formed during cooking dissolve to some extent in the fat giving it an "acquired" sulfur content.

To experimentally test for the existence of such an acquired sulfur fraction, we developed a method for determining trace sulfur in poultry fat (see Materials and Methods) and proceeded to analyze fat samples on the assumption that the acquired sulfur content is equal to the total sulfur content of fat from cooked poultry less whatever intrinsic sulfur there might be in the fat from the raw poultry. The average differences which we found are all positive (Table 4) and thus virtually establishes the existence of an appreciable acquired sulfur fraction in these fat samples from cooked poultry. The conclusion that sulfur compounds migrate into the fat during cooking and remain in it after cooking seems inescapable.

Table 4—Comparison of total sulfur in washed and filtered fat from raw and from cooked poultry.

Sample no.	Origin and type of fat	Total sulfur in fat: Duplicates (μg/g)	Total sulfur in fat: Average (μg/g)
12 C	Simmered fowl	1.27, 1.27	1.27
12 R	Raw fowl	0.87, 0.81	0.84
	Difference		0.43
16 C	Simmered fowl	1.33, 1.35	1.34
16 R	Raw fowl	1.14, 0.52	0.83
	Difference		0.51
17 C	Simmered fowl	1.54, 1.67	1.61
17 R	Raw fowl	1.09, 1.11	1.10
	Difference		0.51
25 C	Drippings from roasted chicken	5.21, 5.38	5.30
25 R	Raw chicken roasters	1.11, 1.22	1.17
	Difference		4.13
30 C	Drippings from roasted turkey	7.72, 7.81	7.77
30 R	Raw turkey	0.74, 0.71	0.73
	Difference		7.04
40 C	Peanut oil from chicken deep fat dryer	6.2, 6.2	6.2
40 R	Peanut oil (fresh unused stock)	0.9, 0.8	0.9
	Difference		5.3

Conceivably, the fat as we sampled it from the raw poultry could have a lower intrinsic sulfur value than the fat which rendered out during cooking. In this case the differences (Table 4) would be attributable, not to acquired sulfur, but to the sampling procedure. But, this extreme possibility seems unlikely because we cannot visualize how intrinsic sulfur, present in fat of raw poultry at relatively low and consistent levels (average values ranged from only 0.73 to 1.17 μg S/g fat from raw, Table 4), should both increase and broaden its range in fat of poultry cooked in several ways (average values range from 1.27 to 7.7 μg S/g fat from cooked, Table 4).

On the other hand, the "acquired sulfur concept" readily explains the remarkable 8 to 14-fold greater quantity of acquired sulfur in fat of drippings from roasted poultry than in fat from simmered poultry (Table 4). Obviously the oil or fat in the drippings of roasted turkey had a better opportunity to acquire sulfur than did fat during the simmering of chicken.

This suggests that the decomposition of protein, sulfur amino acids and other components is more extensive in the drippings of roasting poultry than it is in simmering chicken. This is reasonable when it is recalled that fat in drippings of roasting poultry comes into intimate contact with sedimentary material that is itself rich in flavor, that is hot and concentrated, and that is obviously undergoing thermal decomposition and polymerization. On the other hand, during simmering, in which the temperature does not exceed 100°C and the fat contacts essentially only a dilute water broth we would expect less thermal decomposition and hence less sulfur build-up in the fat.

Table 5—Sulfur distribution in several fractions of drippings from roasted poultry.

Fraction analyzed	Total sulfur: Chicken (μg/g)	Total sulfur: Turkey (μg/g)
Whole homogenized	561	—
Oil (from centrifugation of drippings)	—	12.4
Oil (centrifuged and filtered)	—	11.6
Oil (centrifuged, washed and filtered)	5.3	7.8

The approximately 100 to 1 ratio of sulfur in whole homogenized drippings to sulfur in the washed and filtered oil from the roasted chicken drippings (Table 5) supports our claim that the sediment of roast poultry drippings is probably the principal precursor of the acquired sulfur found in the drip oil. The values for turkey (Table 5) show that small but significant amounts of sulfur were removed from the drip oil by filtering and water washing. This illustrates the importance of carefully defining the steps used in the preparation of fat samples for comparative sulfur analyses.

We have not determined what sulfur compounds occur in the acquired sulfur fraction of fat. Hydrogen sulfide (H_2S) is a possibility because so much of it is formed during cooking (Mecchi et al., 1964). But it seemed unlikely to be a major direct contributor because we would expect little of it to survive the water washing and vacuum filtration steps. Nevertheless we checked this possibility.

Fat from freshly simmered chicken,

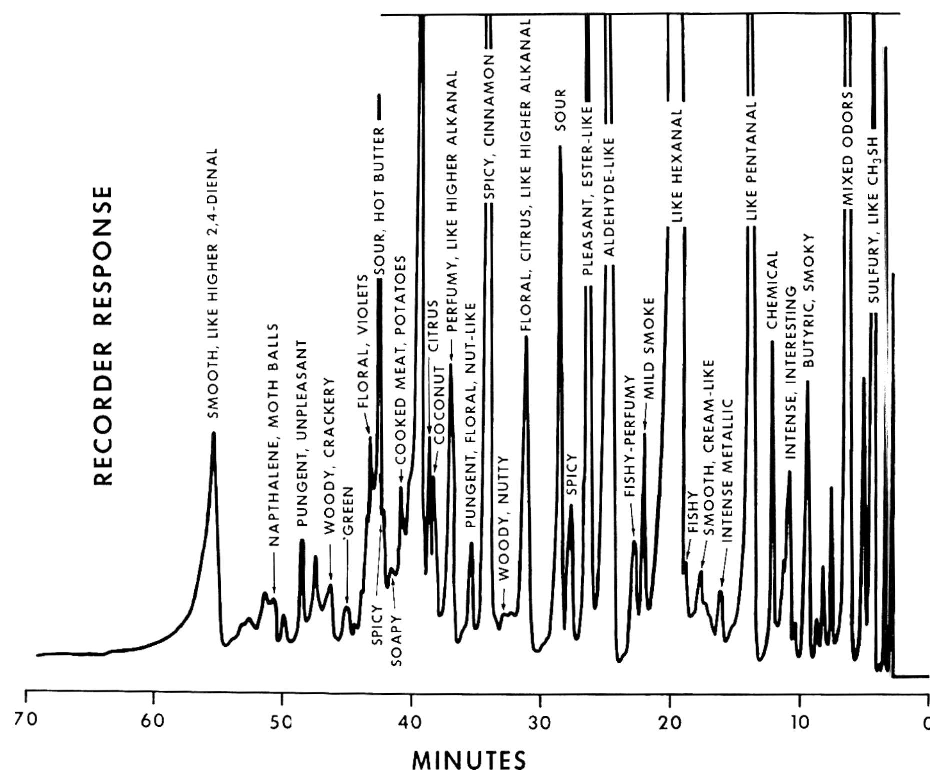

Fig. 4—Gas chromatogram of volatiles isolated from the oil of roasted turkey drippings with descriptions of some of the odors observed as fractions eluted from the column. The 24 ft × 1/8 in. OD stainless steel column was packed with 5% FFAP on 60/80 mesh acid washed and dimethyl dichlorosilane treated chromosorb G and, starting at 60°C, was programmed to a temperature of 200°C at a 2°C/min rate.

filtered crudely through a milk filter, placed into the H_2S analysis apparatus as soon as its temperature had dropped to 65°C and analyzed at 30°C (Sample I, Table 1), contained only 0.008 μg S/g fat which amounts to only 0.1 to 2% of the acquired sulfur values (differences, Table 4). No H_2S at all could be detected in additional fat samples (II, III, Table 1) after vacuum filtration and water washing. Furthermore, the amounts of H_2S that could be driven out of these three samples (Table 1) at 95–100°C also represent very small percentages of the acquired sulfur values (Table 4). Hence practically all acquired sulfur occurs in a non-H_2S form that decomposes only slowly, if at all, to form H_2S at 95–100°C.

There remains the possibility for H_2S to contribute to the acquired sulfur fraction by reacting during cooking to form other fat soluble sulfur compounds. In this connection, the formation of an odorous fat soluble product by reacting H_2S with acetaldehyde in poultry fat has been mentioned (Pippen, 1967). It seems more probable, however, that the acquired sulfur stems from indigenous poultry meat sulfur compounds and their degradation products that become dissolved in the fat during cooking.

Of course, there is no reason why the process of fat solubilization of substances released and formed during cooking has to be restricted to sulfur compounds. Therefore we should not be surprised to find fat of cooked poultry also acquires, during cooking, ingredients that come from non-sulfur containing precursors. To investigate these possibilities, we smelled fractions as they eluted from the column of the gas chromatograph to determine if odor types could be detected that were probably traceable to a non-fat precursor. For these investigations we selected filtered oil from roast turkey drippings (Fraction V, Fig. 1). Many chromatograms were run on this oil and from this a rather extensive list of odor descriptions was compiled.

Space limitations permit showing only a portion of the compiled list of odor descriptions in the illustrative chromatogram (Fig. 4). Fractions having odors like saturated and unsaturated aldehydes probably have their origin principally in fat deterioration. We suspect that the bulk of these types of compounds came directly from peroxide precursors because the peroxide value of the oil fell from about 12 to nearly zero during the 30 min treatment at 175°C involved in the isolation procedure. On the other hand, we observed many interesting odors that probably do not stem from oxidative deterioration of fat. The similarity between many of these odor descriptions and odors reported to stem from the decomposition of amino acids and sugars is evident (Table 6). We list only the potential amino acid precursor in Table 6 because the sugar type is less important than the amino acid type in determining these odor characteristics (Rothe et al., 1963; Kiely et al., 1960; Hodge, 1967).

Table 6—A partial list of odor descriptions recorded as fractions of volatiles from fat of roast turkey drippings were smelled as they eluted from the gas chromatograph. Likely precursors with supporting references are also listed.

Odor description	Possible precursor
Cooked cabbage, methyl mercaptan	Methionine[2,3,6]
Burnt, smoky	Glycine,[5] cystine[11]
Burnt sugar, caramel, chocolate	Arginine, leucine, etc[1]; glycine[5,11]
Walnuts	α-aminobutyric acid[3]
Popcorn	Arginine[1]
Almonds, roast nuts, benzaldehyde	α-amino, α-phenylacetic acid,[6] unsat. fatty acid[10]
Crackers	Proline[1,3,4]
Musty, rotten wood	Isoleucine,[1] glutamic acid[5]
Meaty, cooked potatoes	Methionine[1,4]; lysine[5]; hydroxyproline, cystine[11]
Green potatoes	Lysine,[5] unspecified amino acids[7]
Hot butter	Glutamine,[1] lysine[5]
Cheese	Leucine[1,4]
Naphthalene, "moth balls"	Carotenoids[8,9]
Butter	Leucine, histidine, arginine[11]
Floral, violets	Phenylalanine[1,3]
Coconut	Hydroxy fatty acid in triglyceride[12]

[1] Herz et al., 1960; [2]Ballance, 1961; [3]Rothe et al., 1963; [4]Wiseblatt et al., 1963; [5]El'Ode, et al., 1966; [6]Barnes et al., 1947; [7]Mason et al., 1966; [8]Jones et al., 1948; [9]Erdman, 1961; [10]Kawada et al., 1967; [11]Kiely et al., 1960; [12]Forss, 1967.

The coconut odor was evident in several fractions and suggests lactone formation by heat breakdown of hydroxy acids in turkey fat triglyceride as in heated beef and dairy fat (Boldingh et al., 1962). We have found no report of the occurrence of α-phenyl, α-amino acid in poultry meat. But another possibility for the formation of benzaldehyde through cyclization of unsaturated fatty acids has been outlined (Kawada et al., 1967).

The naphthalene or "moth ball" odor observed (Table 6) recalls our identification of a naphthalene compound in

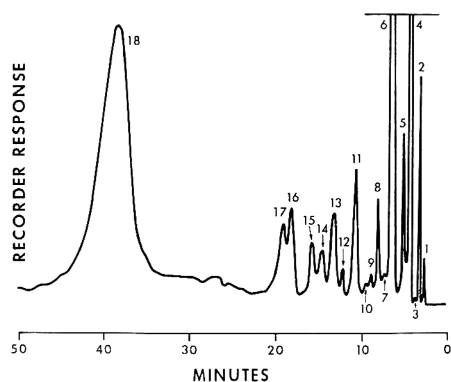

Fig. 5—Chromatogram of lower boiling components isolated from roasted turkey drip oil showing fractions identified for GC Condition I, Table 7. The 24 ft x 1/8 in. OD column, packed with 5% FFAP on 60/80 mesh acid washed and dimethyldichlorosilane treated chromosorb G, was operated isothermally at 55°C.

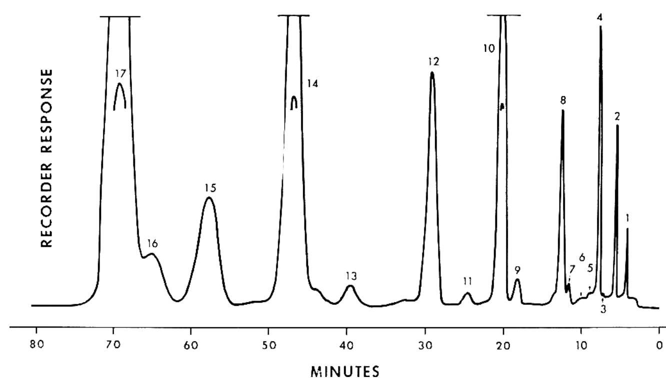

Fig. 6—Chromatogram of lower boiling components isolated from roasted turkey drip oil showing fractions identified for GC Condition II, Table 7. The 16-1/2 ft x 1/8 in. OD column, packed with 80-100 mesh Porapak Q, was operated isothermally at 150°C.

cooked chicken meat volatiles (Nonaka et al., 1967). It is well documented that carotenoids undergo thermal decompositions to give methyl naphthalenes, toluene and xylene (Jones et al., 1948; Erdman, 1961; Borenstein et al., 1966). Doubtless a wide variety of other hydrocarbons and cyclic compounds are also formed in the decomposition of carotenoids. Hence the carotenoids are likely precursors of naphthalene, toluene, xylene and possibly other "unusual" hydrocarbons and benzene derivatives that have been repeatedly found in aroma fractions of foods such as chicken, beef, butter and fish (Nonaka et al., 1967; Merritt et al., 1965; Wick et al., 1965; Forss et al., 1967; Wong et al., 1967).

Supplementing evidence from the odor descriptions, we also found generally excellent agreement between relative retention times of compounds isolated from roast turkey oil and authentic compounds representing degradation products of several amino acids (Table 7 and Figs. 5 and 6). Both degradation products and precursors may exist in the roast turkey drip oil. This appears to be the case for we observed that the quantity of volatile material isolated from the oil depended upon and was proportional to its temperature during the isolation step.

Also, when compared to the oil alone, addition of sediment (Fraction III, Fig. 1) to the oil caused a considerably increased yield of some volatile fractions as determined by both peak size on the chromatogram and by aroma intensity as fractions eluted from the column. The sediment or non-oil portion of roast turkey drippings is obviously loaded with precursors that degrade into volatile components at roasting temperatures.

We also heated authentic amino acids in the oil to determine whether they would degrade under the isolation conditions. A typical result with valine and leucine (Fig. 7) illustrates that these amino acids did indeed degrade in the hot oil to give the expected product. Similarly, glycine and alanine degraded to formaldehyde and acetaldehyde respectively. Methionine, on the other hand, degraded principally to methyl mercaptan and acrolein as reported by Ballance (1961). Whether this amino acid degradation in the hot oil is strictly thermal or is aided by an agent in the oil has not been determined. Certainly this turkey drip oil contains, among other possibilities, peroxides, which typically are active in promoting the Strecker degradation of amino acids (Schönberg et al., 1952).

The area on the chromatogram where we observed an odor reminiscent of cooked meat or potatoes (Fig. 4) also smelled like a commercial sample of methional. The occurrence of methional, a degradation product of methionine, in the drip oil of roasted turkey is consistent with the sulfur build-up we observed in this fat, with the occurrence of methyl mercaptan and acrolein (Ballance, 1961) and with the fact it has been found in roasted turkey by Hrdlička et al. (1965).

Authentic methional chromatographed

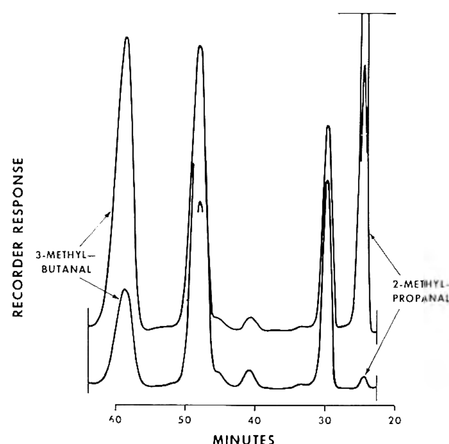

Fig. 7—Chromatogram of volatiles isolated from roasted turkey drip oil illustrating the degradation of valine and leucine in the hot drip oil. Upper curve, oil with 70 ppm each of valine and leucine; lower curve, oil with nothing added. Chromatographic conditions were the same as those described in Figure 6.

Table 7—Relative retention times (RRT)[1] of typical amino acid degradation products and of aroma fractions from drip oil of roasted turkey.

Authentic compound	Possible amino acid precursor	GC Condition I[2]: Turkey drip oil fraction Peak no.[2]	GC Condition I: Turkey drip oil fraction RRT	GC Condition I: Authentic compound (RRT)	GC Condition II[3]: Turkey drip oil fraction Peak no.[3]	GC Condition II: Turkey drip oil fraction RRT	GC Condition II: Authentic compound (RRT)
Formaldehyde	Glycine	5	0.47	0.45	1	0.28	0.28
Acetaldehyde	Alanine	—	—	0.38	2	0.41	0.41
2-Methylpropanal	Valine	7	0.77	0.78	6	2.29	2.21
3-Methylbutanal	Leucine	13	1.61	1.56	11	5.52	5.51
Acrolein	Methionine	9	1.00	1.00	4	1.00	1.00
Methyl mercaptan	Methionine	4	0.33	0.30	3	0.61	0.57

[1] Acrolein = 1.00.
[2] See chromatogram, Figure 5.
[3] See chromatogram, Figure 6.

under these conditions (Fig. 4) decomposed extensively but did give a major peak with a retention time that would place it in the "cooked meat" area of the chromatogram. Co-chromatography of authentic methional and drip oil volatiles also indicated that methional emerged in this area but not as a distinct well separated peak. Although these difficulties prevented us from positively establishing the presence of methional, the aroma and retention time of authentic methional are consistent with its being a contributor to the "meaty" odor observed in this area of the chromatogram (Fig. 4).

CONCLUSIONS

COOKED POULTRY aroma cannot be derived from the fat alone. The characteristic cooked poultry aroma that occurs in fat of cooked poultry is derived from the "lean" portions of the meat. Migration of sulfur containing substances into the fat during cooking supports this concept and suggests that sulfur compounds are important precursors and contributors to this aroma. The dependence of the magnitude of the sulfur build-up in fat upon cooking conditions, the readiness with which authentic amino acids decompose in hot fat and the nature of aroma components found in fat of roasted turkey all suggest that protein, amino acids, sugars and other water soluble components are involved in the formation of the characteristic aroma that accumulates in fat of cooked poultry.

These results do not support the view that lean meat flavor is much the same from one meat to another and that characteristic meat flavors are derived from the fat. Instead, they show that poultry fat contributes to characteristic cooked poultry aroma passively and indirectly through its ability to dissolve and retain aroma components formed and released during cooking. As a corollary, characteristic cooked poultry aroma, including that which appears in the fat of cooked poultry, stems from and is primarily dependent upon the composition of the "lean" portion of the meat.

These results predict, and certainly do not exclude, the possibility that significant aroma can be derived from the connective tissue, protein, water soluble components, meat scraps, etc. that usually accompany the crude fatty tissue of meat and which is often casually referred to as simply "fat." Certainly the ambiguous meaning of the word "fat," particularly as applied to meat, can partly explain why there are divergent opinions about the role fat plays in cooked meat flavor. The results of this study demonstrate and emphasize the importance of working with carefully defined animal fat samples in meat flavor studies.

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Ms. received 7/16/68; revised 10/16/68; accepted 9/8/69.

Presented at 28th Annual Meeting of the Institute of Food Technologists in Philadelphia.

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Hydrogen Sulfide, a Direct and Potentially Indirect Contributor to Cooked Chicken Aroma

SUMMARY—The 35 parts per billion (ppb) H_2S in freshly prepared broth and the 180 to 730 ppb H_2S in meat of freshly simmered, roasted and fried chicken all substantially exceed the 10 ppb H_2S odor threshold in water. Hence, H_2S contributes directly to the aroma of these products. Freezing, thawing and reheating can reduce the H_2S in broth to subthreshold levels thus indicating the transient nature of its direct contribution to aroma. In a model system, H_2S was passed through molten chicken fat containing 5% acetaldehyde. After expulsion of excess H_2S and acetaldehyde the residual highly odorous fat exhibited a fixed sulfur content. These exploratory results, together with related results reported in the literature, suggest that a reaction beween H_2S and acetaldehyde was involved and that such interactions between H_2S and carbonyls in fat could be quite general. Thus H_2S may also contribute to cooked chicken flavor and aroma through the formation of such secondary products.

INTRODUCTION

HYDROGEN SULFIDE continuously evolves from simmering chicken (Mecchi et al., 1964; Klose et al., 1966), and hence contributes to its aroma. But, the dynamic aroma producing system involved in the cooking of chicken must be interrupted and the meat must be cooled before it can be eaten. As the meat cools, H_2S will be produced at a slower rate and there may be appreciable loss of H_2S because of its volatility and reactivity. Consequently, there is no assurance that enough H_2S exists in the poultry meat, when eaten, to contribute significantly to its aroma. Therefore, we determined the quantity of H_2S in chicken broth and in the freshly cooked ready-to-eat meat of boiled, roasted and fried chicken. We also determined the H_2S odor thresholds in water and in chicken broth.

In addition we reacted H_2S with acetaldehyde in chicken fat to explore the potential H_2S has to contribute to poultry flavor indirectly through its ability to react with carbonyl compounds.

MATERIALS & METHODS

Raw material and cooking methods

Chilled unfrozen raw ready-to-cook poultry was obtained from commercial sources, and if not cooked within 24 hr, was bagged in polyethylene, frozen in a blast freezer at −30°F and held at −10°F. Clear chicken broth was prepared by simmering cut-up fowl (1.8 parts by weight) and water (1 part by weight) for 3 hr. The hot broth was passed through two layers of cheese cloth into a separatory funnel from which the aqueous layer was withdrawn and filtered through a milk filter. This freshly prepared broth, if not used the same day, was sealed in cans, frozen and stored at −30°F. Chicken was deep fat fried in peanut oil at 284°F to an internal temperature of 185°F. Roasting was carried out in a rotary electric oven at 375°F to an internal temperature of 185°F.

Odor threshold of H_2S in water and in chicken broth

Water and broth samples being tested for odor were presented to judges at about 25°C in approximately half-full 16 oz Teflon® squeeze bottles cleaned and equipped with straight tubes for sniffing as described by Guadagni et al. (1963). A freshly prepared solution of H_2S, containing an analytically determined amount of H_2S, was added as needed to give the desired H_2S concentration in the test samples. The volume of H_2S solution added amounted to about 2% of broth volume, but, to keep broth components at the same concentration, the broth sample to which no H_2S was added was diluted with a compensating volume of water.

Broths differing in H_2S concentration were presented to the judges as a triangle test. To determine the approximate concentration of H_2S in water required to give detectable H_2S odor in the headspace, judges were given paired samples, one containing only water and the other containing H_2S. Just before the judge made his selection of the sample containing H_2S, he smelled a control sample containing a readily recognizable H_2S odor (20 to 80 ppb H_2S in water) to refresh his memory of the odor type he was being asked to detect.

Quantitative determination of H_2S

The quantity of hydrogen sulfide in chicken broth was determined by the previously described reflux-trap method (Mecchi et al., 1964). To determine H_2S in the meat, a weighed (about 20 g) portion of the cooked muscle taken from the carcass within 5 min after cooking, was immediately placed in 250 ml prechilled (0°C) triple distilled water and blended 30 sec in a closed system at full speed on an Omnimixer. The still cold (7°C) mixture was transferred promptly to the apparatus for H_2S analysis with water rinses and additional water as needed to suspend the meat in a total of 500 ml of water. H_2S in this mixture was determined by passing nitrogen through it for 1 hr at 25°C using the reflux-trap method (Mecchi et al., 1964). By promptly chilling and analyzing the cooked meat at room temperature, formation of additional H_2S due to heating was avoided. Hence, H_2S values obtained this way represent H_2S that was in the meat just after cooking.

Reaction of H_2S with acetaldehyde in chicken fat

Adipose tissue, removed from the partially thawed carcasses of five fowl, was minced while warming on a water bath to 40–50°C. Melted fat was decanted and the residue was extruded at room temperature from a stainless steel cylinder-and-piston-press through a plate having 1/16 in. holes. The extruded tissue was rewarmed (40–50°C) and the liquid fat in it was separated from solid tissue by suction filtration on a coarse sintered glass filter.

The filtrate, combined with the previously decanted fat, was washed thrice with warm (60°C) distilled water in a separatory funnel. After the final washing, the fat was roughly separated from the wash water, centrifuged, decanted from the small amount of water and sediment and suction filtered through a medium porosity sintered glass filter. A portion of this melted fat (30.76 g) was placed in a spherical 100 ml flask equipped with a heating mantle to maintain the fat in the molten state (30–40°C), with a tap-water-cooled condenser to reduce loss of acetaldehyde and with a glass inlet tube extending to the bottom of the flask. Acetaldehyde (1.4 g, freshly distilled) was added to and mixed with this fat to give a clear homogeneous solution. Water washed H_2S from a cylinder was then bubbled through the mixture for 4 hr and nitrogen for 1 hr to expel excess H_2S and acetaldehyde.

The product looked like the original fat but now had a marked sauerkraut-like odor and neither the odor of acetaldehyde or H_2S could be recognized in it. Total sulfur in the product and in the unreacted fat was determined by burning the fat, converting SO_2 formed to SO_4^{-2}, and determining the latter colorimetrically with barium chloronilate. Hydrogen sulfide in the fat was determined by the reflux-trap method of Mecchi et al. (1964).

RESULTS & DISCUSSION

TO DETERMINE the approximate

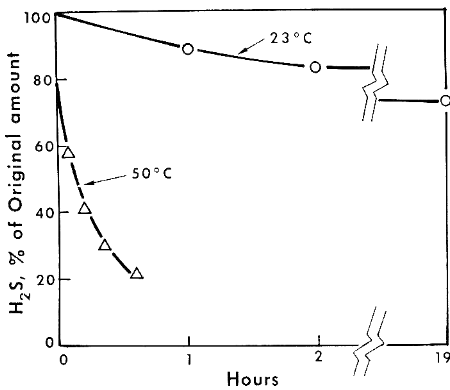

Fig. 1—Hydrogen sulfide retention in pure water at 23 and 50°C.

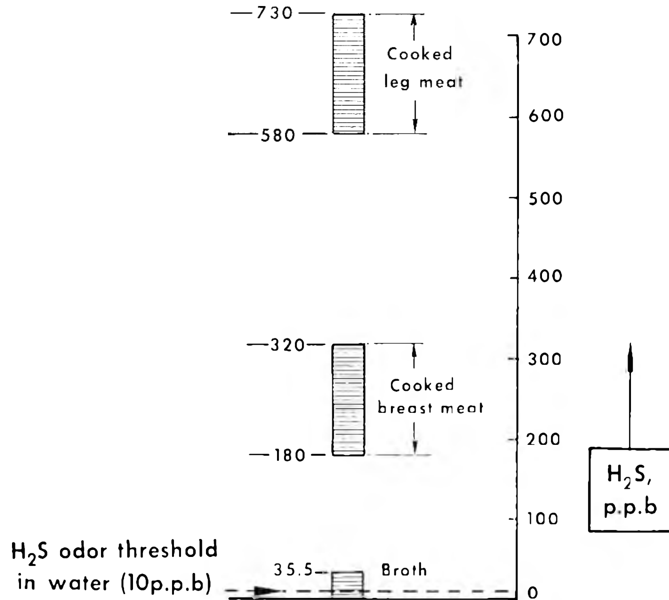

Fig. 2—Hydrogen sulfide range in chciken broth and in freshly cooked meat of simmered, roasted and fried chicken meat as compared to the H_2S odcr threshold in water.

threshold of H_2S in chicken broth and in water, reasonable retention and stability of H_2S in the odor evaluating system had to be assured. The 80% loss of H_2S at 50°C in only 35 min (Fig. 1) ruled out determination of the odor threshold at 50°C, a temperature at which broth or soup is typically consumed. But at 23°C, nearly 90% of the added H_2S was retained 1 hr after addition and over 70% remained even after 19 hr (Fig. 1). Therefore, we determined the H_2S odor thresholds at 23°C and within 1 hr of H_2S addition.

The 180 to 320 ppb H_2S found in cooked breast meat and the 580 to 730 ppb H_2S found in cooked leg meat (Table 1 and Fig. 2) exceeds the 10 ppb H_2S odor threshold in water (Table 2 and Fig. 2) by a factor ranging from 18 to 73. Thus H_2S certainly contributes to the flavor of freshly cooked chicken meat.

The increasing quantity of H_2S found as we progress from broth to breast meat to leg meat (Fig. 2) is consistent with previously reported results showing that the principal H_2S precursors are in the muscle protein and that the more alkaline leg muscle produces more H_2S than does the more acidic breast muscle (Mecchi et al., 1964).

Table 1—Quanity of H_2S in freshly cooked[1] ready-to-eat boiled, roasted and fried chicken meat.

Cooking method	Quantity of H_2S found[2]: Leg meat (ppb)	Breast meat (ppb)
Boiled (1 hr at 100°C)	730	320
Roasted (to 85°C internal temp.)	590	180
Fried (to 85°C internal temp.)	580	180

[1] Samples were taken for analysis within 5 min after completion of cooking.
[2] Values are averages of two determinations.

Table 2—Approximate H_2S concentration required in water at 25°C for odor panel detection of H_2S in the headspace.

H_2S in water (ppb)	Judgments (correct/total)	Correct judgments (%)
40	10/10	100
20	21/21	100
10	22/30	73[1]
5	19/29	65.5[2]
2½	12/19	63.2[2]
1¼	5/9	55.5[2]

[1] Significant at the 0.01% level.
[2] Not significant.

Table 3—Hydrogen sulfide in clear chicken broth.

Sample	H_2S (ppb)
Freshly prepared, analyzed same day at 65°C	35.5
Freshly prepared, frozen, thawed, simmered 5 min and analyzed at 65°C	8
Commercial canned broth, simmered 5 min and analyzed at 65°C	0.1–0.2

The 0.1 to 35.5 ppb H_2S range in chicken broth (Table 3) brackets the 10 ppb H_2S odor threshold (Fig. 2). Certainly the 3.5-fold excess of H_2S in freshly prepared broth (Table 3) over the 10 ppb H_2S odor threshold in water establishes the contribution of H_2S to the flavor of this broth. But, processing steps such as freezing, thawing and reheating can reduce the quantity of H_2S to subthreshold and insignificant levels (Tables 3 and 4).

This may be also true of canning (Table 3), but in this case we cannot be sure that this commercial broth was comparable to the laboratory prepared broth. Obviously, processing steps can cause substantial H_2S loss that is replaced only partly, if at all, by new H_2S formed in the reheating process. While there is plenty of H_2S in freshly cooked chicken meat and in freshly prepared broth to contribute directly to their flavor, there is certainly no guarantee that significant amounts of this volatile and reactive substance will stay very long in these freshly cooked products. This suggests that H_2S values might provide an objective method for determining recency of cooking or as

Table 4—Results showing that an odor difference created by adding 500 ppb H_2S to chicken broth at 24°C is eliminated by subsequently warming the broth to 55°C.

Samples	Correct/total judgments (triangle test): At 24°C (odor)	After warming to 55°C (odor)	After warming to 55°C (taste)
Broth vs. Broth + 500 ppb H_2S[1]	13/16 (P = 0.01)	7/22 (Not sig.)	6/16 (Not sig.)

[1] Two replications.

Table 5—Approximate H_2S concentration required in chicken broth at 25°C for odor panel detection of H_2S in the headspace. Compared were the odor of the broth with the odor of the broth containing added H_2S.

H_2S added to broth (ppb)	Odor panel results,[1] triangle test (correct/total judgments)
42	12/24 (not sig.)
54	15/30 (not sig.)
162	16/31 P = 0.05
486	26/31 P = 0.01

[1] There were 3 replications at the 42 ppb level and 4 replications at all other levels.

Table 6—Total sulfur and sulfide sulfur content of chicken fat before and after treatment with acetaldehyde and H_2S.

	Total sulfur	Sulfur as H_2S
	µg/g fat	µg/g fat
Fat (before treatment)	0.96	—
Fat (after treatment)	65.6	0.25 and 1.67[1]

[1] The 0.25 value was obtained by purging H_2S from the sample at 30°C with nitrogen for 1/2 hr; the 1.67 value was then obtained by continuing to purge the sample at 100°C for 1/2 hr.

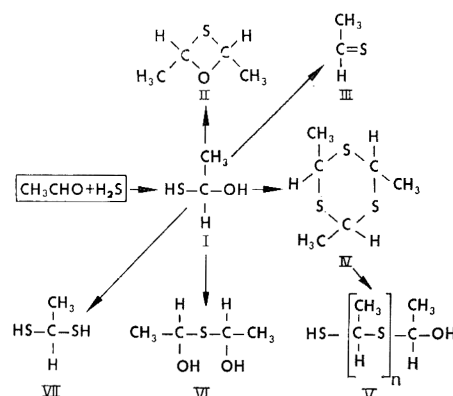

Fig. 3—*Outline of some reactions and products that could result from the reaction of H_2S with acetaldehyde as compiled from the data of Campaigne (1946) and Campaigne et al. (1962).*

an indicator of flavor freshness in cooked chicken.

In the aroma of cooked chicken, H_2S is blended with other odorants which were, of course, absent when the H_2S odor threshold was determined in water. Hence, the odor stimulus of any H_2S present in, or added to, this blend is superimposed upon the odor sensation of the entire blend. Furthermore, in accordance with the Weber-Fechner law (Appell, 1964), the odor intensity of H_2S should increase only arithmetically as its concentration increases geometrically. These considerations explain why the odor of H_2S does not stand out as a distinctly recognizable entity even though H_2S occurs in chicken broth and meat at 3.5 to 73 times the 10 ppb H_2S odor threshold in water. This also explains why the 162 ppb H_2S odor threshold in broth (Table 5) is about 16 times greater than the H_2S odor threshold in water.

Hydrogen sulfide may also contribute indirectly to cooked poultry flavor by forming secondary products. The possibility for forming such products by reactions between H_2S and carbonyls is particularly attractive because these ingredients occur in poultry (Pippen, 1967) and because it is well established that they will react with one another (Campaigne, 1946). Thus, *sym.*-trithiane, reported in cooked chicken by Minor et al. (1965), could form from a reaction between H_2S and formaldehyde (Campaigne, 1946).

The possibility for forming food type flavors by reacting H_2S with low molecular weight carbonyl compounds under mild conditions in triethyl citrate solvent has been pointed out in a patent by Barch (1952). Since triethyl citrate and fat are similar, and some of the carbonyls Barch (1952) used are identical to some of those in poultry (Pippen, 1967), it seems likely that reactions of this type might also take place in poultry fat during cooking. This possibility is also intriguing in view of the recently demonstrated build-up of non-H_2S sulfur in fat of cooked poultry (Pippen et al., 1969). Certainly the 10- to 26-fold greater concentration of carbonyl in the fat than in the water phase of simmered chicken favors fat as the most likely reaction site insofar as carbonyl concentration is concerned. Since H_2S is continuously produced during the cooking of chicken, this essential ingredient is available and present in the fat in finite amount during cooking.

To explore the possibility that H_2S will react with a carbonyl compound dissolved in chicken fat and thus become fixed in the fat in a modified chemical form, we passed H_2S into a 5% solution of acetaldehyde in chicken fat kept at 30–40°C and then purged it with nitrogen to free it of unreacted H_2S and acetaldehyde. The total sulfur in the fat after this treatment increased 68.3-fold over that in the fat before treatment (Table 6). Only 0.25 µg/g fat or 0.4% of this sulfur increase could be attributed to free H_2S as determined by quantitatively measuring H_2S purged from the treated fat at 30°C (Table 6). Therefore, more than 99% of the sulfur that existed in the fat after treatment with acetaldehyde and H_2S had been converted to some form other than H_2S.

Subsequent purging of the treated fat for 1/2 hr at 100°C released additional H_2S equivalent to only 1.67 µg S/g fat or only 2.6% of the sulfur increase. This confirms the essential non-H_2S character of the sulfur that existed in the fat after treatment and further shows that the new sulfur form is remarkably stable at 100°C insofar as its decomposition into H_2S is concerned.

The conversion of H_2S to a modified form of sulfur in the fat and the sauerkraut-like aroma of the fat after the treatment with acetaldehyde and H_2S suggests, but does not prove, that H_2S and acetaldehyde reacted in chicken fat much as they are claimed to react in several ester solvents (Barch, 1952). Assuming that a reaction between H_2S and acetaldehyde did take place in the fat, there are several products that might be formed (Fig. 3). If H_2S-carbonyl reactions can take place in poultry fat, as the preliminary work reported here suggests, it is apparent from the variety of carbonyl compounds that have been reported to occur in poultry (Pippen, 1967), from the seemingly complicated nature of H_2S-carbonyl interaction and from the reported intense food-like character of the odors produced (Barch, 1952), that H_2S-carbonyl interactions taking place during the cooking of poultry represent a considerable potential for the formation of flavor components. Further work is needed to characterize the products formed by these reactions and to determine whether reactions of this type take place in the cooking of poultry.

CONCLUSIONS

HYDROGEN SULFIDE occurs in broth of freshly simmered chicken and in the freshly cooked ready-to-eat meat of simmered, roasted and fried chicken at 3.5 to 73 times the H_2S odor threshold in water and hence contributes directly to the flavor of these products. But, the quantity of H_2S in freshly prepared chicken broth can be reduced to subthreshold levels by freezing, thawing and reheating. Thus, while H_2S certainly contributes directly to the "freshly-cooked" flavor, its transient nature will cause this contribution to diminish as its quantity drops in the cooked product.

Chicken fat, after being treated with acetaldehyde and H_2S and then purged essentially free of these reagents, exhibits both a new aroma and an acquired and fixed sulfur content. These results can be explained by assuming that a reaction between H_2S and acetaldehyde took place in the fat. This leaves open the possibility for H_2S to contribute indirectly to cooked poultry aroma through the formation of secondary products.

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Ms. received 7/16/68; revised 10/16/68; accepted 9/5/69.

Reference to a company or product name does not imply approval or recommendation of the product by the U.S. Department of Agriculture to the exclusion of others that may be suitable.

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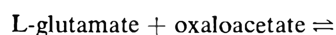
Transaminases of Skeletal Muscle. 1. The Activity of Transaminases in Post-Mortem Bovine and Porcine Muscles

SUMMARY—The activity of glutamic-oxaloacetic transaminase (GOT) and glutamic-pyruvic transaminase (GPT) of bovine and porcine muscle tissue and muscle press juice was determined. The total GPT activity of muscle tissue is about one tenth of the GOT activity. There are no remarkable differences in the activities of GOT and GPT between these slaughter animals and other species (rat, rabbit and man). The GOT activity of the longissimus dorsi muscle of pigs is significantly higher than that of the same bovine muscle. The mitochondrial (GOT_M) and sarcoplasmic isozymes (GOT_S) of GOT in skeletal muscles of cattle and pigs were determined after electrophoretic separation. The ratio GOT_M:GOT_S in skeletal muscle was found to be about 1:1. There is only a small decrease in GOT activty during storage of muscle tissue at 0 or +4°C for several weeks post-mortem. The small activity of GOT_M in the muscle press juice does not substantially change during storage of muscle tissue under these conditions, indicating that there is no drastic change of the mitochondrial structure during aging of meat. Bacterial spoilage of meat, however, results in the release of GOT_M from the mitochondria.

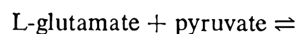
INTRODUCTION

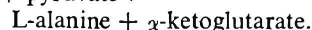
THE MUSCLES of all species of animals contain enzymes which catalyze biological transamination. High concentrations of transaminases have been found in cardiac muscle and liver and lower amounts in skeletal muscles (Tautschold et al. 1966). The transaminases found in muscles are mainly glutamic-oxaloacetic transaminase (GOT; aspartate aminotransferase; E.C. 2.61.1) and glutamic-pyruvic transaminase (GPT; alanine aminotransferase; E.C. 2.6.1.2). GOT catalyzes the reaction:



$$\text{L-glutamate} + \text{oxaloacetate} \rightleftharpoons \text{L aspartate} + \alpha\text{-ketoglutarate},$$

whereas GPT catalyzes the reaction:





$$\text{L-glutamate} + \text{pyruvate} \rightleftharpoons \text{L-alanine} + \alpha\text{-ketoglutarate}.$$

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It is known that stress, muscle dystrophy and other pathological conditions cause a transition of transaminases from the muscle tissue into the blood, increasing the blood transaminases and decreasing the level of muscle transaminases (Laudahn et al. 1968). This fact is used for the diagnosis of certain muscle diseases.

It is possible that the skeletal muscle of animals that have undergone heavy stress before slaughtering or have suffered from certain diseases, might show an abnormally low transaminase activity; hence, the transaminase activity of the post-mortem muscle would be an indicator of ante mortem stress or certain pathological conditions. It would be useful for meat quality studies to have a test that shows whether the muscle is from a normal or from a drastically stressed or pathological animal. Furthermore, this study seems to be of special interest for determining the conditions that cause pale, soft, watery pork.

Gantner et al. (1964) and Körmendy et al. (1965) developed suitable methods for the determination of transaminases in muscle tissue and for studying the properties of transaminases in bovine and porcine muscles. They found that freezing and thawing increase the extractibility of GOT; this is probably due to a damage of muscle mitochondria which results in a release of mitochondrial GOT. At least two isozymes of the GOT are known. One is bound to the mitochondria (GOT_M), the other is localized in the sarcoplasm (GOT_S). The proteins of these isozymes are chemically distinct, differing in molecular weight, chemical structure, isoelectric point, optical absorption, K_m for pyridoxal phosphate, ketoglutarate and aspartate, inhibition by oxaloacetate, heat stability and immunochemical properties (Wada et al., 1964; Trautschold et al., 1966; Martinez-Carrion et al., 1967). Recently Zezelinskaya (1967) suggested that in rat heart and liver, GOT_M is localized mainly in the mitochondrial matrix and GPT in the mitochondrial membrane.

Isozymes of GOT of skeletal muscles have been found in rats (Boyd, 1962; Yamada et al., 1962; Decker et al., 1963; Yoshimata et al., 1964; Boyd, 1966), rabbits (Czok et al., 1960; Pette et al., 1963) and men (Laudahn et al., 1963; Kar et al., 1964; Laudahn et al., 1968) and were separated by starch gel electrophoresis. Körmendy et al. (1965) found two isozymes of GOT in skeletal muscles of cows and pigs by high-voltage electrophoresis on cellulose acetate membranes.

They showed that one of these isozymes is localized in the mitochondria (GOT_M), the other in the sarcoplasm (GOT_S). No evidence for the existence of isozymes of GPT could be demonstrated electrophoretically. However, GPT activity was found in the muscle mitochondria as well as in the sarcoplasm.

The temperature optimum of GOT as well as of GPT lies around 50°C. However, it is not advantageous to measure the transaminase activity at 50° because at this temperature the muscle extract starts to become turbid; activity measurements at 40° are preferred because the optimum temperature is almost reached without heat coagulation of proteins (Gantner et al., 1964).

The earlier work did not present enough data on the activity of total GOT, of GOT isozymes, and of GOT in the skeletal muscle of pigs and cattle; nor enough data on transaminase activity in the muscle press juice. Nothing has been known about the post-mortem stability of transaminases in bovine and porcine muscle. This paper presents information on these topics.

EXPERIMENTAL

Muscle samples

Samples of the M. longissimus dorsi, M. psoas and M. semitendineus of pigs and of the M. longissimus dorsi and M. semitendineus of 5-year old cows were taken immediately after slaughter. Fat and connective tissue were mechanically removed as best as possible.

Extraction of the total transaminase activities

An extraction method already published was used (Gantner et al., 1964; Körmendy et al., 1965). After a one minute homogenization of the muscle tissue in 0.1 M phosphate buffer (pH 7.4) with a Bühler-homogenizer (blendor principle), at least 97% of the total GOT and GPT activity of the tissue was found in the supernatant (centrifugation at 50,000 × G).

Extracts of five parts from the same muscle sample showed an 8% standard deviation from the average value for the GOT activity and 10% for the GPT activity. The supernatant kept at 2°C for 24 hr decreased the GOT and GPT activity slightly (0–15%). Therefore, the extracts should be kept in the frozen state. Freeze-storage for 7 days resulted in a slight activity loss (2–9%).

If the homogenate was centrifuged at 3000 rpm (room temperature) the GOT activity in the supernatant was almost the same as in the supernatant after centrifugation at 50,-000 × G (0°C), indicating that no GOT activity remains bound to particles sedimentating at high speed.

Muscle press juice

Press juice was obtained by pressing samples of unground muscle tissue with a hydraulic press (maximum pressure 10 kg/cm^2). The juice was filtered through a membrane filter (diameter of pores: 600 nm) and centrifuged at 50,000 × G (0°C) for 30 min. The supernatant ("sarcoplasm") may still contain microsomes and other "small grana" but no intact mitochondria. The non-centrifuged muscle press juice contained only very small amounts of particle-bound GOT (i.e., small amounts of mitochondria, because centrifugation at 50,000 × G caused an activity-loss of only about 5%. There was no significant change in GOT activity of centrifuged press juice stored at 2°C for 24 hr.

Storage of frozen muscle press juice for three weeks did not significantly change the GOT activity. A remarkable increase in the GOT_M activity of muscle press juice was obtained by grinding muscle in a commercial meat grinder. The GOT_M activity in the press juice of beef muscle (Longissimus dorsi) e.g., was 3.8% of the total activity before grinding and 12% after. The assumption of Pette (1966), that mincing the tissue in a "latapie mill" would not release the enzymes bound to the mitochondria, might therefore be incorrect for GOT.

Determination of transaminase activities

The transaminases were determined with the coupled enzymic test using methods already described (Bergmeyer, 1963; Körmendy et al., 1965). All reagents needed are commercially available in sets. (GOT and GPT "Test Combination," C. F. Boehringer u. Söhne G.m.b.H. Mannheim, Germany).

These methods were thoroughly checked for interfering factors (Gantner et al., 1964; Körmendy et al., 1965). The standard deviation of transaminase activity in the same muscle extract amounts to 4% of the average value for the GOT activity and 1.2% for the GPT activity. In this paper, the transaminase activities are expressed in Bücher-units per gram of fresh tissue (BU/g).

Separation of GOT isozymes by electrophoresis

The relative proportion of the two GOT isozymes (GOT_M and GOT_S) in muscle extracts or press juice was measured after separation by electrophoresis on cellulose acetate membranes using the method of Körmendy et al. (1965). The bands of isozymes became visible under the ultraviolet lamp after spraying the membrane very lightly with the same mixture of reagents that was used for the determination of GOT activity. The entire membrane containing the isozyme was cut out and extracted with phosphate buffer (pH 7.6); the GOT activity was determined in the extract. A recovery of 95 to 100% of the total activity placed on the membrane before electrophoresis was achieved with this procedure.

RESULTS AND DISCUSSION

Total GOT and GPT activity in bovine and porcine muscle

In the longissimus dorsi muscle of cows (10 animals), the total GOT activity varied from 1860 to 2450 BU/g (average: 2004; s = 301). The longissimus dorsi muscle of pigs (10 animals) varied from 930 to 2797 BU/g (average: 1536; s = 618). Thus, the GOT activity of porcine muscle was significantly lower ($P < 0.05$) than that of bovine muscle. The GPT activity in the longissimus dorsi muscles of pigs and cows varied from 55 to 140 BU/g; this is less than one tenth of the GOT activity. There are not yet enough data available for finding significant GPT differences between the two species.

The GOT and GPT activities in the psoas major and semitendineus muscle of pigs and cattle are of the same magnitude as in the M. longissimus dorsi. In the psoas, however, higher activities (up to 4000 BU/g for GOT and 430 BU/g for GPT) were found. A higher variation in the activity of both transaminases was observed in other kinds of muscle depending on its function. This interesting observation will be discussed in the second paper of this series. There are no remarkable differences in the transaminase activities of skeletal muscle between these slaughter animals and other species (rat, rabbit and man).

The GOT activity in the muscle press juice varied from 1000 to 4000 BU/ml, depending on the type of muscle and unknown factors. Unavoidable variation in the pressure applied in the preparation of press juice may influence the absolute values of GOT activity. As Czok et al. (1960) pointed out, the protein concentration in the muscle press juice is lower than in the sarcoplasm and therefore, the press juice is probably more or less diluted with water from areas of the muscle tissue other than the sarcoplasm. It is, of course, not valid to relate the absolute value of the GOT in the press juice to the total GOT activity in the muscle (activity in the muscle extract after homogenization). The relative activities of the GOT isozymes, however, present valuable information as shown below.

GOT isozymes

The relative proportion of the GOT isozymes in different muscles of pigs and cows was determined after electrophoretical separation. The ratio GOT_M:GOT_S in psoas, longissimus dorsi and semitendineus muscles (beef and pork) was found to be about 1:1. The same ratio was observed by Fleisher et al. (1960) in liver and cardiac muscle and by Pette et al. (1963) in skeletal muscle of rabbits. In the special case of the longissimus dorsi muscle we found in 11 pigs an average of 51.8 (s = 5.48) % GOT_M and in the longissimus dorsi muscle of 10 cows 59.7 (s = 12.1) % GOT_M. The difference between the species is not significant.

It should be mentioned that some researchers detected, by chromatographical or electrophoretical methods more than two GOT isozymes in skeletal muscle of

Table 1—Influence of post-mortem storage of bovine muscle (M. longissimus dorsi) at +4°C on the GOT activity in muscle extract and muscle press juice.

Days post mortem	GOT activity in the muscle extract BU/g	GOT_M activity in muscle press juice Percent of the total GOT activity in the press juice
0	2387	1.8
1	2535	2.8
2	2204	2.6
3	2028	3.8
4	1973	1.8
7	2017	2.5

Table 2—Influence of post-mortem storage of porcine muscle (M. longissimus dorsi) at 0°C on the GOT_M activity in the muscle press juice.

Days post-mortem	0	1	2	7	8	13
GOT_M activity in percent of the total GOT activity in muscle press juice	3.9	4.9	5.5	4.1	5.3	4.7

Table 3—Influence of post mortem storage of porcine muscle (Psoas) at different temperatures on the GOT_M activity in the muscle press juice.

Days post mortem	GOT_M activity in percent of the total GOT activity in press juice	
	storage at +4°C	storage at +22°C
1	6.0	6.9
2	6.6	14.9[1]
3	6.2	36.5[1]

[1] Strong bacterial spoilage.

Table 4—Influence of post-mortem storage of bovine muscle (M. longissimus dorsi) at different temperatures on the GOT_M activity in the muscle press juice.

Days post mortem	GOT_M activity in percent of the total GOT activity in the muscle press juice	
	storage at 0°C	storage at −22°C
0	1.2	1.2
1	—	7.1
2	1.2	32.0[1]
3	—	40.0[1]
7	1.4	—
11	2.1	—
14	3.9	—
21	3.6	—

[1] Strong bacterial spoilage.

rats (Decker et al., 1963) and pig heart (Cordoba et al., 1963; Martinez-Carrion et al., 1967). However, we could find only two isozymes in skeletal muscles by the method used.

Post-mortem changes in the GOT activity

As Table 1 shows, there is a relatively small decrease in the total GOT activity during storage of bovine muscle at 4°C for 7 days. The same small decrease was observed with porcine muscle.

Most of the GOT activity of juice squeezed from muscle immediately after slaughter is due to the presence of GOT_S. There exists only a very small GOT_M activity, which is probably caused by a release of the mitochondrial isozyme at the cut-surface of the muscle cubes. The GOT_M activity in the press juice of porcine muscle is usually a little higher than in the press juice of bovine muscle. There is no remarkable increase in GOT_M activity in the press juice during post-mortem storage of porcine muscle (Tables 2 and 3) and bovine muscle (Tables 1 and 4), at 0°C or 4°C. "Chilled beef," stored at −2°C for 4 weeks, showed a GOT_M activity in the press juice of only 1.5% of the total GOT activity (2800 BU/ml), i.e., about the same activity as in the press juice prepared immediately after slaughter.

The results suggest that other than freezing and thawing, the aging of meat does not cause drastic damage of the mitochondrial structure causing a release of GOT_M. On the other hand, structural changes and some disintegration of mitochondria during storage of muscle were observed (Partmann, 1964), which might be due to the effect of lysosomal enzymes. The portion of the mitochondrial structure, to which the GOT_M is bound, however, seems to remain intact. Therefore, the opinion of Zezelinskaya (1967), that GOT_M is localized mainly in the mitochondrial matrix might be unlikely.

The relatively high post-mortem stability of GOT in nonfrozen beef and porcine muscle at temperature between −2° and +4°C is in agreement with data from Schmidt et al. (1960) and Laudahn et al. (1965) on human muscle.

However, after storage of muscle tissue at +22°C for more than one day, remarkable quantities of the mitochondrial isozyme are detectable in the press juice of porcine (Table 3) and bovine muscle (Table 4). At this temperature the meat was completely spoiled and unpalatable. Apparently, the mitochondrial structure containing bound GOT_M is damaged by the enzymes of microorganisms and muscle.

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Ms. received 6/10/68; revised 10/4/68; accepted 9/5/69

The authors wish to thank Mrs. L. Tetzlaff for her excellent technical assistance and Dr. H. Burmeister, Peoria, Ill., for his advice.

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Transaminases of Skeletal Muscle. 2. Transaminase Activities in White and Red Muscles of Pigs and Cows

SUMMARY—The total activity of glutamic-oxaloacetic transaminase (GOT), the relative activity of the mitochondrial GOT isozyme (GOT_M) and the total activity of glutamic-pyruvic transaminase (GPT) in five different muscles of four pigs and two cows were determined. A highly significant, positive correlation was found between either GOT or GPT activity and the amount of muscle pigments. Tonic ("red") muscles had high transaminase activities and tetanic ("white") muscles had low transaminase activities. However, the relative GOT_M activity (GOT_M activity in percent of the total GOT activity) of porcine muscle was found to be almost constant in the different types of muscle. Likewise, in bovine muscles, higher GOT and GPT activities were found in "red" than in "white" muscles. However, the relative GOT_M activity seemed to be higher in the "red" than in the "white" muscles. A discussion of the transaminase activities and their relationship to muscle function is presented.

INTRODUCTION

GLUTAMIC-OXALOACETIC transaminase (GOT; aspartate aminotransferase; E.C. 2.6.1.1.) is an enzyme that is widely distributed in animal tissues. This key enzyme of the mitochondrial amino acid metabolism is of considerable significance, due to its role in glutamate oxidation. In addition, the elevation of GOT in serum and its decrease in muscle tissue in certain diseases makes the study of this enzyme of particular importance. The concentration of glutamic-pyruvic transaminase (GPT; alanine aminotransferase; E.C. 2.6.1.2.) in animal tissues is considerably less than that of GOT (Hamm et al., 1969).

A study of transaminases in bovine and porcine muscles and their relation to certain pathological conditions and meat quality was recently initiated. Two isozymes of GOT were found by electrophoretical methods in skeletal muscles of both species. One of these isozymes is localized in the mitochondria (GOT_M), the other in the sarcoplasm (GOT_S) (Hamm et al., 1969). Therefore, the GOT of bovine and porcine muscle as well as the GOT of other animal tissues belong to the "type III" enzymes, i.e. to enzymes which are localized in both the sarcoplasm and the mitochondria. The GPT in bovine and porcine muscle presumably belongs to the "type III" enzymes but it was not possible to separate the GPT isozymes by electrophoretical methods (Körmendy et al., 1965).

In Körmendy's study, a remarkable variation in the GOT and GPT activities of different muscles of pigs and cattle was observed. This variation seemed to be related to muscle color. The slow acting "red" muscles (tonic muscles) exert holding functions whereas the fast acting "white" muscles (tetanic muscles) exert discontinuous functions. Therefore, it was assumed that the transaminase activity of skeletal muscle tissue depends on the physiological function of muscle. This relationship was investigated by measuring the activities of GOT, its isozymes and GPT in muscles with different pigment content.

EXPERIMENTAL

THE TOTAL GOT activity from the M. longissimus dorsi, pectoralis profundus, psoas major, extensor carpi radialis and diaphragma muscle taken from 4 pigs and 2 cows immediately after slaughter was determined. The activities of the mitochondrial (GOT_M) and sarcoplasmic isozyme (GOT_S) were measured after separation by electrophoresis.

The total GOT activity in muscle and the absolute and relative activities of the isozymes GOT_M and GOT_S separated by low-voltage electrophoresis of muscle extracts on cellulose acetate membrane were measured by methods described in earlier papers (Gantner et al., 1964; Körmendy et al., 1965; Hamm et al., 1969).

In this paper, the transaminase activity is expressed in Bücher units per gram of fresh tissue (BU/g). The relative GOT_M activity is expressed as a percent of the total GOT activity in the electrophoretic diagram.

The muscle pigments were determined as the total hemin content of tissue according to the method used by Hornsey (1956). These data include not only myoglobin but also hemoglobin, cytochromes, catalase and other heme compounds present in muscle tissue. Most of the hemin, however, originates from myoglobin.

RESULTS

IN THE longissimus dorsi, pectoralis profundus, psoas major, extensor carpi radialis and diaphragma muscles from four pigs and two cows, the total GOT activity and the activity of the mitochondrial (GOT_M) and sarcoplasmic isozyme (GOT_S) were determined after separation by electrophoresis.

The total GOT activity of porcine muscles varied from 1006 to 7371 Bücher-units per gram fresh tissue (BU/g). The mean value of all muscles from the four pigs was 3512 (s = 1638) BU/g. A highly significant correlation (r = +0.94***) between the total GOT activity and the hemin content of muscles was found (Fig. 1). The hemin content ranged from 5.6 to 27.0 mg hemin/100 g tissue (average 13.76; s = 6.16) depending on the muscle function. Variations in water and protein content were rather small.

Activity of GOT_M in porcine muscles varied from 636 to 3736 BU/g. A highly significant correlation (r = +0.92***) existed between GOT_M activity and the pigment content of muscle tissue. The higher the total GOT activity the higher is the GOT_M activity. However, the ratio, GOT_M:GOT_S and the GOT_M activity in percent of the total GOT activity (relative GOT_M activity) are remarkably constant. The mean value of the relative GOT_M activity of all the pig muscles was 53.2% (s = 8.2). Therefore, no significant correlation exists between the relative GOT_M activity and the pigment content of muscles (r = +0.20) or between the relative GOT_M activity and the total activity of tissue (r = +0.20).

The GPT activity of the porcine muscles varied from 55 to 521 BU/g. The fact that GPT activity of the skeletal muscles is about ten times lower than the GOT activity agrees with our earlier findings (Hamm et al., 1969). A highly significant correlation (r = 0.89***) between the GPT activity and the pigment contents of muscles was observed (Fig. 2). The GPT activity increases with rising GOT activity (r = 0.89***) (Fig. 3).

The muscles with the lowest pigment content, namely the longissimus dorsi muscles ("white" fibers predominant), showed the lowest GOT and GPT activities. On the other hand, dark-red diaphragma muscles (mainly "red" fibers)

had by far the highest transaminase activities. The transaminase activities and pigment contents of M. extensor carpi radialis and M. psoas range between these two "extreme" muscle types. The pectoralis major muscle consists of a "red" part and a "white" part. An investigation of the red and the white portion was made and the results are plotted in Figures 1, 2 and 3. For example, the light part of the M. pectoralis major (5.4 mg hemin/100 g) had a GOT activity of 1585 BU/g and GPT activity of 114 BU/g, whereas the dark part of the same muscle (11.4 mg hemin/100 g) had a GOT activity of 3796 BU/g and a GPT activity of 261 BU/g.

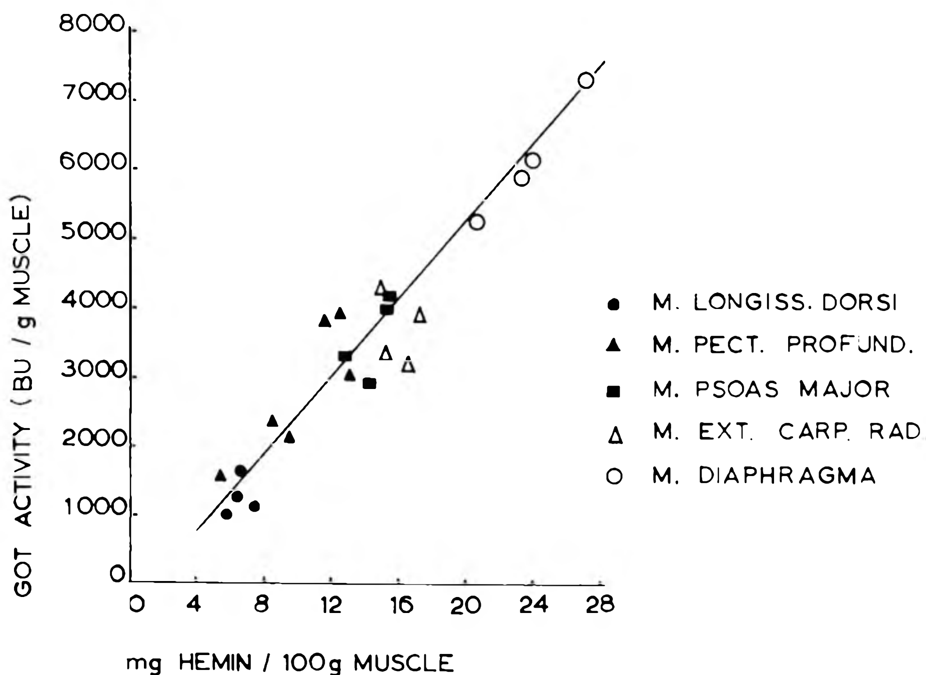

Fig. 1—Correlation between the total GOT activity and the muscle pigments of five different porcine muscles (four animals).

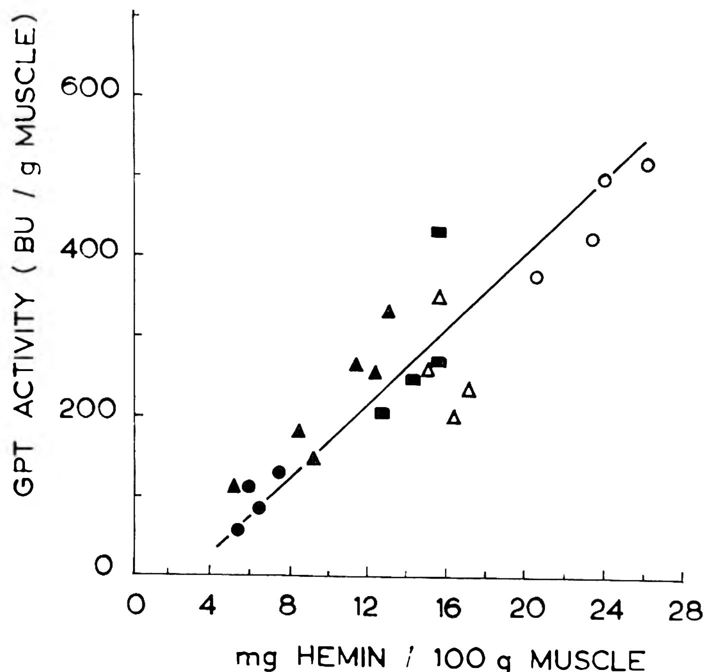

Fig. 2—Correlation between the GPT activity and the muscle pigments of five different porcine muscles (four animals). The signs are explained in Figure 1.

The correlation between transaminase activities and heme content of porcine muscles for the four examined animals were similar: the activity values lay close to the regression line (Fig. 1 and 2).

The GOT and the GPT activity of bovine muscles was significantly greater in highly pigmented muscle tissue. (Fig. 4). The pigment content of all bovine muscles was much higher than that of the corresponding porcine muscles. The GOT and GPT activity, however, was of about the same magnitude as in porcine muscle (Fig. 4). Diaphragm muscles had the highest values of transaminase activity and pigment content and the longissimus dorsi and pectoralis profundus muscles the lowest values. The relative differences in transaminase activities and pigmentation of these bovine muscles were similar to porcine muscles. The other three muscle types had intermediate values. A two-fold increase in pigment content of porcine muscles corresponded to a seven fold increase in total GOT, GOT_M and GPT activities. In the case of bovine muscles, however, the same increase in pigment content resulted in a much lower rise of transaminase activity (two to four times) (Fig. 4).

The relative GOT_M activity of the five different bovine muscles examined are shown in Table 1. These values were higher for animal II than for animal I. A great variation in the relative GOT_M activity of bovine muscles was already observed (Hamm et al., 1969). There appears to be a relative increase of GOT_M activity with rising pigment content of muscles. This relationship between GOT_M and pigment content could not be observed with porcine muscles as previously mentioned. In the longissimus dorsi muscle of animal II relatively high values of muscle pigment and GOT_M activity were obtained.

It could be possible that the transaminases of red and white muscle, particularly the GOT, might not be identical.

Table 1—Relative GOT_M activity and muscle pigments in different bovine muscles of two animals.

Kind of muscle	GOT_M activity in percent of total GOT activity		mg hemin/100 g tissue	
	animal I	animal II	animal I	animal II
longissimus dorsi	58.5	80.5	18.3	30.6
pect. profund.	54.9	77.0	19.2	23.5
psoas	55.3	65.2	20.9	28.5
ext. carp. rad.	64.3	72.4	25.9	33.3
diaphragma	74.7	83.1	33.0	47.6

With regard to this possibility, the patterns of the electrophoresis of extracts from red and white porcine muscle and of a mixture of both extracts were compared. The pattern of the mixture showed only two bands (GOT_M and GOT_S) just as the single extracts; the rate of migration of the two isozymes was exactly the same in all three cases. Therefore, the GOT enzymes in the red and white muscle seem to be identical or almost identical proteins.

DISCUSSION

THE DATA show that porcine and bovine muscles with a high content of "red" fibers (high hemin content) exert a higher transaminase activity than muscles with a high content of "white" muscle fibers (low hemin content). Lawrie (1953) found a significant correlation between myoglobin content of skeletal muscles and the amount of succinic dehydrogenase or cytochrome oxidase. These enzymes are specific for the function of mitochondria. Hence, high GOT and GPT activity is characteristic for skeletal muscles with a high number of mitochondria and a predominantly aerobic metabolism; whereas, muscles with a relatively small number of mitochondria and a predominantly anaerobic metabolism exert a low transaminase activity.

These observations on skeletal muscles of slaughter animals agree well with findings of other authors on the skeletal muscle of other species. A higher GOT activity was found in the M. semitendineus and M. soleus ("red") than in the M. semimembraneus and M. adductor magnus of rabbits ("white") (Pette et al., 1963; Amberson et al., 1964; Bücher et al., 1965; Pette, 1965). The flexor muscles of dogs and rabbits had lower GOT and GPT activity than the extensor muscles (Dorogan et al., 1961). Kleine (1967) found higher GOT and GPT activities in thorax and abdominal muscles ("red" muscles) than in the M. deltoides bracchialis and M. semimembraneus ("white" muscles). According to Laudahn et al., (1968), the average of GOT and GPT activities is significantly higher in human trunk muscles than in the extremities muscles. The level of GOT activity is higher in the flight muscle of Locusta migratoria than in the flexor tibis muscle of this insect (Pette et al., 1962).

GOT is a typical enzyme of the normal mitochondrial function, whereas e.g. glutamic dehydrogenase represents an enzyme for special mitochondrial functions (Pette, 1965). The metabolic function of GOT is essentially related to the main pathway of energy supply; its position in the mitochondrial enzyme-activity patterns as they were presented by Pette (1965), suggests its role as an auxiliary enzyme of the citric acid cycle.

GPT is regarded to be an enzyme related to mitochondrial functions in muscle (Pette et al., 1963) and to the glycolytic enzymes. This relationship is the same in red and white muscles (Czok et al., 1960).

In the case of cross-striated muscles, differentiation of structure and function is reflected largely by enzyme-activity patterns (Pette, 1966). Differences between transaminase activities of "red" and "white" porcine muscles are not due to a significant change in the mitochondrial and extramitochondrial enzymes ratio. The GOT_M activity increase to the same

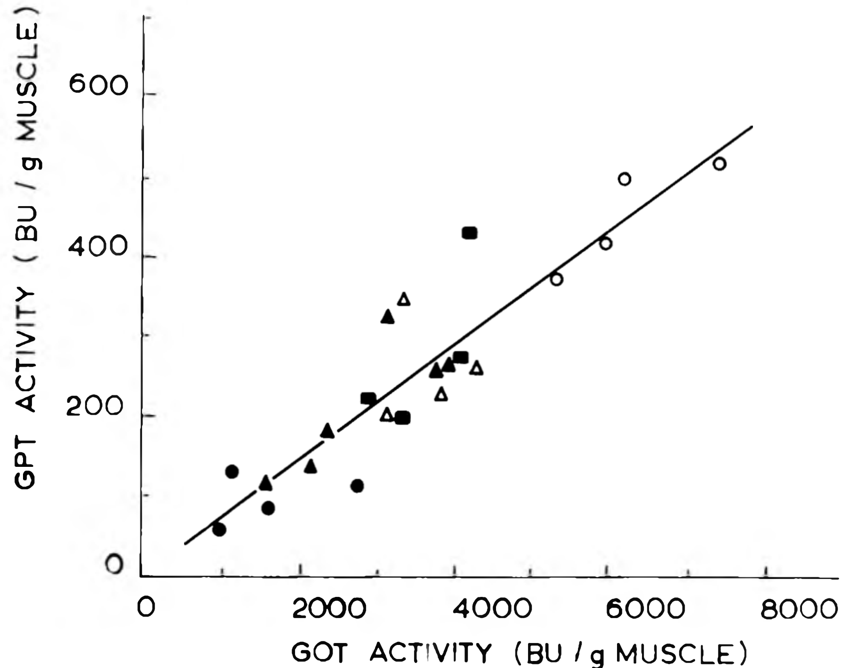

Fig. 3—Correlation between the total GOT activity and the GPT activity of five different porcine muscles (four animals). The signs are explained in Figure 1.

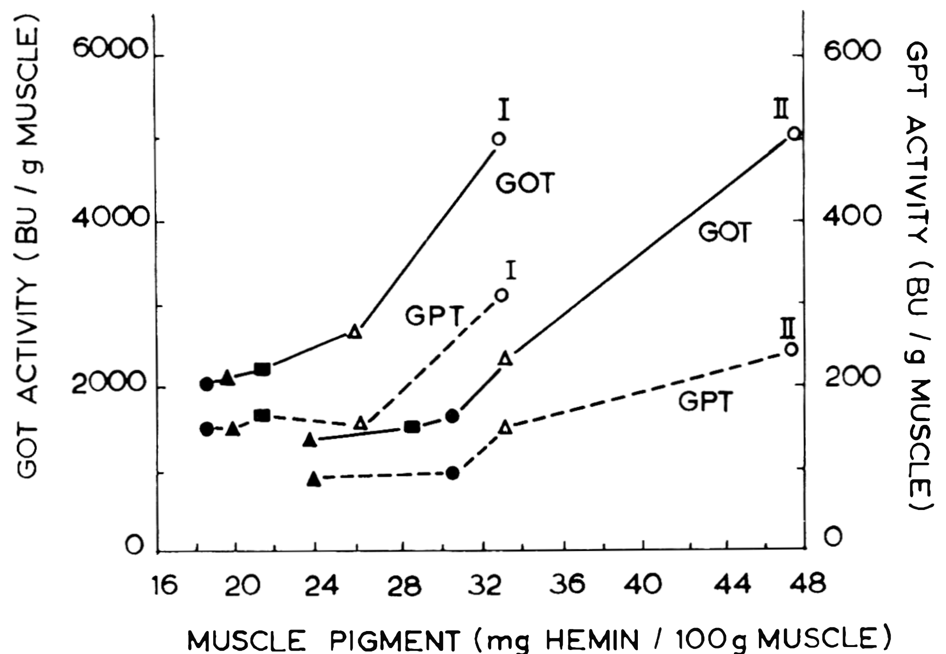

Fig. 4—Correlation between the transaminase activities and the muscle pigments of five different bovine muscles (two animals). The signs are explained in Figure 1.

extent as the total GOT activity. Therefore, the ratio $GOT_M:GOT_S$ is 1:1 independent of the function of porcine muscle. A similar observation was made on rabbit muscles by Pette et al. (1963). These authors (Pette et al., 1963; Bücher et al., 1965) point out, that certain "multi-located" enzymes as GOT and GPT catalyze the same reaction in sarcoplasm and mitochondria. The enzymes have a close functional relationship and are localized on both sides of the mitochondrial barrier.

The ratio $GOT_M:GOT_S$ in "white" and "red" bovine muscles seems to differ from each other. This ratio is higher in "red" muscles than in "white" muscles (Table 1). Although there are not yet enough data available to draw a final conclusion, it is apparent that cattle have a higher aerobic activity in their "red" muscles than the less active pigs. This could be the reason for other observed differences between cattle and pigs e.g. the stronger increase of GOT activity in porcine muscle with increasing pigment content. In order to obtain more information on differences in muscle metabolism between cattle and pigs, the proportion-constant and specific enzyme groups of the glycolytic chain, the fat catabolism, the citric acid cycle and the respiration chain have to be determined.

The results presented in this paper lead to the conclusion that the same kind of muscle must be analyzed for comparisons of transaminase activity in individual animals and animal species.

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Ms. received 6/10/68; revised 10/4/68; accepted 9/5/69.

The author wishes to thank Mrs. L. Tetzlaff for her excellent technical assistance and Dr. H. Burmeister, Peoria, Ill. for his advice. Also Dr. W. Woltersdorf for providing the different types of muscles.

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Transaminases of Skeletal Muscle. 3. Influence of Freezing and Thawing on the Subcellular Distribution of Glutamic-Oxaloacetic Transaminase in Bovine and Porcine Muscle

SUMMARY—Freezing and thawing of bovine and porcine muscle cause a remarkable release of the mitochondrial isozyme of the glutamic-oxaloacetic transaminase (GOT_M) from the mitochondrial structures resulting in an increase of GOT_M activity in the muscle press juice. The lower the freezing temperature the stronger is this effect. Repeated freezing and thawing increases the release of GOT_M. It is considered that the level of GOT_M activity in the muscle press juice indicates the extent of mitochondrial damage. On the basis of these results a simple and rapid routine method was developed which allows a reliable differentiation between nonfrozen and frozen and thawed meat.

INTRODUCTION

GANTNER et al. (1964) observed that freezing and thawing of skeletal muscle tissue of pigs increase the activity of glutamic-oxaloacetic transaminase (GOT; aspartate aminotransferase; E.C. 2.6.1.1) and glutamic-pyruvic transaminase (GPT; alanine aminotransferase; E.C. 2.6.1.2) in extracts from prerigor and postrigor muscle when using the Potter-Elvehjem homogenizer. It is well known that mitochondrial enzymes are more easily extractable after repeated freezing and thawing of tissues. The existence of a mitochondrial isozyme (GOT_M) and of a sarcoplasmic isozyme (GOT_S) of GOT in skeletal muscles of pigs and cattle has been demonstrated (Körmendy et al., 1965; Hamm et al., 1969). Therefore, it was expected that freezing and thawing of skeletal muscle tissue results in a release of the mitochondrial isozyme into the intermitochondrial space.

A study of the influence of freezing and thawing on the subcellular distribution of the GOT activity seems to be of interest for two reasons: The extent of the release of GOT_M from the mitochondrial structure by freezing might present some information on the degree of damage of mitochondria by the freezing process. Furthermore, the appearance of certain

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mitochondrial enzymes in the muscle press juice as a result of freezing and thawing could lead to a method for differentiation between nonfrozen meat and frozen and thawed meat.

EXPERIMENTAL

Muscle samples

Samples of skeletal muscles of pigs and cattle were taken at different times after slaughter. Usually the longissimus dorsi muscle was excised. Fat and connective tissue were mechanically removed as best as possible, 200 to 250 g of the muscle tissue were wrapped in cellophane foil and frozen at temperatures between −18° and −20°C. After freeze-storage at −20°C for different periods of time, the samples were thawed at room temperature.

Methods for measuring the distribution of the GOT isozymes

The following methods were discussed in earlier papers (Gantner et al., 1964; Körmendy et al., 1965; Hamm et al., 1969): Extraction of the total GOT activity from the muscle tissue; preparation of the muscle press juice; determination of the transaminase activities; separation of the GOT isozymes by electrophoresis on cellulose acetate membranes; isolation of muscle mitochondria; extraction of transaminases from the mitochondria.

All data given in Tables and Figures are mean values of double-determinations, the standard deviation being 4% of the average values.

In this paper the transaminase activities are expressed in Bücher units per gram of nonfrozen or frozen and thawed tissue (BU/g) or per ml press juice. The GOT_M activity is usually expressed in percent of the total GOT activity in the electrophoretic diagram.

A routine method for differentiation

Five to 10 ml of muscle press juice are obtained by pressing the sample of lean meat with a hydraulic press (maximum pressure: 10 kp/cm²). The juice is filtrated through a filter paper and then through a membrane filter No. MF 50 (Membranfiltergesellschaft Göttingen, Germany). One ml or less of the filtrate is diluted with the same volume of 0.1 *M* phosphate buffer (pH 7.5), 0.005 ml of this fluid is placed on the center of a cellulose-acetate membrane-foil (39 × 315 mm; Schleicher and Schüll, 3354 Dassel, Germany) as a line. After electrophoresis (0.05 *M* phosphate buffer, pH 7.5) at 100 to 150 V (measured at the ends of the membrane) for at least 12 hr or after high-voltage electrophoresis at about 1000 V and 20-30 mA (+3°C) for 60 to 90 min, the foil is taken out and—still wet—sprayed with the following reagent: The content of one bottle "GOT-Monotest" (C.F. Boehringer und Söhne, GmbH., Mannheim, Germany) and about 3 mg NADH dissolved in a mixture of 2.75 ml 0.1 M phosphate buffer (pH 7.5) and 2.0 ml 0.005% pyridoxal phosphate solution (the spray reagent should not be kept cold for longer than 2 or 3 days).

If necessary, the spraying can be repeated one or two times. Observed under an ultraviolet lamp with a filter for 365 nm, the electrophoretic pattern from nonfrozen meat shows only one dark bond (GOT_S) before a brightly fluorescent background, whereas the pattern from frozen and thawed meat shows in the cathodic range a second distinct band (GOT_M).

Table 1—Influence of freezing (−18°C) on the GOT activity in muscle tissue and muscle press juice.

Species	Postmortem period before freezing days	Time of freeze-storage	GOT activity in the total tissue BU/g: before freezing	GOT activity in the total tissue BU/g: after freezing and thawing	GOT activity in the muscle press juice BU/ml: before freezing	GOT activity in the muscle press juice BU/ml: after freezing and thawing
cow	0	8 days	1864	1888	1944	2535
cow	5	1 day	1686	1774	1854	2134
		2 months		1344		3978
		4 months		1040		—
		6 months		760		4133
pig	5	6 months	1419	1130	1503	2465
pig	7	1 day	1284	1460	2289	2004
		2 months		1256		3928
		4 months		1130		—
		6 months		854		4003

RESULTS

Influence of freezing and freeze-storage on total GOT activity

The influence of freezing and thawing of bovine and porcine muscle at −20°C on the total GOT activity of tissue (extractable activity after destruction of muscle cells by grinding the tissue in a Bühler-homogenizer; Gantner et al., 1964) was small (Table 1). After two months freeze-storage (−20°C), the GOT activity decreased 14% for porcine muscle and 24% for beef muscle; after six months freeze-storage, the activity decreased 42% and 57% respectively (Table 1).

Freezing and thawing of bovine and porcine muscle tissue resulted in most cases in a remarkable increase of total GOT activity in the muscle press juice (Table 1). Despite the decrease of the GOT activity in the total tissue during freeze-storage, higher GOT activities are found in the muscle press juice with progressing time during freeze-storage of the muscle tissue. During 6 months freeze-storage of bovine or porcine muscle, the total GOT activity in the muscle press juice increases about 100% (Table 1).

Influence of freezing and thawing of muscle tissue on the electrophoretic pattern

Electrophoresis of the press juice of nonfrozen, nonground porcine muscle re-

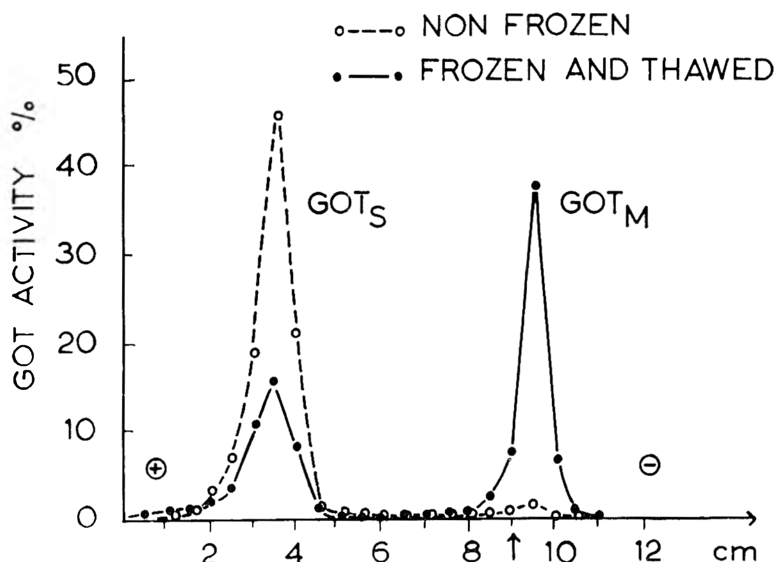

Fig. 1—Low-voltage electrophoresis of the press juice of nonfrozen and frozen porcine muscle. Psoas muscle, excised 5 days post-mortem and and frozen at −20° for 20 days.—Ordinate: GOT activity in percent of the total GOT activity; arrow: start line.

Table 2—Influence of freezing (—20°) and thawing of muscle tissue on the activity of GOT isozymes in the muscle press juice.

Species	Treatment of tissue	Number of animals	Activity of GOT isozymes in percent of the total GOT activity in the muscle press juice				Percent recovery of the GOT activity after electrophoresis (average)
			GOT_S		GOT_M		
			$\bar{x}$	s	$\bar{x}$	s	
cattle	nonfrozen	15	98.0	1.83	2.0	1.83	88.2
cattle	frozen	14	71.9	6.43	28.1	6.46	90.8
pig	nonfrozen	10	94.0	4.81	5.0	2.47	83.0
pig	frozen	10	64.9	8.26	35.1	8.26	82.7

Table 3—Influence of freezing temperature on the activity of GOT isozymes in the muscle press juice. Time of freezing: 24 hr.

Tempera-ture of freezing	Beef muscle		Porcine muscle	
	GOT_M BU/ml	GOT_S BU/ml	GOT_M BU/ml	GOT_S BU/ml
nonfrozen	44	1653	44	1034
−4°C	44	1545	48	940
−8°C	123	852	120	962
−12°C	233	1289	237	1195
−20°C	320	1318	266	911
−40°C	481	1792	459	1602

sulted in only one GOT band which migrated to the anode (Fig. 1). This result is in agreement with our earlier findings (Körmendy et al., 1965; Hamm et al., 1969). After freezing at −20°C and freeze-storage, however, a second GOT band appeared which migrated slowly to the cathode (Fig. 1). This second band represents the mitochondrial isozyme of GOT (GOT_M, Hamm et al., 1969). Muscle mitochondria are apparently damaged by freezing and thawing in such a way that the GOT isozyme, which seems to be bound to the mitochrondria, is released. The same was observed with bovine muscle.

Influence of freezing and thawing on the activity of GOT isozymes

Muscles of pigs and cattle were frozen at different times after slaughter at −18° to −20°C and stored at these temperatures for different periods. After thawing, the GOT isozymes were electrophoretically separated; the foil was sprayed very lightly with the test mixture to make the localization of the isozymes visible. The entire band containing the isozyme was cut out and the pieces were extracted by phosphate buffer; the GOT activity of the extracts was determined. A recovery of 83 to 91% of the total activity placed on the membrane before electrophoresis was achieved (Table 2).

Table 2 shows a general survey of the influence of freezing and thawing of different bovine and porcine muscles on the activity of GOT isozymes in the muscle press-juice for different periods of freeze-storage. In the press juice of nonfrozen muscle tissue a very small but significant GOT_M activity was found which was higher in porcine muscle than in beef muscle. Most of the GOT activity (94 to 98%), however, was due to the activity of the sarcoplasmic isozyme. Freezing and thawing caused a considerable increase in the relative GOT_M activity. This increase was somewhat greater in porcine muscle than in bovine muscle.

The release of mitochondrial GOT isozyme into the muscle press juice by freezing and thawing was not influenced either by time after slaughter when the muscle sample was frozen or by the time of freeze-storage (Figs. 2 and 3).

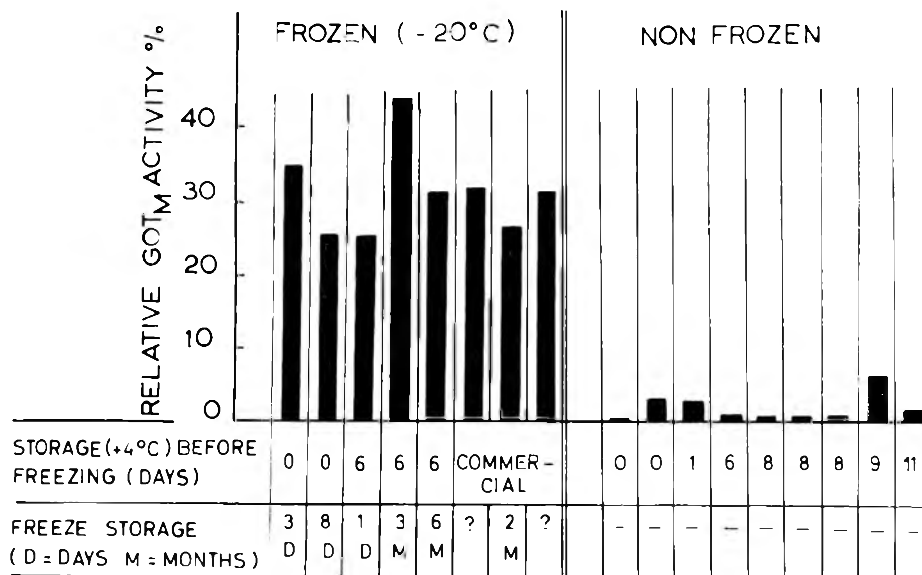

Fig. 2—Relative GOT_M activity in the press juice from nonfrozen and frozen bovine muscles (M. longissimus dorsi).—Ordinate: GOT activity in percent of the total GOT activity.

Influence of freezing temperature on the subcellular distribution of GOT

Small cubes of bovine and porcine muscle (length of edge about 1 cm) were frozen in a freezer at different temperatures between −4° and −40°C for 24 hr. The temperature inside the samples was not measured and the exact rate of freezing was not determined. In these experiments the rate of freezing increased with decreasing temperature of freezing. The GOT_M activity in the muscle press juice increased considerably and continuously with falling temperature (Table 3). The GOT_S activity, however, increased only at temperatures lower than −20°C but the relative increase with falling temperature was much smaller than for GOT_M. As Figure 4 shows, the relative GOT_M activity in the muscle press juice increased remarkably between −4° and −20°C. Freezing the muscle cubes at −197°C by dipping them in fluid nitrogen resulted in an additional strong increase in the absolute and relative GOT_M activity in the muscle press juice of the thawed tissue (Fig. 4). The relative increase of the GOT_S activity by freezing in fluid nitrogen was not significant.

This result suggests that at lower freezing temperatures, the release of GOT from mitochondrial structures is stronger. A preliminary experiment was carried out with a mitochondrial fraction isolated from porcine muscle. The mitochondrial fraction was suspended in tris buffer (pH 7.6)—sucrose (0.3 M) solution. The suspension was frozen (2 ml for each temperature of freezing) and kept in a frozen state for 24 hr. After thawing the suspension at room temperature and centrifuging at 20 000 × G, the total GOT activity in the supernatant was determined (Table 4). By decreasing the temperature from −4° to −12°C, the GOT activity in the supernatant increased about 140%. No further increase in GOT activity in the supernatant was observed between −20° and −40°C. This experiment does not

Table 4—Influence of freezing of mitochondria suspensions on the GOT activity in the supernatant (Bücher units related to the amount of mitochondria isolated from 40 g pig muscle tissue).

Temperature of freezing of mitochondria suspended in tris buffer-saccharose	GOT activity in the supernatant BU
nonfrozen	3.0
−4°C	2.6
−8°C	3.4
−12°C	7.3
−20°C	7.3
−40°C	7.6

answer the question of how much of the total mitochondrial activity is released by freezing and thawing.

Influence of repeated freezing and thawing on the subcellular distribution of muscular GOT

Samples of bovine muscles were frozen and thawed several times. Repeated freezing and thawing caused a further increase in the total GOT activity in the press juice of thawed muscle. If a frozen muscle sample was twice refrozen and thawed, a further increase in the GOT activity within the press juice of 32% was observed (Table 5), while the relative GOT_M activity increased about 40%. For this reason it must be considered, that repeated freezing and thawing causes a stronger release of the mitochondrial isozyme into the press juice than a single freezing cycle.

Influence of grinding the tissue

Grinding of bovine muscle in a commercial meat grinder increased the total GOT activity in the muscle press juice (Table 6). The level of activity of both isozymes, GOT_M and GOT_S is increased and the relative GOT_M activity in the press juice of ground muscle is considerably higher than in the press juice obtained from nonground tissue. This indicates a damage of mitochondrial structures by mincing. Freezing and thawing of ground muscle does not cause an increase in GOT_S activity in the press juice of the thawed pulp. The GOT_M activity,

Table 5—Influence of repeated freezing (—20°C) and thawing on the GOT activity and relative GOT_M activity in the press juice of bovine muscle.

Treatment of muscle tissue	Time of each freezing period days	Total GOT activity BU/ml	GOT_M activity in percent of the total GOT activity
nonfrozen	—	2455	2.6
once frozen	3	2936	19.8
twice frozen	4	3667	25.4
three times frozen	4	3868	27.7

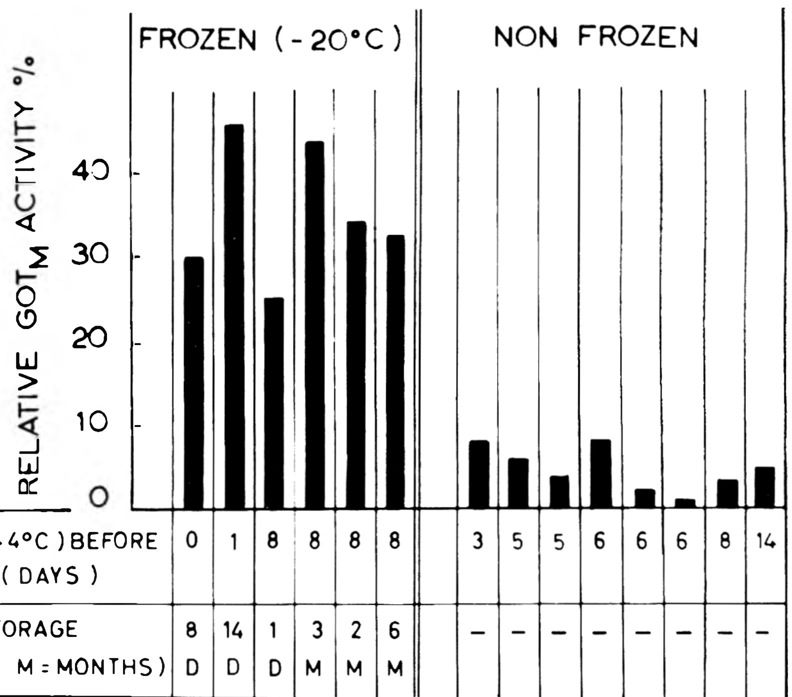

Fig. 3—Relative GOT_M activity in the press juice from nonfrozen and frozen porcine muscles. (M. longissimus dorsi).—Ordinate: GOT activity in percent of the total GOT activity.

Table 6—Influence of freezing (—20°C for 24 hr) of non-ground and ground bovine muscle tissue on the activity of GOT and GOT isozymes in the muscle press juice.

Treatment of muscle tissue	Total GOT activity BU/ml	GOT_M activity BU/ml	GOT_M activity in percent of the total GOT activity	GOT_S activity BU/ml
nonground, nonfrozen	2180	82	3.8	2098
ground, nonfrozen	3504	416	11.9	3088
ground, frozen	3350	1187	35.4	2163

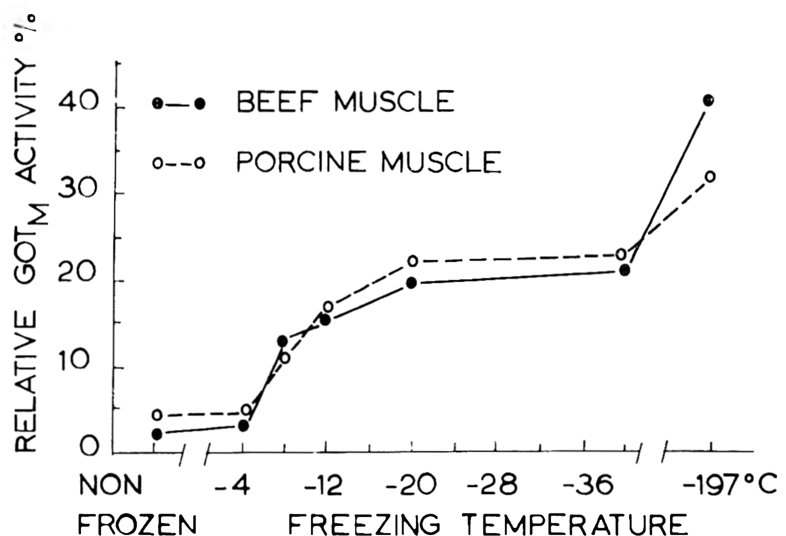

Fig. 4—Influence of freezing at different temperatures on the relative GOT_M activity in the muscle press juice.—Ordinate: GOT_M activity in percent of the total GOT activity.

however, is increased by about the same extent as was observed after freezing and thawing of nonground tissue (Table 6).

Method for differentiation

On the basis of the results mentioned above a simple and rapid routine method was developed which allows a reliable differentiation between nonfrozen meat and frozen and thawed meat. The procedure (Hamm et al., 1966) is described under "Experimental Methods." This method is applicable to beef and pork, no matter what kind of muscle is used. The time of post-mortem storage has no influence. The activity of GOT_M in the press juice of cold-stored meat is so low (Hamm et al., 1969; also see Figs. 2 and 3 in this paper) that after electrophoresis and spraying only one band (GOT_S) appears on the cellulose acetate membrane. If the meat is spoiled by microorganisms to a completely unacceptable state, a GOT_M band in addition to the GOT_S band, will be found in the press juice (Hamm et al., 1969). After storage at 0° to −2°C for several weeks, the meat still remains fresh.

Concerning the method of freezing and thawing, the same positive reaction—i.e., two distinct bands—were obtained when the meat was frozen prerigor or postrigor. The test is positive at all temperatures of freezing which are commercially used (−12°C and lower). Insufficiently frozen meat, i.e., meat frozen at temperatures above −8°C shows only a slight positive reaction (faint GOT_M band). The time of freeze-storage does not influence the test. Frozen meat from Argentina which was frozen for at least 6 months gave strong positive reaction. Also, repeated freezing and thawing does not influence the test. This test does not indicate whether the frozen meat was temporarily thawed.

DISCUSSION

THE DATA presented in this paper show that freezing and thawing of bovine and porcine muscle causes a remarkable release of the mitochondrial isozyme of glutamic-oxaloacetic transaminase (GOT_M) resulting in an increase in GOT_M activity in the muscle press juice. The lower the freezing temperature the stronger the effect.

There is no published information, as far as is known, on changes in the subcellular distribution of GOT by freezing and thawing animal tissues except an observation of Dawson (1966) who found that the rate of efflux of GOT and other enzymes from chicken muscle is markedly influenced by freezing and thawing. The rate of efflux was increased by lowering the freezing temperature from −15° to −70°C.

The release of other enzymes from mitochondria by freezing was observed. Lusena (1965; Lusena et al., 1966) determined the release of glutamate dehydrogenase (enzyme of the mitochondrial matrix) and 3-hydroxybutyrate dehydrogenase (enzyme of the mitochondrial membrane) from liver mitochondria by freezing and thawing. He found that the temperature interval between −5° and −20°C maximum enzyme release occurred at about −15°C.

The release of mitochondria-bound enzymes by freezing and thawing is probably due to damage of the mitochondria. It is known that the cellular organization including the nucleus, the subcellular organelles, mitochondria, ribosomes and lysosomes and the endoplasmic reticulum can be damaged to varying degree by freezing and thawing (Tappel, 1966). Lusena (1965) concluded from his enzyme studies that freeze-damage of liver mitochondria occurs in two steps: one is rapid and involves changes in the membrane structure, the other is slow and causes a release of the matrix-enzymes. According to Lusena et al. (1966), the partial release of enzymes from liver mitochondria by freezing and thawing is not associated with a partial structural modification of all mitochondria but rather with drastic structural changes in only some of these.

Partmann (1964) observed with the electron microscope changes of the muscle mitochondria during freeze-storage of red muscles which seemed to be due to a damage of the lipoproteins of mitochondrial membranes. On the other hand, micrographs of other muscles did not reveal mitochondrial damage whereas we have found a release of GOT_M by freezing of many kinds of muscles in different species, e.g., in chicken muscle; the results obtained with chicken muscles will be published later. Therefore we believe that the release of GOT_M indicates changes in mitochrondrial structures not demonstrable by electron micrographs.

The effect of freezing temperature on the release of GOT_M from mitochondrial structures leads to the problem of the influence of different kinds of ice formation. Slow freezing of tissues and cellular suspensions generally results in large crystals which are located entirely in extracellular areas. Rapid freezing, on the other hand, results in numerous small crystals distributed uniformly throughout the specimens (see the reviews of Fennema, 1966, and Love, 1966). Fennema mentioned in his review that intracellular ice formation (rapid freezing) is usually lethal; "it seems somewhat inconsistent that intracellular ice formation should be quite acceptable in the case of food materials and totally inacceptable for materials in which viability is desired."

In our opinion, this is not contradictory. The large extracellularly formed ice crystals in slowly-frozen tissues distort the cells without severe damage of cell membranes and organelles necessary for the viability of the cell. Changes of the gross-structure, some mechanical damage of cell walls and a limited denaturation of proteins by the raised ion concentration or other factors may contribute to a loss of water-holding capacity and other factors of meat quality. Quick-freezing, however, changes the fine-structure of the muscle cells rather than the gross-morphology.

It is expected, that a damage of cell organelles which strongly injures the viability of cells will influence meat quality factors, as water-holding capacity or tenderness to a lower extent than the formation of large ice crystals (Rapatz et al., 1959). In slowly frozen tissue, the mitochondria and their internal structure are essentially unaltered. In contrast, the morphological damages within cells undergoing rapid freezing are numerous and drastic (Karon et al., 1965). The mitochondrial envelopes are broken with the loss of matrix granules and disruption of cristae. According to the hypothesis of Karon et al. (1965), water remaining in the cell after development of large ice crystals during slow-freezing as lattices and clathrates serves to strengthen protein membranes; during rapid freezing, however, intracellular ice formation governs not only the free water but also portions of the bound water, thereby weakening the lattice structures and promoting the denaturation and the subsequent breakdown of membranes.

The conditions of very "rapid freezing" (about 40°C/sec) have never been given in the normal techniques of freezing meat or other foods. They were likewise not given in the experiments presented in this paper; even freezing of small muscle cubes (length of edge 1 cm) in fluid nitrogen did not realize very "rapid freezing" in the whole tissue. Of course, there might have been thinner or thicker layers of "rapid frozen" tissue on the surface of the muscle samples in our experiments but the center of the samples certainly consisted of "slow frozen" tissue. Our data do not allow any final conclusions concerning the influence of the rate of freezing. Presently further investigation on this topic is being done in our laboratory.

The release of GOT_M from the mitochondrial membranes might be caused by the combined effect of various factors: ion concentration by formation of large ice crystals (Fennema, 1966; Love, 1966), violent dehydration of proteins and lipoproteins forming the mitochondrial structure (Luyet et al., 1936) and the destruction of protecting lattices of bound water (Karon et al., 1965). The latter

idea is not supported by the results of Riedel (1968) who could not find an influence of the rate of freezing on the amount of bound hydration water in beef and fish muscle.

The process of freezing and thawing itself does not significantly change the total GOT activity of muscle tissue. In some cases even an increase in the GOT activity was observed. It is known that rapid freezing "unmasks" sites of enzyme activity in situ (Melnick, 1964, 1965). Increase in activity of some enzymes of mitochondria is found when they are disrupted as in freezing and thawing (Tappel, 1966), but it apparently is not known if GOT belongs to the enzymes showing this "latency-phenomenon." The remarkable decrease of the total GOT activity of skeletal muscle during freeze-storage is not surprising, because much information is available in the literature indicating a denaturation of tissue proteins during freeze-storage which might be mainly due to the effect of the high ionic strength of the remaining, nonfrozen fluid in the tissue (Fennema, 1966).

Not only the absolute and relative GOT_M activity but also the absolute GOT_S activity in the muscle press juice is usually increased by freezing and thawing of muscle tissue. Therefore, the freezing process promotes the efflux of this intermitochondrial enzyme which might be caused by a damage of the walls of muscle cells. It is not surprising that freezing and thawing did not in all cases cause an increase of the total GOT activity in the press juice because it was shown by Love (1966) that the influence of ice formation on the rupture of cell walls is a complicated process which discontinuously changes with increasing rate of freezing. With respect to this effect of ruptured cell walls, freezing and thawing seem usually to exert a similar effect as mincing.

Amberson et al. (1964) concluded from their experiments that only the press juice from nonground muscle tissue and not an extract of minced tissue represents the composition of the normal sarcoplasmic matrix. They observed that the GOT and lactic dehydrogenase (LDH) activities of homogenates of the whole muscle are always found to be greater than those obtained in the press juices and they concluded that substantial fractions of these two enzymes must be bound to the ultrastructure and released by mincing and homogenizing the tissue. However, another explanation of this phenomenon also seems to be possible which was not discussed by these authors. It is possible that the cell membranes exert a "filter-effect" retarding the efflux of certain enzymes of the sarcoplasmic matrix.

If the cell walls are destroyed by mincing, these enzymes can easily migrate into the supernatant. This consideration leads to the question whether the increase in the absolute and relative GOT_M activity in muscle press juice by freezing and thawing, is not caused by a damage of mitochondrial structures but by a "filter-effect" of the cell walls in such a way, that the efflux of GOT_M through the cell walls of nonfrozen tissue is more strongly retarded than that of GOT_S. Consequently, freezing would abolish the effect of the cell wall.

We believe that the increase in the relative GOT_M activity in the muscle press juice caused by freezing and thawing is not due to changes in such a "filter-effect" of the cell wall but to damages of the mitochondria for the following reasons: (a) it is known that GOT_M activity is not present in the sarcoplasmic matrix of nonfrozen muscle; (b) the molecular weight of the GOT_M protein is about the same as that of the GOT_S protein the latter being slightly higher (Wada et al., 1964); it seems to be unlikely that one of the two proteins can pass through the cell wall while the other one cannot; (c) freezing and thawing of ground muscle tissue increases the relative GOT_M activity in muscle press juice by about the same extent as freezing and thawing of nonground tissue (Table 6); (d) GOT_M was demonstrated by differential centrifugation to be localized in the mitochondrial fraction (Körmendy et al., 1965).

In our opinion the relative GOT_M activity in the muscle press juice can be used as an indicator for the damage of muscle mitochondria and as a method for differentiation between nonfrozen and frozen and thawed meat. The GOT_M isozyme which can be easily separated from the GOT_S isozyme by electrophoresis, is particularly appropriate for these purposes because on the one hand this enzyme shows a high post-mortem stability and is not released from the mitochondrial structure during cold-storage of muscle. On the other hand it is easily released from mitochondrial structures by freezing and thawing. All reagents for the photometric determination of the GOT activity are commercially available.

As far as we know, there exists no other reliable method for differentiating between nonfrozen meat and frozen and thawed meat than the procedure which we described under "Experimental Methods." It is nearly impossible to carry out these differentiations by histological methods. (Rapatz et al., 1959). With previously developed chemical methods (Turmel, 1957; Massi, 1960; Radan, 1965) all results were not reliable.

We found in preliminary experiments that succinic dehydrogenase (SDH) and glutamic dehydrogenase (GLDH), two enzymes which are localized only within the mitochondria, cannot be used as indicators for freezing and thawing meat.

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Ms. received 6/10/68; revised 10/4/68; accepted 9/10/69.

The authors wish to thank Mrs. L. Tetzlaff for her excellent technical assistance and Dr. H. Burmeister, Peoria, Ill. for his advice.

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A Texture Profile of Foodstuffs Based upon Well-defined Rheological Properties

SUMMARY—Many attributes contribute to texture, and panel tests should be so arranged that all these attributes are analyzed. The texture profile concept used by Szczesniak and her co-workers is critically examined, and several modifications are proposed. The new scheme consists of primary, secondary and tertiary categories. These terms are not used in the philosophical sense proposed by Locke. Primary attributes are analytical composition, particle size and size distribution, particle shape, air content etc. There are only three secondary attributes viz., elasticity (E), viscosity (η) and adhesion (N). The tertiary characteristics are basically the responses most often used in sensory analysis of texture. Tertiary characteristics are derived from a complex blending of two or more secondary attributes. The former can be regarded as falling within a three dimensional continuum which has the secondary attributes as coordinate axes. Consequently all tertiary attributes can be represented by rectangular coordinates of the form (αE, $\beta\eta$, γN) where α, β, and γ represent the respective magnitudes of the three secondary attributes. Since solids, semisolids and fluids have characteristic values of these attributes, it should be possible to predict panel responses from mechanical strain-time tests, which are carried out at the approximately constant rate of shear operative during mastication, and adhesion tests.

INTRODUCTION

TEXTURAL qualities play an important role in the consumer's response to a product. When a laboratory panel gives a sensory evaluation of texture, their responses are usually in terms of "good" or "poor" texture or some intermediate condition, or of ratings on an arbitrarily selected texture scale. The generalized term "texture" comprises several interrelated attributes, and no information about them, or their relative importance, is obtained from such tests.

It is now well established that instruments used to assess texture do not all measure the same property; e.g., a cone penetrometer measures the hardness of margarine, whereas a study of its flow properties through an extrusion capillary viscometer provides more reliable information about its spreadibility. It follows that if the data obtained from such tests are correlated independently with panel evaluation of "texture on the palate" the two correlation coefficients will not be the same, and one of them may not be statistically significant at the 1%, or even 5%, level. Strictly speaking, an instrumental measurement should be correlated with that attribute of texture which the measurement procedure most closely resembles in its action, using the rank correlation principle.

Szczesniak (1963) has proposed a classification of textural characteristics based on the view that these characteristics are recognized according to a definite pattern viz., first bite, mastication and residual impression (Brandt et al., 1963).

This paper critically examines the Szczesniak (1963) texture profile, and suggests various modifications so as to place it on a more basic rheological foundation. It also indicates how it may be possible to predict panel response to sensory evaluation of texture from a limited number of physical measurements.

REVIEW OF SZCZESNIAK'S TEXTURE PROFILE

SZCZESNIAK et al. (1963a) suggested that textural attributes could be subdivided into three principal categories—mechanical, geometrical and "others," the last named consisting mainly of analytical characteristics, e.g., moisture content, fat content, etc. The first two categories are related to the initial and masticatory impressions on the palate respectively during sensory evaluation of texture. Each category was subdivided further into primary parameters and secondary parameters, the latter being defined as parameters "that could be adequately described by two or more of the primary terms."

This classification is unsatisfactory for various reasons, which are given below:

(1) If one accepts the quoted definition for a secondary parameter, then all so-called mechanical primary parameters are indeed secondary parameters, because they depend upon a blend of primary parameters which fall within the geometrical and "others" categories.

(2) Brittleness is defined "as the force with which the material fractures. It is related to the primary parameters of hardness and cohesiveness. In brittle materials, cohesiveness is low and hardness can vary from low to high." Furthermore, hardness is defined "as the force necessary to attain a given deformation," which is strictly speaking a definition of "firmness," and cohesiveness "as the strength of internal bonds making up the body of the product."

Hard materials, in point of fact, have a high modulus of elasticity (E), which depends on the net potential energy of attraction between the basic structural elements of the material, i.e., on forces of cohesion. Brittle materials behave as hard materials with high E until the applied stress exceeds the value of the rupture stress, when cracks become visually apparent in the sample. When the attraction is due to strong primary bonds only, and there are no weak secondary bonds present, a material is brittle (Houwink, 1958). Thus both hardness and cohesion are related to elasticity.

Biscuits (cookies) are a practical example of brittleness. These contain very fine cracks due to the internal strains which develop from uneven contraction during the final stage of baking (Matz, 1962). Therefore, it should be possible to interpret brittle fracture in biscuits in terms of dislocation theory and crack propagation, the latter proceeding with a high velocity when the yield stress is exceeded. In plastic materials, on the other hand, the rate of crack propagation depends on the stress developed in the plastic zone at the tip of the crack (Kennedy, 1962).

By analogy with two-dimensional brittle fracture theory for metals (Kennedy, 1962), and other materials, the surface energy increases by 4rS when a fracture occurs, where S is the surface energy/unit area, and r is the radius of the crack. When a crack develops it reduces the strain energy/unit thickness by $\pi r^2 \tau / 2E$, where τ is the applied stress, and E is now the elastic shear modulus. Assuming that these two factors are the only ones involved in crack propagation, the crack will spread only when the reduction in strain energy associated with an increase in r is greater than the increase in surface

energy.

At equilibrium

$$\frac{d}{dr}\left[4rS - \pi r^2\tau^2/2E\right] = 0 \qquad [1]$$

and the criterion for crack propagation is that

$$\tau = [4ES/\pi r]^{1/2} \qquad [2]$$

(3) Adhesiveness is defined correctly, viz., "the work necessary to overcome the attractive forces between the surface of the food and the surface of other materials with which the food comes in contact (e.g., tongue, teeth, palate, etc.)." Stickiness is given as one of the popular terms related to this, but it depends not only on the adhesion forces but also on the forces of cohesion between the basic structural components. The relative importance of these two types of forces, which are collectively termed "hesion" (Claassens, 1958) depends on their magnitude. When the adhesion forces are larger than the cohesion forces, part of the food on the palate will adhere to the teeth etc. as they move upwards following the initial biting motion. On the other hand, when the adhesion forces are smaller than the cohesion forces the food particles will not be retained on the teeth.

For a solid food material, the rupture process can be treated in terms of the crack propagation model referred to under [2], when r is large with respect to the thickness of the crack.

In the case of a Newtonian fluid, one can apply Stefan's law (Bikerman, 1960) for the viscous flow of liquid between two surfaces when one of the surfaces is raised. This leads to

$$\frac{dD}{dt} = \frac{2FD^3}{3\eta R} \qquad [3]$$

where D is the distance between two surfaces of radius R, η is the viscosity of the liquid, and F is the force required to produce separation. Eq. [3], shows that F is directly proportional to the rate at which separation occurs. If the rate of separation is very high, i.e., dD/dt is very large, there may be a clean break between the surfaces of the teeth, etc., and the fluid, or alternatively some of the fluid may adhere to the teeth etc. When the fluid is non-Newtonian, and exhibits plastic flow, Eq. [3] cannot be applied, since the viscosity now depends on the operative rate of shear. The non-Newtonian flow of many materials can be defined by a power law type equation over several decades of shear rate ($d\sigma/dt$), and

$$\frac{d\sigma}{dt} = \frac{1}{\eta^{\#}}(F - f)^n \qquad [4]$$

where f is the yield value, n is a constant which is specific to the material and $\eta^{\#}$ now refers to the viscosity at a specified rate of shear. For such materials Eq. [3] becomes

$$\frac{dD}{dt} = \frac{AF^nDD_0^{n+1}}{\pi^n R^{3n+1}(\eta^{\#})^n} \qquad [5]$$

where A, an infinite convergent series, is a function of n and D/D_0, where D_0, the limiting thickness is equal to $2\pi R^2f/F$. Most non-Newtonian fluids show a value of n which is greater than unity, and for these F is less dependent on the rate of separation than are Newtonian fluids.

(4) The definitions of elasticity ("the rate at which a deformed material goes back to its undeformed condition after the deforming force is removed"), chewiness ("energy required to masticate a solid food product to a state ready for swallowing") and gumminess ("the energy required to disintegrate a semisolid food product to a state ready for swallowing") have little fundamental significance (Drake, 1966). Strictly speaking, elasticity is the "the property of a material by virtue of which, after deformation and upon removal of stress, it tends to recover part or all of its original size, shape, or both" (Reiner et al., 1967). Furthermore, Szczesniak's definition of elasticity is misleading because, for example, in a creep compliance-time study at constant low stress, the sample may not show an instantaneous elastic recovery on removal of the stress which is identical with the instantaneous elastic deformation exhibited when the stress was applied. This phenomenon is known as elastic fatigue (Reiner, 1960).

MODIFIED TEXTURE PROFILE

IN THE LIGHT of these criticisms of

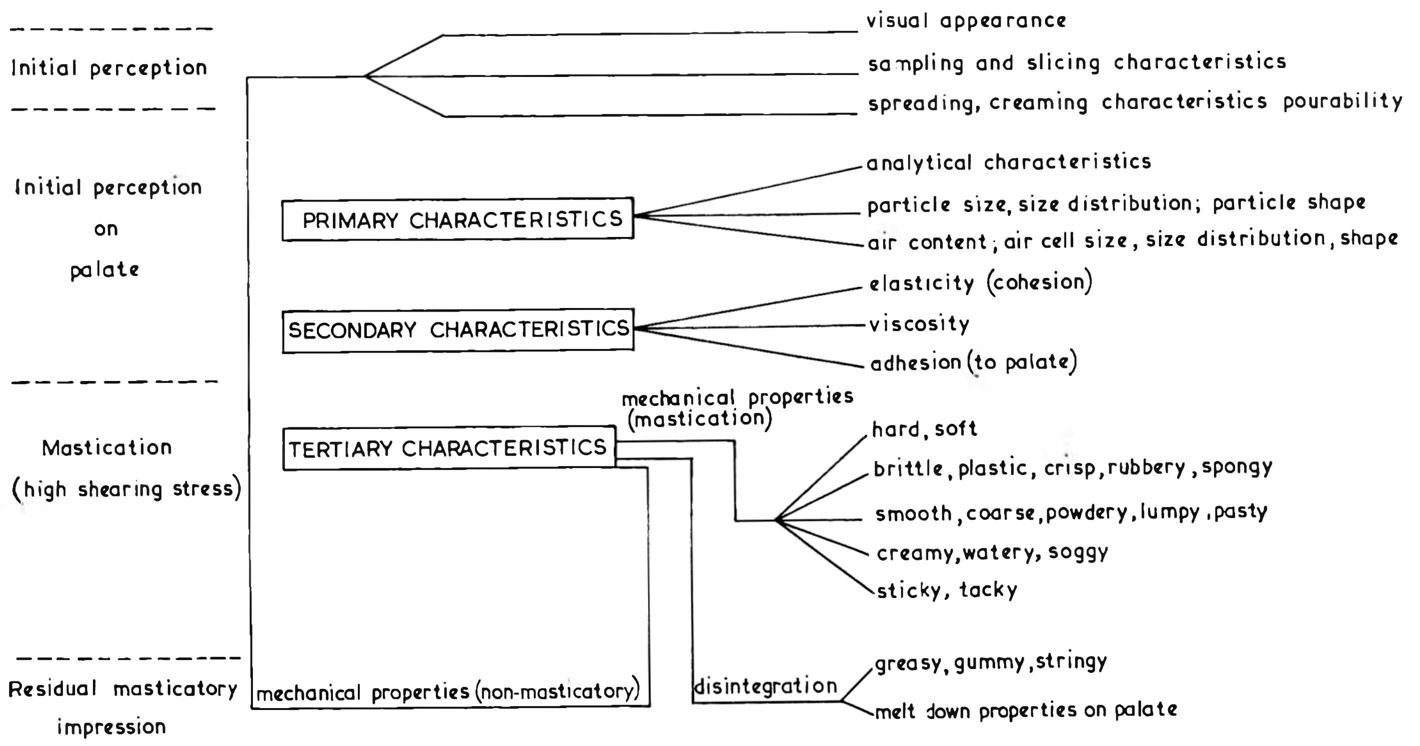

Fig. 1—Modified texture profile.

Szczesniak's (1963) texture profile, and also because it has been shown that textural attributes can be treated in a more rheological way, several modifications are proposed. Figure 1 gives the amended version. No distinction is drawn now between analytical, geometrical and mechanical attributes. The only criterion for the new classification is whether a characteristic is a fundamental property, or whether it is derived by a combination of two, or more, attributes in unknown proportions. Thus, the properties previously labelled geometric and analytical characteristics are now introduced into the primary category. All other attributes are derived from these. The basic rheological parameters, elasticity, viscosity and adhesion, form the secondary category, and the remaining attributes are established as a tertiary category, since they are a complex mixture of these secondary parameters.

The tertiary category can be subdivided further according to the type of mechanical process involved, viz., mastication, disintegration following mastication or the nonmasticatory mechanical treatment of the sample prior to sensory assessment on the palate. The last of these three processes controls the very first sensory impression received. General terms used to define tertiary, masticatory and disintegratory attributes (Fig. 2) were selected from housewife panel assessments of, and comments on, various food products. The terms adopted are those which occurred most frequently in the responses of the panel members. It is interesting to note that the same words appeared most frequently during word association tests which were given to a panel of 100 people by Szczesniak et al. (1963b) to determine "their degree of texture consciousness and terms used to describe texture."

Figure 2 indicates how these tertiary terms are distributed between solid, semisolid and liquid foods. The subdivisions are inevitably somewhat arbitrary and are employed for ease of tabulation. The terms solid, semisolid and fluid do not form a distinctive grade of characteristics.

On the basis of the classifications proposed in Figures 1 and 2, attributes which should be scored in the sensory assessment of the texture of cakes or ice cream, for example, are listed in Table 1. The questionnaire is divided into three sections comprising initial impression, mastication and residual impression. Each attribute should be judged using a rating scale, i.e., by "a method for securing and recording a judgement concerning the degree to which a stimulus material possesses a specified attribute . . ." (Inst. Food Technologists, 1964).

Since all attributes are rated in the same way using an arbitrary scale of n points, although the value of n may vary for the different attributes, one cannot deduce from these scores whether all attributes contribute equally to the overall rating of texture, or whether some are considered more important than others. It is suggested, therefore, that some indication of their relative importance may be obtained by asking panel members to indicate which attributes had the greatest influence on their overall texture rating.

PREDICTION OF PANEL RESPONSES

SZCZESNIAK et al. (1963a) obtained good correlations between panel ratings of hardness, brittleness, chewiness, gummi-

Table 1—Textural attributes to be scored during panel assessment of ice cream and cake textures.

		Ice Creams	Cake
1. Initial impression (general handling of sample)	hardness	×	×
	elasticity	×	×
	crumbliness		×
2. Masticatory impression	smoothness	×	
	creaminess	×	
	viscosity	×	
	tackiness	×	×
	spongy/plastic/rubbery		×
	heavy/light		×
	ease of disintegration (chewiness)		×
	moistness		×
	melt down properties on palate	×	
	residual impression of greasy or nongreasy coating to palate	×	
3. Any other comments about texture			
4. Overall rating of texture			
5. Factors scored which most influenced overall assessment of texture			

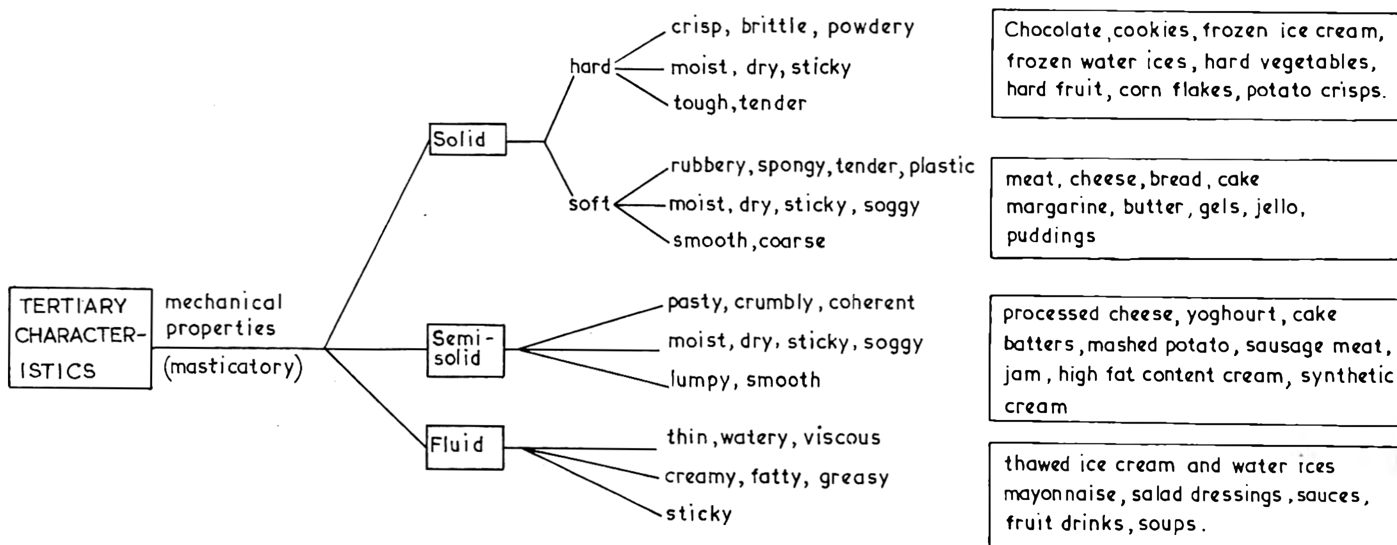

Fig. 2—Panel responses associated with masticatory tertiary characterisitics of modied textural profile.

Table 2—Magnitude of the secondary characteristics associated with some tertiary characteristics.

Tertiary textural characteristic	E	η	N
hard, tough	very high	high	
hard, brittle (e.g., biscuits)	low	very low	
plastic	low	high	
rubbery (e.g., gels)	low	high	variable
semisolids (e.g., cake batters, high fat cream)	low	medium	variable
fluid (e.g., mayonnaise, salad dressings)	nil	low	variable

ness and adhesiveness, and relevant measurements with a modified M.I.T. denture tenderometer (Friedman et al., 1963). Satisfactory correlations were also obtained between panel ratings of viscosity and viscosity measurements with a Brookfield viscometer at fairly high rates of shear. Some of these correlations is of doubtful significance because of the odd way in which the textural attributes were defined. Bourne (1966) attempted a dimensional analysis of some of these attributes but was not wholly successful.

Scott Blair (1949, 1960) suggested that some of the less specific terms associated with texture are amenable to dimensional analysis. For example, following Nutting, the attribute, firmness (ψ), can be defined at constant stress in terms of stress, strain and time. For a viscous fluid

$$\psi = \eta = \tau\sigma^{-1}t^{1} = ML^{-1}T^{-1} \quad [6]$$

and, for an elastic solid

$$\psi = E = \tau\sigma^{-1}t^{0} = ML^{-1}T^{-2} \quad [7]$$

so that, in general

$$\psi = \tau\sigma^{-1}t^{k} = ML^{-1}T^{(k-2)} \quad [8]$$

where k, the "dissipation coefficient," has a value of 1 for viscous fluids and a value of 0 for elastic solid. Materials that fall between these two categories show fractional values of k. In this way it is possible to define textural characteristics by a limited number of physical terms.

This concept has some similarity to the relationship between secondary and tertiary characteristics quoted in Figure 1, because the tertiary characteristics describing the mechanical masticatory responses not involving stickiness depend on elasticity and viscosity, which in turn derive from the τ-σ-t behavior. Although there is evidence that τ varies with the hardness of the food being masticated (Neil Jenkins 1966), it remains approximately constant during mastication of a particular food, apart from some increase towards the end of the chewing period. Thus, the criterion really is the change in σ with t at constant τ, as in a creep compliance-time study. Since $d\sigma/dt$ is not constant, but decreases as t increases, and some tertiary textural attributes relate to higher values of $d\sigma/dt$ than do others, the information obtained by calculating E and η from the $\sigma - t$ data is more detailed than the latter as they stand.

The secondary characteristic of adhesion must be incorporated now. Each tertiary textural characteristic enumerated in Figures 1 and 2 is a complex mixture of two or three secondary characteristics, so that in theory the former can be regarded as located in a three dimensional continuum which has the three secondary characteristics as coordinate axes. The position of each tertiary characteristic at the operative shearing stress can thus be defined by rectangular coordinates of the form (αE, $\beta\eta$, γN), where N is the adhesion of the food material to the palate, and α, β and γ, define the location of the attribute with respect to the three coordinate axes.

Both α and β decrease as one passes from a hard solid to a semi-solid and then to a fluid, while γ varies with the degree of stickiness. All solids are characterized by a high α, although it will be higher for a nonbrittle solid than for a brittle one. The magnitude of β also depends on whether the solid is tough or brittle. Table 2 illustrates the general levels of α, β and γ which are associated with some textural characteristics. One can envisage boundary lines running through the three dimensional continuum, as in a Phase Rule diagram, so as to define the transition from possession of a particular characteristic to its absence.

If the relevant elasticity and viscosity data are derived from a creep compliance-time study at a constant shearing stress, then they comprise the instantaneous elastic shear modulus, the elasticity moduli associated with retarded elasticity and the viscosity moduli associated with retarded elasticity plus the Newtonian viscosity, respectively. This approach is based upon the belief that the simplest mechanical model to depict the rheological behavior is a Hookean spring and dashpot in series (Maxwell element) with a spring and dashpot in parallel (Kelvin-Voigt unit) which together constitute a Burgers body.

In principle it should be possible to predict panel responses to sensory assessment of textural characteristics from adhesion and mechanical strain-time tests which are made at the shearing stresses operating during mastication. All responses on the texture profile questionnaire should be characterized by the particular values of α, β and γ found experimentally for the food material. Adhesion could be determined, for example, from the force required to pull a cylindrical piece of fresh meat away from the food with which it had been originally in contact along an extended flat interface for a definite time. Calculation of the viscoelastic parameters, from creep compliance-time studies for melted ice creams which had been assessed as "good" texture or "poor" texture by a panel has already indicated that the values of all the parameters are significantly higher for the "good" texture samples (Whitehead et al., 1967).

It should be remembered that classical elasticity theory deals with very small strains, so that when considering the much larger strains involved in mastication the accepted definition of strain may not be valid. The equations for the six tensor strain components now assume greater complexity, so that it is very difficult to express the state of strain in tensorial form.

The present discussion ignores the moistening of food structure which ensues from the production of saliva following the stimuli provided by the presence of food in the mouth, and by the masticatory pressure on the teeth (Neil Jenkins, 1966). Presumably penetration by saliva profoundly weakens the structure, so that when comparing two food samples the differences are substantially reduced.

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Ms. received 2/28/68; revised 12/17/68; accepted 9/5/68.

The author is indebted to Dr. G.W. Scott Blair for a most stimulating and helpful discussion of this paper.

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Studies on Enzymic Activity of Rat Liver Subcellular Fractions

SUMMARY—Mitochondrial and lysosomal preparations from rat livers were examined for the presence of a number of particulate bound enzymes, previously described as being present in mitochondria. The study sought to determine whether these enzymes were truly mitochondrial or partly associated with lysosomes which are contaminants of most mitochondrial preparations. The amino transferases were of particular interest because of their possible involvement in the metabolism of amino acids which are concentrated within lysosomes. It was shown that particulate aspartate amino transferase and alanine amino transferase are truly mitochondrial enzymes. Particulate glutathione reductase was shown to be distributed between lysosomes and mitochondria, the specific activities in the two particles being almost equal. Possible activation of catheptic protein hydrolysis is discussed. Lysosomes were shown not to contain the following enzymes: isocitrate dehydrogenase, glycerol-3-phosphate dehydrogenase, serine dehydratase, glucose-6-phosphate dehydrogenase, xanthine oxidase, aldehyde oxidase and fumarate hydratase. Lysosomes were unable to incorporate amino acids into proteins but were able to incorporate acetate ^{14}C into fatty acids. The importance of this process as a possible mechanism for controlling or altering the structure of the lysosomal membrane is discussed. Relationships of these findings to some properties of meats are discussed.

INTRODUCTION

MAJOR hydrolytic enzymes of animal tissues are organized within subcellular organelles, the lysosomes (Straus, 1967). Lysosomal enzymes include cathepsins, nucleases, glycosidases and lipases active in degrading respectively, proteins, nucleic acids, mucopolysaccharides and complex lipids. Lysosomal enzymes appear to be important in many hydrolytic processes occurring in meats, including postmortem autolysis, aging and tenderization, and in production of flavor constituents especially amino acids, nucleotides and sugars.

Although knowledge of lysosomal enzymes is available for many commercially important animals (Tappel, 1966) including the ox, hog, chicken and fish, detailed investigations of enzymes are limited to lysosomes isolated from laboratory animals.

Presently available knowledge on isolation of lysosomes and mitochondria and studies of enzymes of protein hydrolysis and amino acid conversions make rat liver the ideal experimental tissue. The laboratory rat has been most widely used for studies of subcellular organelles and enzymes with the understanding that the basic knowledge gained has wide application.

Previous studies on the subcellular distribution of enzymes have shown many enzymes to be associated with the mitochondrial fraction. Limitations of the fractionation methods available made it impossible to determine if contaminating subcellular particles in this fraction, e.g., the lysosomes, also have similar enzyme activities. Improved methods for isolating the various subcellular particles have made it possible to describe their various enzyme activities more accurately. Beaufay et al. (1959) were able to show that glutamate, malate and β-hydroxybutyrate dehydrogenases were associated with the mitochondria. The distribution of amino transferases and other enzymes previously associated with the mitochondrial fraction is less well defined. Rowsell et al. (1963a, 1963b), studying the distribution of amino transferases in rat kidney and liver, showed that their mitochondrial preparations also contained most of the lysosomal cathepsin.

Lysosomes contain a large pool of free amino acids (Tappel et al., 1965; Beck, 1967), which possibly undergo further metabolism within these particles. Therefore, this investigation sought to determine if amino transferases and other particulate enzymes involved in amino acid metabolism were present in lysosomes as well as in mitochondria. Some of the free amino acids in animal tissues and meats appear to be related to lysosomal protein hydrolysis and the subsequent metabolism of these amino acids. Mitochondria incorporate amino acids into proteins (McLean et al., 1958; Roodyn et al., 1961) and acetate into fatty acids (Hulsman, 1960; Wakil et al., 1960). Because of the importance of these processes to the dynamic state of membranes, it was important to determine if lysosomes also possess these activities.

The possible association with lysosomes of another partially particulate bound enzyme, glutathione reductase (Rall et al., 1952), was studied. Such an association might be indirectly important in the hydrolysis of proteins by lysosomal enzymes by providing a pool of glutathione for both the direct activation of the sulfhydryl cathepsins B and C and the breaking of sulfhydryl bonds of proteins (Narahara et al., 1959; Libenson et al., 1964), thus making them more accessible to the ac-

tion of cathepsins. Catheptic enzymes are important topics in recent research on postmortem changes in bovine (Randall et al., 1967) and chicken muscle (Martins et al., 1968; Berman, 1967).

EXPERIMENTAL

Tissue fractionation

Mitochondrial, microsomal and lysosomal fractions were prepared from the livers of fasted male Sprague-Dawley rats by methods previously described (Shibko et al., 1963; Sawant et al., 1964). Lysosomal-mitochondrial fractions in a range of composition can be obtained by this method (Sawant et al., 1964). Fractions used for these studies ranged from light mitochondria, containing low levels of lysosomes, to fractions containing 85% lysosomes.

For a similar method of lysosome preparation, the protein content and acid phosphatase activity have been determined for each fraction obtained during the procedure (Ragab et al., 1967). Some typical activities of lysosome fractions prepared by the Ragab et al. (1967) procedures in mμmoles substrate hydrolyzed/minute/mg protein are: acid phosphatase, 580; cathepsin B, 30; and cathepsin C, 700–1000. Mitochondrial and soluble cytoplasm fractions were obtained as previously described (Ragab et al., 1967).

For studying the distribution of glutathione reductase, the procedure of Sawant et al. (1964) was modified as follows: The cellular debris and nuclei were removed from the homogenate. Fractions 2, 3, 4, 5, 6, 7 and 3 were obtained by subjecting the supernatant to successive centrifugations at 30,200, 43,-400, 59,000 121,000, 173,000, 270,000 and 6,000,000 $g \times$ min. Fractions 4 (59,000 $g \times$ min) and 5 (121,000 $g \times$ min) were suspended in 0.25 *M* sucrose and subjected to further fractionation A, B, C and D by successive centrifugations at 30,200, 43,400, 77,100 and 202,000 $g \times$ min, respectively.

The pellets obtained were suspended in 0.25 *M* sucrose and used for enzyme assay. To release lysosomal enzymes, the suspended particulate fractions were treated by freezing and thawing 10 times, or by suspending in deionized water, or by adding 0.1% Triton X-100 to the suspensions. Broken lysosomes were separated into soluble and membrane fractions by centrifugation at 6,000,000 $g \times$ min.

Enzyme assays

Malate dehydrogenase (Ochoa, 1955), glutamate dehydrogenase (Hogeboom et al., 1953) and lactate dehydrogenase (Kornberg, 1955) were determined by direct spectrophotometric measurement of the disappearance of DPNH. Alanine amino transferase (glutamic pyruvic transaminase) was assayed spectrophotometrically using the pyridine nucleotide linked lactate dehydrogenase system (Wróblewski et al., 1956). Aspartate amino transferase (glutamic oxaloacetate transaminase) was determined by coupling the oxaloacetate formed with the pyridine nucleotide linked malate dehydrogenase (Karmen, 1955).

All reactions were recorded for 4 min or more at 25°C and all followed zero order kinetics. These enzyme measurements were accurate to about ±10%. The level of pyridine nucleotides used in coupling reactions was 25% greater than that indicated in the references given. The amount of coupling enzymes added to the reaction systems was three times in excess of that required to complete the reaction.

Glutathione reductase was measured by the method of Racker (1955). Succinoxidase was determined by measuring the oxygen uptake polarographically (Hamilton et al., 1963). Cytochrome c was added to the reaction system to ensure maximum reaction rates. Acid phosphatase and β-glucuronidase were determined by the methods of Gianetto et al. (1955).

Incorporation of ^{14}C labeled amino acids and acetate

^{14}C-labeled lysine was obtained from Schwartz Biochemical Research Co. and algal protein hydrolysate from New England Nuclear Co. Sodium acetate-1-^{14}C was obtained from Nuclear Chicago.

For measurements of incorporation of labeled amino acids into subcellular fractions, a reaction system similar to that described by Roodyn et al. (1961) was used. After purification, the protein was dissolved in Hyamine and its radioactivity determined using a scintillation counter.

The method used to study the incorporation of labeled acetate into fatty acids was similar to that used by Hulsman (1960). A large amount of ATP was substituted for oxidizable substrate. The reaction system used for incorporation of acetate-1-^{14}C was of the following composition: 9.5 μM KCl, 3 mM EDTA, 50 μM potassium phosphate buffer (pH 7.5), 5 μM $MgCl_2$, 50 μM ATP, 3 μM acetate (1 μc), 0.18 μM Coenzyme A, 5–10 mg of particulate protein, 0.2 μM TPN, 10 μM glucose-6-phosphate and glucose 6-phosphate dehydrogenase. Incubation was for 1 hr at 25°C.

The reaction was stopped by addition of 5 ml of 5 *N* alcoholic KOH; this mixture was saponified at 90°C for 15 min, then acidified with HCl and extracted with pentane. After washing the organic phase, solvent was removed and the radioactivity in the residue was counted in a scintillation counter.

RESULTS

Distribution of dehydrogenases and amino transferases

Dehydrogenases and amino transferases have previously been shown to be concentrated mainly in the mitochondrial or soluble fractions. Therefore a careful evaluation of the mitochondrial content of the lysosomal-mitochondrial fractions was essential. Glutamate dehydrogenase, an enzyme previously characterized as a mitochondrial enzyme and which exhibits latency similar to that of lysosomal enzymes, was chosen as the best measure of mitochondria.

Table 1 shows the glutamate dehydrogenase activity of a number of fractions varying in mitochondrial content. These can be compared with the values obtained for two purified mitochondrial preparations. The mitochondrial content of the lysosomal-mitochondrial fractions used here varied from 15% to almost 100%. The specific activities of alanine amino transferase, aspartate amino transferase, and malate dehydrogenase in these preparations were compared with their specific activities of glutamate dehydrogenase. It is apparent that the activities of these enzymes are directly related to the mitochondrial content of the preparations. Examination of the kinetic properties of aspartate amino transferase of the lysosomal-mitochondrial fractions showed that they were identical with those of the enzyme from mitochondria.

Isocitrate dehydrogenase, glycerol-3-phosphate dehydrogenase, serine dehydratase, glucose-6-phosphate dehydrogenase, xanthine oxidase, aldehyde oxidase and fumarate hydratase were shown not to be present in lysosomes. Lactate dehydrogenase is a well characterized enzyme of the soluble cytoplasm. The low activity of this enzyme in the lysosomal-mitochondrial and mitochondrial fractions can be used as a measure of the efficiency of washing away the cytoplasmic components.

Table 1—Enzymes of subcellular fractions.

Enzyme	Lysosomal-mitochondrial[1]						Mito-chondrial[1]		Soluble cytoplasm[1]	
Glutamate dehydrogenase	12.8	13.5	58.2	67	84	100	79	122	4.1	4.9
Malate dehydrogenase	4.3	10	24	91	88	48	139	137	115	85
Lactate dehydrogenase	—	—	—	8.2	11.4	—	5.0	8.0	115	310
Aspartate amino transferase	24.2	73	127	126	148	170	178	269	54	93
Alanine amino transferase	1.4	3.2	2.6	6.7	9.0	4.8	10.5	12.0	4.1	4.9

[1] μmoles substrate utilized/100 mg protein/min.

Distribution of particulate glutathione reductase

In preliminary studies, the specific activity of glutathione reductase was found to be fairly constant for a series of preparations containing variable amounts of lysosomes and mitochondria. Figure 1 shows the distribution of glutathione reductase in a number of particulate frac-

Table 2—Distribution of glutathione reductase in subcellular fractions of rat liver.

Fraction	Specific activity[1]	Total enzyme activity (%)
Homogenate	52	100
Mitochondria	20	14.3
Lysosomes	22	0.7
Microsomes	9	6.0
Soluble cytoplasm	69	79.0

[1] mμ moles TPNH oxidized/min/mg protein.

Table 3—Distribution of β-glucuronidase, glutamate dehydrogenase and glutathione reductase between the soluble and membrane fractions of lysosomes and mitochondria.

Particle	Enzymes	Total Activity (%)	
		Membrane	Soluble
Lysosome	β-Glucuronidase	28	81
	Glutathione reductase	65	28
Mitochondria	Glutamate dehydrogenase	19	80
	Glutathione reductase	50	39

tions isolated from rat liver. Measurements of enzyme activities characteristic of the subcellular particles were used to calculate the contribution of each type of particle to the total activity of the fractions. The enzymes analyzed for this purpose were lysosomal acid phosphatase, mitochondrial succinoxidase and glutamate dehydrogenase, and microsomal glucose-6-phosphatase.

Particulate fractions 4 and 5, which have the highest glutathione reductase specific activity, were found also to have the highest lysosomal acid phosphatase specific activity and to contain the least mitochondria, as indicated by specific activities of succinoxidase and glutamate dehydrogenase. Further fractionation of fractions 4 and 5 showed that high glutathione reductase specific activity corresponded to high acid phosphatase specific activity and decreased mitochondrial enzyme specific activities. These results indicate that particulate glutathione reductase is derived from both mitochondria and lysosomes.

Table 4—Incorporation of acetate-1-^{14}C into lysosomal and mitochondrial fractions.[1]

Reaction system	Lysosomes[2]	Mitochondria[2]
Complete system	3.0	2.0
ATP	0.3	0.18
CoA	0.9	0.5
TPNH	1.0	0.7
Complete system membrane only	0.2	0.2

[1] Mean values of 4 experiments are given.
[2] mμ moles/mg protein/100 sec.

Table 2 shows that the specific activities of glutathione reductase in the lysosome-rich fraction is equal to that in the mitochondrial fraction. Separation of the mitochondrial and lysosomal fractions into soluble and membranous fractions showed that more than 50% of the glutathione reductase activity was associated with the membrane fractions (Table 3).

The possibility that the enzyme associated with the particulate fractions was adsorbed onto the surface of the particles was ruled out because little enzyme activity was detected when intact preparations of lysosomes were measured for this activity, the activity becoming detectable only after disruption of the particles. Physical trapping of the soluble cytoplasm seems unlikely since it was found that the specific activity of the glutathione reductase remained essentially the same after five resedimentations from sucrose. The specificity of the lysosomal enzyme for TPNH and glutathione was demonstrated by using DPNH in place of TPNH or cystine in place of glutathione. In either case, no activity was observed.

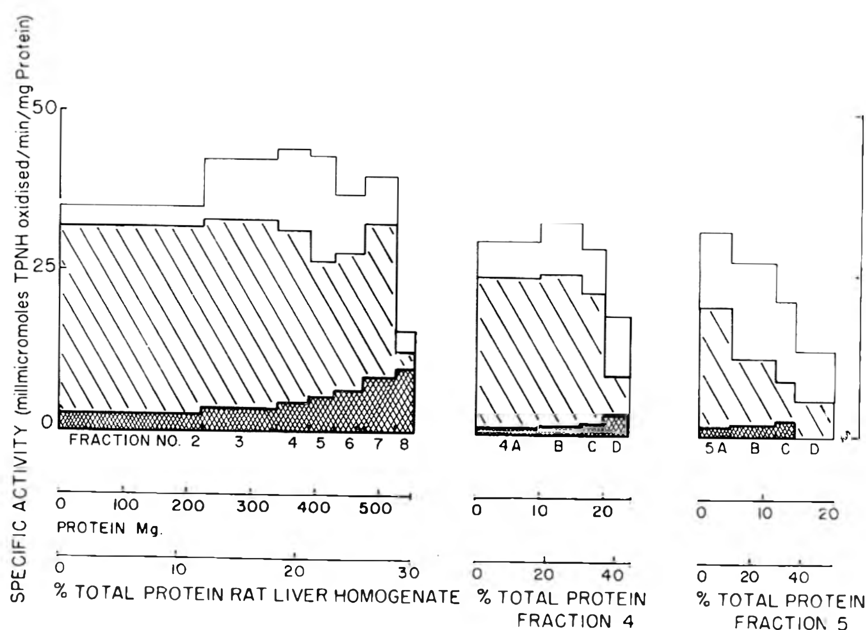

Fig. 1 Distribution of glutathione reductase in subcellular fractions of rat liver. Fractions are described in the text. Lysosomal □, mitochondrial ▨, and microsomal (cross hatching), glutahione reductase.

Incorporation of ^{14}C-labeled amino acids into lysosomal proteins

Incubation of lysosomal and mitochondrial preparations with ^{14}C-labeled amino acids derived from hydrolyzed algal protein or with ^{14}C-labeled lysine in the presence of ATP and with medium A, as described by Roodyn et al. (1961), led to incorporation of some activity into the protein of mitochondria. No activity was detected in the lysosome-rich fraction when corrections were made for mitochondrial content based on the glutamate and succinoxidase activities of the preparation.

Incorporation of ^{14}C-labeled acetate into lysosomal fatty acids

Incubation of lysosomal and mitochondrial preparations with sodium acetate-1-^{14}C, ATP, Coenzyme A and TPNH resulted in the incorporation of ^{14}C into long chain fatty acids. The incorporation of ^{14}C into these fractions was dependent upon the presence of TPNH, Coenzyme A and ATP (Table 4).

Disrupting the particles by freezing and thawing led to loss of ability to incorporate the ^{14}C. Testing of the separated membrane fractions of lysosomes and mitochondria showed that they were inactive. The amount of ^{14}C incorporated by the lysosomal fraction was slightly greater than that observed for the mitochondrial fraction. This indicates that the incorporation of ^{14}C into the lysosomal fraction is not due to the mitochondrial content of the preparation. The lysosome preparations used had average acid phosphatase specific activities of 585 mμmoles phosphate/mg protein/min. Mitochondrial content, as measured by glutamate dehydrogenase and succinoxidase activity, was found to be 15%.

DISCUSSION

THE EXPERIMENTAL results provide evidence that particulate aspartate amino transferase and alanine amino transferase are truly mitochondrial enzymes. Therefore, the activities of these enzymes in lysosomal-rich fractions are due entirely to mitochondrial content. Thus present knowledge indicates that the amino acids produced in the lysosomes by protein hydrolysis will not be metabolized further until they diffuse out and are acted upon by mitochondrial and cytosol enzymes.

If particulate glutathione reductase were only mitochondrial, its activity should parallel that of succinoxidase and glutamate dehydrogenase with concomitant increase in activity with increase in mitochondrial enzymes. The results obtained here indicate that lysosomes also contain glutathione reductase. It is not known if the particulate enzyme is different from the form occurring in the soluble cytoplasm, as has been observed with other enzymes with a similar bimodal distribution. Because of the small total amount in the lysosomes (Table 2) these questions were not pursued further.

The role of glutathione in breaking sulfhydryl bonds (Narahara et al., 1959) and activating sulfhydryl enzymes, such as cathepsins B and C, indicates the possible importance of glutathione reductase in the hydrolysis of proteins by lysosomes. Present knowledge (Tappel, 1968) of the pathway for hydrolysis of proteins by lysosomal enzymes indicates the important role of cathepsins B and C and their required sulfhydryl activation. Sulfhydryl activation might come through many routes, including that of glutathione reductase in lysosomes and soluble cytoplasm.

The incorporation of ^{14}C-acetate into long chain fatty acids by lysosomes may represent either a net synthesis or an elongation of short chain fatty acids by successive additions of acetyl CoA. These processes may be involved in lysosomal membrane metabolism during formation of cytolysomes and in autophagy. In analogous systems where the metabolism of the whole cell has been studied, the passage of particles into the cell and autophagy seem to have common characteristics with respect to changes in the metabolism of components of the membrane, particularly lipid substances.

Sbarra et al. (1960) observed incorporation of radioactivity into lipids of phagocytic cells. There was no net increase in lipid during phagocytosis. New or altered lipid was formed to replace that part of the membrane broken down during phagocytosis. Similarly, the changes in permeability of the internalized membrane in pinocytic vacuoles of amoeba described by Chapman-Andresen et al. (1955) may represent another phase in the lipid metabolism of the membrane. Since lysosomes appear to have a similar lipid composition to mitochondria and microsomes (Tappel et al., 1965), acetate incorporation may be due to common enzyme activities of these subcellular entities.

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Ms. received 2/8/68; revised 11/14/68; accepted 9/8/69.

This research was supported by Public Health Service Research Grant AM 05609 from the National Institute of Arthritis and Metabolic Diseases.

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Quantitation of Flavorful Food Components Using Isotope Dilution

SUMMARY—Radioactively-labeled flavor compounds are added to a food system prior to reduced-pressure distillation. The example used is benzaldehyde, which in the distillate is converted to its corresponding 2,4-dinitrophenylhydrazone. Separation of the labeled hydrazone from other material present is accomplished by thin-layer chromatography. Recovered material is quantitated using ultraviolet spectroscopy. Employing isotope monitoring data, the native aldehyde is calculated via isotope dilution. The method is adaptable to flavor compounds of sufficient volatility to be recovered under 5 mm Hg and 65°C, and which are stable or can be converted to a stable form for purification. The potential for quantitation of several components during an experiment is briefly discussed.

INTRODUCTION

CHEMICAL characterization of food flavors has necessitated the development of microtechniques for isolation and purification. Such analytical tools as gas chromatography and mass spectrometry have proven to be excellent devices for separating and identifying the isolated flavor mixtures (Teranishi et al., 1963; Mason et al., 1967; Gianturco et al., 1966). Self et al. (1963) have shown, however, that the volatile aroma components of a number of foods exhibit similar qualitative composition. These workers stated that differences in flavor of certain foods may lie in the relative quantitative pattern of the chemical components, rather than the qualitative presence of one or more unique components. Such an analogy might be drawn between the flavors of roasted peanuts and chocolate. Carbonyls and substituted pyrazines have been shown to be prominent in the qualitative chemical makeup of the flavor of both products (Mason et al., 1966, 1967; Rizzi, 1967; Boyd et al., 1965), yet it is quite evident each has its own unique flavor and aroma properties.

The object of the present research was to develop a technique for quantitation of flavor components which could be applied to virtually any food system. Isotope dilution seemed a likely tool, as variations in physical conditions of fractional distillation, extraction and chromatography of flavor compounds, as well as the degradation or interaction of components during isolation, could be accounted for in one step. Benzaldehyde, a flavorful carbonyl found in roasted peanuts (Mason et al., 1967) was taken as the example. This compound would be difficult to quantitate under most circumstances due to its relative instability.

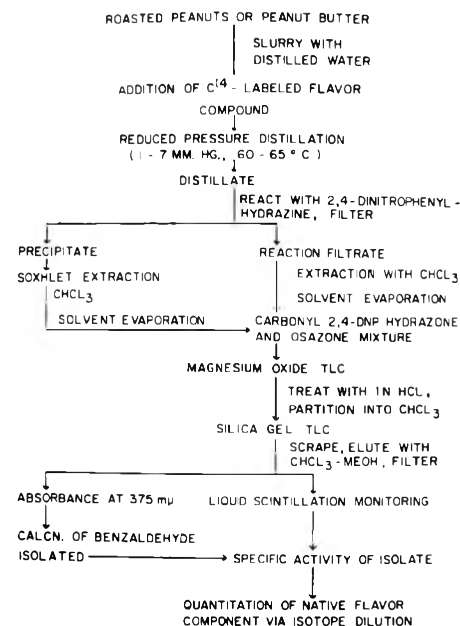

Fig. 1—Flow diagram. Quantitation of benzaldehyde from roasted peanuts via isotope dilution.

EXPERIMENTAL

Reagents

All solvents were reagent grade. Those utilized for dilution of labeled aldehyde, carbonyl analysis or extraction of hydrazones were rendered carbonyl-free by refluxing with 2,4-dinitrophenylhydrazine and trichloroacetic acid, followed by distillation.

2,4-dinitrophenylhydrazine reagent (2,4-DNP-HCl) was prepared by dissolving 5×10^{-3} moles hydrazine per liter of 2N HCl.

Unlabeled benzaldehyde (UB) was vacuum distilled, sealed under nitrogen and stored overnight in the dark at 0°C. Gas-liquid chromatography (GLC) indicated a purity exceeding 99%.

Labeled benzaldehyde (carbonyl ^{14}C, spec. act. 21.4 μc/mg) was obtained from Nuclear Chicago Radiochemical Division. The chemical purity by GLC was 99%. Radiochemical purity of the aldehyde semicarbazone was 100%. Upon removal from the shipping vial the isotope was mixed with freshly-distilled UB and the mixture was redistilled once again.

200 μl quantities were sealed under dry nitrogen in single service vials. The vials were wrapped in aluminum foil and stored in the dark at 0°C until use. Specific activity of the stored labeled aldehyde (IB) ranged from $1-2 \times 10^4$ dpm/mg, the final activity being determined by the quantity of UB in which the isotope was mixed prior to distillation.

Apparatus

Magnesia thin-layer chromatographic plates were prepared according to the procedure of Schwartz (private communication, 1967). Baker magnesium oxide ("suitable for chromatographic use"), analytical grade Celite and water (7:3:50, w/w/v) were slurried, spread onto 20 × 20 cm plates in 500 μ layers and allowed to stand for two days at room temperature prior to use. Silica gel PF plates were prepared in 375 μ layers immediately prior to use and dried at 100°C for 1 hr. Samples were applied to preparative thin-layer chromatograms with a TLC Sample Streaker from Applied Science Laboratories.

Radioactive monitoring was accomplished with a Packard Model 3002 Tri-Carb Liquid Scintillation Spectrometer equipped with automatic external standardization. The scintillation medium was prepared by dissolving 4.0 g 2,5-diphenyloxazole (PPO) and 0.05 g 1,4 - bis - 2 - (5 - phenyloxazolyl) - benzene (POPOP) in 500 ml toluene. Ten ml of this solution was mixed with an additional 10 ml of toluene containing the hydrazone to be monitored. The isolated derivative was usually counted for 100-min intervals, which allowed compilation of sufficient counts to have a statistical counting error of less than 1.5%. When monitoring free aldehyde prior to distillation, sufficient toluene was added to the solution to be counted to make a 10 ml volume. Phosphor was then added, and the sample was counted.

A Cary Model 15 ultraviolet-visible spectrophotometer was used for measurement of absorption spectra.

USDA-approved plastic food color guides for peanut butter were obtained from Magnuson Engineers, Inc., San Jose, California.

Procedure

A flow diagram of the procedure is given in Figure 1. Commercial peanut butter or freshly-roasted extra large Virginia-type peanuts ground to the consistency of peanut butter were used in the experiments. The color grade of each blend was determined prior to distillation by visual comparison to

the color guides. The peanut product was slurried with distilled water in a blendor, 500 g product plus 2 l water per charge. Quantities of peanut butter used ranged from 6 kg in early experiments to as little as 1.5 kg in the last experiments. The peanut slurry was added to a 20 l reservoir carboy. A slight positive flow of nitrogen was maintained in the carboy during the subsequent holding period.

A weighed quantity (100 mg ± 0.5) of labeled benzaldehyde was made to 100 ml volume with ethanol. Three to five ml of the thoroughly-mixed solution were added to the reservoir carboy. An identical volume was pipetted into each of three screw-cap vials for radioactive monitoring. One ml of the aldehyde solution was diluted to 50 ml with benzene, and the carbonyl content was determined on three aliquots employing the procedure of Henick et al. (1954).

Concentrations of isotope were determined from a standard curve prepared simultaneously, using freshly-distilled UB. Specific activity of the isotope was determined from the scintillation and carbonyl data. An increase in specific activity of isotope in excess of 5% of previous samples from the same lot was taken as evidence of oxidation, and the results were invalidated.

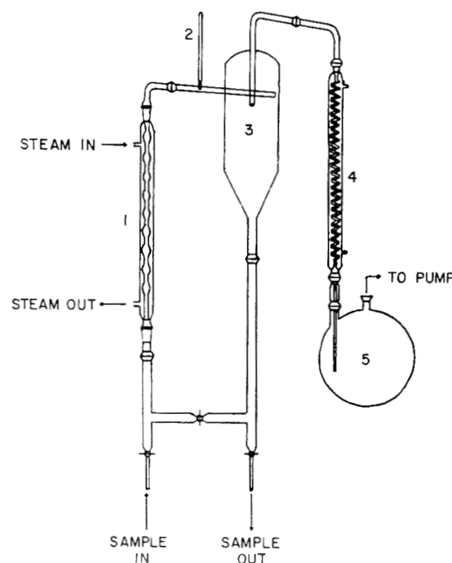

Fig. 2—Low pressure cyclonic evaporator. (1) Heat exchanger; (2) Thermometer; (3) Cyclonic evaporation chamber; (4) Griffin condenser; (5) Wet ice trap.

Following addition of the isotope to the carboy, the contents were stirred for 15 min to insure complete dispersion of IB.

The slurry was fractionally distilled in a cyclonic evaporation apparatus adapted from Lindsay et al. (1965) and Bartholomew (1949). A sketch of the apparatus is shown in Figure 2.

This apparatus was maintained at 1–7 mm Hg pressure during the distillations, with 5 psi steam pressure at the inlet of the heat exchanger. The distillate trapping system included two wet ice traps for the aqueous distillate, followed by several traps containing dry ice-ethanol or liquid nitrogen. The latter traps were placed in the train as a means of protecting the pump from aqueous vapors as only the distillate from the first wet ice trap was employed in further experimentation.

The distilling chamber was designed to hold approximately 3 l of slurry per charge. Temperature of the slurry rapidly rose to 60–65°C, at which point the charge was allowed to cycle until reduced to about half its original volume. The residual liquid from each spent charge was drained into a waste flask attached to the system. 3 to 4 l of distillate could be obtained from an original 10 l of slurry.

The aqueous distillate obtained was combined with an equivalent quantity of the 2,4-DNP-HCl reagent. This mixture was stirred for 72 hr, at which time 2,4-pentanedione was added to react with excess hydrazine reagent. The solution was then filtered. The lemon-colored filtrate was extracted several times with 0.1 volumes of chloroform. The precipitate was extracted from the filter paper in a Soxhlet extractor using chloroform. The combined extracts were then evaporated to dryness.

The hydrazone mixture was thereafter submitted to preparative thin-layer chromatography. Initial separations were made on magnesium oxide plates developed in hexane-$CHCl_3$ (85:35). The benzaldehyde area was removed from the plate into water, released from the adsorbent with 1 *N* HCl and extracted into chloroform. Chromatography on silica gel plates with a system of CCl_4:$CHCl_3$ (17:3) followed. The upper end of each plate was left exposed to the atmosphere in the manner of Libbey et al. (1964) such that there was continuous long term movement of solvent across the plate. 6 to 7 hr development offered a sufficient separation to recover the benzaldehyde band easily from the plate.

The adsorbent was subsequently mixed with $CHCl_3$-MeOH (5:1) and filtered through sintered glass. Solvent was removed under vacuum, then the derivative was made to volume with $CHCl_3$. The absorption spectrum from 350–400 mμ was obtained to assure purity, then the absorbance at the visible maximum (375 mμ) was obtained. Similarly, a sample of the solution was evaporated to dryness in a counting vial. The residue was dissolved in 10 ml of toluene, phosphor solution was added and the solution was monitored for radioactivity.

Concentration of aldehyde recovered was calculated from the formula (Day et al., 1960):

$$\text{Mg aldehyde} = \frac{A \times MW \times 10^3}{\epsilon \times \text{Dilution Factor}}$$

where

A = absorbance
MW = molecular weight (1.06×10^2 for benzaldehyde)
ϵ = molar absorptivity index

Native benzaldehyde in the product was calculated from the formula (Aronoff, 1956):

$$M_1 = \frac{M^* (S^* - S_\Sigma)}{S_\Sigma}$$

where

M^* = mg isotope added to system prior to distillation
S^* = specific activity of isotope
S_Σ = specific activity of isolated aldehyde

Control distillations with distilled water were conducted by adding known quantities of both IB and UB to 10 L distilled H_2O, and proceeding through the entire isolation procedure. Control experiments with the roasted peanut system were divided into two parts. In the first part only IB was added to the system prior to distillation. In the second part known quantities of IB and UB were added prior to distillation. Isolation and quantitation of the aldehyde in both systems were then performed. From results of native aldehyde present in the system (part one), it was possible to determine by difference the recovery of added aldehyde (part two).

RESULTS & DISCUSSION

ISOLATION of flavor compounds from natural systems by such techniques as low temperature vacuum distillation (Lindsay et al., 1965), steam distillation (Tharp et al., 1960), solvent extraction (Patton, 1961; Arnold et al., 1966) and headspace analysis (Bassett et al., 1962) involves disadvantages for quantitation such as poor yields, alterations in natural ratios of flavor components, artifact production and possible reaction of components during isolation. The facility of isotope dilution lies in the fact that once a known quantity of pure isotope is added to a system, native and labeled compound can be expected to behave similarly.

In the present system, it is assumed that the fineness of particle size overcomes the factor of native aldehyde being trapped in micelles. The flavor molecules are of such size that any isotope effects in reactions during isolation would be minimal. As indicated by Aronoff (1956): "It will be noted that quantitative isolation of M (labeled additive) is not necessary, but that *purified* M is mandatory. Indeed, M need not be isolated in weighable quantity if an indirect method of obtaining the mass, e.g., spectrophotometry, may be used." Precautions were thus taken to keep benzaldehyde as free as possible from the effects of oxygen and light. Rapid weighing, mixing under nitrogen and immediate pipetting were practiced. Variation in the specific activity within lots of benzaldehyde was found to exceed 5% in only one instance.

The benzaldehyde band on silica gel plates was quite discrete and this material indicated λ_{max} = 375 mμ. Jones et al. (1956) reported: λ_{max} = 378 mμ, ϵ = 2.83×10^4. Authentic benzaldehyde 2,4-

Table 1—Results of recovery studies for benzaldehyde in roasted peanuts.

Experiment[1]	Sample	USDA color grade no.	Added aldehyde IB (mg)	Added aldehyde UB (mg)	Added aldehyde Spec. act. (dpm/mg)	Recovered aldehyde Wt (mg)	Recovered aldehyde Spec. act.	Native UB (mg/kg)[2]	Recovered UB (mg)	% Recovered UB
A-1	Freshly roasted	4	1.13	—	11,256	0.22	565	3.08	—	—
A-2	Freshly roasted	3	6.65	—	11,507	0.44	5,806	1.26	—	—
A-3	Commercial peanut butter	3–4	5.53	—	11,917	0.20	11,104	0.09	—	—
A-4	Commercial peanut butter	3–4	5.50	—	11,130	0.11	4,066	2.09	—	—
C-1	Distilled water	—	3.17	3.18	15,254	0.07	7,362	—	3.39	106.7%
C-2[1]	Commercial peanut butter	2	4.94	—	16,728	0.95	11,499	1.53	—	—
C-2[2]	Commercial peanut butter	2	5.65	5.39	15,311	2.19	6,380	1.53[3]	5.58	103.5%

[1] A = analytical; C = control.
[2] Also, expressed as parts per million.
[3] Value taken from C-2[1].

DNP hydrazone prepared in this laboratory and recrystallized to constant melting point (238–39°C) was found to have: $\lambda_{max} = 376\ m\mu$, $\varepsilon = 2.99 \times 10^4$. Calculations were made on the basis of the latter data.

Table 1 lists the results obtained from several experiments. The distilled water control experiment yielded 106.7% recovery of UB. The critical necessity of adding IB and UB to the system immediately following one another was reflected in a single experiment. In this experiment UB was added approximately 20 min prior to the isotope. Recovery of the unlabeled compound was less than 25%, indicating that although the system was under nitrogen pressure, dissolved oxygen and/or trace metals were acting to rapidly oxidize the dilute solution of aldehyde. It is not known whether the situation would be as critical in the peanut slurry.

Data on recovery of added UB in the control peanut system (C-2[2]) was dependent on the accuracy of quantitation of native aldehyde in the product (C-2[1]). Any error in quantitation of native aldehyde would subsequently be reflected in the calculation of recovery of unlabeled compound, in addition to any normal experimental error in the C-2[2] distillation.

In view of this, the recovery of 103.5% is considered quite acceptable. Recoveries of greater than 100% on both distilled water and peanut control systems, however, lead to speculation that in spite of all precautions some aldehyde oxidation is occurring prior to monitor of free carbonyl. This can be seen in the fact that although the number of radioactive disintegrations would not decrease, less than a theoretical amount of free aldehyde would be found in the carbonyl analysis. In calculating specific activity, therefore, the result would be high.

The concentration of native benzaldehyde may be related to the extent of roast. The USDA color grade of the product would approximate heat treatment given the peanuts; however, the time of storage after processing of the commercial peanut butter was unknown, so cannot be taken into account in this work. The effect of roasting conditions on concentration of several aldehydes of flavor significance will be the subject of a further paper.

Quenching of scintillation by highly-colored molecules is rather commonplace in isotope monitoring (Walter et al., 1966; Peng, 1960; DeBersaques, 1963; Ross et al., 1963). It was anticipated that such difficulty might be encountered with the hydrazone derivatives in this research. It was found, however, that counting efficiencies of 65% or above could be obtained by varying the concentration of isolated aldehyde monitored for radiation (see Fig. 3).

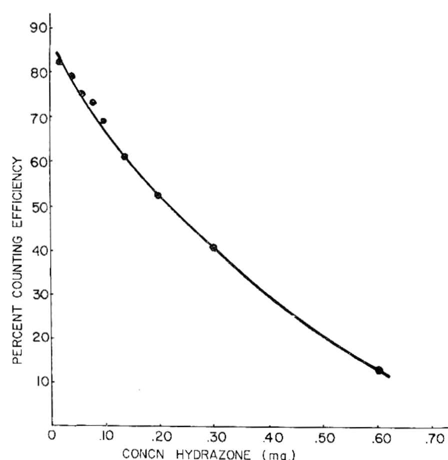

Fig. 3—Color quenching effect of benzaldehyde 2,4-DNP hydrazone in liquid scintillation isotope monitoring. Specific activity 3 × 10³ dpm/mg.

With the complexities of food systems, one faces much difficulty in quantitation of flavor components. The method described herein seems readily adaptable to such situations. As described for carbonyls, the method could be used for quantitation of several components simultaneously. The resolution of mixtures using the thin-layer chromatographic method is so indicated. The method should also be adaptable to other components of food systems, providing methods for purification and quantitation are available.

The multitude of chemical components isolated from food systems particularly heat-processed food systems, makes the task of quantitative flavor analysis an unenviable one; yet it is possible in many instances to select compounds on the basis of their aroma properties. By careful selection it may be possible to apply quantitative procedures in such a manner as to elucidate the innermost mysteries of flavor chemistry.

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Ms. received 3/28/68; accepted 9/5/69.

Paper No. 2638 of the Journal Series of the North Carolina State University Agricultural Experiment Station, Raleigh, North Carolina.

The author is indebted to E.B. Williams for technical assistance in this work. Discussions of isotope procedures with W.M. Walter and A.E. Purcell greatly facilitated planning and execution of the research.

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A Research Note

Changes of Some Protein Fractions of Beef Muscle Postmortem

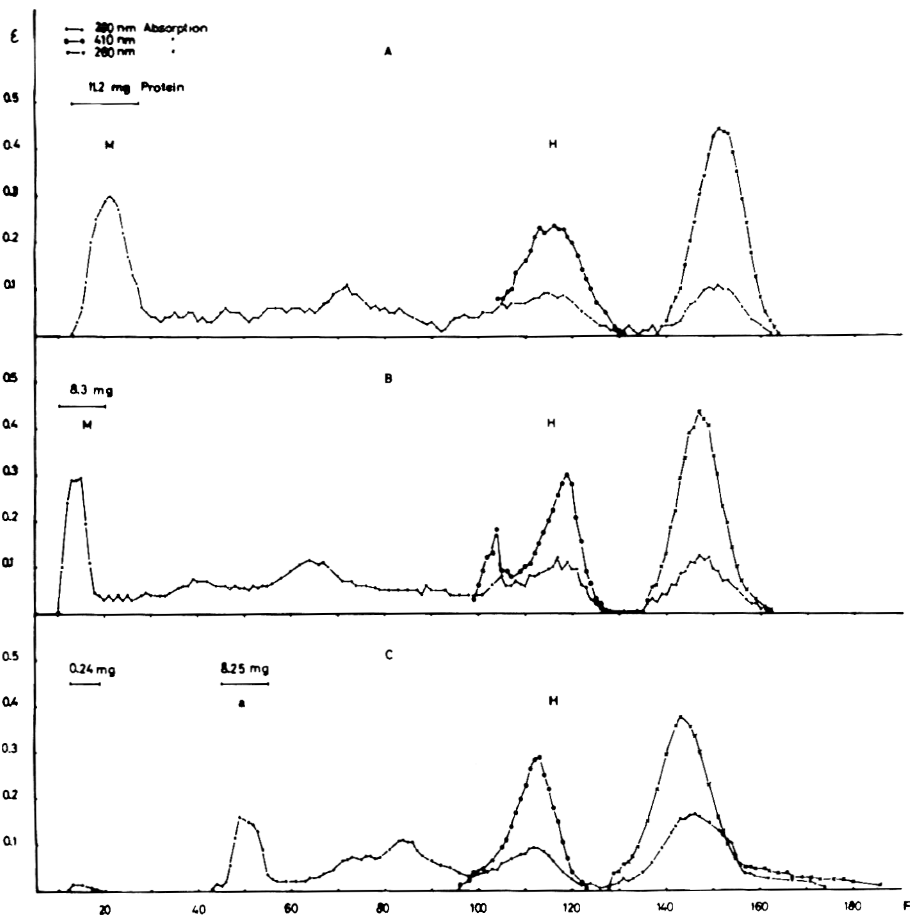

Fig. 1—Gel filtration of total extract of beef muscle at different times postmortem.

A = fresh muscle
B = muscle 5 days old
C = muscle 7 days old
Extraction buffer: 0.5 M KCl, 0.001 M Tris and 0.1 M pyrophosphate
Elution buffer: 0.5 M KCl with 0.0001 M Tris
Rate of elution: 12–15 ml/hr
Gel: Sephadex G 200
Column: 1000 x 25 mm

LITTLE IS KNOWN about postmortem proteolytic reactions in muscle. It has been reported (Fujimaki et al., 1965; Soloviev et al., 1964), that myosin decreases during postmortem aging, but the degradation products remain unidentified. We have confirmed the disappearance of myosin and the appearance of degradation products after 4 days, one of which was still present after 7 days. By means of a tissue press (Harvard Apparatus Co., Dover, Mass.) followed by gel filtration (Pharmacia, Uppsala, Sweden), myosin was isolated in the same manner as was major degradation product (Fig. 1).

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Both proteins (M and a) were characterized by their peptide maps (fingerprint) after tryptic digestion (Crestfield et al., 1962; Jones, 1964; Szent-Györgyi, 1952), by ATP-ase activity (Kielly et al., 1956), by SH-groups content (Benesch et al., 1950), by sedimentation constant and binding capacity with actin (Kuschinski et al., 1951). The myosin exhibited no change in viscosity following addition of ATP (Kuschinski et al., 1951; Mommaerts, 1952) and was apparently free of actin. The degradation product exhibited a reduction of ATP-ase activity and SH-groups content, to values approximately half those of the myosin after aging 6 days. The sedimentation constant decreased from 6.2 to 4.1.

The comparison of peptide maps (Cellulose powder MN 300 HR. Macherey und Nagel, Düren, Germany on 350 × 320 mm plates) of myosin and of the degradation product showed a lack of 18 peptide spots of the original 75 myosin peptides and the appearance of 8 new peptides in the degradation product. The other peptides appear identical according to their location. Remarkable is a yellow colored basic proline peptide (stained with ninhydrine) on both peptide maps. Although it is probable that myosin can be regarded as the origin of the degradation product because the only ATP-ase activity of the gel filtration fractions was found in fraction a, the final proof can be given only after investigation of the other proteins of muscle and connective tissue. A microbial cause can be excluded.

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Ms. received 5/23/68; revised 4/21/69; accepted 9/2/69.

The author thanks Prof. Walter Jennings for his stylistic advice.