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JOURNAL
of FOOD SCIENCE

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TRACE COMPONENTS OF THE FLAVOR FRACTION OF MAPLE SYRUP. V. J. FILIPIC, J. C. UNDERWOOD & C. J. DOOLEY. *J. Food Sci.* **34**, 105–110 (1969)—Several previously undetected flavor-related compounds were found in trace amounts. Among these were the aromatic compounds acetovanillone, guaiacyl acetone and vanilloyl methyl ketone. Sugar degradation products found were furfural, hydroxymethylfurfural, lactic acid and levulinic acid. Other components were the C_7 to C_9 aliphatic acids and oxalic, fumaric and malic acids. All of the acids occurred as ethyl esters resulting from unintentional esterification during extraction.

ELECTROPHORETIC COMPARISON OF ANTHOCYANIN PIGMENTS IN EIGHT VARIETIES OF SOUR CHERRIES. J. H. von ELBE, D. G. BIXBY & J. D. MOORE. *J. Food Sci.* **34**, 113–115 (1969)—Cyanidin-3-gentiobioside and cyanidin-3-rhamnoglucoside were found to be the major anthocyanin pigments in eight varieties of sour cherries—Marasca Moscata, Wczesna Z Prinn, Del Nord, Königliche Amarelle, Triaux, Schattenmorelle, Marasca di Ostheim and Flemish Red, using electrophoresis and paper chromatography with Montmorency pigments as standards. All except Flemish Red contained cyanidin-3-monoglucoside as a minor component. A second minor component of cyanidin-3-diglycoside was found in Wczesna Z Prinn, Del Nord, Schattenmorelle, and Marasca di Ostheim. In all cases, relative concentrations of major pigments approximated those of Montmorency.

POST-MORTEM CHANGES IN SUBCELLULAR FRACTIONS FROM NORMAL AND PALE, SOFT, EXUDATIVE PORCINE MUSCLE. 1. Calcium Accumulation and Adenosine Triphosphatase Activities. M. L. GREASER, R. G. CASSENS, E. J. BRISKEY & W. G. HOEKSTRA. *J. Food Sci.* **34**, 120–124 (1969)—Myofibrillar, mitochondrial, heavy sarcoplasmic reticulum and light sarcoplasmic reticulum fractions were isolated by differential centrifugation of homogenates from normal and pale, soft, exudative (PSE) porcine muscle at various times post-mortem. The oxalate-stimulated calcium accumulating ability declined 5-10 fold by 24 hr post-mortem. The major part of this decline occurred in the first hour after death in fractions from PSE muscle but was more gradual in the normal fractions. The ATPase activities of normal and PSE fractions obtained at death did not differ significantly.

COMPARATIVE RATES OF IMP DEGRADATION IN UNFROZEN AND FROZEN-AND-THAWED (SLACKED) FISH. B. KEMP & J. SPINELLI. *J. Food Sci.* **34**, 132–135 (1969)—English sole and rainbow trout showed slight increases in the rate of IMP degradation when they were frozen and then thawed within 48 hr. Silver salmon and halibut that were frozen and then thawed within 48 hr showed no change in the rate of IMP degradation. Halibut, however, that was frozen and stored at −20°F for 3 months showed a slight decrease in the rate of IMP degradation after it was thawed; but king salmon handled under the same conditions did not. The method of kill or freezing the fish either pre- or post-rigor did not alter the rate of IMP degradation after the fish was thawed.

LIPID OXIDATION AND PIGMENT CHANGES IN RAW BEEF. BARBA E. GREENE *J. Food Sci.* **34**, 110-113 (1969)—Lipid oxidation in ref erated, raw ground beef is accompanied by rancid odors which easily detectable after cooking as well as before. Antioxidants, incluc butylated hydroxyanisole and propyl gallate, prevent lipid oxidation flavor changes. They also protect color by retarding the formation metmyoglobin. Air tight vs. oxygen permeable packaging is investiga

CONNECTIVE TISSUES FROM NORMAL AND PSE PORCINE MUSC 1. Chemical Characterization. P. E. McCLAIN, A. M. PEARSON, J. BRUNNER & G. A. CREVASSE. *J. Food Sci.* **34**, 115–119 (1969)—epimysial connective tissue from normal and PSE (pale, soft and exu tive) porcine muscle was chemically characterized. Samples from muscles exhibited markedly lower turbidity values ($P < 0.01$) than th from normal muscles. No significant differences were found in ultim pH values. The epimysium from PSE muscles contained significa higher ($P < 0.05$) quantities of salt soluble tropocollagen and a grea amount of heat labile collagen ($P < 0.01$) than that from normal tissu No significant differences in α- and β-subunit composition, plasma hydrc proline levels or amino acid composition were evident between the sources of epimysial connective tissues.

POST-MORTEM CHANGES IN SUBCELLULAR FRACTIONS FROM NORM AND PALE, SOFT, EXUDATIVE PORCINE MUSCLE. 2. Electron Microsco M. L. GREASER, R. G. CASSENS, E. J. BRISKEY & W. G. HOEKST *J. Food Sci.* **34**, 125–132 (1969)- Myofibrillar, mitochondrial, heavy sar plasmic reticulum, and light sarcoplasmic reticulum fractions were lated from homogenates of normal and pale, soft, exudative (PSE) porc muscle at 0 and 24 hr post-mortem and examined by electron microsco No differences were observed between normal and PSE myofibrils tained at death. PSE myofibrils prepared at 24 hr post-mortem had m granular appearing filaments and wider Z lines than normal myofibrils 24 hr.

EFFECT OF FEEDING AND WITHDRAWAL OF MENHADEN OIL ON T ω3 AND ω6 FATTY ACID CONTENT OF BROILER TISSUES. D. MILL K. C. LEONG & P. SMITH JR. *J. Food Sci.* **34**, 136-141 (1969)—Anal was made of the fatty acid composition of liver, adipose fat, thigh a breast muscles of broilers fed corn-soy commercial-type of diets conta ing one of two levels of fish oil (2.5 or 5.0%). The oil was subsequer continued, withdrawn or replaced with yellow grease 2, 3 or 4 we before termination of the experiment at the 8th week. The tissue c tents of four ω-3-type fatty acids (20:4, 22:5 and 22:6) were increa in relation to the number of weeks menhaden fish oil was included in diet and were significantly correlated to organoleptic scores. Liver h highest total content of the ω3 fatty acids; the adipose fat, the least; muscles, intermediate.

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.ATIVE DIFFERENCES IN TENDERNESS OF BREAST MUSCLE IN NOR- L AND TWO DYSTROPHIC MUTANT STRAINS OF CHICKENS. D. W. 'ERSON & A. L. LILYBLADE. *J. Food Sci.* **34**, 142–145 (1969)—Cooked ıples of pectoralis major muscles from one normal and two mutant ıtrophic lines of chickens were compared for tenderness using a me- nical device. The muscles from one of these lines, characterized by tial atrophy, were found to be significantly more tender than normal scles, whereas the muscles from the second mutant line, characterized hypertrophy, were much tougher than normal muscles. Cooking weight ıes were much greater in both of the dystrophic mutant lines than in normal line. Some relationships between composition of the three scle types and their textural qualities are discussed.

STUDY OF SARCOPLASMIC PROTEINS OF PORCINE MUSCLE BY ıRCH GEL ELECTROPHORESIS. L. L. BORCHERT, W. D. POWRIE & I. BRISKEY. *J. Food Sci.* **34**, 148–153 (1969)—There were no discerni- differences between the starch gel electrophoretograms of sarcoplasmic teins extracted from pre- and post-rigor muscle. Likewise, there were visually detectable differences between the starch gel electrophoreto- ms of sarcoplasmic proteins extracted from normal and pale, soft, dative (PSE) muscles. Furthermore, the preservation of normal color ı gross morphology through liquid nitrogen ($L\text{-}N_2$) treatment did not ə rise to electrophoretic differences when compared with either normal pale, soft, exudative (PSE) muscle. Although sarcoplasmic protein ıbility diminished in PSE muscle, this loss did not necessarily manifest lf in the preferential denaturation of a specific sarcoplasmic protein.

SESSMENT OF GREEN TUNA: DETERMINING TRIMETHYLAMINE OX- AND ITS DISTRIBUTION IN TUNA MUSCLES. M. YAMAGATA, K. RIMOTO & C. NAGAOKA. *J. Food Sci.* **34**, 156–159 (1969)—To ess green meat of tuna in preparation for canning, it is necessary to ermine the trimethylamine oxide (TMAO) content of the raw meat. A ple and rapid TMAO determination method combined with a reduc- ı method, which converts TMAO to trimethylamine (TMA), was devel- ed. Using this method, the distribution of TMAO content in the scles of yellowfin tuna was investigated to determine the most suitable ıpling portion of the fish. A recommended practical method of sam- ıg and determining TMAO for assessing green tuna is presented.

RCOLEMMAE FROM CHICKEN SKELETAL MUSCLE. 1. Preparation. O. HULTIN & C. WESTORT. *J. Food Sci.* **34**, 165–171 (1969)—Sev- l factors which govern the ability to prepare sarcolemmae largely free other cellular components have been studied in this report. The pose was to obtain a procedure which would allow variation of pre- ative conditions over a wide range so that the enzymatic constitution sarcolemmae could be studied. A major step involved aging of the ised whole muscle for 4 hr at 0–4°C. Protein solubilization during ıg is not involved nor are there major changes in several classes of osphorus compounds.

SMOKE FLAVOR AS RELATED TO PHENOL, CARBONYL AND ACID CONTENT OF BOLOGNA. L. J. BRATZLER, M. E. SPOONER, J. B. WEATHERSPOON & J. A. MAXEY. *J. Food Sci.* **34**, 146–148 (1969)—Phenol, carbonyl and acid determinations were made on layers using bologna that had been smoked during processing. Corresponding samples were evaluated for intensity of smoke flavor by taste panel. Correlation coefficients between phenols, carbonyls, acids and taste panel evaluation of smoke flavor intensity were 0.81, 0.37 and 0.32, respectively. Thus, phenols appear to be the principal contributors to smoke flavor and aroma.

ISOLATION OF STRAWBERRY ANTHOCYANIN PIGMENTS BY ADSORPTION ON INSOLUBLE POLYVINYLPYRROLIDONE. R. E. WROLSTAD & T. B. PUTNAM. *J. Food Sci.* **34**, 154–155 (1969)—A procedure for isolation of anthocyanin pigments from a water extract of strawberries by adsorption on insoluble polyvinylpyrrolidone followed by elution with methanolic HCl is described. This technique gives a high recovery of the total anthocyanin pigments in a nonselective manner without any evidence of degradation.

OXIDATION EFFECTS IN A FREEZE-DRIED GELATIN-METHYL LINOLEATE SYSTEM. A. ZIRLIN & M. KAREL. *J. Food Sci.* **34**, 160–164 (1969)—Freeze-dried model systems containing gelatin and methyl linoleate were incubated at 50°C. Incubation for 5–10 days in absence of water caused a drop in viscosity of gelatin solution, an increase in solubility in ethanol-rich solvents, and other changes indicative of oxidative degradation of the protein. There were no such changes when the linoleate was extracted prior to incubation. High relative humidities during incubation minimized the above changes but in some cases led to partial insolubilization of gelatin.

SARCOLEMMAE FROM CHICKEN SKELETAL MUSCLE. 2. Properties. H. O. HULTIN & C. WESTORT. *J. Food Sci.* **34**, 172–176 (1969)—Sarcolemmae are usually identified solely by morphological characteristics. We have determined several chemical and enzymic properties of sarcolemmae from chicken breast muscle. The phospholipid content of the sarcolemmae was low, but this may be because the plasma membrane represents only a small portion of the sarcolemma. The sarcolemmae either have many of the properties of microsomes or are contaminated with subcellular particles rich in certain constituents normally considered to be associated with microsomes, viz., RNA and glucose-6-phosphatase. Lactate dehydrogenase was shown to be associated with the sarcolemmae.

ABSTRACTS:
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TENDERIZATION OF CHICKEN MUSCLE: THE STABILITY OF ALKALI-INSOLUBLE CONNECTIVE TISSUE DURING POST-MORTEM AGING. D. de FREMERY & I. V. STREETER. *J. Food Sci.* **34**, 176–180 (1969)—Alkali-insoluble connective tissue of raw breast and thigh muscles from chicken broilers (measured by alkali-insoluble hydroxyproline) was unchanged during post-mortem aging. When breast muscles were cooked after short or long aging periods, no connective tissue changes resulted from aging. Hence, there is no correlation with shear resistance (toughness) which decreases markedly during post-mortem aging. Breast and thigh shear resistance was highest at 3 to 4 hr post-mortem; breast muscle reached a minimum at 12 hr and remained unchanged for 8 days; in thigh muscle tenderization continued throughout the 8-day period.

MOLECULAR PROPERTIES OF POST-MORTEM MUSCLE. 6. Effect of Temperature on Protein Solubility of Rabbit and Bovine Muscle. H. M. CHAUDHRY, F. C. PARRISH JR. and D. E. GOLL. *J. Food Sci.* **34**, 183–191 (1969)—The effects of temperature on the actin-myosin interaction in rabbit and bovine muscle during rigor and post-rigor aging were investigated. Muscle was stored at four different temperatures (2°, 16°, 25° and 37°) corresponding to three types of post-mortem shortening: cold, minimal and high temperature. Although time and temperature of post-mortem storage caused sizable alterations in protein solubility, these alterations could not be directly related to changes in tenderness or sarcomere length or to species differences in the effects of temperature on post-mortem shortening. Myofibrillar protein extracts did not exhibit any typical actomyosin peak in the analytical ultracentrifuge.

SEMIAUTOMATED THIOCYANATE METHOD FOR DETERMINING PEROXIDE VALUES OF LIPIDS. J. A. STARKOVICH & W. T. ROUBAL. *J. Food Sci.* **34**, 194–195 (1969)—A semiautomated liquid-flow arrangement, designed for use in monitoring peroxide values of extracted oils, is presented. Twenty samples per hour can be measured by means of a system using conventional manifold tubing and AutoAnalyzer components.

TANNINS OF GRAIN SORGHUM: LUTEOFOROL (LEUCOLUTEOLINIDIN), 3′,4,4′,5,7-PENTAHYDROXYFLAVAN. E. C. BATE-SMITH & V. RASPER. *J. Food Sci.* **34**, 203–209 (1969)—The principal tannin of sorghum is a leuco-anthocyanin yielding luteolinidin (3′,4′,5,7-tetrahydroxy-flavylium) when heated with mineral acid. The precursor, luteoforol, has most of the properties of 3′,4,4′,5,7-pentahydroxyflavan prepared by reduction of eriodictyol. Luteoforol, when treated with concentrated mineral acid in the cold, gives a purple color with λ_{max}550nm. The results with a number of varieties of sorghum are compared with those obtained by the AOAC Folin-Denis method. The contribution of luteoforol to the "tannin" so determined varies from 1 to $\lesssim$ 25%. Except for one sample of Kaffir corn, which contained leucocyanidin as well as luteoforol, no other tannins were detected. The "tannin" content varied widely (from 0.05 to 0.67% as tannic acid), a white-skinned variety having the least.

THE DETECTION AND ENUMERATION OF Clostridium perfringens IN FOODS. W. M. HALL, J. S. WITZEMAN & R. JANES. *J. Food Sci.* **34**, 212–214 (1969)—A method that uses SPS agar and incorporates an improved egg yolk agar and nitrate motility medium has been developed for the enumeration and confirmation of vegetative cells and/or spores of *Clostridium perfringens* in foods. The method is based upon several diagnostic criteria and can be completed within 48 hr.

THE USE OF CRYOSCOPY IN ENZYMATIC STUDIES. W. F. SHIPE & S BREDDERMAN. *J. Food Sci.* **34**, 181–182 (1969)—The feasibility of u: cryoscopy as a practical tool for measuring changes in water-sol components of five enzyme-substrate mixtures was investigated. Ce lase, pectinase, pectin esterase, alpha-amylase and beta-amylase w studied. Viscosity measurements were run simultaneously with freez points as a conventional method for determining enzymatic acti Measurements taken at periodic intervals revealed changes in t viscosity and freezing-point values. A comparison of the two sets values can be useful in assaying the purity of commercial enzyme pre rations.

CHANGES IN MOLECULAR WEIGHT OF PECTIN DURING METHYLATI WITH DIAZOMETHANE. C. J. B. SMIT & E. F. BRYANT. *J. Food Sci.* 191–193 (1969)—Molecular weight values decreased with increas esterification. By using acid and enzymatic demethylation, it was sho that (1) change in molecular weight during methylation is a result depolymerization and (2) a change in the percent methoxyl groups pr ably has very little effect on the change in molecular weight dur methylation or demethylation with pectins having an ester con between 60 and 95%. Gel characteristics of the prepared samp changed with a change in methoxyl content or molecular weight.

MOLECULAR PROPERTIES OF POST-MORTEM MUSCLE. 7. Changes Nonprotein Nitrogen and Free Amino Acids of Bovine Muscle. F. C. P RISH, JR., D. E. GOLL, W. J. NEWCOMB II, B. O. de LUMEN, H. CHAUDHRY & E. A. KLINE. *J. Food Sci.* **34**, 196 202 (1969)—Prote sis and its relationship to tenderness were studied by measuring r protein nitrogen (NPN), free amino groups, and shear resistance due post-mortem aging of bovine muscle. Both NPN and free amino gro increased during post-mortem aging, indicating some degradation of p tein and/or peptides. However, neither the increase in NPN nor f amino groups was related to post-mortem tenderization since the f amino groups increased only after most of the improvement in tenderr had occurred. Evidence for and against the role of cathepsins in p mortem tenderization is discussed and possible sites of post-mortem alt tions of myofibrils are indicated.

DESTRUCTION OF TRICHINA LARVAE IN COOKED PORK ROASTS. A CARLIN, C. MOTT, D. CASH & W. ZIMMERMANN. *J. Food Sci.* **34**, 2 212 (1969)—Roasts were cooked to end-point temperatures of 120, 1 130, 135, 140, 145, 150 or 160°F in electric household ranges at o temperatures of 200, 325 or 350°F. Samples of the cooked roasts w artificially digested; the recovered larvae were examined for viabi Also samples were fed to rats; 4 weeks later the rats were killed examined for presence of trichinae by ingesting aliquots of ground tissue. All larvae were destroyed in roasts cooked to 140°F or higl viable larvae were found in some of the roasts cooked to 135°F . in all of the roasts cooked at 130°F or lower. Thus the thermal de point of trichinae is between 135° and 140°F.

MEASUREMENT OF THE RELATIVE SWEETNESS OF SELECTED SWE ENERS AND SWEETENER MIXTURES. H. STONE & S. M. OLIVER. *J. F Sci.* **34**, 215–222 (1969)—Relative sweetness of the simple sugars, sucr dextrose and fructose, and the synthetic sweeteners, calcium cyclam and sodium saccharin, was measured by magnitude estimation. Chang the reference or reference concentration resulted in shifts in the rela sweetness values for a sugar; however, these were consistent at all c centrations tested. Mixtures of dextrose-fructose, dextrose-sucrose sucrose-fructose were studied at several concentration combinations.

V. J. FILIPIC,* J. C. UNDERWOOD and C. J. DOOLEY
Eastern Utilization Research and Development Division, ARS
U. S. Department of Agriculture, Philadelphia, Pennsylvania 19118

Trace Components of the Flavor Fraction of Maple Syrup

SUMMARY—An exhaustive chloroform extraction of maple syrup removed the maple flavorants. The extract was analyzed in part by a gas chromatograph-mass spectrometer tandem procedure. Several previously undetected flavor-related compounds were found in trace amounts. Among these were the aromatic compounds acetovanillone, guaiacyl acetone and vanilloyl methyl ketone. These aromatics could have resulted from the ethanolysis of ligneous material previously reported in maple sap. Sugar degradation products found were furfural, hydroxymethylfurfural, lactic acid and levulinic acid. These indicate that the products of caramelization also are part of the maple flavorants.

Acids found, in addition to those above, were the C_6 to C_9 aliphatic acids and oxalic, fumaric and malic acids. All of the acid occurred as ethyl esters resulting from unintentional esterification during extraction. The C_8 to C_9 acids may be artifacts perhaps derived from the vegetable oil used as antifoaming agent in syrup processing.

INTRODUCTION

EARLIER PUBLICATIONS have described the use of chloroform to isolate the maple flavor from the sugar in maple syrup (Underwood *et al.*, 1961, 1963). Vanillin, syringaldehyde and dihydroconiferyl alcohol were separated and identified from chloroform extracts, using both column and gas chromatography. Later, through the use of more refined techniques, the more volatile ketolic compounds, methylcyclopentenolone, acetoin and acetol were identified in such an extract (Filipic *et al.*, 1965).

However, many small peaks on the gas chromatograms of the chloroform extracts made in these studies indicated the presence of more flavor components in trace amounts. To identify as many of these compounds as possible analysis has been made of a chloroform extract of maple syrup. The concentrated extract and some individual gas chromatographic fractions were subjected to a GLC-mass spectrometer tandem procedure which proved most successful with the more volatile components of the extract. This paper reports the results of the work covering isolation and identification of 34 different compounds, a few of which are only tentatively identified.

EXPERIMENTAL

Preparation of the chloroform extract

To insure obtaining the maximum amount of the chloroform soluble materials from the maple syrup, a more rigorous extraction procedure was used in this study than in earlier work. A No. 1 or A grade commercial maple syrup containing a high level of distinctive maple flavor was used for this study.

Forty gal of syrup were extracted in approximately 3 gal batches with analytical grade chloroform using an all-glass, steam-heated, 50 L liquid-liquid extraction apparatus. Each 3 gal batch was extracted continuously for 24 hr with fresh solvent from the 25 L reservoir of $CHCl_3$ in the extraction. Thus, the syrup was subjected to a longer period of treatment at a higher temperature (25–60°C) than earlier extractions. Further, the $CHCl_3$ solubles removed from the syrup were subjected to the temperature of boiling $CHCl_3$ (60°C) in the solvent reservoir.

The solvent in the reservoir was used to treat three batches of syrup before it was removed for a fresh charge. The 25 L of solvent thus obtained was concentrated to about 4 L in the solvent reservoir of the apparatus by distilling off $CHCl_3$ using steam heat. The four 4 L volumes of extract resulting from the treatment of the 40 gal of syrup were combined and allowed to evaporate at room temperature in a hood through which a stream of air was moving. The extract was thus concentrated to 2 L at which point the solution became saturated in respect to the least soluble of the constituents in it. This concentrate was stored in a glass-stoppered bottle.

Ether-treated chloroform extract

In earlier work with $CHCl_3$ extracts of maple syrup, it was learned that much improved GLC separations could be made if the lignin-like material in $CHCl_3$ were removed. Therefore, a 500 ml aliquot of the gross $CHCl_3$ concentrate was shaken with 2000 ml of peroxide-free diethyl ether and the precipitate filtered off using a sintered glass filter. The filtered solution was then concentrated to 100 ml as described for the original $CHCl_3$ extract. This purified extract was again stored in a glass-stoppered bottle for the GLC work.

Gas chromatographic fractionation

Thirty-five gas chromatographic fractions were collected by a previously described technique (Filipic *et al.*, 1965). A dual column chromatograph with the F & M Model 720 thermal conductivity detector was used. The dual columns were packed with 20% Carbowax 20M on 60–80 mesh acid-washed Chromosorb W and the oven programmed from 50°C to 240°C at 4° per min. The initial temperature was held for 16 min after injection to permit complete elution of the solvent and the final temperature for 50 min to allow for elution of the major high boilers. The reproducible chromatogram obtained is illustrated in Fig. 1. Fig. 2, 3 and 4 show the temperature range of each fraction in greater detail.

Fractions 1–9 were rechromatographed on the same Carbowax 20M column; fractions 10–34 on a silicone SE-30 column. As in the previous study, subfractions were collected in melting point capillary tubes. In all cases

* Deceased.

where sufficient isolate was collected, the infrared and mass spectra were obtained. In addition, fractions 1–14 were analyzed by the combined gas chromatogram-mass spectrometer technique.

This gas chromatograph was an F & M 810 equipped with a flame ionization detector and a 1:10 splitter at the column exit. A portion of the effluent from the chromatograph was diverted into the ion source of a CEC-103 C mass spectrometer via a heated valving system. No helium separator was used and that amount of effluent sufficient to raise the pressure to 7×10^{-6} torr was introduced.

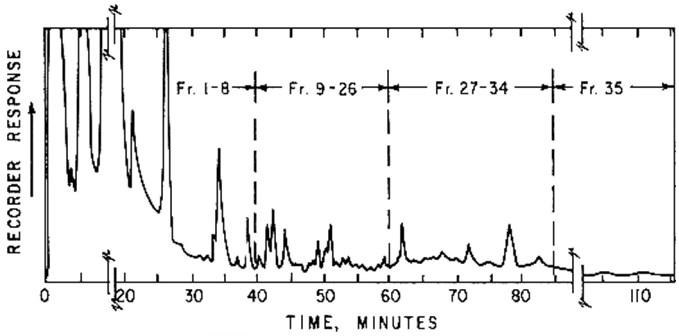


Fig. 1. The chromatogram of the exhaustive extract of maple syrup (attenuation 4x).

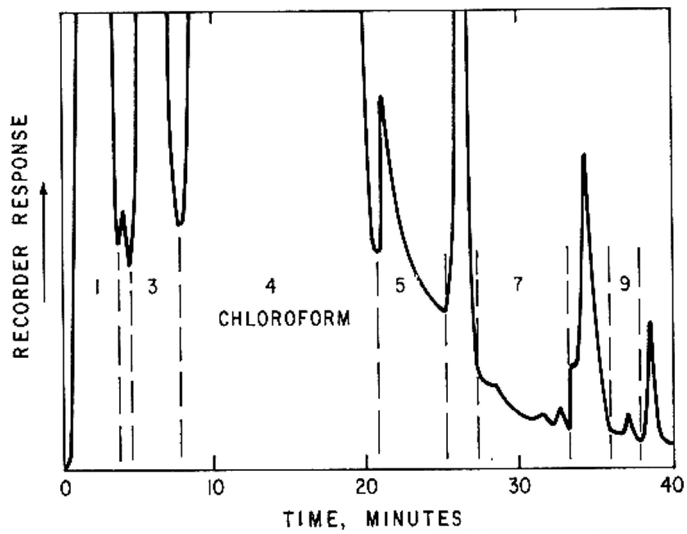


Fig. 2. Fractions 1–10, illustration of retention range of collection.

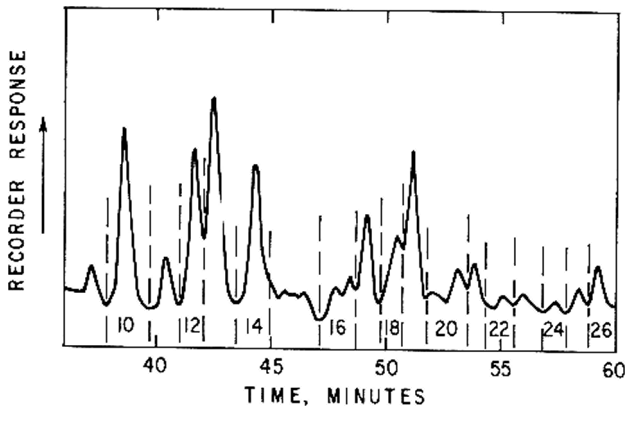


Fig. 3. Fractions 9–26, illustration of retention range of collection.

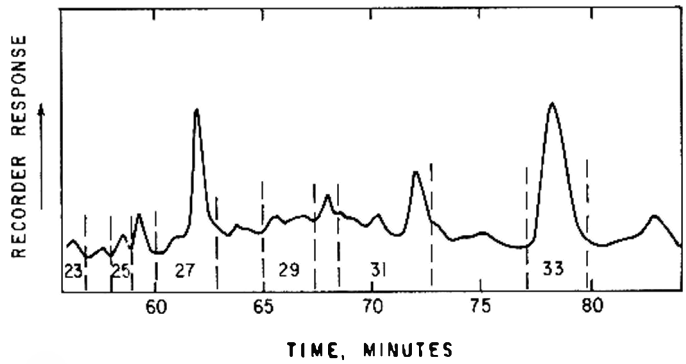


Fig. 4. Fractions 23–34, illustration of retention range of collection.

RESULTS AND DISCUSSION

THE COMPOSITION of all the gas chromatographic fractions is summarized in Table 1. Whenever possible the spectral methods of identification were supplemented either by cochromatography of a standard with the unknown or by a comparison of retention times.

Spectral interpretations

The majority of the compounds listed in Table 1 are the products of ethanolysis resulting from the prolonged extraction by refluxing chloroform containing 1% ethanol as a preservative. In spite of this solvent interaction most of the compounds identified can be classified into four major categories: solvent-related impurities, contaminants and artifacts, constituents of ligneous origin and constituents of carbohydrate origin.

Three of the components cannot be so described; these were the ethyl esters of nonvolatile acids present in maple syrup (Porter *et al.*, 1951) diethyl malate, diethyl fumarate and ethoxydiethylsuccinate. Some of the identifications are tentative because standards were not available for comparison of spectral and chromatographic data.

A component of fraction 26 is suspected to be ethoxymethylfurfural (EMF) mainly on the basis of infrared and mass spectral comparisons with hydroxymethylfurfural (HMF). The infrared spectrum of EMF differed notably from that of HMF by the absence of a hydroxyl band near 3.0 μ and the appearance of a strong band at 9.1 μ, in the C-O-C asymmetric stretching region of aliphatic ethers (Colthup *et al.*, 1964). However, the normally strong hydrogen stretching vibrations of the furan ring in the 12–13 μ region were of low to medium intensity, possibly due to impurity of the isolate. The mass spectrum showed a parent peak of 154 with some of the prominent fragments having masses of 125 (M-29), 109 (M-45), 97 (M-57) and 39 which would be expected from this type of substituted furan (Grigg *et al.*, 1965) as illustrated in Fig. 5.

The major component of fraction 30 was suspected to be ethoxy diethyl succinate, which could result from the further ethanolysis of diethyl malate. Some corroboration of this assumption can be elicited from a comparison of the

Table 1. Composition of GLC fractions and bases of identification.

Fraction number	Constituents	Spectral methods		GLC Methods	
		Infrared	Mass spec.	20 M	SE 30
1	Ether*	....	G	C	....
2	Ethyl acetate*			C	C
3	Ethyl alcohol*	S	G, M	R	...
4	Chloroform*		G	R	...
5	Two silicones*		G	....	...
6	Diethyl carbonate*	S	G, M	C	...
7	Ethyl valerate	...	G	R	
	Several silicones*	....	G	....	....
8	A chlorinated artifact*	...	G	....	...
	A silicone	...	G	....	...
	Ethyl caproate	...	G	R	....
9	Acetol*	....	G	C	C
10	Ethyl lactate*	S	G	R	...
	Ethyl heptoate	....	G	R	
11	A chlorinated artifact*	....	G	..	..
12	Phenetole	....	G	R	...
	A silicone*	....	G	...	....
13	Ethyl octanoate	....	G	R	...
	A chlorinated artifact*	....	G	....	....
14	Diethyl oxalate*	....	G	R	...
	Furfural	....	G	R	..
	1,1,2,2-tetrachloroethane	....	G	R	.
15	Ethyl nonanoate*	....	M	R	..
	A silicone	K	..	....	.
16	Two silicones*	K	M	....	....
17	Ethyl levulinate*	....	M	R	....
18	A hydrocarbon*	K	M	....	....
19	Diethyl fumarate*	K	M	R	....
20	Unknown* (1)	....	M	....	....
21	A hydrocarbon*	K	M	....	....
22	Unknown* (2)	....	M	....	....
23	Methylcyclopentenolone*	....	M	C	R
24	No major component	....	....	....	....
25	Unidentified*	....	...	....	...
26	Ethoxymethylfurfural (5)	K	M	....	
27	Diethyl malate*	K	M	C	R
28	No major component		....	...	....
29	No major component	....	....	....	....
30	Ethoxydiethyl succinate* (5)	K	M	....	....
	Ethyl palmitate	K	M	R	....
31	Para ethoxy phenol*	K	M	R	....
	Ethyl phthalate	K	M	R	....
32	Hydroxymethylfurfural	K	M	C	....
	Ethyl stearate	K	M	R	R
	Ethyl oleate	K	M	R	R
33	Vanillin*	K	M	C	R
34	Acetovanillone	K	M	C	R
	Guaiacyl acetone	K	M	....	....
	Ethyl vanillate	K	M	C	R
	Unknown (3)		M	....	....
	Chloropropyl guaiacol (5)	K	M	....	
35	Vanilloyl acetyl	K	M	....	...
	Syringaldehyde*	K	M	C	R
	Dihydroconiferyl alcohol*	K	M	C	R
	Unknown (4)	....	M	....	....
	Butylphthalylbutylglycollate	K	M	....	....

* Major constituent in fraction.
S Sealed cell.
K Micro KBr pellet.
G Tandem GC-MS.
M Mass spectrograph of a collected GLC fraction.
C Standard cochromatographed with sample.
R Retention time of sample and standard agree.
(1) MS indicates molecular weight of 176.
(2) MS indicates molecular weight of 170.
(3) MS indicates molecular weight of 224.
(4) MS indicates molecular weight of 210.
(5) Identification is tentative because no standard was available for comparison of spectral and chromatographic data.

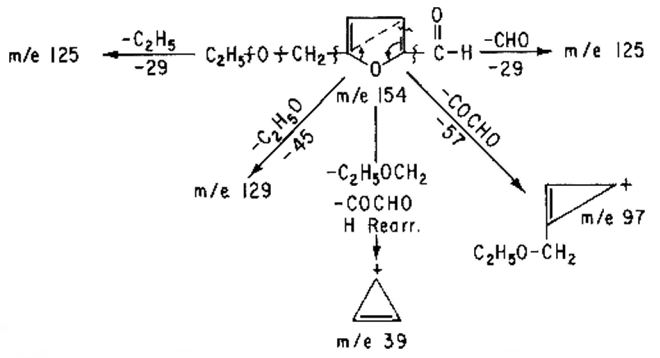
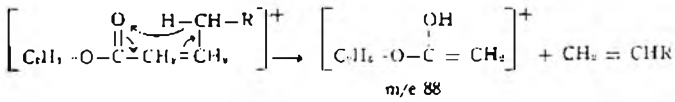


Fig. 5. Probable mass spectral fragmentations of ethoxymethylfurfural.

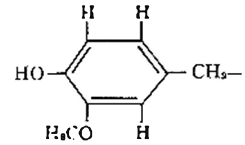
spectra in Fig. 6. Again the hydroxyl band is greatly diminished in the unknown. Also the band at 12.75 μ which could be ascribed to the CH_2 rocking frequency of an ethyl group (Colthup *et al.*, 1964) is significantly more intense in the unknown.

The mass spectrum is identical to that of diethyl malate with two slight exceptions, an increase in a small m/e 81 (the significance of which we have not deduced) and the disappearance of a small mass at 88. The m/e 88 is commonly found in the spectra of ethyl esters of fatty acids and can occur when a gamma hydrogen is available (Budzikiewicz *et al.*, 1964) as shown:



This type of fragmentation can occur with diethyl malate due to the availability of the hydroxyl hydrogen, but cannot occur and is absent in the spectra of diethyl fumarate and maleate. The parent peaks in the mass spectra of all four of these compounds are virtually absent and cannot be used to aid in structural identification.

The evidence for the tentative identification of chloropropylguaiacol in fraction 34 is based mainly on its mass spectrum. A strong parent peak at 200 m/e with a p + 2 peak of about one-third the intensity indicated a monochloro compound. The fragmentation at m/e 137 and below was very similar to compounds having the structure:



The > $C\text{-}CH_2\text{-}CH_2\text{-}Cl$ group results in two bands (related to the presence of Cl) due to rotational isomers. The planar C-C-C-Cl trans zig-zag form absorbs near 726 cm^{-1} and the gauche C-C-C-Cl form near 649 cm^{-1} (Colthup *et al.*, 1964). Bands at these positions were detected in the infrared spectrum of this isolate. However, these bands were weaker than expected, possibly due to impurity of the isolate.

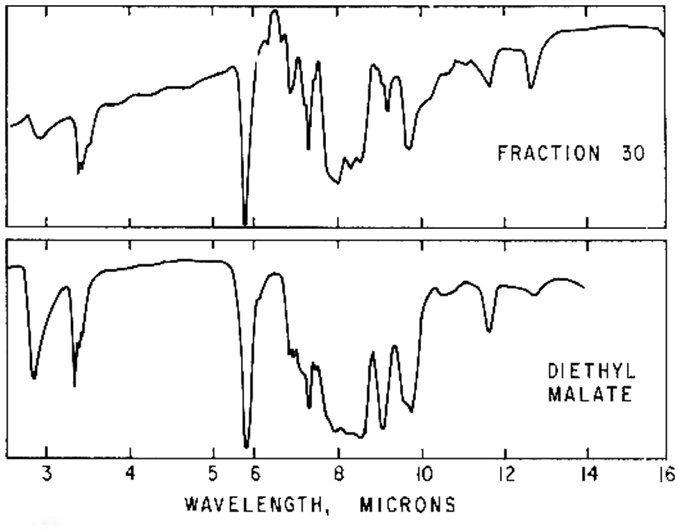


Fig. 6. A comparison of the infrared spectra of fraction 30 and diethyl malate.

Related experimental observations

Another observation may tend to support the foregoing conclusions. In prior extracts, the peak due to dihydroconiferyl alcohol (DHCA) was always more intense in the first chromatogram of the day. But in this extract, a peak in fraction 34 showed that effect and there was no increase in the DHCA peak. Apparently some nonvolatiles in the extracts slowly decompose in the injection port (kept at 140–160°C) to yield DHCA. In this extract, in which a large number of chlorinated artifacts were detected, a chlorinated decomposition product rather than DHCA, was the result.

Mass spectral analysis indicated that trace amounts of alpha ketols were apparently converted to diketones by the gas chromatographic fractionation. The p-2 peaks were far more intense at low concentrations than the expected parent peaks. This was true whether the compound was introduced to the ion source via the tandem GLC system or by the normal sample introduction system. This effect was noted with acetoin, acetol and 5-hydroxymethylfurfuryl, which is an alpha ketol via conjugation.

The final concentrate was also observed to be far less stable than prior extracts. In Fig. 7, the chromatogram of fraction 9 through fraction 23 of the extract, fractionated in this study, is compared to the same chloroform extract immediately after treatment with a fresh batch of ether. In the fresh batch, fractions 9 and 23, which contain acetoin, acetol and methylcyclopentenolone, are much larger. Fractions 17 and 19 which contain ethyl esters of levulinic and fumaric acids are much smaller.

Solvent-related impurities

Four of the compounds listed in Table I are related to the use of ether and chloroform solvents. The chloroform used for extraction contained 1% ethanol as a preservative. Whenever a heated maple syrup was extracted with this solvent, a relatively large amount of diethyl carbonate formed. Apparently, the solvent also contained a trace of 1,1,2,2 tetrachloroethane impurity. The mass spectra indicated the presence of several chlorinated artifacts, one of which is suspected to be chloropropyl guaiacol.

Contaminants and artifacts

The maple syrup analyzed in this study was of commercial origin and was extracted in large scale equipment.

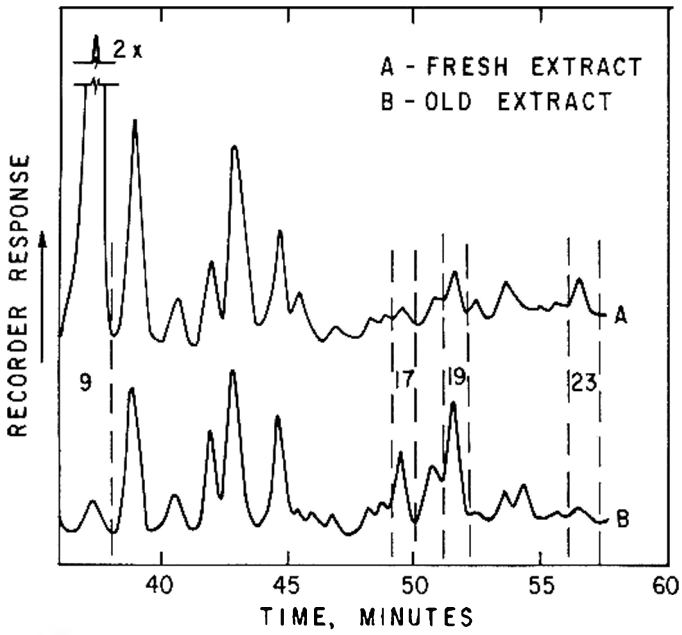


Fig. 7. Comparison of chromatograms obtained from fresh and old extracts of maple syrup.

For one or both of these reasons the extract contained a large number of trace contaminants consisting mainly of unidentified silicones and hydrocarbons. Phthalate esters were another type of contaminant; butylphthalyl butyl glycolate and diethyl phthalate (possibly not a contaminant, see below) are two that were identified in this extract.

Some of the ethanolysis products resulted from interaction with a contaminant. Corn oil was used as an antifoaming agent in the processing of the maple syrup extracted in this study. This accounts for the presence of ethyl oleate, palmitate and stearate. The ethyl esters of the C_5 to C_9 acids were also present, ethyl nonanoate in the greatest concentration. Such an effect points to the breakdown of unsaturated fatty acids, oleic and linoleic, present in the oil. The long chain fatty acid in a previous extract in which extensive ethanolysis did not occur probably was nonanoic acid (Filipic *et al.*, 1965). This compound was the major component in fraction 15 of that prior extract. Fig. 8 is a reproduction of the center portion of the chromatogram obtained with that extract. Phenol, a possible contaminant, was previously detected in unreacted form (fraction 14, Fig. 8) and this time in the form of two ethanolysis products, phenetole and paraethoxy phenol.

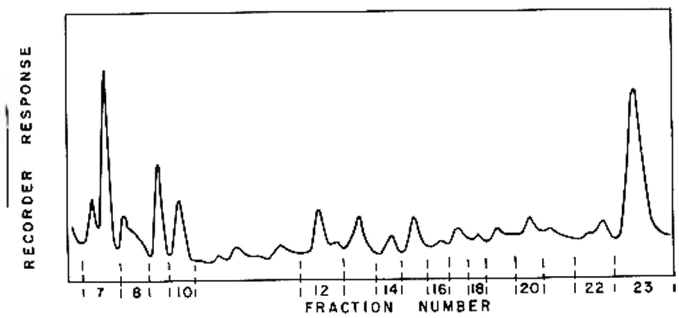


Fig. 8. Fractions 7-23, from a chromatogram of a different maple syrup extracted under milder conditions.

Constituents related to lignin

Vanillin, syringaldehyde and dihydroconiferyl alcohol have been previously reported and ascribed to degradation of ligneous material (Underwood *et al.*, 1964). Acetovanillone (in fraction 34) also is a lignin degradation product of frequent occurrence (Brauns *et al.*, 1960). Vanillin, ethyl vanillate, vanilloyl acetyl and guaiacyl acetone are all products to be expected from the ethanolysis of lignin (Fujii *et al.*, 1966). The unknown in fraction 34 (M.W., 224) may very well be another of Hibbert's ketones, one of the ethoxyguaiacylpropanones. Oxalic and phthalic acids, present here as ethyl esters, are among the chief oxidation products of lignin and wood (Pearl *et al.*; Brink *et al.*, 1966).

Constituents of carbohydrate origin

As might be expected, ethanolysis products of carbohydrate degradations were also present—ethyl lactate, ethyl levulinate and ethoxymethylfurfural. Other products of probable carbohydrate origin were acetol, furfuryl, methylcyclopentenolone and hydroxymethylfurfural.

In the chloroform extracts of any maple syrup that has been freshly extracted under milder conditions than here employed, the predominant constituent is acetol. Methylcyclopentenolone has always been a primary constituent. Inspection of the infrared data from our previous study indicates that the primary component in fraction 8 (Fig. 8) was furfural. Hydroxymethylfurfural has been found in this laboratory to be a major constituent of high flavored maple syrup (maple syrup given additional heat treatment).

Constituents of known flavor effect

In a recent review, Hodge (1967) describes the desirable degree of sugar caramelization to be that point at which fragrant notes are added to the sweetness of the sugar and before further heat treatments develop burnt, bitter and acrid flavors that overcome the sweet. In the same review a table describes the odor and taste of various carbohydrate caramelization and dehydration products. Of such products in the mild chloroform extraction of maple syrup, acetol has a sweet taste and other constituents such as acetoin, furfural and methylcyclopentenolone impart desirable characteristics. Compounds such as levulinic acid and hydroxymethylfurfural which impart sour and bitter flavor notes are present in low concentrations.

Of the compounds deriving from ligneous material in the maple sap, vanillin is apparently the most important with respect to flavor contribution. Because of the very wide range of odor and taste thresholds, one must be very careful in relating flavor effects to the concentration of the flavor components. However, in this case, methylcyclopentenolone (fraction 12, Fig. 8) and vanillin, (fraction 23, Fig. 8) two of the prominent constituents in the previous mild extraction, impart desirable notes to the flavor complex and are known to have extremely low flavor thresholds (Dow Chemical Company 1966).

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Lipid Oxidation and Pigment Changes in Raw Beef

SUMMARY—Two major sources of nonmicrobial deterioration in prepackaged raw meats are the development of off-odors and off-colors. The relationship of these changes to polyunsaturated fatty acid oxidation in the meat was investigated. Lipid oxidation was measured by the thiobarbituric acid test; pigment changes, by reflectance spectrophotometry. Lipid oxidation was found to produce detectable off-odors in raw and subsequently cooked beef. Anaerobic packaging to prevent oxidation of myoglobin and in turn, lipids, appeared to be useful only if packaging (and oxygen removal) could be carried out rapidly and if meat contained sufficient enzyme activity to establish anaerobic conditions quickly and to completely reduce metmyoglobin. Propyl gallate and butylated hydroxyanisole, even under aerobic conditions, offered substantial protection to the fresh meat pigments and at the same time effectively inhibited lipid oxidation.

INTRODUCTION

A COMMON PROBLEM in marketing prepackaged meats is the development of an undesirable brown color after the meat has been cut. This is due to oxidation of the red meat pigments oxymyoglobin (MbO_2) and myoglobin (Mb) to the brown ferric metmyoglobin (MetMb). In addition, there is evidence (Younathan et al., 1960; Brown, et al., 1963) that ferric heme pigments can catalyze oxidation of the tissue lipids in meat. Free radical intermediates from this reaction can decompose hemes, causing loss of color (Haurowitz et al., 1941). In cooked meats, lipid oxidation produces a stale or "rancid" odor. Rancid odors associated with lipid oxidation would also be expected in raw meat, but this problem has not yet been investigated.

To retard both pigment and lipid oxidation in raw meat, two approaches may be taken: (1) preventing MetMb formation to eliminate the lipid oxidation catalyst, and (2) preventing lipid oxidation, by means of an antioxidant to protect the pigment from lipid oxidation intermediates.

The first approach focuses attention on enzymatic reduction of MetMb. Stewart et al. (1965a) have shown that enzymes in raw meat can reduce MetMb under anaerobic conditions. Wrapping the meat in an oxygen-impermeable film usually produces anaerobiosis and pigment reduction. When MetMb has been completely reduced, provided no additional oxygen enters the package, no further oxidation of pigment or lipids should occur. One disadvantage of this approach is that reduced Mb is purple, which is not the typical color the consumer associates with fresh meat. At the present time most meat is packaged in air permeable films. The pigment is in the familiar bright red form of MbO_2. The free exchange of oxygen, however, makes the meat more susceptible to oxidation of both pigment and lipids. An antioxidant might inhibit lipid oxidation and still permit aerobic packaging. If this type of treatment also afforded pigment protection, it might be more advantageous from the standpoint of consumer appeal.

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The present work was done to determine whether lipid oxidation produces rancid odors in raw beef and to determine whether an antioxidant and/or anaerobic packaging can prevent both lipid and pigment oxidation.

EXPERIMENTAL PROCEDURE

BEEF TOP ROUND was used, purchased locally with no knowledge of its previous history. The meat was trimmed of adipose tissue and ground twice in a Hobart grinder fitted with stainless steel and chrome plated parts to avoid contamination with metal ions. Thirty ppm chlortetracycline was thoroughly mixed with the meat immediately after grinding. This eliminated the possibility of off-odors due to bacterial spoilage. The antioxidants tested were butylated hydroxyanisol (BHA), 0.01%; propyl gallate (PG), 0.01%; sodium tripolyphosphate (PP), 0.5%. PG and PP were added as aqueous solutions; BHA as an emulsion (Sustane E, Universal Oil Products Co.). Samples of uniform weight and surface area were packaged either in impermeable Saran (Dow,) or in "Prime Wrap" (Goodyear), which is a highly oxygen-permeable film commonly used by supermarkets for wrapping meat cuts. They were stored at approximately 4°C on a tray in a single layer.

MetMb was measured by reflectance spectrophotometry as described by Stewart et al. (1965b) on a Bausch and Lomb "Spectronic 505" recording spectrophotometer. Lipid oxidation was measured by the thiobarbituric acid (TBA) test (Tarladgis et al., 1960). Results are expressed as "TBA number," meaning mg of malonaldehyde per 1,000 g of meat. A trained panel evaluated the samples for rancid odors. Significance of differences was tested by the Wilcoxon matched-pairs signed-ranks test (Siegel, 1956).

RESULTS

Effect of lipid oxidation on odor of meat

The first experiment was designed to determine whether lipid oxidation actually produces off-odors in raw meat. Samples were stored in the oxygen-permeable wrap. Table 1 gives an example of the results. The BHA- and PG-treated samples were rated significantly higher on sensory scores than the control samples. A TBA number of 0.5 to 1.0 in cooked meat is considered the range in which odor can first be detected (Tarladgis et al., 1959). Odor was first evaluated in raw meat and later in samples that had been cooked after several days of storage in the raw state. Cooking did not destroy the rancid odor. To determine whether BHA and PG could continue protection after the meat was cooked, samples were stored for two days after cooking and assayed again. The results, not presented here, showed that cooking did not destroy the antioxidant properties of these compounds.

Table 1—Effect of antioxidants on odor scores and TBA numbers of raw and subsequently cooked beef.

Experiment	Sample	Days stored	Odor score[1]	TBA No.
1	Control (raw)	2	3.5	5.7
1	Polyphosphate (raw)	2	3.9	4.8
1	BHA (raw)	2	4.9	0.6
2	Control (raw)	8	3.5	12.5
2	PG (raw)	8	5.6	0.9
2	Control (cooked)[2]	8	3.7	16.4
2	PG (cooked)[2]	8	5.4	0.5

[1] All samples with BHA or PG significantly different from controls. Sample with BHA significantly different from sample with polyphosphate. A score of 6 = no detectable off odor; 1 = strong off odor.
[2] Cooked after 8 days of storage raw.

Sodium tripolyphosphate has been used effectively as an antioxidant in cooked meat and fish (Tims et al., 1958; Ramsey et al., 1963). Johnson (1966) found that in raw meat, the phosphate groups were hydrolyzed presumably by phosphatases in the muscle. In order to use PP as an antioxidant it was necessary to first heat the meat to at least 70°C. The data in Table 1 further illustrate its ineffectiveness as an antioxidant in raw meat.

Effect of antioxidants and wrapping material on lipid oxidation and MetMb formation

Table 2 shows the effects of antioxidants on lipid oxidation (TBA number) and MetMb formation in top round samples wrapped in both oxygen permeable and oxygen impermeable films. In this experiment Saran wrap was sufficient to reduce MetMb and prevent its accumulation. By the second day the meat in these packages had turned a deep purple color. The TBA numbers for the untreated sample, while high for essentially anaerobic storage, did not change to any extent after the first day. Once the pigment was reduced and little residue oxygen remained in the package, no further lipid oxidation took place. The high TBA number probably developed while the meat was being packaged and before the pigment had reduced.

TBA numbers were quite low in the sample with added BHA. It was added shortly after the meat was ground so that time involved in preparation and for MetMb reduction would not affect lipid oxidation in this sample. A later experiment helped to substantiate this. TBA tests were run immediately after grinding the meat and then again shortly after packaging. During this time the TBA number of unprotected samples had more than quadrupled. Keskinel et al. (1964) found a similar increase in TBA number immediately after grinding raw beef.

Wrapping the meat in Saran did not always result in MetMb reduction and lipid oxidation inhibition. When samples that did not undergo reduction after packaging in Saran were tested for MetMb-reducing activity (MRA) by the method of Stewart et al. (1965b), activity was barely detectable. Anaerobic packaging can apparently be useful only if sufficient reducing activity is present in the meat.

In examining the effects of antioxidants on lipid and pigment oxidation, the ineffectiveness of PP is again illustrated (Table 2). However, in all cases both BHA and PG effectively protected against lipid oxidation. With aerobic packaging they also offered substantial protection against MetMb formation. After one week, samples containing either of these antioxidants and packaged in Prime Wrap were still a good red color.

Table 2—Effect of antioxidants and wrapping material on MetMb development and lipid oxidation in refrigerated raw beef.

Experiment	Antioxidant	Wrapping material	Days stored	TBA No.	% MetMb
1	None	Saran	1	2.6	52
	None	Saran	7	3.0	0
	None	Prime Wrap	1	5.0	49
	None	Prime Wrap	7	13.3	66
	Polyphosphate	Saran	1	2.1	29
	Polyphosphate	Saran	7	1.9	0
	Polyphosphate	Prime Wrap	1	3.5	40
	Polyphosphate	Prime Wrap	7	7.7	50
	BHA	Saran	1	0.6	48
	BHA	Saran	7	0.5	0
	BHA	Prime Wram	1	0.7	31
	BHA	Prime Wrap	7	0.6	33
2	None	Prime Wrap	8	6.2	52
	BHA	Prime Wrap	8	0.4	36
	PG	Prime Wrap	8	0.7	36

DISCUSSION

THESE RESULTS have demonstrated the importance of lipid oxidation in raw beef in relation to both color and odor. The odor produced from this reaction in raw beef had not previously been recognized as resulting from lipid oxidation. The fact that the odor remains after cooking the meat places further importance on this type of spoilage.

Anaerobic packaging can effectively prevent MetMb formation and rancidity, providing sufficient MRA is present in the meat. The great variability in MRA between different samples of meat suggests a need for research into factors limiting this enzyme action. Genetic or environmental factors or slaughter conditions undoubtedly play a part in this variability. Work is presently in progress to determine the substrate(s) for MetMb-reducing enzymes in meat. Addition of suitable substrates either directly to the meat or indirectly to the whole animal could result in meat with uniformly high MRA.

Anaerobic packaging is unnecessary if an antioxidant is added to the meat. BHA and PG inhibit lipid oxidation and retard MetMb formation. In this way, the meat pigment can be in the familiar oxygenated form. This method offers the additional advantage of preventing rancidity in the cooked product. A number of recent studies (among them, Lewis et al., 1962, and Roubal et al., 1966) have shown that intermediates from oxidizing lipids can damage proteins and enzymes. Antioxidants may exert their effect on meat color by protecting reducing enzymes as well as heme pigments from damage due to lipid oxidation intermediates.

We performed additional experiments to investigate this possibility, using the method of Stewart et al. (1965a). While more MRA was retained in stored samples treated with an antioxidant, the effect was not great enough to completely account for the amount of MetMb inhibition that occurred. In addition to protecting enzyme proteins, antioxidants may also protect heme proteins. Heme pigments are more susceptible to oxidation if the globin has been denatured (Lemberg et al., 1949). Studies by Nishida et al. (1965) and Little et al. (1968) indicated, however, that destruction of the heme moiety of hemoglobin may be the main point of attack by lipid hydroperoxides rather than denaturation of the protein portion. Measurement of total pigment might provide evidence of heme protection.

Initial MRA may be important to antioxidant treated samples, too. During these experiments enzymically "active" samples were observed to brown more readily in aerobic packaging than less active samples. At the same time, antioxidant-treated "active" samples remained a brighter red than the same samples from a less active piece of meat. Antioxidants may prevent oxidation of the heme iron, although this is more difficult to substantiate.

Samples of meat with a very high pH (6.2) have been found to remain red under aerobic conditions even without an antioxidant. Rancidity does not develop in this case. Such meat is usually associated with extremely high reducing activity. It is apparently just on the border line of "dark-cutting" beef, but it does oxygenate and it is not unduly sticky in texture. Finding a means of producing animals to give this type of meat would be extremely valuable. Perhaps some of the pre- and post-slaughter treatments proposed for reducing PSE in pork would be helpful with beef also.

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Electrophoretic Comparison of Anthocyanin Pigments in Eight Varieties of Sour Cherries

SUMMARY—Electrophoresis and paper chromatography were used to establish the identity and the relative pigment concentrations in eight varieties of sour cherries using the pigments of Montmorency cherries as standards: (1) Marasca Moscata, (2) Wczesna Z Prinn, (3) Del Nord, (4) Königliche Amarelle, (5) Triaux, (6) Schattenmorelle, (7) Marasca di Ostheim and (8) Flemish Red. It was found that all varieties contained cyanidin-3-gentiobioside and cyanidin-3-rhamnoglucoside as their major anthocyanin pigments. All varieties except Flemish Red contained cyanidin-3-monoglucoside as a minor component. A second minor component of cyanidin-3-diglycoside was found in Wczesna Z Prinn, Del Nord, Schattenmorelle and Marasca di Ostheim. In all cases, the major pigments were approximately in the same relative concentrations as previously found in Montmorency cherries.

INTRODUCTION

SOUR CHERRIES (*Prunus cerasus* L.) derive their characteristic red color from anthocyanin pigments. During processing, these pigments are subject to great changes which, in general, are undesirable. Several varieties of sour cherries have been propagated at the University of Wisconsin Experimental Farm, Sturgeon Bay, Wis., from scions obtained from the U. S. Department of Agriculture's Plant Introduction Garden, Chico, Calif. These varieties were introduced as a possible source of disease-resistant commercial stock. Differences of pigment proportion or structure among these varieties might also be a source of more stable color in cherry products.

The two major anthocyanin pigments of sour cherries (*Prunus cerasus* L., var. Montmorency) were identified as cyanidin-3-gentiobioside and cyanidin-3-rhamnoglucoside (Li et al., 1956). A third and minor pigment was identified as cyanidin-3-monoglucoside by Schaller et al. (1968).

The ionic nature of anthocyanins has been firmly established. Paper electrophoresis has been applied to the calculation of the isoelectric point of cyanidin-3-rhamnoglucoside and cyanidin-3-gentiobioside from Montmorency cherries (Markakis, 1960). Under the influence of 7 v/cm for 5 hr at room temperature in an orthophosphoric acid-sodium phosphate buffer, these pigments were separated from an ethanol extract of cherries. Markakis also observed that acetic acid, formic acid and their sodium salts in concentrations from 0.01 to 0.1 *M* were suitable buffers for electrophoretic separation of anthocyanins.

Since methods for separation of anthocyanins with paper chromatography were first enumerated (Bate-Smith, 1948), they have found increasing use. Some researchers find R_f values in paper chromatography systems to be sufficient for identification (Geissman et al., 1955; Harborne, 1958). In the work reported here, both paper chromatography and paper electrophoresis are used to establish the identity of anthocyanin pigments in eight varieties of sour cherries.

MATERIALS AND METHODS

THE EIGHT varieties of sour cherries (*Prunus cerasus* L.) obtained from the University of Wisconsin Experimental Farm, used in the study of anthocyanin pigments, were: (1) Marasca Moscata, (2) Wczesna Z Prinn, (3) Del Nord, (4) Königliche Amarelle, (5) Triaux, (6) Schattenmorelle, (7) Marasca di Ostheim and (8) Flemish Red. The unpitted cherries were frozen immediately after harvest and stored at −23°C until used.

Paper electrophoresis was carried out with a Gelman Power Supply Rapid Electrophoresis Chamber and Whatman 3 MM chromatography paper. The electrophoresis chamber was modified by attaching a 26-gauge platinum wire to the electrodes and running it the full length of the cell near the exterior edges in order to obtain uniform migration rates throughout the chamber. Whatman 3 MM chromatography paper was cut into 23- × 26-cm sheets and allowed to equilibrate in the electrolyte system at 6°C for 4 hr. Citric acid (0.1 *M*, pH = 2.00, μ = .007 at 25°C) was used as an electrolyte.

Strips of skin ca 1.5 mm in width were removed from the frozen cherries and placed on the equilibrated paper at the positive pole. Electrophoresis was carried out for 6 hr at 6°C under a voltage gradient of 15 v/cm. The papers were dried at room temperature and a representative 3.8-cm strip was cut parallel to the direction of migration. This strip was scanned by a Spinco model RB Analytrol densitometer. The unit was equipped with 550 mμ interference filters, a ½-mm slit width, and a B-5 cam. The trace obtained was used to determine pigment migration rates and relative concentrations. Migration of the pigments was measured from the point of application to the maximum peak height of the individual pigment. The area under the peak was assumed to be proportional to the amount of pigment present and was determined from the integrator of the analytrol.

Identification of the pigments was accomplished by determining R_f values using the known pigments of Montmorency cherries as standards. Purification and identification were carried out as follows: 175 g of frozen whole cherries were lyophilized and ground in a Waring Blendor along with 300 ml of a solution consisting of ethanol, water and concentrated HCl (90:9:1 v/v/v). The macerate was filtered under vacuum through Whatman No. 1 filter paper. The filtrate was concentrated under vacuum to about 50 ml at 30°C. This solution was streaked on Whatman 3 MM chromatography paper and developed descendingly for 4 hr with 0.01% HCl as the solvent.

The bands of pigment were cut and eluted with methanol, water and acetic acid (18:1:1 v/v/v). The eluate was concentrated under vacuum at 30°C and rechromatographed twice. To determine the R_f values, the pigments were spotted on Whatman No. 1 chromatography paper and developed descendingly at 20°C in the following systems:

1. n-butanol, acetic acid, water (4:1:5 v/v/v); papers were equilibrated with the lower phase for 24 hr and developed with the upper phase for 14 hr.
2. Concentrated HCl, water (1:99 v/v); developed for 6 hr.
3. Water, acetic acid, concentrated HCl (82:15:3 v/v/v); developed for 8 hr.
4. Acetic acid, water (3:17 v/v); developed for 8 hr.

RESULTS AND DISCUSSION

ELECTROLYTES were prepared from several organic acids and their salts. All electrolytes tested produced satisfactory separations. Buffered electrolytes increased the separation of the pigment fractions. However, 0.1 *M* citric acid was chosen as the electrolyte since, upon drying of the papers, salts of organic acids used in the preparation of buffers caused yellowing of the papers and interference with densitometer readings. Separations were best when electrophoresis was carried out at 6°C. Several methods of pigment application were explored for paper electrophoresis:

1. direct application of an ethanol extract prepared from whole cherries;
2. application of extracts obtained from ion exchange resins;
3. application of skins.

The direct application of the ethanol extract yielded extremely weak bands, of which only the two major pigments were readily discernible. Efforts to concentrate the extract to increase the amount of pigments were unsuccessful, since vacuum concentration resulted in an increase in other alcohol extractable constituents which made separation impossible.

To remove nonpigment constituents, water extracts of pigment were applied to ion exchange resins (Dowex 50 W-X4 or Amberlite CG-50 Type I). The pigments were removed from the resins with absolute methanol containing small amounts of HCl (von Elbe et al., 1968). The eluates obtained from the resins gave excellent separations, but relative pigment concentrations could not be reproduced. Therefore, these methods were abandoned in favor of the simpler application of skin sections.

Extraction of the pigments from skins by electrophoresis was accomplished within 0.5 hr and complete separation occurred within 5 to 6 hr. The varieties; Montmorency, Marasca Moscata, Königliche Amarelle and Triaux, showed three distinct bands; Wczesna Z Prinn, Del Nord, Schattenmorelle and Marasca di Ostheim showed four bands; Flemish Red showed only two bands. Bands were numbered 1 to 4, with band 1 having the fastest migration rate. Table 1 summarizes the data obtained by electrophoresis. The migration rates shown in Table 1 are the average of six determinations. Within the error of the experiment, it can be concluded that bands 1, 3 and 4 found in Marasca Moscata, Wczesna Z Prinn, Del Nord, Schattenmorelle and Marasca di Ostheim are identical to those pigments found in Montmorency cherries (Schaller et al., 1968). The migration rates found in Königliche Amarelle, Triaux, and Flemish Red were slightly lower. R_f values obtained by paper chromatography in four solvent systems showed that pigment bands 1, 3 and 4 were identical in all varieties. The bands were identified as cyanidin-3-gentiobioside, cyanidin-3-rhamnoglucoside and cyanidin-3-monoglucoside.

The reason for the slightly slower migration rates found in some varieties seems to be that these varieties were poorly pigmented, making it necessary to use 5-mm sections, instead of the usual 1.5-mm sections, to obtain enough pigments for analysis. The increase in width required more time for the pigment to migrate from the skins, thus lowering the rate.

Table 1. Electrophoretic migration of sour cherry pigments: 15 v/cm, 6°C, 0.1 *M* citric acid as electrolyte, 6 hr.

Variety	Migration rate[1]—cm per sec per v/cm × 10^6			
	Band 1 Cyanidin-3-gentiobioside	Band 2 Cyanidin-3-diglycoside	Band 3 Cyanidin-3-rhamnoglucoside	Band 4 Cyanidin-3-monoglucoside
Montmorency	3.11 ± .06	....	1.69 ± .06	.92 ± .06
Marasca Moscata	3.13 ± .06	....	1.77 ± .06	.92 ± .06
Wczesna Z Prinn	3.07 ± .06	2.43 ± .06	1.65 ± .06	.87 ± .06
Del Nord	3.20 ± .06	2.58 ± .06	1.75 ± .06	.93 ± .06
Schattenmorelle	3.20 ± .06	2.64 ± .06	1.80 ± .06	.93 ± .06
Marasca di Ostheim	3.11 ± .06	2.63 ± .06	1.72 ± .06	.93 ± .06
Königliche Amarelle	3.05 ± .06	....	1.52 ± .06	.68 ± .06
Triaux	3.05 ± .06	....	1.58 ± .06	.87 ± .06
Flemish Red	2.98 ± .06	....	1.77 ± .06	

[1] Average of six determinations.

Varieties Wczesna Z Prinn, Del Nord, Schattenmorelle and Marasca di Ostheim showed an additional minor band referred to as band 2. The R_f values obtained in the solvent systems used indicated that the pigment was identical to cyanidin-3-diglycoside as obtained by the method described by von Elbe et al. (1968).

In all cases, bands 1 and 3 were found to be the major pigments, and the relative concentrations, based on six determinations, were approximately equal to the amounts previously found in Montmorency cherries (Schaller et al., 1968). The presence of cyanidin-3-diglycoside seems to be the most significant difference in pigmentation.

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Connective Tissues from Normal and PSE Porcine Muscle. 1. Chemical Characterization

SUMMARY—The epimysial connective tissue from normal and PSE (pale, soft and exudative) porcine muscle was chemically characterized. Samples from PSE muscles exhibited markedly lower turbidity values ($P < 0.01$) than those from normal muscles. No significant differences were found in ultimate pH values. The epimysium from PSE muscles contained significantly higher ($P < 0.05$) quantities of salt soluble tropocollagen and a greater amount of heat labile collagen ($P < 0.01$) than that from normal tissues. No significant differences in α- and β-subunit composition, plasma hydroxyproline levels or amino acid composition were evident between the two sources of epimysial connective tissues.

INTRODUCTION

THE LITERATURE on the biochemistry, physiology and histology of the PSE (pale, soft and exudative) condition in porcine muscle is well documented in relation to muscle mass (Briskey, 1964). However, very little has been reported on the relationship of the PSE condition to the connective tissues.

Sayre *et al.* (1963a) demonstrated that in severe cases, PSE muscles lose their connective tissue attachments and can be easily pulled from the carcass. Briskey (1963) postulated that a relationship existed between the develop-

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ment of the PSE condition and the loss of intermuscular binding in PSE hams. McClain *et al.* (1968) reported an altered or decreased ground substance content in the epimysium from PSE muscles.

This study was, therefore, carried out in order to chemically characterize connective tissues from PSE muscles.

EXPERIMENTAL METHODS

Selection and treatment of samples

The *l. dorsi* muscles of 12 market weight pigs were classified as normal or PSE on the basis of subjective evaluation and by the turbidimetric method of Hart (1962). The epimysium was removed from the anterior portion of the loin in the region of the 3rd and 4th ribs at 48 hr post-mortem. Samples were dissected free of adhering fat and muscle, frozen in liquid nitrogen and stored at −20°C for subsequent chemical studies.

Turbidity and pH determinations

The turbidity method of Hart (1962) was used to determine the severity of or relative freedom from the PSE condition. A 10 gm sample was extracted in distilled water for 18 hr at 2°C. The water extracts were acidified with disodium phosphate-citrate buffer at pH 4.6, and the degree of turbidity was measured in a Spectronic 20 spectrophotometer at a wavelength of 600 mμ.

Equal volumes of muscle tissue and distilled water were mixed in a Waring blender, and pH readings were made with a Beckman Zeromatic pH meter to the nearest 0.01 pH unit.

Extraction and purification of salt and acid soluble collagen

The salt and acid extraction procedure was a modification of the methods of Gross (1957) and Harkness *et al.* (1953). Samples were extracted for 18 hr at 2°C in 0.45 M sodium chloride. The salt soluble collagen was removed by centrifugation and filtration. The residue was then extracted for 18 hr at 2°C in 0.15 M citrate buffer at pH 3.7 and the acid soluble fraction isolated as above. Aliquots of the salt and acid extracts were removed for hydrolysis and hydroxyproline determinations. The remaining acid extractable collagen was purified according to the method of Rubin *et al.* (1965).

α- and β-subunit composition of acid soluble collagen

The subunit composition of the purified acid soluble extracts was determined by disc gel electrophoresis according to a modification of the method of Nagai *et al.* (1954). The major modification of the above system involved the use of cyanogum (E. C. Apparatus Co.) for making the gels instead of the usual acrylamide and the N-N'- methylenebisacrylamide. Photopolymerization with riboflavin was also omitted. Prior to applying the samples to the disc gel tubes, sucrose was added to a final concentration of 5%. The sucrose sample solution was then layered directly on the spacer gel. The α- and β-subunit composition was determined on the destained gels utilizing a Canalco, Model F, Electrophoresis Microdensitometer with an electronic integrator.

Thermal labile collagen

Heat labile collagen was determined according to the method of Verzar (1964). Samples were placed in 0.9% saline solution and heated in a water bath for 10 min at 65°C. The supernatant was isolated by centrifugation and filtration. Hydroxyproline was determined on the supernatant and the residue according to the procedure of Woessner (1961) as modified by McClain *et al.* (1965).

Amino acid analysis and plasma hydroxyproline levels

Amino acid analysis was performed on 24 hr acid hydrolyzates from pooled samples of the purified acid soluble collagen using a Beckman Amino Acid Analyzer, Model 120B (Moore *et al.*, 1958). Blood samples were taken at the time of exsanguination. Samples were collected in heparinized centrifuge tubes and centrifuged for 40 min at 2000 rpm. The plasma was frozen and stored at −20°C for subsequent hydroxyproline determinations, which were carried out as outlined earlier. The plasma hydroxyproline values were from pigs utilized in another phase of the investigations on PSE muscle, and therefore, were not from the same animals as those used in the connective tissue studies.

RESULTS AND DISCUSSION

Turbidity and pH values

Table 1 lists the turbidity and ultimate (48 hr) pH values for the *l. dorsi* muscles from the pigs used in this study. The mean turbidity value for the PSE muscles was 92.00 as compared to 26.67 for the normal muscles (Table 1). The markedly lower turbidity for the PSE samples indicated that much of the protein in the PSE muscles had been precipitated.

Table 1. Turbidity values and ultimate pH of normal and PSE *l. dorsi* muscle.

PSE			Normal		
Animal No.	Turbidity[1] value	Ultimate[2] pH	Animal No.	Turbidity[1] value	Ultimate[2] pH
1	99.00	5.52	2	52.00	5.50
4	95.50	5.60	3	33.00	5.55
6	93.00	5.43	5	35.00	5.40
8	98.50	5.40	7	18.00	5.52
10	68.50	5.50	11	12.00	5.45
14	97.50	5.52	13	10.00	5.60
Mean	92.00	5.49		26.67	5.50
Std. dev.	11.72	0.06		16.24	0.08

[1] % transmittance.
[2] Ultimate pH was taken at 48 hr post-mortem.

Results of the turbidimetric measurements were also in good agreement with subjective evaluations. Analysis of variance revealed a highly significant ($P < 0.01$) difference between the turbidity values of the normal and PSE muscles. Results agree with those of McLoughlin (1963) and Sayre *et al.* (1963b), who reported that the sarcoplasmic proteins in PSE muscles were severely denatured and lost much of their solubility as a result of rapid glycolysis at high temperatures.

The mean ultimate pH values for the normal and PSE muscles are also listed in Table 1. The difference in pH values for the PSE and normal muscles was not statistically significant, indicating that low ultimate pH is probably not responsible for the occurrence of the PSE condition.

Acid and salt soluble collagen

Normal epimysial connective tissue was found to contain an average of 3.20% of salt soluble and 0.31% of acid soluble collagen, while the average values for the epimysium from PSE tissues were 4.29 and 0.24%, respectively (Table 2). There was significantly ($P < 0.05$) more salt soluble collagen in the PSE tissues than in the normal. The difference in acid soluble collagen was small and not significant.

It is difficult to pinpoint the cause of the increased tropocollagen solubility in the connective tissues from PSE muscles. The fact that only the salt soluble or newly synthesized tropocollagen was high in PSE connective tissues could indicate increased collagen anabolism. In light of Bowes *et al.* (1966) finding of an immature type collagen in the hides of rapidly growing sheep and beef, it is possible that the connective tissues of animals developing the PSE condition are also more immature than those of normal animals of the same age.

α- and β-subunit composition of acid soluble collagen

The α- and β-subunit composition was determined by disc gel electrophoresis on purified acid soluble extracts of the epimysial connective tissue from normal and PSE muscles. A typical densitometer tracing of the α- and β-subunit patterns obtained from the disc gels is shown in Fig. 1. The acid soluble collagen from normal tissues contained 38.46% of the α-subunits and 61.54% of β-subunits. The corresponding values for PSE tissues were 37.92 and 62.08%, respectively (Table 3).

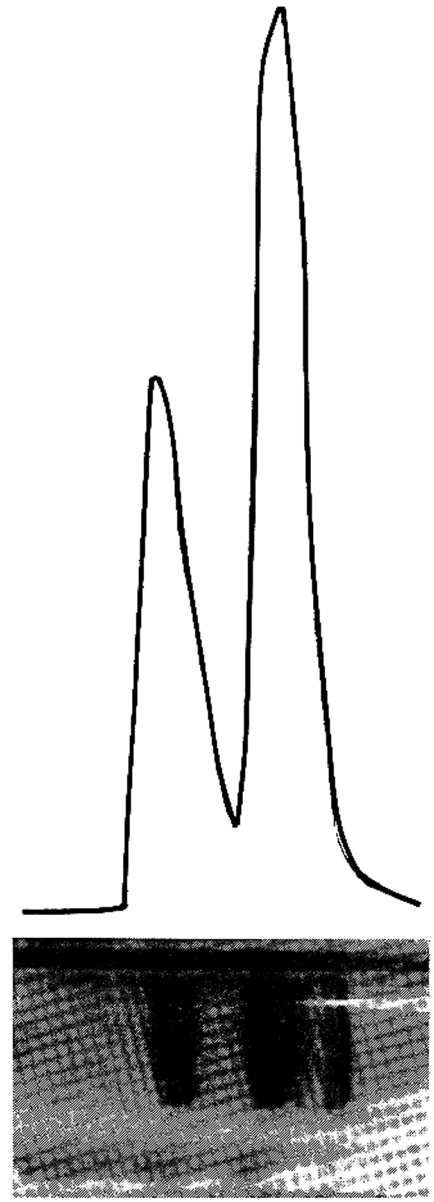

Fig. 1. Typical densitometer tracing of α- and β-subunit patterns upon disc gel electrophoresis of acid soluble collagen. The α-peak is on the left and the β-peak on the right.

Table 2. Percentage of acid and salt soluble collagen in epimysial connective tissues from normal and PSE *l. dorsi* muscles.

PSE			Normal[1]		
Animal No.	Salt soluble[1]	Acid soluble[1]	Animal No.	Salt soluble[1]	Acid soluble[1]
1	4.16	0.22	2	2.63	0.21
4	3.93	0.25	3	3.41	0.28
6	4.66	0.25	5	4.52	0.51
8	4.10	0.24	7	3.30	0.28
10	5.37	0.31	11	2.62	0.27
14	3.57	0.21	13	2.75	0.27
Mean	4.29	0.24		3.20	0.31
Std. dev.	0.63	0.03		0.72	0.10

[1] As % of total collagen.

Table 3. Percentage of α- and β-subunits in acid soluble collagen of epimysial connective tissues from normal and PSE *l. dorsi* muscles.

PSE			Normal		
Animal No.	% α[1]	% β[1]	Animal No.	% α[1]	% β[1]
1	40.00	60.00	2	38.00	61.00
4	36.50	63.50	3	38.25	61.75
6	40.00	60.00	5	40.00	60.00
8	35.50	64.50	7	39.00	61.00
10	38.00	62.00	11	36.00	64.00
14	37.50	62.50	13	38.50	61.50
Mean	37.92	62.08		38.46	61.54
Std. dev.	1.66	1.66		1.22	1.22

[1] As percentage of total purified acid soluble collagen from densitometer tracings of disc gels.

Analysis of variance revealed that the difference between normal and PSE tissues in α- and β-subunit composition was not statistically significant. However, it must be remembered that there was a significantly greater amount of salt extractable collagen in the connective tissues from PSE muscles. Salt soluble collagen is composed mainly of noncross-linked α-components (Picz *et al.*, 1963). Therefore, if the sample had not received a prior salt extraction, the acid soluble fraction from the PSE tissues would have had a lower percentage of β-components, thus indicating decreased intramolecular cross-linking.

Similar results have been reported by Martin *et al.* (1961), who extracted acid soluble collagen without prior salt extraction from the skins of lathyritic rats. These authors suggested that there is a defect at the molecular level in the maturation of collagen in lathyritic animals. It is possible that a similar maturation defect might be responsible for the altered solubility characteristics of the collagen from PSE tissues observed in the present study.

Heat labile collagen

The results of a heat solubility study on the epimysium from normal and PSE tissues is shown in Table 4. These data revealed that the collagen content of the porcine epimysial connective tissues was approximately 250 mg. It is also apparent that 22.98% of the collagen from PSE tissue was released upon heating as compared to 15.02% from the normal tissues. These results agree very well with the work of Verzar (1964), who reported 25% heat labile collagen in tendons from 3 yr-old rats. There are, however, differences in the amount of heat labile collagen due to animal age (Verzar, 1964; Goll *et al.*, 1964). Hill (1966) found 22.0% of heat labile collagen in collagenous residues isolated from the loose connective tissues of 5-mo-old hogs, as compared to 3.8% from old sows.

Analysis of variance revealed that there was a highly significant difference ($P < 0.01$) between the percentage of heat labile collagen in the normal and PSE tissues. There results indicated that the epimysium from normal tissue had greater numbers or an increased strength of cross-links as compared to that from PSE tissues (Goll *et al.*, 1964; Hill, 1966).

Plasma hydroxyproline levels

It was noted that most of the heparinized plasma samples contained a precipitate after freezing and thawing. LeRoy *et al.* (1964) also reported the formation of a precipitate in frozen and thawed heparinized plasma. They found that removal of the precipitate reduced the protein-bound hydroxyproline level by 30%. Although hydroxyproline levels in the present study agree very well with those reported by the above workers, it is entirely possible that some protein-bound hydroxyproline was lost due to precipitation.

The results of this study (Table 5) revealed a slightly

Table 5. Plasma hydroxyproline levels in normal and PSE pigs.

PSE		Normal	
Animal No.	Plasma hydroxyproline[1]	Animal No.	Plasma hydroxyproline[1]
11	7.30	3	11.80
2	9.65	4	9.10
17	7.80	6	9.10
18	14.15	9	10.80
19	8.15	13	9.90
Mean	9.41		10.14
Std. dev.	2.79		1.16

[1] Values are expressed in μg of hydroxyproline per ml of plasma.

Table 4. Heat solubility parameters of epimysial connective tissues from normal and PSE *l. dorsi* muscles.

PSE					Normal				
Animal No.	Total collagen[1,2]	Heat soluble collagen[1,3]	Residual collagen[1,4]	% Heat soluble collagen	Animal No.	Total collagen[1,2]	Heat soluble collagen[1,3]	Residual collagen[1,4]	% Heat soluble collagen
1	251.54	55.81	195.74	22.23	2	265.64	31.50	235.36	11.20
4	262.63	64.76	197.88	24.62	3	268.69	42.62	226.07	15.89
6	256.18	72.46	183.72	28.29	5	247.01	37.75	209.26	15.25
8	262.17	54.73	207.43	20.88	7	232.93	38.24	194.68	16.58
10	224.93	39.83	185.09	17.70	11	249.84	42.75	207.09	17.10
14	284.72	69.00	215.71	24.19	13	257.65	36.42	221.23	14.15
Mean	257.04	59.43	187.60	22.98		253.82	38.21	215.62	15.02
Std. dev.	19.42	11.90	12.47	3.61		13.44	4.21	14.71	2.14

[1] Expressed as mg/gm connective tissue.
[2] Total collagen is the total of the heat soluble and residual collagen.
[3] Heat soluble collagen was determined by the method of Verzar (1964).
[4] Residual collagen was the fraction remaining after determining the heat soluble collagen.

Table 6. Partial amino acid composition of acid soluble collagen from the epimysium of normal and PSE muscles.

Amino acids	Normal[1]	PSE[1]
Lysine	38.1	36.8
Histidine	9.9	9.4
Arginine	51.3	50.3
Hydroxylysine	6.5	6.7
Aspartic acid[2]	61.5	60.0
Threonine	26.2	25.7
Serine	50.9	48.9
Glutamic	85.6	84.8
Proline	150.4	159.7
Glycine	295.1	301.9
Alanine	103.0	106.1
Valine	30.2	29.1
Methionine	5.9	6.4
Isoleucine	17.1	16.1
Leucine	39.8	37.5
Tyrosine	9.7	9.3
Phenylalanine	18.9	17.7
Total residues	1,000.1	1,006.4

[1] Values are given as residues per 1,000 residues—average of two determinations.
[2] Values for aspartic acid are high due to interference from hydroxyproline.

higher plasma hydroxyproline level in the normal animals. However, analysis of variance showed that this difference was not statistically significant.

Amino acid analysis

Results of analysis of the amino acids in pooled samples of purified acid soluble collagen extracted from the epimysium of normal and PSE muscles are shown in Table 6. The results of this analysis were limited by the fact that standard runs revealed that hydroxyproline was eluted off the column at the same time as aspartic acid.

Although hydroxyproline exhibited only minimum absorption at 570 mμ, where the aspartic acid-ninhydrin complex is measured, the value for aspartic acid is undoubtedly high. In addition, it was not possible to quantitatively determine the hydroxyproline content in this analysis. However, the percent hydroxyproline was determined utilizing the colorimetric method of Woessner (1961). Using this method, values of 13.10 and 13.25% hydroxyproline were found for normal and PSE acid soluble collagen, respectively.

The partial amino acid composition of the acid soluble collagen from normal and PSE epimysium was very similar. Thus, variation in the primary structure of the collagen molecule was probably not responsible for the differences between PSE and normal epimysium in the salt soluble and heat labile fractions.

The results of this study have shown that, in addition to changes in muscle proteins, the connective tissue proteins are also altered in muscles developing the PSE condition. The high values for salt soluble and heat labile collagen indicate a less highly cross-linked or more immature type of collagen in the connective tissues from PSE muscles.

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Post-Mortem Changes in Subcellular Fractions from Normal and Pale, Soft, Exudative Porcine Muscle. 1. Calcium Accumulation and Adenosine Triphosphatase Activities

SUMMARY—Myofibrillar, mitochondrial, heavy sarcoplasmic reticulum and light sarcoplasmic reticulum fractions were isolated by differential centrifugation of homogenates from normal and pale, soft, exudative (PSE) porcine muscle at various times post-mortem. Calcium uptake was measured using a solution containing $^{45}Ca^{++}$. The oxalate-stimulated calcium accumulating ability of the subcellular fractions declined 5–10 fold between 0 and 24 hr post-mortem. The major portion of this decline occurred in the first hour after death in fractions from PSE muscle but was more gradual in the normal fractions. The ATPase activities of normal and PSE fractions obtained at death did not differ significantly. These activities increased with time post-mortem in most normal fractions but decreased in those from PSE muscle. The subcellular site of ATP hydrolysis post-mortem was discussed. The results obtained point to the potential importance of the relaxing factor in muscle post-mortem.

INTRODUCTION

MUSCLE UNDERGOES a large number of biochemical changes post-mortem. Discontinuance of the blood supply causes muscle to become anaerobic and it consequently converts glycogen to lactic acid in an attempt to maintain its adenosine triphosphate (ATP) level (Bate-Smith, 1948; Bendall, 1951). The build-up of lactic acid causes the pH to decline, creatine phosphate and ATP are concomitantly depleted, and the muscle goes into rigor mortis (Bendall, 1960).

In pig muscle post-mortem, certain muscles have an extremely rapid rate of glycolysis after death (Briskey *et al.*, 1961; Lawrie, 1960; Wismer-Pedersen *et al.*, 1959). This high glycolytic rate causes the pH to decline rapidly, and a pale, soft, exudative (PSE) condition of the muscles presumably results from low pH while muscle temperature is still high (Wismer-Pedersen *et al.*, 1961; Sayre *et al.*, 1963).

Attempts to explain the cause of this condition have prompted research on the elucidation of glycolytic control (Briskey *et al.*, 1966), the effect of ante-mortem stress (Kastenschmidt *et al.*, 1964), hormones (Ludvigson, 1957; Cassens *et al.*, 1966), electrical stimulation (Hallund *et al.*, 1965), and the contribution of stimulation and anoxia to rapid glycolysis (Lister *et al.*, 1968).

An aspect which has not been examined in relation to this problem is the possibility of the involvement of the relaxing factor. The relaxing factor, originally discovered by Marsh (1951), consists of fragments of the sarcoplasmic reticulum which have the ability to remove Ca^{++} ions from solution by an ATP-dependent transport process (Ebashi *et al.*, 1962; Hasselbach *et al.*, 1961).

Since a small amount of Ca^{++} is known to be necessary to activate the contractile proteins, it has been postulated that contraction occurs when the sarcoplasmic reticulum releases part of its bound Ca^{++} into the sarcoplasm (Ebashi, 1961; Weber *et al.*, 1963). When there is no longer a stimulus for contraction, the sarcoplasmic reticulum pumps the Ca^{++} out of the sarcoplasm and the muscle relaxes. Thus the sarcoplasmic reticulum controls contraction and relaxation by controlling the concentration of free Ca^{++} in the muscle cell.

It has been demonstrated that the relaxing factor can reduce myofibrillar ATPase activity by 10-fold (Baird *et al.*, 1960; Marsh, 1952), and this reduction is probably due to its Ca^{++} transporting ability.

The rapid glycolysis that occurs in some muscles post-mortem might be postulated to be a manifestation of a rapid ATP breakdown. Accelerated ATP splitting might occur as a result of (1) activation of the sarcoplasmic reticulum adenosine triphosphatase (ATPase) or (2) the inability of the relaxing factor to control myofibrillar ATPase activity.

It has recently been shown that the relaxing factor loses part of its calcium accumulating ability relatively rapidly after death in porcine muscles with a normal pH decline (Greaser *et al.*, 1967). The present study was conducted to determine if changes in calcium accumulating ability and ATPase activity of different subcellular fractions of muscle are related to the rate of pH decline post-mortem.

EXPERIMENTAL

Sample preparation

Ten Poland China pigs were assigned to one of two groups on the basis of rate of pH decline and development of normal or pale, soft, exudative characteristics in their muscle post-mortem (Forrest *et al.*, 1963). Samples were removed from the longissimus dorsi within 5 min after exsanguination and at ½, 1, 3 and 24 hr post-mortem. Following exsanguination and evisceration of the pigs, the carcasses were stored at 4°C beginning about 30 min post-mortem and continuing throughout the remaining sample periods. Samples were homogenized in a Waring blendor in 4 volumes of ice cold 0.1M KCl and 5 mM histidine (pH 7.2) in six 15 sec bursts. All subsequent preparative procedures were conducted at 0 to 4°C.

The homogenate was separated sequentially by differential centrifugation into the following subcellular fractions: myofibrils (including nuclei, connective tissue and undisrupted fiber segments) sedimented at 1,000 × g for

20 min; mitochondria sedimented at 8,000 × g for 20 min; heavy sarcoplasmic reticulum sedimented at 30,000 × g for 60 min; and light sarcoplasmic reticulum sedimented at 60,000 × g for 60 min.

The myofibrillar fraction was resuspended in 0.1M KCl-5 mM histidine, filtered through cheesecloth to remove large connective tissue and muscle fragments, and recentrifuged at 1,000 × g for 20 min. The resulting myofibrillar pellet and the other sedimented fractions were resuspended in KCl-histidine prior to assay.

Calcium accumulation

Measurement of Ca^{++} uptake was performed in a medium consisting of 0.1M KCl, 5 mM histidine (pH 7.2), 5 mM ATP, 5 mM $MgCl_2$, 5 mM $K_2C_2O_4$ and 0.1 mM $CaCl_2$ (containing 0.1 μc $^{45}Ca^{++}$). The following concentrations of the resuspended material were used in the assay: mitochondria—0.05 mg/ml; heavy sarcoplasmic reticulum—0.02 mg/ml; and light sarcoplasmic reticulum—0.10 mg/ml. Samples were incubated for 15 min at 22–24°C and the particulate material removed by filtration through Millipore filters—type HA, 0.45 μ average pore diameter (Martonosi *et al.*, 1964).

The radioactivity of an aliquot of the supernate was determined by liquid scintillation counting and compared with appropriate standards to determine the amount of Ca^{++} bound by the proteins.

ATPase activity

The determination of ATPase activity was conducted at 25°C in a medium consisting of 0.1M KCl, 5 mM histidine (pH 7.2), 5 mM ATP, 5 mM $MgCl_2$ and 0.1 mM $CaCl_2$. The following protein concentrations were used: myofibrils—1.0 mg/ml; mitochondria—0.4 mg/ml; heavy sarcoplasmic reticulum—0.3 mg/ml; and light sarcoplasmic reticulum—0.4 mg/ml. Aliquots were removed at 0, 2, 4 and 8 min and added to ice cold trichloroacetic acid (final concentration of 5%). After centrifugation to remove the denatured protein, the supernate was analyzed for inorganic phosphate (Fiske *et al.*, 1925).

Other methods

Protein concentrations were determined using the biuret procedure with bovine serum albumin as a standard (Gornall *et al.*, 1949). Muscle pH measurements were made using a combination electrode placed directly on the freshly cut surface of the muscle (Briskey, 1964). ATP was treated with Dowex 50 × 8 (H^+ form) to remove any contaminating Ca^{++} (Seidel *et al.*, 1963). Distilled water which had been passed through a mixed bed ion exchange resin was used for all solutions.

RESULTS AND DISCUSSION

CALCIUM ACCUMULATING ABILITIES of the different subcellular fractions at the various times post-mortem are shown in Table 1. The highest specific activity was found in the heavy sarcoplasmic reticulum fraction, with a value of 2.20 μmoles Ca^{++} bound per mg protein in the samples isolated from normal muscle. This value was comparable to that found by other workers with preparations from rat and rabbit muscle under similar assay conditions (Carsten *et al.*, 1964; Hasselbach *et al.*, 1966; Martonosi *et al.*, 1964). It also agreed with the results of a previous study using porcine muscle (Greaser *et al.*, 1967).

The mitochondrial Ca^{++} accumulating ability was about half that of the heavy sarcoplasmic reticulum fraction. Although mitochondria remove Ca^{++} from solution (Carafoli *et al.*, 1965; Rossi *et al.*, 1964), mitochondria inhibitors decrease Ca^{++} uptake of rabbit muscle mitochondria by less than 10% (Weber *et al.*, 1966). These inhibitors completely suppress Ca^{++} accumulation by mitochondria from tissues other than muscle. Five mM sodium azide, which

Table 1. Calcium accumulation by the subcellular fractions from normal and pale, soft, exudative (PSE) porcine muscle.

Time post-mortem (hr)		μmoles Ca^{++}/mg protein[1]				
		0	½	1	3	24
Mitochondria	Normal	0.93 ±0.12	0.87 ±0.08	0.76 ±0.11	0.54 ±0.07	0.24 ±0.04
	PSE	0.67 ±0.06	0.31 ±0.02	0.23 ±0.05	0.15 ±0.04	0.10 ±0.03
	Significance	NS	P<.01	P<.01	P<.01	NS
Heavy sarcoplasmic reticulum	Normal	2.20 ±0.09	2.09 ±0.23	1.83 ±0.20	1.25 ±0.15	0.40 ±0.04
	PSE	1.85 ±0.15	1.06 ±0.16	0.64 ±0.06	0.42 ±0.06	0.15 ±0.03
	Significance	NS	P<.01	P<.01	P<.01	P<.01
Light sarcoplasmic reticulum	Normal	0.37 ±0.06	0.28 ±0.03	0.17 ±0.08	0.20 ±0.06	0.08 ±0.07
	PSE	0.16 ±0.03	0.11 ±0.02	0.11 ±0.03	0.10 ±0.03	0.10 ±0.02
	Significance	P<.05	P<.01	NS	NS	NS

[1] Values given are the mean ± the standard error.

blocks mitochondrial calcium accumulation (Fanburg *et al.*, 1965), inhibited the calcium accumulating ability of three mitochondrial preparations from pig muscle by less than 5%.

Contaminating sarcoplasmic reticulum fragments thus appear to play an important part in the calcium accumulation by the mitochondria fraction. The presence of a considerable number of small vesicles as observed in electron micrographs of the muscle mitochondria fraction also supports this contention (Greaser *et al.*, 1967).

The calcium accumulating ability of the light sarcoplasmic reticulum fraction was much lower than that found in the other two fractions (Table 1). Also the total protein yield of this fraction was considerably less than in the mitochondrial or heavy sarcoplasmic reticulum fractions (Greaser *et al.*, 1967). Thus, any calcium accumulation by this fraction would appear to be of little quantitative significance in relation to post-mortem changes.

The calcium accumulating ability decreased with time post-mortem in all fractions from both normal and PSE animals (Table 1). The rate of decline for the normal animals was similar to that found in a previous study (Greaser *et al.*, 1967). In all three fractions activities decreased to about 60% of the initial activity during the first 3 hr and to approximately 20% by 24 hr post-mortem.

The loss of calcium accumulating activity from the fractions from PSE muscle was dramatic. By 5 min post-mortem the calcium accumulating ability had declined in all three subcellular fractions although the decrease was statistically significant only in the light sarcoplasmic reticulum fraction. However, by 30 min post-mortem the activities of the PSE mitochondrial and heavy sarcoplasmic reticulum fractions had fallen to about 50%. By 3 hr the calcium accumulating ability of these two fractions had fallen to about 20%, a level comparable to that of the corresponding normal samples at 24 hr. The values for the PSE light sarcoplasmic reticulum were low initially and remained fairly constant with time post-mortem.

ATPase activities are shown in Table 2. The myofibrillar fraction was assayed in addition to the three fractions which were used to measure Ca^{++} accumulating ability. Under the conditions used in the assay, the ATPase specific activities were similar in the myofibrillar, mitochondrial and heavy sarcoplasmic reticulum fractions at death, while the light sarcoplasmic reticulum was much less. There was no significant difference in ATPase activity between normal and PSE muscle in any fraction immediately post-mortem.

With increasing time after death, however, some interesting changes occurred. With the myofibrils from normal muscle, the ATPase activity remained relatively constant during the first 3 hr but was significantly higher at 24 hr. The increase in activity with time post-mortem is somewhat difficult to explain, although it has been observed previously with bovine myofibrils (Goll *et al.*, 1967) and bovine actomyosin (Robson *et al.*, 1967; Herring, 1967). The extraction of part of the A band material from myofibrils which were obtained soon after death but not from those obtained at the later intervals might be related to the difference in activity (Greaser *et al.*, 1969).

The change in ATPase activity of PSE myofibrils with time post-mortem was considerably different from that of normal myofibrils. The activity dropped significantly within 30 min after death and by 24 hr had reached a

Table 2. ATPase activity of the subcellular fractions from normal and pale, soft, exudative (PSE) porcine muscle.

Time post-mortem (hr)		μmoles P_i/min/mg protein[1]				
		0	½	1	3	24
Myofibrils	Normal	0.136 ±0.027	0.120 ±0.012	0.120 ±0.013	0.158 ±0.019	0.218 ±0.014
	PSE	0.135 ±0.013	0.090 ±0.013	0.072 ±0.012	.072 ±0.014	0.063 ±0.014
	Significance	NS	NS	P<.05	P<.01	P<.01
Mitochondria	Normal	0.156 ±0.026	0.164 ±0.028	0.148 ±0.019	0.118 ±0.010	0.176 ±0.007
	PSE	0.139 ±0.016	0.087 ±0.011	0.084 ±0.007	0.060 ±0.003	0.067 ±0.009
	Significance	NS	P<.05	P<.01	P<.01	P<.01
Heavy sarcoplasmic reticulum	Normal	0.114 ±0.022	0.149 ±0.035	0.164 ±0.028	0.222 ±0.038	0.225 ±0.037
	PSE	0.138 ±0.016	0.132 ±0.004	0.109 ±0.016	0.108 ±0.019	0.104 ±0.013
	Significance	NS	NS	NS	P<.05	P<.01
Light sarcoplastic reticulum	Normal	0.039 ±0.005	0.037 ±0.003	0.038 ±0.003	0.062 ±0.009	0.136 ±0.020
	PSE	0.029 ±0.005	0.047 ±0.008	0.064 ±0.007	0.064 ±0.005	0.060 ±0.008
	Significance	NS	NS	P<.05	NS	P<.01

[1] Values given are the mean ± the standard error.

level which was less than half the initial activity. This change parallels the loss of myofibrillar protein solubility and the loss of water-binding ability as shown in previous studies of PSE muscle (Sayre *et al.*, 1963; Scopes, 1964).

In the preparations obtained from normal muscle, there was no consistent change in mitochondrial ATPase activity with time post-mortem (Table 2). With the PSE mitochondrial fraction, the ATPase activity declined significantly within 30 min after death and remained lower at subsequent time periods, with a pattern very similar to that found in PSE myofibrils.

The heavy sarcoplasmic reticulum behaved somewhat differently. The ATPase activity of this fraction from normal muscle increased with increasing time post-mortem. This result may not be too surprising since several workers have shown that the ATPase activity of sarcoplasmic reticulum preparations increases with aging *in vitro*, and that its ATPase activity often increases as its calcium accumulating ability decreases (Inesi *et al.*, 1965; Nagai *et al.*, 1965; Sreter *et al.*, 1964). The PSE heavy sarcoplasmic reticulum ATPase activity remained relatively constant with time post-mortem, with only a slight but nonsignificant decrease in the three latter time periods.

The ATPase activity of the light sarcoplasmic reticulum increased with time post-mortem for both normal and PSE fractions. However, this increase was much more pronounced in the normal than in the PSE fractions. The explanation for these changes is not readily apparent, although it may be that with time post-mortem the light sarcoplasmic reticulum fraction gains an increasing proportion of sarcoplasmic reticulum fragments with higher ATPase activities. A larger proportion of membrane fragments in 24 hr light sarcoplasmic reticulum fractions than in 0 hr ones has been observed by electron microscopy and would support this conclusion (Greaser *et al.*, 1969).

The decline in muscle pH with time post-mortem is shown in Table 3. The pH declined much more rapidly in muscle which developed the PSE characteristics than in normal muscle, confirming many previous studies (Bendall *et al.*, 1962; Briskey *et al.*, 1961; Sayre *et al.*, 1963; Wismer-Pedersen, 1959; Wismer-Pedersen *et al.*, 1961).

There appeared to be a close relationship post-mortem between rate of pH decline in the muscle and loss of calcium accumulating ability in the subcellular fractions. There are several possible explanations for this relationship. The first may be that the inactivation of the relaxing factor leads to a more rapid myofibrillar ATPase activity which depletes the creatine phosphate and ATP reserves and stimulates glycolysis, resulting in a more rapid pH decline. A second possibility may be that the increasing acidity in the muscle has a specific inactivating effect on the Ca^{++} accumulating ability of the relaxing factor. A third explanation may be that partial inactivation of the relaxing factor leads to a more rapid pH decline, which in turn accelerates the inactivation. This latter idea encompasses the essential features of the first two possible explanations. Unfortunately on the basis of the present results, there is no way to differentiate between these possibilities.

Table 3. Normal and pale, soft, exudative (PSE) muscle pH.[1]

Time post-mortem (hr)	0	½	1	3	24
Normal	6.4	6.2	6.1	5.6	5.4
PSE	6.3	5.6	5.3	5.3	5.4

[1] Values given are means from 4 normal and 6 PSE animals.

The present study of the ATPase activities of the different subcellular fractions of muscle may suggest new insights into the site of ATP degradation in muscle post-mortem. The rate of ATP turnover after death can be calculated by considering the rate of lactate accumulation and changes in concentrations of ATP and creatine phosphate. Values of approximately 1.0 and 1.6 μmoles ATP split per minute per gram muscle have been estimated for normal and pale, exudative muscle, respectively (Bendall *et al.*, 1963). Bendall (1960) has postulated that the majority of the ATP breakdown after death is carried out by a sarcoplasmic ATPase. This sarcoplasmic ATPase is now known to consist mainly of fragments of the sarcoplasmic reticulum.

Assuming that there are approximately 5 mg of sarcoplasmic reticulum per gram of muscle (Ohnishi *et al.*, 1964) and that the heavy sarcoplasmic reticulum ATPase activity is 0.20μM per mg protein per minute (a value which is higher than that found in the present study), the ATP degradation rate would be 1.0 μmole per gram per min. This value would account for the observed rate of ATP breakdown in normal muscle but could not explain the increased ATP flux of PSE muscle.

Similar calculations for the potential ATP splitting rate can also be determined for myofibrils. If a value for myofibrillar ATPase activity of 0.13 μM per mg per min (Table 2) is taken and the content of myofibrillar protein is 110 mg per gram of muscle (Helander, 1957), the maximal rate of ATP breakdown would be 14 μmoles/g/min which far exceeds the observed rate. Even in a relaxed or inactivated state, whether produced by the relaxing factor or chelators of Ca^{++}, the myofibrillar ATPase activity is usually not reduced more than 10-fold (Marsh, 1952; Weber *et al.*, 1962). Thus in the relaxed state the myofibrillar ATPase activity might be about 1.4 μmoles/g/min, a value which could explain more than the observed rate of ATP degradation in normal muscle and a major portion of the required rate in PSE muscle.

Although the ionic strength and Mg-ATP concentrations used in the assay were similar to those under physiological conditions, *in vitro* ATPase activities can not be confidently assumed to apply exactly to the proteins *in situ*. However, the fact that a maximal sarcoplasmic reticulum ATPase and a minimal myofibrillar ATPase give ATP breakdown rates which are comparable to the observed *in situ* degradation suggests that the myofibrils may play a more important role than was previously suggested.

The requirement for nearly maximal suppression of the myofibrillar ATPase activity and the demonstrated ability of the relaxing factor to carry out this suppression points to the potential importance of relaxing factor stability post-mortem.

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Post-Mortem Changes in Subcelllular Fractions from Normal and Pale, Soft, Exudative Porcine Muscle. 2. Electron Microscopy.

SUMMARY—Myofibrillar, mitochondrial, heavy sarcoplasmic reticulum, and light sarcoplasmic reticulum fractions were isolated from homogenates of normal and pale, soft, exudative (PSE) porcine muscle at 0 and 24 hr post-mortem and examined by electron microscopy. No differences were observed between normal and PSE myofibrils obtained at death. PSE myofibrils prepared at 24 hr post-mortem had more granular appearing filaments and wider Z lines than normal myofibrils at 24 hr. The PSE heavy sarcoplasmic reticulum fraction obtained at death had a higher proportion of granular material than the same fraction from normal muscle. Several structural differences between the other PSE and normal fractions were also observed, especially at 24 hr post-mortem. This study indicated that the composition of the subcellular fractions changed with time post-mortem and that this change should be considered when analyzing biochemical data from these fractions. However, the differences observed could not explain the large changes in calcium accumulating ability that have been shown to occur post-mortem.

INTRODUCTION

USE OF THE ELECTRON MICROSCOPE in the study of biological tissues has led to many important concepts about cellular function and organization. In addition to observations on intact cells, this technique has been particularly valuable in checking the composition and purity of subcellular fractions (Schneider, 1964). Therefore it was logical that electron microscopy could provide information about post-mortem changes in muscle in addition to the biochemical observations reported in the preceding paper (Greaser *et al.*, 1969). The purpose of this study was to determine if the composition and morphology of pig muscle subcellular fractions changed post-mortem and if the changes were related to the rate of pH decline after death. An attempt was also made to relate the visual appearance of these fractions with changes in biochemical properties.

EXPERIMENTAL

SAMPLES OF THE longissimus dorsi of Poland China pigs which could ultimately be classified as normal or pale, soft and exudative (PSE) were obtained within 5 min after exsanguination and at 24 hr post-mortem. Classification was based on a color score rating system (Forrest *et al.*, 1963). Experimental material was obtained from two animals in each group. Muscle homogenization and subcellular fractionation were performed according to the procedures described in the preceding paper (Greaser *et al.*, 1969). The separated myofibril, mitochondria, heavy sarcoplasmic reticulum, and light sarcoplasmic reticulum pellets were fixed for 2 hr in an ice cold solution containing 3% Biological Grade glutaraldehyde, 0.1*M* sodium cacodylate (pH 7.4), and 1 m*M* $CaCl_2$.

Following rinsing with 0.2*M* sucrose, 0.1*M* sodium cacodylate (pH 7.4), and 1 m*M* $CaCl_2$ for 2 hr, the pellets were removed from the centrifuge tubes, cut into 1 mm cubes and transferred to a fixative containing 1% osmic acid, 0.1*M* sodium cacodylate (pH 7.4), and 1 m*M* $CaCl_2$ for a period of 1 hr. They were then dehydrated in ethyl alcohol and propylene oxide and embedded in an Epon-Araldite mixture. Some samples were soaked overnight in a 1:1 mixture of 5% aqueous uranyl acetate and 95% ethanol prior to dehydration; this treatment enhanced the contrast greatly and caused no other observable change.

Thin sections were obtained with a Porter-Blum MT-2 ultramicrotome, mounted on uncoated copper grids, and stained with lead citrate (Reynolds, 1963). Electron micrographs were obtained with a Siemens Elmskop I electron microscope operating with an accelerating voltage of 80 KV. Sections from at least three blocks of each subcellular fraction, experimental animal, and post-mortem time period were observed and photographed in order to assure representative sampling. The differences reported were consistent for both animals in each group.

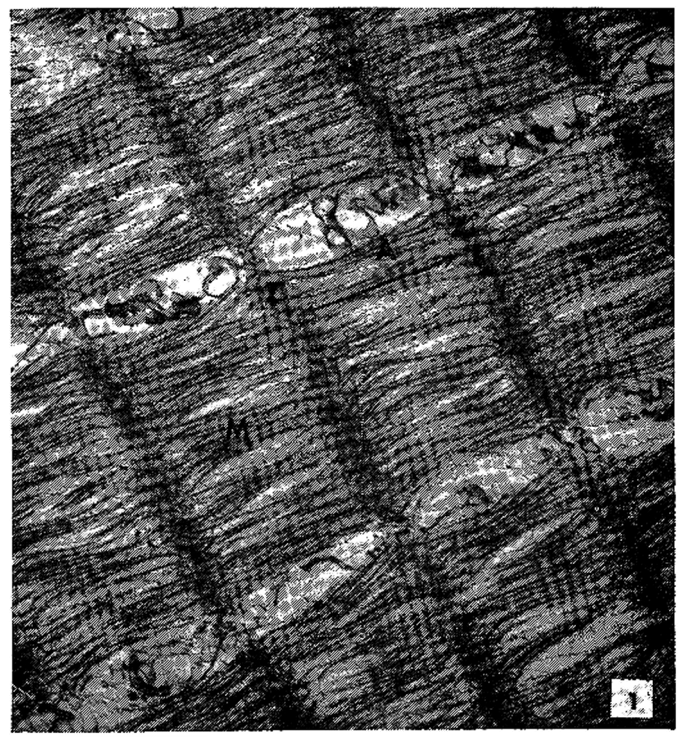

Fig. 1. Myofibril fraction from normal muscle isolated immediately after death. These myofibrils were highly contracted with no I band present. (×25,000).

RNA was determined using the orcinol method (Schneider, 1957). Glycogen was isolated using the procedure of Pfleiderer (1963) and analyzed following hydrolysis by the glucose oxidase method.

RESULTS AND DISCUSSION

Micrographs of the myofibril fraction isolated and fixed immediately after death are shown in Fig. 1–3. There were usually several types of myofibrils present in each sample. Fig. 1 shows the most common structure. The myofibrils were highly contracted with no I band present and with a dense band at the Z lines. This supercontracted conformation probably resulted from the disruption during homogenization which released divalent cations and which in turn stimulated contraction in the presence of the residual muscle ATP. Although this shortening was of the same magnitude as that occurring in leaf muscle during rigor at 2°C (Stromer *et al.*, 1967a,b), there was no evidence of displacement of M line segments as was seen by these workers.

The next most common pattern, although comprising less than 10% of the total, was that of myofibrils in a relaxed state as shown in Fig. 2. The appearance and banding pattern was similar in many respects to that typically observed in fixed, embedded intact muscle preparations. The Z lines were well preserved and displayed the typical zig-zag appearance (see arrow 1) (Knappeis *et al.*, 1962, Kelly, 1967). The major apparent difference from intact muscle was that the filaments at the A-I border were somewhat tangled and disoriented, causing this boundary to be less clearly defined (arrow 2). This localized and seemingly specific disorganization appeared to result from some condition during the separation procedure.

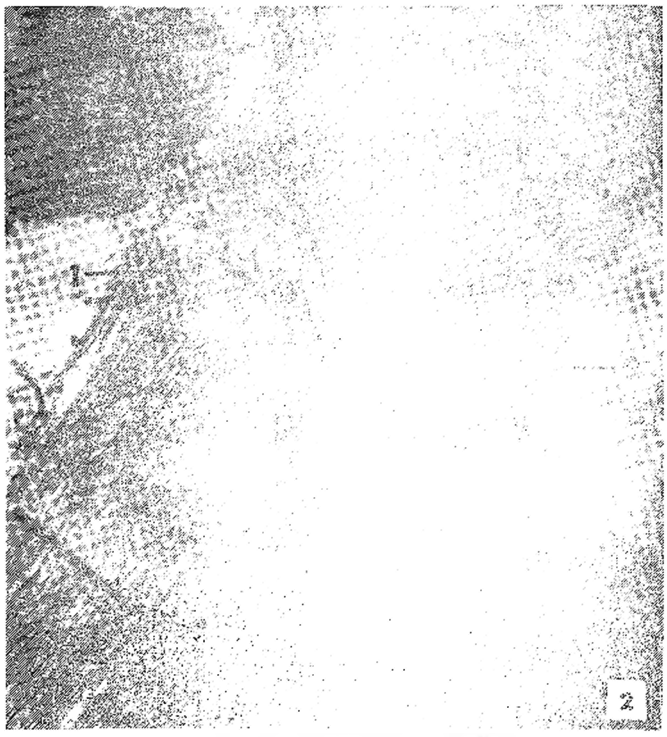

Fig. 2. Myofibril fraction isolated immediately after death. The myofibrils here in a relaxed state. Note the zig-zag pattern of the Z line (arrow 1) and the indistinct A-I border (arrow 2). (×25,000).

Fig. 3. Myofibril fraction from normal muscle isolated immediately after death. Part of the A band material appeared to be extracted. The M line was still present (see arrow). (×25,000).

A third pattern of myofibrillar structure which was often observed in the 0 hr samples is shown in Fig. 3. The A band material appeared to be partly extracted, with many of the thick filaments missing or broken. There was also extensive tangling and disorganization of the thin filaments. However, the extra material on the center of the thick filaments which gives rise to the M line could still be observed (see arrow). This extraction of A band material was probably the result of the combined effects of the residual muscle ATP and the salt of the homogenization medium, since it never occurred in the samples examined post-rigor.

Figs. 1–3 were obtained with samples of normal muscle. PSE myofibrils yielded identical results, both in the types of myofibrils observed and in the proportion of each type present.

Fig. 4 is a micrograph of the 24 hr myofibril fraction from normal muscle. This figure gives the pattern typical of nearly all myofibrils observed. Neither the supercontracted nor the extracted types of myofibrils which were observed in the 0 hr samples were seen at 24 hr. The myofibril structure appeared to be very similar to that observed with intact fresh muscle, with clearly defined A and I bands, Z lines, and M lines present. There was also evidence of an N line in most of the micrographs, although its appearance in the figure shown was less clear. The major structural change in the 24 hr myofibrils compared to intact fresh muscle was the breakage and loss of material from the Z line and the adjacent thin filaments (see arrows). This type of post-mortem change has been previously observed; however, its cause has not been explained (Cassens *et al.*, 1963).

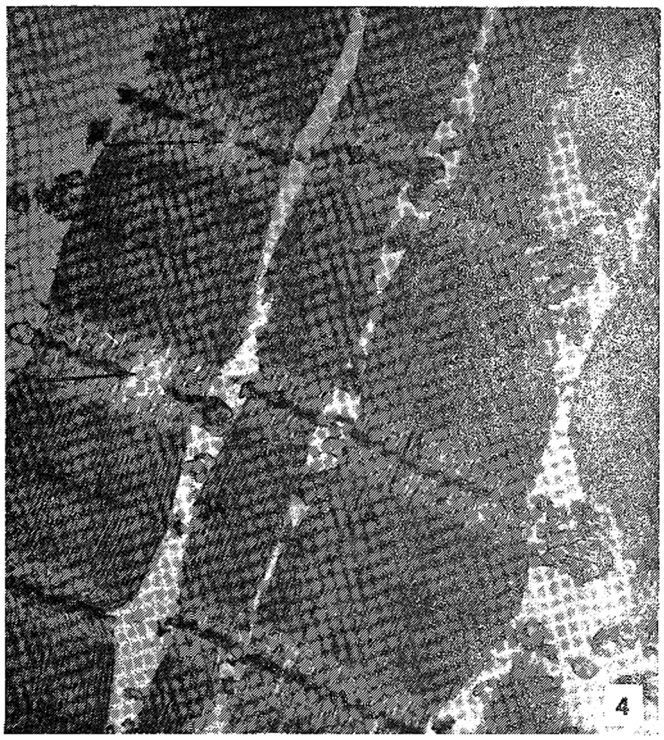

Fig. 4. Myofibril fraction from normal muscle isolated at 24 hr post-mortem. There was some breakage and loss of material in the region of the Z lines (see arrows). (×25,000).

A micrograph typical of the 24 hr PSE myofibrils is shown in Fig. 5. Although these myofibrils had structural patterns similar to normal 24 hr samples in most respects, there were two major differences. The first difference was the markedly increased width of the Z lines. The zig-zag structure was obscured and it appeared that there was a precipitation of material of unknown origin in this region. It is possible that this material was precipitated sarcoplasmic protein as has been postulated previously (Bendall *et al.*, 1962). Another possibility might be that the material consists of some myofibrillar protein that has become solubilized and then reprecipitated in the Z line region. It might also consist of Z line proteins which became disorganized. The present study does not differentiate between these or other possibilities.

The second major difference observed with PSE myofibrils was the distinctly granular appearance of the filaments, which confirmed previous work with PSE muscle (Cassens *et al.*, 1963). This granularity might be due to either precipitated sarcoplasmic proteins or changes in the conformation and properties of the myofibrillar proteins themselves. It may be related to the decreased myofibrillar protein solubility and the lesser water-holding capacity which has been found with PSE muscle (Sayre *et al.*, 1963; Bendall *et al.*, 1962). It might also be important in explaining the decreased ATPase activity of these samples compared to normal 24 hr myofibrils as shown in the previous paper (Greaser *et al.*, 1969).

The mitochondria fraction from 0 hr normal muscle is shown in Fig. 6. The mitochondria appeared very similar to those found in intact muscle (Shafig *et al.*, 1966; Slautterback, 1966) and those isolated from heart and liver (Munn *et al.*, 1965; O'Hegarty *et al.*, 1966; Wlodawer *et al.*, 1966). The outer membrane appeared to be intact and the internal membranes were arranged into vesicular and tubular configurations. The samples always contained many smaller membrane fragments in addition to the intact mitochondria. These smaller vesicles probably consisted

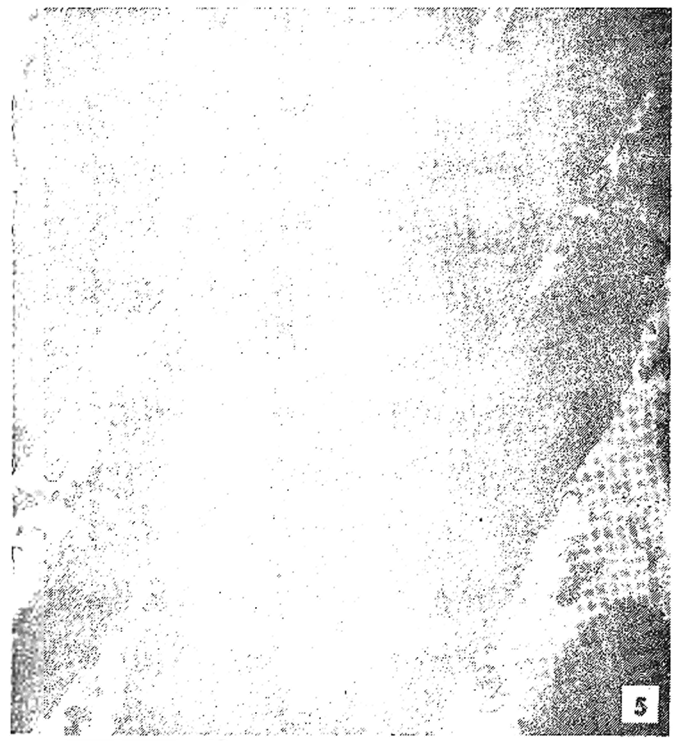

Fig. 5. Myofibril fraction from PSE muscle isolated at 24 hr post-mortem. Note the wide Z lines and the granular appearance of the myofilaments. (×25,000).

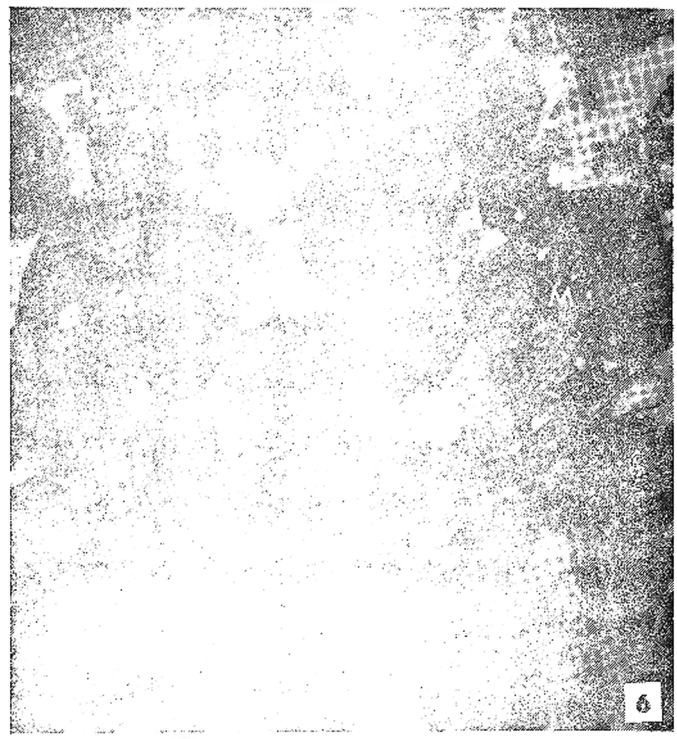

Fig. 6. Mitochondria fraction from normal muscle isolated immediately after death. The mitochondria (M) appeared to be well preserved. (×25,000).

of submitochondrial pieces and also larger sarcoplasmic reticulum fragments. The general appearance of this fraction was similar to that observed with pig muscle mitochondria (Greaser *et al.*, 1967).

Fig. 7 is a micrograph of the 0 hr PSE mitochondria fraction. Most of the mitochondria had conformation patterns like those from normal muscle. However, some were markedly swollen and had a decreased matrix density (see arrow). There was also a smaller proportion of intact mitochondria in the fractions isolated from PSE muscle. Thus it appeared that there may be important morphological changes that occur rapidly after death in PSE muscle or that the mitochondria become more susceptible to damage during homogenization. Although these differences seemed to be consistent in the samples examined, they apparently were not related to the calcium accumulating ability or ATPase activity since there were no statistical differences in these parameters as shown in the previous paper (Greaser *et al.*, 1969).

The 24 hr normal mitochondria fraction is shown in Fig. 8. The proportion of identifiable mitochondria fraction was much less than in the 0 hr samples. Most of the mitochondria were very swollen with a very low matrix density (see M1). However, an occasional mitochondrion appeared fairly well preserved with evident cristae still present (see M2). The majority of material present in this fraction consisted of small membrane fragments with heterogenous sizes and forms.

Fig. 9 shows the 24 hr PSE mitochondria fraction. The mitochondria in this fraction had a highly altered form in comparison to those observed in any of the other samples. The predominant configuration consisted of a gray, nonmembranous matrix surrounded by a variable number of concentric membrane forms. These membranes were triple layered, with two dark lines separated by a layer of lesser density. The appearance of these peculiar forms may be due either to the low pH-high temperature conditions that occur in PSE muscle or to inherent differences in their structural organization. The importance of this observation to the understanding of the PSE problem is unknown.

The 0 hr normal heavy sarcoplasmic reticulum fraction is shown in Fig. 10. It contained mostly membranous material in the form of vesicles (V) and tubules (T). In addition there was some small granular material (G) and occasional filaments (F). The appearance of this fraction was in general agreement with observations using similar preparations from rabbit muscle and with a previous study using pig muscle (Nagai *et al.*, 1960; Ebashi *et al.*, 1962; Sreter, 1964; Greaser *et al.*, 1967).

The composition of the 0 hr PSE heavy sarcoplasmic reticulum fraction, however, appeared to be different (Fig. 11). There was a considerably higher proportion of granular material (G) in this fraction than that from normal muscle. This observation was consistent in all sections examined. The granules are probably either ribosomes or glycogen particles, and the results of chemical determinations to identify this material will be discussed later.

The 24 hr heavy sarcoplasmic reticulum fractions from normal and PSE muscle are shown in Figs. 12 and 13, respectively. Both samples consisted predominantly of membranous material with no apparent differences between them. The 24 hr normal fraction appeared nearly identical to its 0 hr counterpart. The only difference in the 24 hr PSE fraction from the 0 hr was the absence of the granular material. In both cases there was no evidence

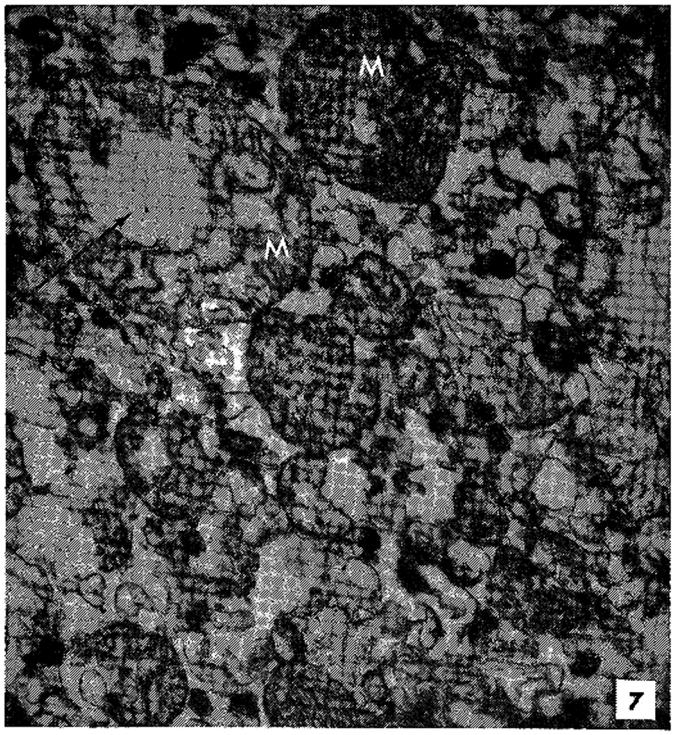

Fig. 7. Mitochondria fraction from PSE muscle isolated immediately after death. Some of the mitochondria (M) were swollen (see arrow). (×25,000).

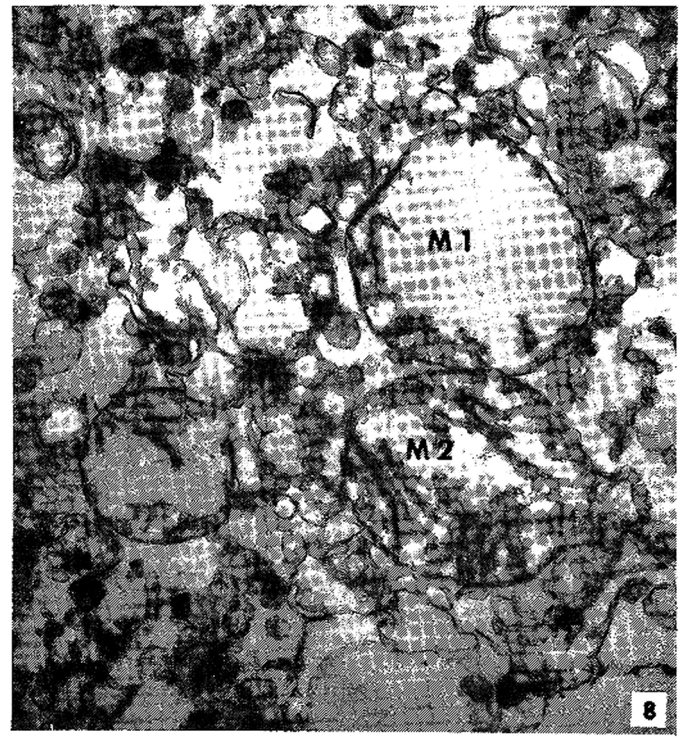

Fig. 8. Mitochondria fraction from normal muscle isolated at 24 hr post-mortem. Most of the mitochondria were quite swollen (see M1). Occasional mitochondria appeared reasonably well preserved (M2). (×25,000).

for a structural change that could explain the 5 to 10-fold decrease in calcium accumulating ability that occurs between 0 and 24 hr in these fractions (Greaser *et al.*, 1969).

Fig. 14 is a micrograph of the 0 hr normal light sarcoplasmic reticulum fraction. This fraction was quite heterogenous in nature with filaments (F), vesicles (V) and granules (G) making up most of the material observed. The proportion of each of these major constituents varied considerably depending on the region of the block from which sections were obtained and on the area of the section observed. The filamentous material resembled the thick and thin filaments found normally in the myofibrils, both in size and in relative position assumed.

The presence of this type of material in subcellular fractions from muscle appears to be more likely when the samples are homogenized in a salt solution (Muscatello *et al.*, 1961). These filaments probably originated from either fragmented myofibrils or dissolution and reprecipitation of myofibrillar proteins. It is possible that they may constitute part of the material which is missing from some of the myofibrils (see Fig. 3).

The 0 hr PSE light sarcoplasmic reticulum fraction is shown in Fig. 15. Its composition was indistinguishable from the corresponding fraction from normal muscle although the figure shown has a larger proportion of granules and vesicles and a smaller proportion of filaments. However, micrographs with appearances identical with Fig. 14 were also obtained with this fraction from PSE muscle.

The 24 hr light sarcoplasmic reticulum fractions from normal and PSE muscle are shown in Figs. 16 and 17. The fraction from normal muscle consisted of a large proportion of granular material with a few flattened vesicles. The appearance of these granules was similar to that seen in the 0 hr heavy sarcoplasmic reticulum fraction from PSE muscle. These vesicles were often arranged in stacks

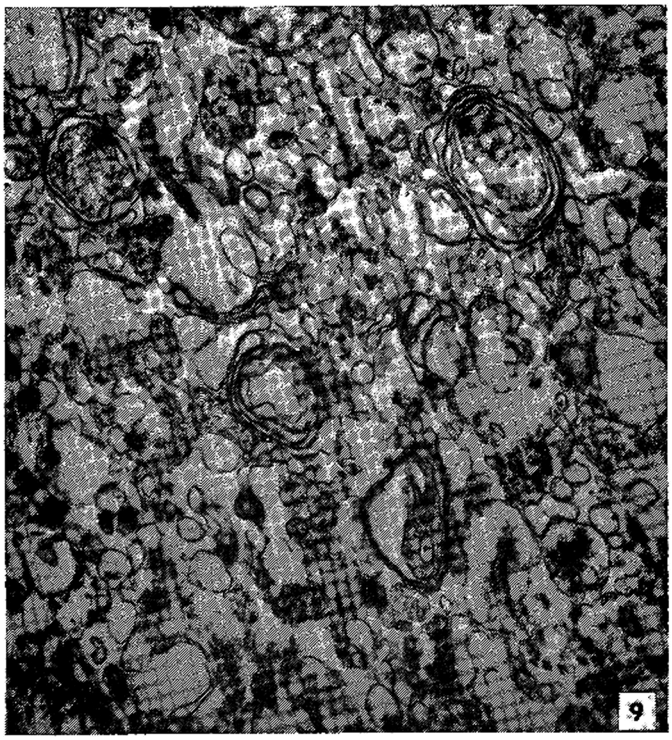

Fig. 9. Mitochondria fraction from PSE muscle isolated at 24 hr post-mortem. Many of the membranes were arranged in a circular configuration. (×25,000).

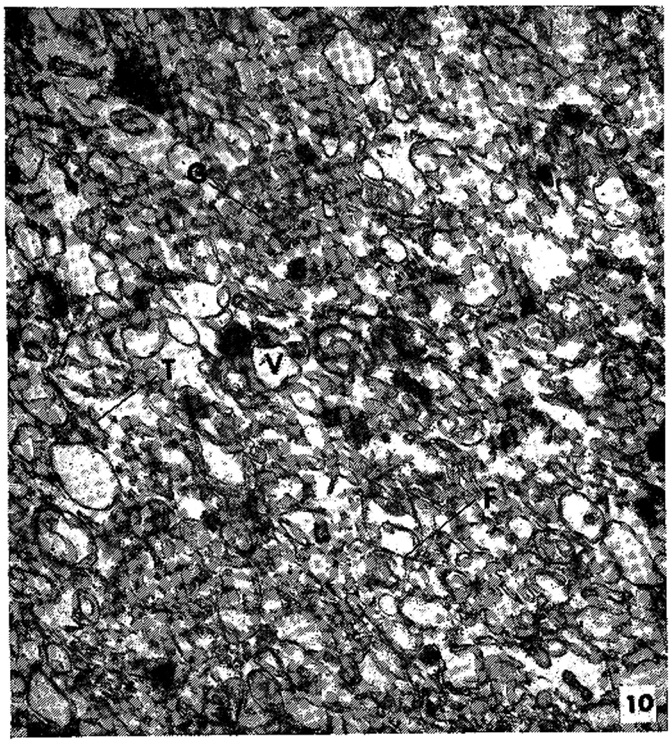

Fig. 10. Heavy sarcoplasmic reticulum fraction from normal muscle isolated immediately after death. It contained vesicles (V), tubules (T), filaments (F), and granules (G). (×25,000).

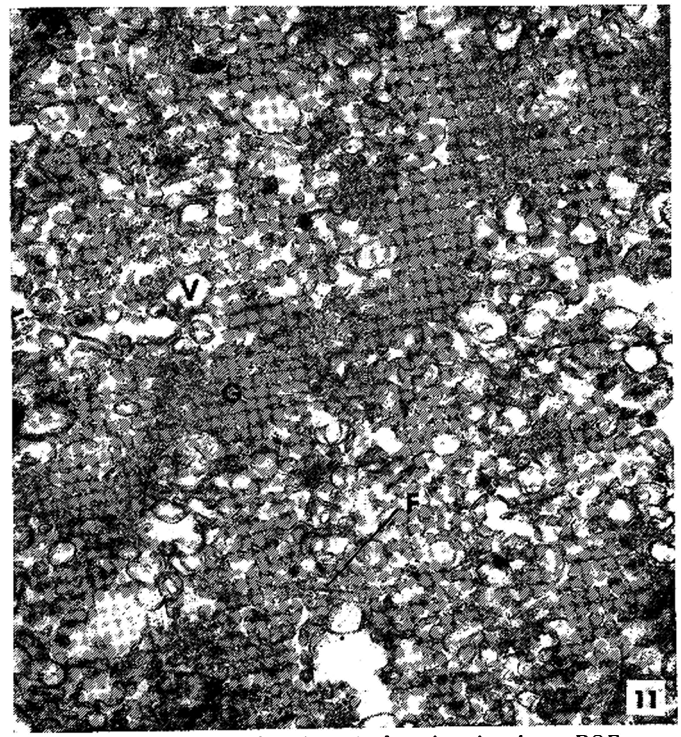

Fig. 11. Heavy sarcoplasmic reticulum fraction from PSE muscle isolated immediately after death. Note the large proportion of granules present. (×25,000).

and were different from those found in other fractions either at 0 or 24 hr.

The PSE light sarcoplasmic reticulum fraction at 24 hr post-mortem had a much different appearance. It consisted predominantly of small vesicles with a few isolated filaments. The content of granules was extremely limited, with most of the material observed being practically devoid of this constituent. The underlying cause of these differences is not apparent.

An attempt was made to determine if the granules which were found in several fractions consisted of either ribo-

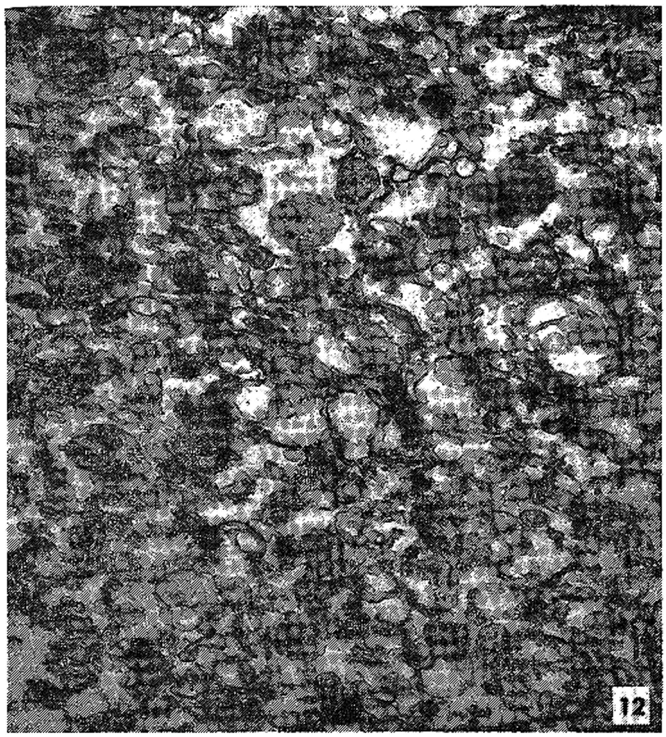

Fig. 12. Heavy sarcoplasmic reticulum fraction from normal muscle isolated at 24 hr post-mortem. Its appearance was very similar to the 0 hr sample. (×25,000).

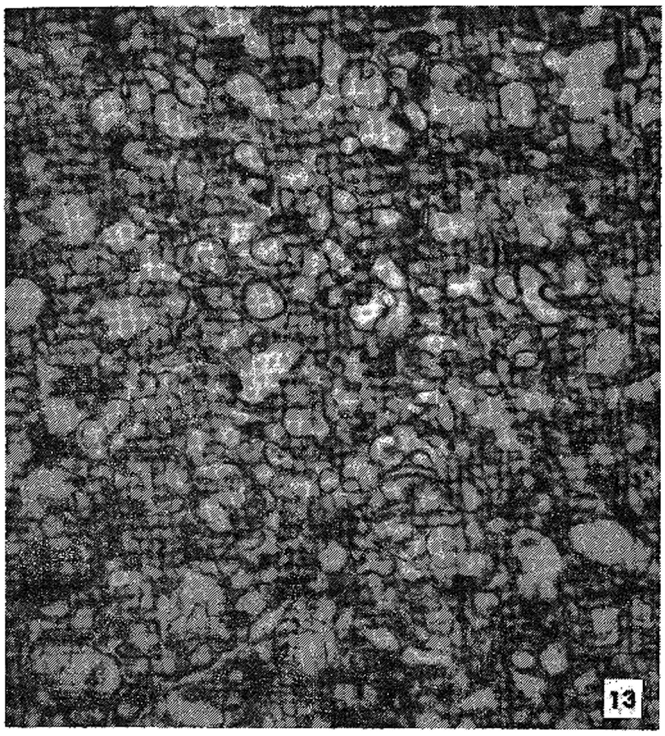

Fig. 13. Heavy sarcoplasmic reticulum fraction from PSE muscle isolated at 24 hr post-mortem. The only major difference from the 0 hr PSE fraction was the absence of the granules. (×25,000).

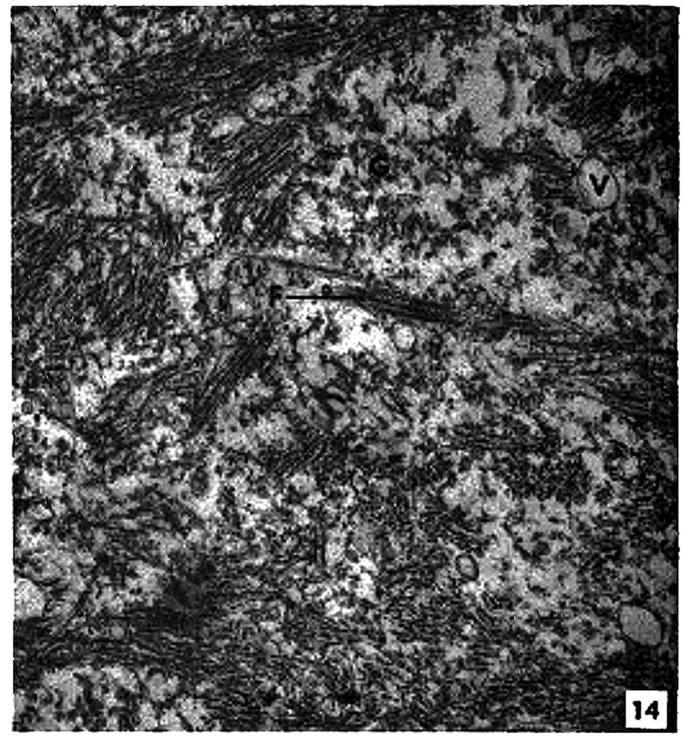

Fig. 14. Light sarcoplasmic reticulum fraction from normal muscle isolated immediately after death. This fraction contained filaments (F), vesicles (V), and granules (G). (×25,000).

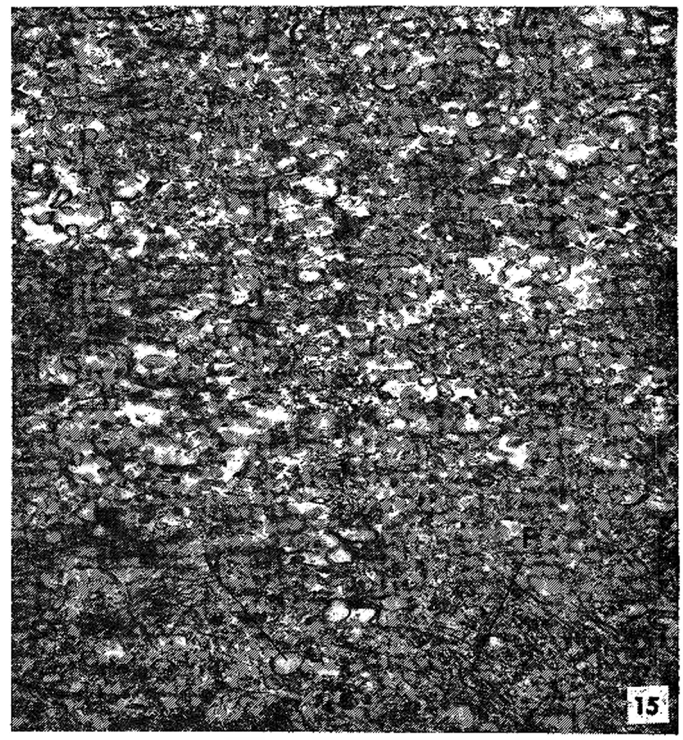

Fig. 15. Light sarcoplasmic reticulum fraction from PSE muscle isolated immediately after death. (×25,000).

somes or glycogen particles. The results of an RNA analysis indicated that there was no difference in RNA concentration between the normal and PSE muscle fractions at either 0 or 24 hr post-mortem. Glycogen levels were found to be greater in the 0 hr PSE heavy sarcoplasmic reticulum fraction than in the same fraction from normal muscle. However, there was no difference in glycogen level between the normal and PSE 24 hr light sarcoplasmic reticulum fractions. Therefore the data are not conclusive. However, if both the RNA and glycogen results are considered, the evidence appears to favor glycogen or something other than RNA as the material which make up the granules. Further work will be necessary to establish their precise identity.

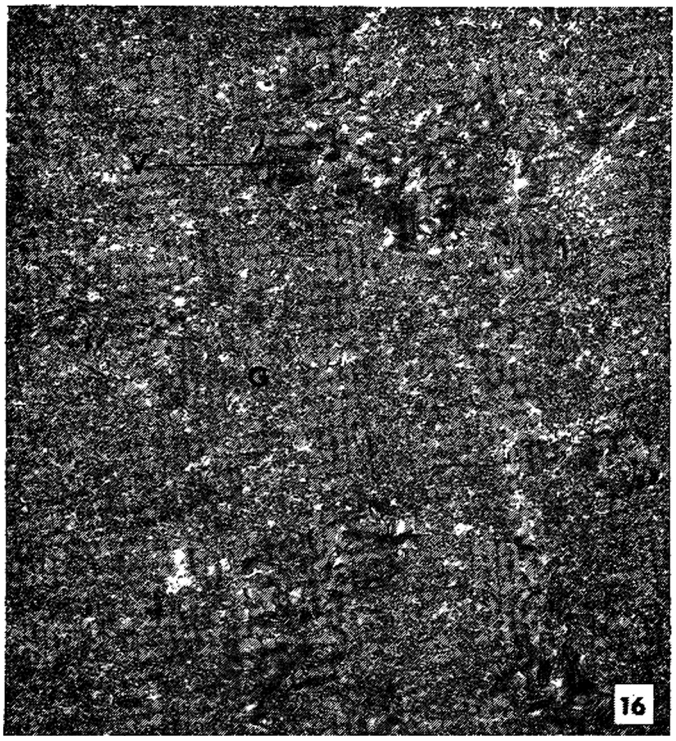

Fig. 16. Light sarcoplasmic reticulum fraction from normal muscle isolated at 24 hr post-mortem. It consisted predominantly of granules (G) and a few flat vesicles (V). (×25,000).

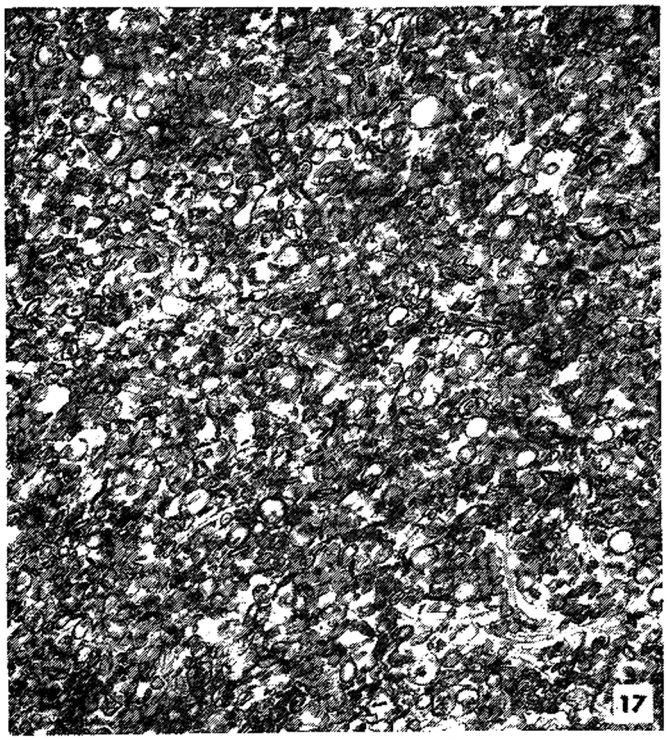

Fig. 17. Light sarcoplasmic reticulum fraction from PSE muscle isoloted at 24 hr post-mortem. Small vesicles were the major material in this fraction. (×25,000).

The results of this study indicated that there were several structural and compositional differences between subcellular fractions from normal and PSE muscle, both immediately after death and at 24 hr post-mortem. Therefore caution should be exercised in making biochemical comparisons on subcellular fractions obtained from post-mortem muscle even though the fractions were obtained using identical procedures. The differences observed, however, could not entirely explain the changes in calcium accumulating ability or ATPase activity that have been shown to occur post-mortem (Greaser *et al.*, 1969).

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Comparative Rates of IMP Degradation in Unfrozen and Frozen-and-Thawed (Slacked) Fish

SUMMARY—The comparative rates of IMP degradation between fresh and frozen-and-thawed (slacked) fish were compared on six different species of fish. Several factors that could contribute to a rate change of IMP degradation were evaluated. These included freezing temperatures, time in frozen storage, pre- and post-rigor freezing, and method of killing the fish.

English sole and rainbow trout showed slight increases in the rate of IMP degradation when they were frozen and then thawed within 48 hr. Silver salmon and halibut that were frozen and then thawed within 48 hr showed no change in the rate of IMP degradation. Halibut, however, that was frozen and stored at—20°F for 3 months showed a slight decrease in the rate of IMP degradation after it was thawed; but king salmon handled under the same conditions did not.

The method of kill or freezing the fish either pre- or post-rigor did not alter the rate of IMP degradation after the fish was thawed.

No loss of IMP occurred in fish (halibut) stored at —20°F. Over one-third of the original IMP content was lost in halibut stored at +15°F after 3 months of storage.

These results show that there is no significant difference in the rate of IMP degradation between fresh and slacked fish. The flavor-contributing effect of IMP in slacked fish therefore should be the same as in fresh fish, provided the fish was frozen and stored at or near a temperature of —20°F.

INTRODUCTION

MUCH OF THE FISH sold as fresh is in reality frozen fish that has been thawed prior to sale (slacked fish). Acceptability in this type of product is related both to the quality of the frozen fish and to the storage life of the fish after it is thawed.

One of the most important factors in producing acceptable processed foods is the preservation of the fresh flavor characteristics. Recent studies on fish flavor have established the importance of nucleotides, especially IMP (inosine monophosphate) to the flavor of good quality fresh fish (Hashimoto, 1964). In species in which IMP is rapidly degraded, flavor losses during iced storage have been related to the degradation of this compound (Miyauchi *et al.*, 1964). Other work shows that irradiated fish containing IMP levels above the taste threshold (0.7 μM/g) were preferred to fish containing less than this amount (Spinelli *et al.*, 1968). Recently Groninger *et al.* (1968) showed that, if nucleotidase activity was blocked in fish by treatment with EDTA, the treated fish were preferred to untreated fish.

The formation and degradation of IMP is the result of a series of enzymatic reactions. It is known that freezing, thawing and frozen storage can alter the enzymatic activity of tissue considerably. For example, freezing can result in the inactivation of some enzyme systems or conversely in the inactivation of others (Chilson *et al.*, 1965; Grant *et al.*, 1966; Partmann, 1961).

If, as a result of freezing, a significant change was induced in the rate of IMP-degradation in slacked fish, this change could have an effect on the ultimate quality and acceptability of the fish. The work presented here has as its primary objectives: (1) comparison of the rates of IMP-degradation during refrigerated storage of frozen-thawed fish and fresh fish; (2) examination of the effects that processing conditions such as freezing temperatures, condition of fish prior to freezing and frozen storage times

and temperatures may have on the rate of IMP degradation during the refrigerated storage of frozen-thawed fish.

MATERIALS AND METHODS

Fish

Sole. English sole *(Parophrys vetulus)* and Dover sole *(Microstomus pacificus)* were obtained from "day boat" fisheries when not more than 24 hr old.

Rainbow trout. Rainbow trout *(Salmi gairdneri)* were obtained from a local trout farm. They were killed at the laboratory by stunning.

Salmon. Silver salmon *(Oncorhynchus kisutch)* and king salmon *(Oncorhynchus tshawytscha)* were obtained from local boats and were 1 to 3 days old when landed.

Halibut. Pacific halibut *(Hippoglossus stenolepsis)* were obtained from commercial boats and were approximately 7 days old when landed. Halibut used in the experiments on determining the effect of the methods used to kill fish were caught by Bureau personnel and were 7 days old when landed.

Sample preparation

Samples for freezing studies on salmon and halibut were prepared as follows: The fish were cut into ¾-in.-thick steaks. One group was reserved for an iced control and the other was frozen. Rainbow trout were split longitudinally into two portions. One portion from each fish was used as an iced control and the other was frozen. English sole and Dover sole were divided into lots of six fish. One lot was used for unfrozen controls and the others were frozen at the desired temperature. All samples were wrapped in foil prior to freezing or iced storage.

Freezing and thawing

Unfrozen control samples were held at 34°F. Frozen samples were cooled to 0°F and −20°F by air blast and to −40°F in a plate freezer. Additional samples of English sole were frozen to −300°F by immersion in liquid nitrogen. At the end of the desired storage period, the frozen samples were thawed and held in a cold room at 34°F.

IMP analysis

At suitable intervals, perchloric acid extracts were prepared from the frozen-thawed samples and the corresponding unfrozen samples. For 0 time values, samples were removed from the frozen samples prior to thawing. IMP content of the extracts was determined by the method of Spinelli *et al.* (1966).

RESULTS

Effect of the freeze-thaw cycle

To determine the effect of the freeze-thaw cycle alone, samples of various species were frozen at different temperatures and held in frozen storage for not longer than 48 hr.

Effect of freezing temperatures. The temperature to which the fish were frozen appeared to have no significant effect on the rate of IMP degradation after thawing. In addition to the −20°F and −40°F temperatures used, English sole samples were also frozen to 0°F and −300°F. The rate of IMP degradation in these samples was similar to that in fish frozen at −20°F.

Differences among species. The effect of freezing and thawing was not significant on the subsequent rate of IMP degradation in slacked halibut, Dover sole and salmon (Figs. 1, 2, 3). In English sole and rainbow trout, freezing and thawing slightly accelerated the rate of IMP degradation (Figs. 4, 5). In thawed English sole, IMP levels of about 1 μM/g were obtained after 2 days of storage, whereas it took 3 days for the iced-storage control samples to drop to that concentration. In rainbow trout,

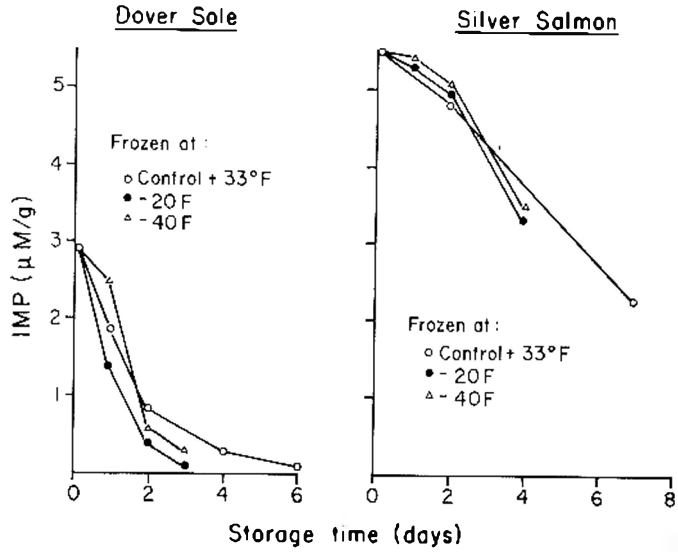

Fig. 1. Comparative rates of IMP degradation between frozen-and-thawed and unfrozen Dover sole and silver salmon.

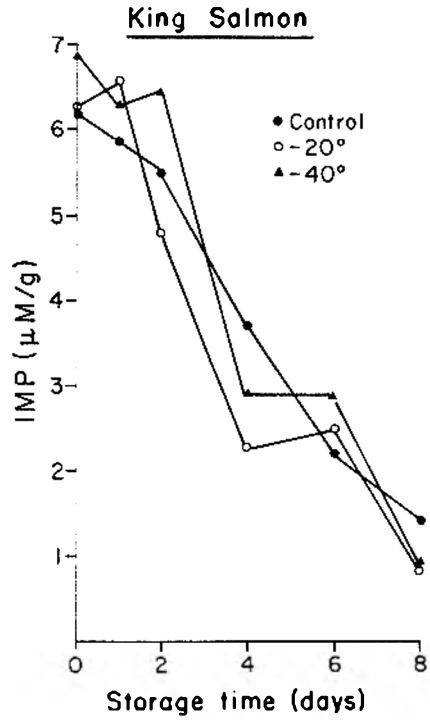

Fig. 2. Comparative rates of IMP degradation between frozen-and-thawed and unfrozen king salmon. Control sample held at 34°F.

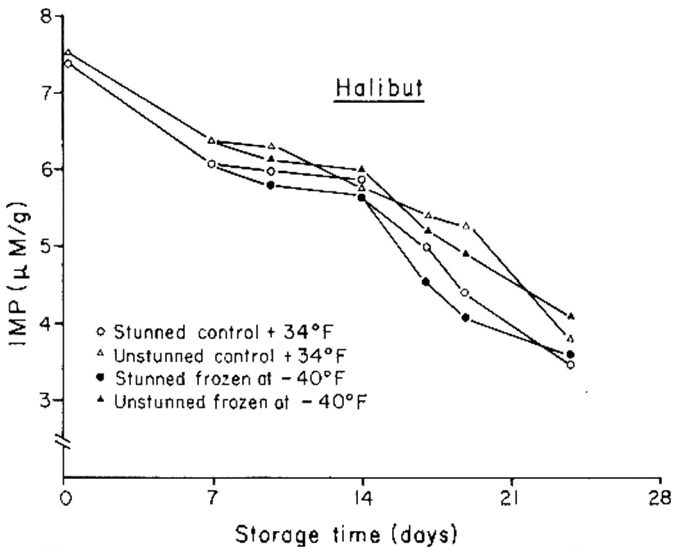

Fig. 3. Comparative rates of IMP degradation between frozen-and-thawed and unfrozen halibut. Rates are shown for both stunned and unstunned halibut.

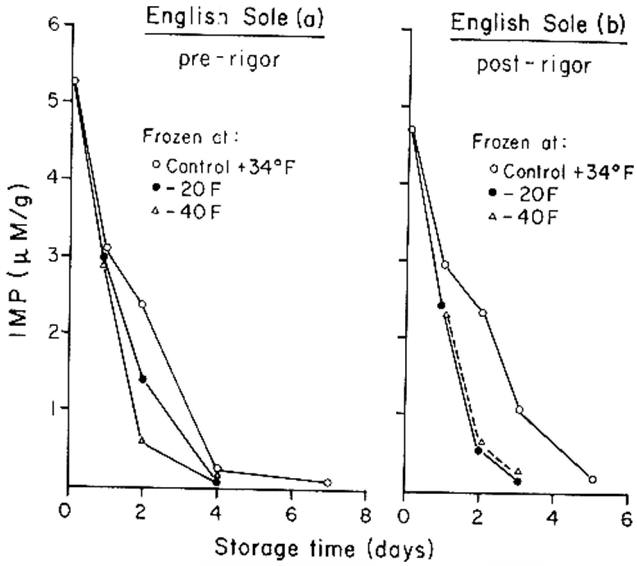

Fig. 4. Comparative rates of IMP degradation between pre- and post-rigor frozen-and-thawed and unfrozen English sole.

an acceleration in the rate of IMP degradation was noted after 3 days of storage. After 3 days, the IMP levels in the thawed samples fell sharply, while the control samples maintained a gradual rate of IMP loss. After 6 days of storage, there was a pronounced difference in the IMP levels of the samples. The thawed samples contained approximately 0.5 μM/g of IMP, while the control samples contained 2.0 μM/g.

The effect of frozen storage and storage temperatures

The effect of storage temperatures and longer storage periods were examined using king salmon and halibut.

Samples of king salmon were frozen and held for 3 months at −20°F and at −3°F. The rate of IMP degradation after thawing was unaltered in both cases (Fig. 6).

Halibut kept in frozen storage at −20°F showed a decrease in IMP degradation after 3 months of storage (Fig. 7). After 9 days, the slacked sample had an IMP content of 3.0 μM/g while the iced control samples contained approximately 1.5 μM/g. In samples held at +15°F, the rate of IMP degradation was similar to that of the controls. At this temperature, however, the initial IMP content of the sample that had been frozen was approximately 2 μM/g lower than the control, indicating the IMP had been degraded during the storage period. Similar results have been reported by Jones (1963) and Dyer *et al.* (1966). These investigators found that nucleotidases were active in haddock, cod and swordfish at temperatures above −14°C but inactive at temperatures lower than −20°C.

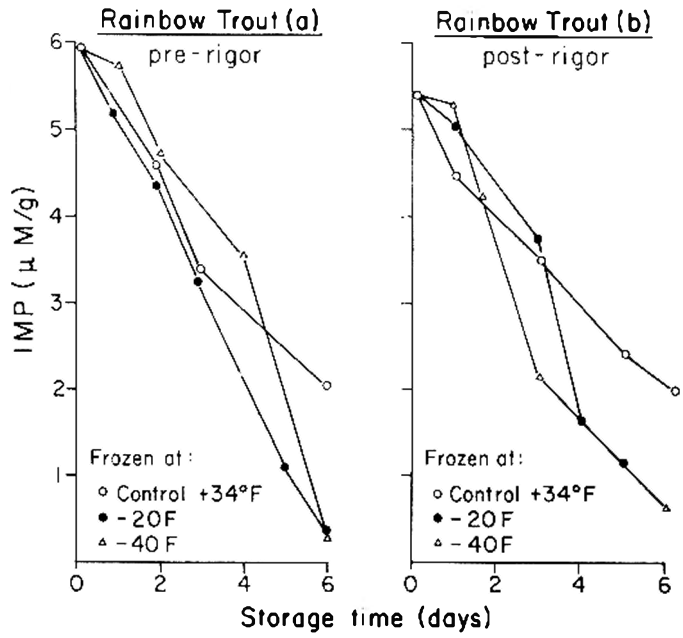

Fig. 5. Comparative rates of IMP degradation between pre- and post-rigor frozen-and-thawed and unfrozen rainbow trout.

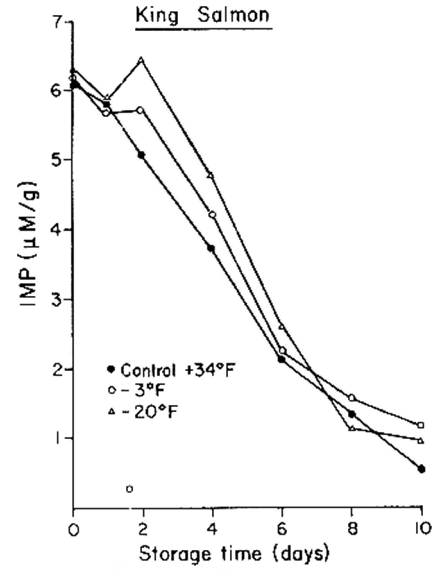

Fig. 6. Comparative rates of IMP degradation between frozen-and-thawed (frozen and stored for 3 months at −20°F and −3°F) and unfrozen king salmon.

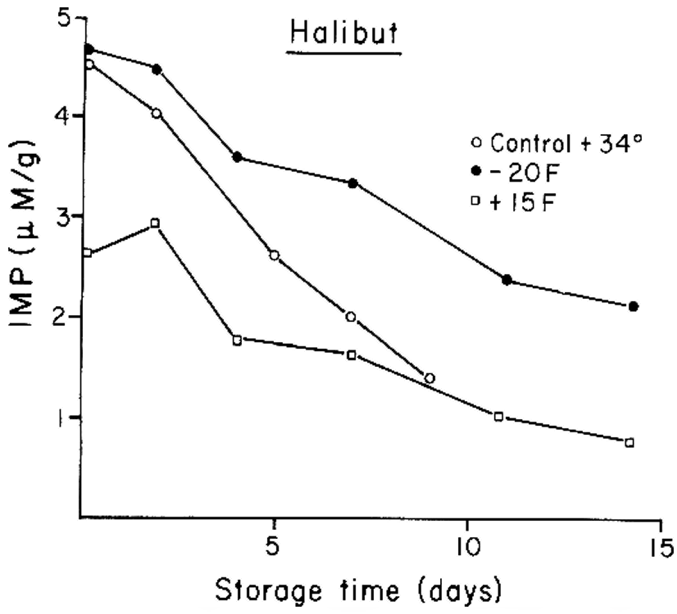

Fig. 7. Comparative rates of IMP degradation between frozen-and-thawed (frozen and stored for 3 months at −20°F and +15°F) and unfrozen halibut.

The effect of fish condition prior to freezing

Pre- and post-rigor freezing. The effect of pre-rigor freezing on the rate of IMP degradation in slacked fish was studied using English sole and rainbow trout. The rate of IMP degradation in these samples was similar to that of samples frozen post-rigor (Figs. 4, 5), indicating that neither ATPase nor the nucleotidases were affected by freezing the fish pre-rigor.

Effect of methods used to kill fish

Although most commercial fish die by suffocation, some investigators believe that the method used to kill fish has an influence on the quality (Tretsven, 1968).

To determine if the method used to kill had any effect on the rate of IMP degradation, samples were obtained from halibut that had been killed by stunning and by allowing them to suffocate.

The results of this experiment are shown in Fig. 3. There was no significant difference in the rate of IMP degradation of the fish that had been killed by stunning and the ones that had been killed by suffocation. At the end of 24 days of iced storage, the differences in IMP levels were only 0.3 μM/g in the iced samples and 0.5 μM/g in the slacked samples.

These experiments indicate that the rate of IMP degradation in slacked and iced fish is not altered significantly by the chemical changes in tissue induced by stress or rigor.

DISCUSSION

FREEZING DOES NOT materially affect the rate of IMP degradation in fish after it is thawed. Insofar as the flavor-enhancing properties of IMP are concerned, losses in the flavor of slacked fish attributable to the degradation of this compound should be similar to those in unfrozen fish.

However, during storage at poor temperatures, IMP may be degraded resulting in a low IMP content in the slacked fish. The implications of quality deterioration due to IMP degradation during frozen storage have been discussed by Jones (1963). It should be pointed out that poor storage temperatures superimpose other quality changes, i.e., texture and rancidity, on those resulting from flavor loss, thereby making it difficult to single out a particular factor as being responsible for alterations in the quality of slacked fish.

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Effect of Feeding and Withdrawal of Menhaden Oil on the ω3 and ω6 Fatty Acid Content of Broiler Tissues

SUMMARY—Analysis was made of the fatty acid composition of liver, adipose fat, thigh and breast muscles of broilers fed corn-soy commercial-type of diets containing one of two levels of fish oil (2.5 or 5.0%). The oil was subsequently continued, withdrawn or replaced with yellow grease 2, 3 or 4 weeks before termination of the experiment at the 8th week. The tissue contents of four ω-3-type fatty acids (20:4, 20:5, 22:5 and 22:6) were increased in relation to the number of weeks menhaden fish oil was included in the diet. (The first number indicates the chain length of carbons, the second indicates the number of double bonds. The number following ω (omega) indicates the carbon position of the first double bond as counted from the terminal methyl group.)

Of the four tissues studied, the liver had the highest total content of the ω3 fatty acids; the adipose fat, the least. The muscles had intermediate values; the breast containing more than the thigh. Withdrawal of fish oil at the 4th week resulted in favorable organoleptic evaluation. A more favorable evaluation was obtained if yellow grease was substituted in place of fish oil for the last 4 weeks. The organoleptic scores were highly significantly correlated with the content of 20:5 ω3, 22:5 ω3 and 22:6 ω3 but it was less significantly correlated with the content of 20:4 ω3. An inverse relation existed between the content of the ω3 with that of the ω6 fatty acids.

INTRODUCTION

BIOCHEMICALLY, the unsaturated fatty acids may be classified into several families of which the three major ones are linolenate, linoleate and oleate families. The fatty acids of these families have the first double bond at either the 3rd, 6th or 9th carbon (counting from the terminal methyl group as containing the first carbon) and are therefore referred to as the ω3, ω6 or ω9 families respectively by Mohrhauer et al. (1963a,b). According to Klenk et al. (1962) and Ackman (1964), the major long-chain polyunsaturated fatty acids of fish oils belong to linolenate (ω3) family, consisting primarily of the fatty acids: 18:3ω3, 18:4ω3, 20:4ω3, 22:6ω3.

Of all the families, only the ω3 and ω6 fatty acids are of exogenous origin, in that the parent acids (18:3ω3 and 18:2ω6) or any other member of these two families must be of dietary origin. They, therefore, are considered as nutritionally essential fatty acids to the animal body for various physiological functions.

Mohrhauer et al. (1963a,b) established the pathways of conversion of fatty acids within the same family and indicated the nonconversion of one family to another. However, after assimilation, the fatty acids of the ω3 and ω6 families can be elongated or desaturated within the metabolic pathway specific for each family. In addition, these two fatty acid families are known to compete metabolically for the same enzymes that desaturate or alter the chain length of the fatty acids.

The deposition of long-chain polyunsaturated fatty acids in broilers due to the ingestion of fish oils has been demonstrated by Edwards et al. (1963) and by Marion et al. (1963) and is in accordance with the previously observed phenomenon that dietary fat can influence the fatty acid composition of tissues. Subsequently, Miller et al. (1967a,b) showed that the flesh of broilers fed refined and concentrated fractions of menhaden oil with extremely low fat basal diet (containing glucose and soybean meal) also contained considerable quantities of long-chain polyunsaturated fatty acids which were identified as ω3 and ω6 fatty acids. These workers reported that ω3 fatty acids were found to be correlated to off-flavor in the broiler flesh.

Subsequently, it was deemed desirable to also obtain data associated with the feeding and withdrawal of commercial menhaden oil to broilers raised on practical type of diets (corn and soy meals), the inherent fat of which contained predominantly linoleic (ω6) acid, in contrast to the high levels of ω3 fatty acids in menhaden fish oil.

The objectives of this study were, therefore, (1) to determine the deposition pattern of the ω3 and ω6 families of fatty acids in broiler tissues as affected by the ingestion of fish oil concomitant with a practical broiler diet, (2) the influence of the withdrawal of menhaden fish oil or its replacement by yellow grease at various time periods prior to marketing and (3) the relation of ω3 and ω6 fatty acids content in the tissues to the organoleptic evaluation of the broiler meat.

MATERIALS AND METHODS

TO ATTAIN these objectives, the research was conducted in three phases: first, the feeding of 2.5 and 5% levels of menhaden fish oil to broilers for 4, 5, 6 or 8 weeks followed by either complete withdrawal or substitution of the fish oil with yellow grease; second, the gas-liquid chromatographic (GLC) analyses of the fatty acids in the lipids extracted from thigh and breast muscles, liver and adipose fat; and third, the organoleptic evaluation of the broiler flesh (legs, breast and wings) for any unacceptable flavor that may have resulted from the dietary treatments. The ω3 and ω6 fatty acids composition of these tissues was compared statistically with the organo-

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Table 1. Composition of diets for broilers fed menhaden oil at different growth periods.

Components	22% Protein starters 1st 4 weeks composition in %			20% Protein finishers 2nd 4 weeks composition in %		
Added fat	0	2.5	5.0	0	2.5	5.0
Ground yellow corn	62.5	56.0	49.5	66.5	60.0	53.5
Soybean meal (50%)	27.5	28.5	29.5	23.5	24.5	25.5
Menhaden fish meal	5.0	5.0	5.0	5.0	5.0	5.0
Alfalfa meal (20%)	2.0	2.0	2.0	2.0	2.0	2.0
Dicalcium PO_4	1.5	1.5	1.5	1.5	1.5	1.5
Ground limestone	0.5	0.5	0.5	0.5	0.5	0.5
Salt (NaCl)	0.3	0.3	0.3	0.3	0.3	0.3
Trace minerals + Se (1 ppm)	0.1	0.1	0.1	0.1	0.1	0.1
Vitamins + ethoxyquin (2.5 ppm) + procaine penicillin (2 g)	0.5	0.5	0.5	0.5	0.5	0.5
Methionine hydroxy analogue	0.1	0.1	0.1	0.1	0.1	0.1
Cellulose	0	3.0	6.0	0	3.0	6.0
	100.0	100.0	100.0	100.0	100.0	100.0

leptic scores obtained for the broiler flesh for possible correlation.

Feeding phase

Chicks. Day-old Arbor Acre broiler cockerels were randomly allotted to two pens of five chicks per diet. The chicks were kept in electrically heated brooder batteries for the first 4 weeks and then were transferred to growing batteries for the second 4 weeks. At the end of the 8-week experiment, the broilers were slaughtered, dressed, eviscerated and cut along the back bone into halves. They were frozen and stored at −20°C for lipid extraction, GLC analysis and organoleptic evaluation.

Diets. Two "commercial-type" diets—a starter and a finisher containing 5% menhaden fish meal, were fed in sequence as shown in Table 1. The 22% protein isocaloric starter diet was fed for the first 4 weeks and the 20% protein isocaloric finisher for the last 4 weeks. Table 2 shows the experimental design which includes a total of 15 dietary treatments. The treatments consist of different dietary fish oils (2.5 or 5.0%) which were fed for various times (4, 5 or 6 weeks) at which time the fish oil was either continued, withdrawn completely or replaced with an equivalent dietary level of yellow grease.

The control contained no added fat. Of the remaining 14 diets, seven received menhaden oil at the 2.5% level and the other seven at the 5% level. Each fish oil level was fed to two groups of chicks for the entire 8 weeks. Both the 2.5 and 5% level of menhaden oil were discontinued in a pair of diets at each of the following time periods: the 4th, 5th or 6th week of the experiment. Subsequently chicks fed one diet of the pair were continued for the remaining weeks of the 8-week experimental period on the basal diet (containing no added fat) while the other group was fed either the 2.5 or 5.0% level of yellow grease substituted for the menhaden oil for the remaining weeks.

Fat supplements. The menhaden fish oil used in this study was of commercial production stabilized with 0.0125% of ethoxyquin. The yellow grease, stabilized with butylated hydroxytoluene and butylated hydroxyanisol, was a commercial product sold to manufacturers of broiler feed.

Extraction and analysis of lipids in tissues and diets

Thigh and breast muscles, liver and adipose tissues of broilers were extracted and analyzed by gas-liquid chromatography to determine the effect of the various diets on the fatty acid composition of the lipids of these tissues.

Composite samples of each of four tissues (liver, adipose fat, thigh muscles and breast muscles) collected from chicks fed the 15 dietary treatments were extracted five times in a gallon-size Waring Blendor for 3 minutes with

Table 2. Organoleptic evaluation of broilers fed menhaden oil during different growth periods.

Treatments	Fish oil level (%)	Weeks fish oil fed	Grease level (%)	Weeks grease in place of fish oil	Organoleptic score[1]
1	0		0		1.4
2	2.5	4	0	4	1.9
3	2.5	5	0	3	1.6
4	2.5	6	0	2	2.1
5	2.5	8	0		3.7
6	5	4	0	4	1.8
7	5	5	0	3	2.5
8	5	6	0	2	3.0
9	5	8	0		3.8
10	2.5	4	2.5	4	1.5
11	2.5	5	2.5	3	2.4
12	2.5	6	2.5	2	1.9
13	5	4	5	4	1.4
14	5	5	5	3	2.9
15	5	6	5	2	2.8

[1] Average score of 12 judges.
Scoring system: 1 indicates entirely acceptable.
2 indicates probably acceptable.
3 indicates questionable flavor.
4 indicates probably unacceptable.
5 indicates definitely unacceptable.

three volumes of chloroform-methanol (2:1) mixture as proposed by Folch et al. (1957). These five extracts of tissue were filtered, freed of solvent by rotary-vacuum distillation and combined. The resulting concentrate was redissolved in chloroform and kept in a 25-ml bottle filled with chloroform and stored under nitrogen at −20°C until analyzed. Extracts of the basal diets, menhaden fish oil and yellow grease were similarly prepared.

The extracted lipids were converted to methyl esters by methanolysis according to the method of Ganglitz et al. (1963). Thin-layer chromatography was used to check the completion of methanolysis as suggested by Malins et al. (1960) of the lipids prior to the analysis of their methyl esters by gas-liquid chromatography.

The methyl esters were analyzed with a gas chromatograph (F & M Biomedical model 400) equipped with a hydrogen flame ionization detector. The temperature of the column was 165°C and that of the detector was 200°C. The samples (0.1 μl) of methyl ester were injected through a rubber septum into a flash heater maintained at 285°C. Primary standards of methyl esters were chromatographed for comparative analytical identification as was done by Gruger et al. (1964). The relative amount of each fatty acid methyl ester was obtained as area percent.

Organoleptic evaluation

The frozen birds were thawed and then roasted in open pans for 45 min. at 400°F in a gas oven. Halves of four different broilers from each dietary treatment were organoleptically evaluated by an experienced panel of 12 members. Each panelist evaluated similar anatomical portions of the broilers at each of eight daily taste tests. The parts evaluated were the drumstick, wing, upper and lower portions of the thigh, and upper and lower portions of the breast. On each day, the panelists evaluated flesh from five treatments (the control basal-diet group and from four other dietary treatments).

The following system of numerical scoring was used; 1 to indicate entirely acceptable; 2 probably acceptable; 3 questionable flavor; 4 probably unacceptable; 5 definitely unacceptable.

RESULTS AND DISCUSSION

Fatty acid composition

The results and discussion in this report are limited to ω3 and ω6 fatty acids, although as many as 28 different fatty acids of several families were identified and quantitated by GLC analyses.

Basal diets and supplementary fats. The ω3 and ω6 fatty acid compositions of the basal diets (starter and finisher), menhaden fish oil and yellow grease are given in Table 3. About half of the fatty acids of the basal diet lipids were ω6 fatty acids, mostly linoleic (18:2ω6) acid. The six major ω3 fatty acids totaled 8.2%, and were derived mostly from the fish meal in the basal diet. Some of the 18:3ω3 was derived from the other dietary feedstuffs. Unpublished data, obtained from a previous study in this laboratory of similar diets when fish meal was excluded, show that only 18:3ω3 was found and the other five ω3 fatty acids (18:4, 20:4, 20:5, 22:5 and 22:6) were not detected. Menhaden oil contained slightly more than 39% of the six ω3 fatty acids and 2.4% of two ω6 fatty acids. The yellow grease used in this study contained 7% of ω6 fatty acids and 1.2% of ω3 fatty acids, almost entirely 18:3ω3.

Table 3. ω3 and ω6 fatty acid content of basal diet and of supplementary fats.

	Fatty acid concentration in:		
	Basal diet fat (%)	Menhaden fish oil (%)	Yellow grease (%)
ω3 fatty acids			
18:3 ω3	3.2	1.3	0.8
18:4 ω3	0.5	3.5	0.2
20:4 ω3	0.2	1.9	0.1
20:5 ω3	2.2	16.2	0.1
22:5 ω3	0.4	2.6	
22:6 ω3	1.7	13.2	
Total	8.2	38.7	1.2
ω6 fatty acids			
18:2 ω6	50.0	1.4	6.8
20:4 ω6	0.3	1.0	0.4
Total	50.3	2.4	7.2

Tissue lipids. Tables 4 and 5 show the content of the ω3 and ω6 fatty acids respectively of the four tissues (liver, breast and thigh muscles and adipose fat) excised from the broilers fed the 15 dietary treatments. In the group fed the basal diets (which contained 5% fish meal), the six ω3 fatty acids totaled 12% of the liver lipids. The breast muscle lipid contained almost the same quantity of ω3 fatty acids as the liver, while the thigh had less than one-half the quantity, and the adipose had less than 2% detected only as 18:3ω3 and 18:4ω3.

About one-fourth of the fatty acids content in each of the four tissues obtained from the basal-fed broilers were members of the ω6 family. Linoleic acid (18:2ω6) was the major component of the ω6 fatty acid total, but its content decreased as the arachidonic acid (20:4ω6) became more prevalent. The 20:4ω6 content varied from the low level of ½% in the adipose fat to about 2% in the thigh muscles, to 3 to 6% in the breast muscle, and to the highest level of (5 to 9%) in the liver. Each tissue apparently has its own characteristic range of the 20:4ω6 fatty acid.

When menhaden oil was added to the feed, the six ω3 fatty acids increased slightly in the depot fat, increased considerably in the thigh muscles, very pronouncedly in breast muscles and even more so in the livers. Evidently each tissue has its own range of assimilation of the ω3 fatty acid family although the range was about the same whether 2.5 or 5% levels of menhaden oil were fed for the same periods of time. Presumably the upper limit of deposition was probably reached with the dietary level of 2.5% menhaden oil in 8 weeks. However, when the menhaden oil was discontinued at the 4th week, (regardless whether yellow grease was or was not included in the finisher diet) the ω3 fatty acid content found in the tissues of the 8-week-old broilers was only slightly higher than those found in the tissues of the basal diet group. The ω3 fatty acid content increased progressively with the 5-, 6- and 8-week feeding of fish oil. The continuous feeding of the oil for

8 weeks resulted in increased deposition of ω3 fatty acid content in the tissues of chicks to twice as much as when the oil is fed for 4 weeks. Obviously, the longer the fish oil was fed, the greater was the deposit of the ω3 type of fatty acids.

The feeding of menhaden oil for an 8-week period resulted in a reduced content of 20:4ω6 to about one-half the basal level found in both the liver and breast muscle lipids. Only a one-fourth reduction was obtained in the thigh. This depression of the conversion of linoleic acid to its metabolites by feeding fish oil is known to occur owing to the presence of members of the linolenic (ω3) family, which reportedly interfere with synthesis of 20:4ω6 from 18:2ω6 according to Edwards et al. (1963) and Miller et al. (1967b). Previously Machlin (1962) and Mohrhauer et al. (1963a) demonstrated that linolenic acid per se hinders the conversion of 18:2ω6 to 20:4ω6. The latter investigators attributed the hindrance of the conversion to enzyme competition.

Marion et al. (1965) reported that the long-chain polyunsaturated fatty acids are concentrated chiefly in the phospholipids and to a lesser extent in the neutral fats. Of the four tissues studied, the phospholipid content is highest in the liver, lower in the breast and thigh muscles, and lowest in the adipose fat. Obviously the same relationship exists for the polyunsaturate content of these tissues, a fact which explains the low levels of polyunsaturated fatty acids found in the adipose fat. Machlin et al. (1961) showed that the depot fat of broilers fed fat-free diet contained no 18:2, 18:3, 20:4 and 20:5 (families not indicated) but only 18:2 (ω6 presumably) appeared in the depot fat when 2% safflower oil was included in the diet. Neudoerffer et al. (1967) demonstrated that the tissues of turkeys fed 5% anchovy oil varied in the content of the different classes of phosphatides and each class had its own characteristic composition of the ω3 fatty acids.

Organoleptic evaluation

Nilson (1954), Carlson et al. (1957), Dansky (1962), Leong et al. (1964) and Hardin et al. (1964) have shown

Table 4. ω3 fatty acids of broiler tissues as affected by dietary fat.

Treatment No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Menhaden oil, %	0	2.5	2.5	2.5	2.5	5	5	5	5	2.5	2.5	2.5	5	5	5
Weeks	0	4	5	6	8	4	5	6	8	4	5	6	4	5	6
Yellow grease, %	0	0	0	0	0	0	0	0	0	2.5	2.5	2.5	5	5	5
Weeks	0	4	3	2	0	4	3	2	0	4	3	2	4	3	2
							ω3 fatty acids (%) in liver lipids								
18:3	0.5	0.5	0.5	0.5	0.3	0.4	0.6	0.5	0.6	0.4	0.4	0.6	0.6	0.6	0.5
18:4	0.1	0.1	0.1	0.2	0.1	0.1	0.2	0.1	0.3	0.1	0.1	0.3	0.1	0.3	0.2
20:4	0.1	0.1	0.1	0.3	0.2	0.1	0.3	0.4	0.3	0.1	0.2	0.4	0.4	0.3	0.1
20:5	1.5	2.5	2.7	3.2	6.3	4.1	5.0	2.8	5.0	7.6	3.6	4.4	2.7	4.4	5.5
22:5	0.5	1.1	1.5	1.6	3.3	1.5	1.9	1.5	3.1	1.4	1.6	1.7	1.4	2.1	2.2
22:6	9.7	9.1	9.8	10.5	19.5	12.8	12.6	11.9	20.2	13.5	15.4	15.0	12.6	16.1	19.5
Total	12.4	13.4	14.7	16.3	29.7	19.0	20.6	17.2	29.5	23.1	21.3	22.4	17.8	23.7	28.0
							ω3 fatty acids (%) in breast lipids								
18:3	1.2	1.2	1.0	1.1	1.6	0.9	0.9	[1]	1.0	0.9	0.8	0.9	0.9	0.9	1.1
18:4	0.3	0.6	0.3	0.4	1.1	0.2	0.4	[1]	0.7	0.2	0.2	0.3	0.3	0.4	0.5
20:4	0.9	1.5	0.5	0.6	1.5	0.6	1.1	[1]	1.2	0.5	0.4	0.8	0.2	0.3	0.3
20:5	1.4	1.6	2.2	2.6	7.6	2.3	3.0	[1]	5.9	1.8	1.9	2.7	2.6	3.1	3.5
22:5	2.5	2.4	2.3	2.2	2.7	2.5	2.5	[1]	3.3	2.6	2.8	2.8	3.4	2.8	2.3
22:6	5.5	4.8	5.7	5.6	9.7	6.0	6.2	[1]	10.7	6.5	6.7	7.1	8.8	7.9	5.8
Total	11.8	12.1	12.0	12.5	24.2	12.5	14.1		22.8	12.5	12.8	14.6	16.2	14.4	13.5
							ω3 fatty acids (%) in thigh lipids								
18:3	1.6	1.4	1.5	1.5	1.5	1.5	1.5	1.5	1.6	1.5	1.4	1.5	1.2	1.5	1.6
18:4	0.2	0.1	0.2	0.2	0.2	0.5	0.5	0.8	0.4	0.3	0.3	0.3	0.3	0.5	0.8
20:4	0.1	0.1	0.2	0.3	0.5	0.2	0.3	0.5	0.6	0.1	0.2	0.3	0.1	0.3	0.4
20:5	0.7	1.0	1.5	2.4	3.9	1.8	2.6	3.6	5.5	1.0	1.2	2.0	0.8	2.2	3.5
22:5	0.9	1.0	1.2	1.7	2.6	1.5	2.0	2.8	2.8	1.1	1.2	1.5	0.9	2.0	2.0
22:6	2.0	2.0	2.2	3.2	5.4	2.8	3.7	5.9	6.5	2.2	2.5	2.9	1.8	4.3	4.2
Total	5.5	5.6	6.8	9.3	14.1	8.3	10.6	15.1	17.4	6.2	6.8	8.5	5.1	10.8	12.5
							ω3 fatty acids (%) in adipose lipids								
18:3	1.4	1.6	1.8	1.9	1.4	1.3	1.8	1.9	1.7	1.6	1.6	2.3	1.3	1.7	1.7
18:4	0.2	tr	0.6	0.5	0.1	tr	0.8	0.7	0.4	tr	0.8	1.0	0.2	0.2	0.8
20:4		tr	0.1	0.2	tr	tr	0.2	0.3	0.1	tr	0.2	0.4	0.1	0.3	0.4
20:5		0.1	1.0	1.4	0.1	0.1	1.7	1.8	0.5	0.1	1.4	3.0	0.2	1.6	2.2
22:5		0.1	0.5	0.7	0.1	0.1	0.8	0.9	0.3	0.1	0.6	1.3	0.1	0.6	0.8
22:6		0.1	0.5	0.6	0.3	0.1	0.8	0.8	0.4	0.1	0.6	1.4	0.1	0.6	0.8
Total	1.6	1.9	4.5	5.3	2.0	1.6	6.1	6.4	3.4	1.9	5.2	9.4	2.0	5.0	6.4

[1] Lost.

Table 5. ω6 fatty acids of broiler tissues as affected by diet.

Treatment No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Menhaden oil, %	0	2.5	2.5	2.5	2.5	5	5	5	5	2.5	2.5	2.5	5	5	5
Weeks	0	4	5	6	8	4	5	6	8	4	5	6	4	5	6
Yellow grease, %	0	0	0	0	0	0	0	0	0	2.5	2.5	2.5	5	5	5
Weeks	0	4	3	2	0	4	3	2	0	4	3	2	4	3	2
							ω6 fatty acids (%) in liver lipids								
18:2	16.9	16.0	17.6	15.8	14.8	16.5	17.0	16.2	14.4	16.4	16.4	16.2	17.7	16.5	16.4
20:4	7.8	6.9	6.8	6.3	4.7	8.0	6.8	7.3	4.0	9.1	9.2	6.8	8.2	7.2	7.0
Total	24.7	22.9	24.4	22.1	19.5	24.5	23.8	23.5	18.4	25.5	25.6	23.0	25.9	23.7	23.4
							ω6 fatty acids (%) in breast lipids								
18:2	20.8	17.8	20.7	20.6	17.5	19.8	18.8	[1]	13.7	19.0	18.7	17.6	18.1	16.7	17.7
20:4	5.6	2.9	3.1	2.6	2.8	3.4	2.7	[1]	3.2	3.8	3.5	2.8	4.7	3.1	2.3
Total	26.4	21.7	23.8	23.2	20.3	23.2	21.5		16.9	22.8	22.2	20.4	22.8	19.8	20.0
							ω6 fatty acids (%) in thigh lipids								
18:2	23.0	21.6	22.4	22.3	20.9	21.7	20.9	20.3	15.6	21.8	20.8	20.0	18.6	19.0	18.5
20:4	2.2	1.8	1.7	1.6	1.6	1.9	1.7	2.5	1.7	1.9	1.8	1.4	1.6	2.0	1.6
Total	27.2	23.4	24.1	23.9	22.5	23.6	22.6	22.8	17.3	23.7	22.6	21.4	20.2	21.0	20.1
							ω6 fatty acids (%) in adipose lipids								
18:2	24.0	37.0	23.5	23.5	30.7	37.5	23.2	22.0	23.7	43.9	20.8	22.9	18.2	19.4	19.1
20:4	0.4	0.5	0.3	0.4	0.5	0.4	0.4	0.4	0.3	0.5	0.3	0.5	0.2	0.5	0.5
Total	24.4	37.5	23.8	23.9	31.2	37.9	23.6	22.4	24.0	44.4	21.1	23.4	18.4	19.9	19.6

[1] Lost.

that excessive dietary levels of menhaden oil taint the flavor of broilers. The taste-test results obtained in this experiment of the flesh of broilers fed the various diets are shown in Table 2. The organoleptic scores are evidently influenced by the length of the feeding period of menhaden oil, as indicated by the low scores associated with the control diet, as shown by early discontinuation of the oil or by its replacement in the diet with yellow grease weeks before the birds were slaughtered.

Flesh of birds fed the practical broiler diet with no fat addition (Treatment 1) for the entire 8-week period was scored 1.4 which indicated that these broilers were almost entirely acceptable to the panelists. The other extreme of the organoleptic evaluation score in this study was obtained when the birds were fed either 2.5 or 5% menhaden fish oils (Treatments 5 and 9) for the entire 8-week feeding period. The average organoleptic scores for both groups fed fish oil 8 weeks were practically identical, 3.7 and 3.8, respectively, which were probably unacceptable.

The discontinuation of the 2.5 and 5% levels of menhaden oil (Treatments 2 and 6) at the 4th week resulted in flesh being probably acceptable to the panel; which scored them as 1.9 and 1.8 respectively. The substitution of yellow grease for the fish oil at this time resulted in a favorable evaluation indicated by the panel scores of 1.5 and 1.4 for Treatments 10 and 13, respectively. In effect, the organoleptic scores of these four treatments were similar to each other as indicated by the fact that the discontinuation of fish oil 4 weeks prior to slaughtering at the age of 8 weeks resulted in flesh which was evaluated by the panel to be acceptable.

Interestingly, Carlson et al. (1957) reported that the elimination of menhaden oil (1 or 2% levels) from the diet at the 7th week of a 10-week experiment resulted in a considerable improvement in the flavor but not complete acceptance. Their observation of a slight unacceptability may have been due to the longer period (7 weeks) of feeding the fish oil and the shorter period (3 weeks) of not feeding fish oil in contrast to the current experiment of 4 weeks of oil feeding and 4 weeks of no oil addition to the diets. Similarly, Edwards et al. (1965) reported that the off-flavor of broilers fed 2% menhaden oil was decreased considerably by the inclusion of poultry fat or corn oil and was decreased to a lesser degree by the dietary addition of beef fat. They also found that the withdrawal of 6% menhaden oil for the final 2 weeks of the 7-week experiment and the inclusion of 6% beef fat in its place improved the flavor somewhat. However, to our knowledge no reports have been published on the effect of a 4-week discontinuance of fish oil or its replacement by a dietary fat low in ω3 fatty acids on the correlation of the flavor acceptability with the deposition of the ω3 and ω6 families in various tissues.

Correlation of organoleptic scores with the ω3 and ω6 fatty acids content in four tissues

The continuous feeding of the menhaden oil at either the 2.5 or 5% dietary level resulted in orpanoleptic scores which were highly correlated ($P < .01$) with the quantities of 20:5ω3, 22:5ω3 and 22:6ω3 fatty acids deposited in the muscles (Table 6). The same significant relation was found for the 20:4ω6 content in the thigh muscles but not in the breast muscles. Although the livers were not included in the taste test, a highly significant correlation of the 22:5ω3 and 22:6ω3 fatty acid content in the liver was found in comparison with the organoleptic scores ob-

Table 6. Correlation of organoleptic scores with ω3 and ω6 fatty acids content in four tissues.

	Liver	Breast	Thigh	Adipose
	Complete feeding of fish oil			
18:3 ω3	NS[1]	NS	NS	NS
18:4 ω3	NS	NS	NS	NS
20:4 ω3	*	NS	**	NS
20:5 ω3	*	**	**	NS
22:5 ω3	**	**	**	NS
22:6 ω3	**	**	**	NS
18:2 ω6	—**	—**	—**	NS
20:4 ω6	—**	NS	NS	NS
	Complete withdrawal or replacement of fish oil			
18:3 ω3	NS	NS	NS	*
18:4 ω3	**	NS	NS	NS
20:4 ω3	NS	NS	**	*
20:5 ω3	NS	*	*	*
22:5 ω3	*	NS	**	**
22:6 ω3	**	NS	**	*
18:2 ω6	— *	— *	NS	NS
20:4ω6	NS	—**	NS	NS

[1] NS No significant correlation.
* Positive correlation at 5 percent level of significance.
** Positive correlation at 1 percent level of significance.
—* Negative correlation at 5 percent level of significance.
—** Negative correlation at 1 percent level of significance.

tained from the muscles. The 20:4ω3 and 20:5ω3 fatty acid contents in the liver were slightly less correlated ($P < .05$) with the flavor.

These correlations, no doubt, are a reflection of the high content in the muscles and liver of these four long-chain polyunsaturated fatty acids derived from feeding menhaden oil. On the other hand, the minute quantities of 20:4ω3, 20:5ω3, 22:5ω3, and 22:6ω3 fatty acids in the adipose fat were not significantly correlated with the organoleptic scores.

A negative highly significant correlation ($P < .01$) was found between the organoleptic score and the 18:2ω6 content in the muscles and liver when the fish oil was fed the entire 8-week period. This decreased content of 18:2ω6 in these tissues probably occurred because of its dilution in the diet by the fish-oil fatty acids and consequently a reduced quantity accumulated in the tissues. With respect to the 20:4ω6 content, a negative correlation was found in the liver. In the other tissues 20:4ω6 was present in small quantities perhaps due to the metabolic inhibition effect of the ω3 family on the ω6 fatty acids.

The withdrawal of dietary fish oil resulted in the loss of many of the correlations for the ω3 fatty acids content with the organoleptic score, especially for the breast muscles. The ω3 content of the thighs, however, was significantly correlated to the organoleptic score. The adipose ω3 fatty acids, because of low and variable content and perhaps poorer mobilization during withdrawal, was found to be correlated to taste evaluation.

The negative correlation of 18:2ω6 content with the organoleptic evaluation was less pronounced while the 20:4ω6 correlation was high for the breast but not for the liver.

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Relative Differences in Tenderness of Breast Muscle in Normal and Two Dystrophic Mutant Strains of Chickens

SUMMARY—Cooked samples of *Pectoralis major* muscles from one normal and two mutant dystrophic lines of chickens were compared for tenderness using a mechanical device. The muscles from one of these lines, characterized by partial atrophy, were found to be significantly more tender than normal muscles, whereas the muscles from the second mutant line, characterized by hypertrophy were much tougher than normal muscles. Cooking-weight losses were much greater in both of the dystrophic mutant lines than in the normal line. Some relationships between composition of the three muscle types and their textural qualities are discussed.

INTRODUCTION

NUMEROUS STUDIES have been made in the attempt to relate tenderness of meat to specific components of muscle tissue and to the effects of pre- and post-mortem conditions on these components. At present, no satisfactory explanation of meat tenderness can be made on the basis of the content of or changes in specific chemical constituents. One possible approach to the problem of relating muscle tissue composition to the textural properties of meat is to study genetic mutations which alter the composition of muscle. One of these rare mutations which affects the composition of specific muscle occurs in the chicken with hereditary muscular dystrophy (Asmundson et al., 1956; Julian et al.,1963). In this mutant, the white muscles are particularly affected, especially the superficial pectoralis which, because of its relatively large size and accessibility, is a most convenient muscle to study.

Several variants of the dystrophic mutant have been obtained by selection (Asmundson et al., 1966). In all of these, the pectoralis muscles differ distinctly in composition from the normal pectoralis. Two of these, however, are of particular interest because the composition of the pectoralis differs between the dystrophic strains. In line 307, this muscle is atrophic and infiltrated with fat, whereas in line 308 the large breast muscle is hypertrophic and quite low in fat content. All dystrophic pectoralis muscles have a much higher water content (fat-free basis) than normal pectoralis. A detailed discussion of the composition of these muscles is not presented here since it has been published elsewhere (Peterson et al., 1968).

Data have been published which show that the line 307 (atrophic) pectoralis muscles when cooked are considerably more tender than normal pectoralis muscles (Scholtyssek et al., 1967).

The present experiment was conducted because sufficient numbers of line 308 birds had been obtained that it became possible to compare the tenderness characteristics of the hypertrophic muscles with those of the other two strains (normal and atrophic).

EXPERIMENTAL

GROUPS of male chickens from three New Hampshire lines—normal (line 200), hypertrophic (line 308) and atrophic (line 307)—were slaughtered at ages of 9 weeks and 19 weeks. They were bled by an outside neck cut, scalded at 54°C for 2 min, machine picked, eviscerated and chilled in drained ice overnight (minimum 16 hr), frozen and stored at −17°C.

Some of the 9-week-old birds were stored for 12 weeks and some for 22 weeks. Groups of 19-week-old birds were stored for 8 weeks and 25 weeks. The reasons for this were twofold: (1) the conditions of the experiment were in part determined by the fact that only small numbers of mutant birds could be obtained at any one time because of their rarity and (2) in order to study possible effects of storage time on tenderness.

It was found subsequently that in each age group, significant differences in tenderness could be ascribed only to genetic strain and not to period of storage. Because of this, all data from birds of a specific strain were combined within each age group regardless of storage time. The number of birds per group varied from 9 to 15, depending on availability of the different strains.

The chickens were thawed in a refrigerator at 3°C for 44 to 48 hr. The left and right pectoralis muscles from each of the birds were dissected out. The entire muscles were flattened between two aluminum plates, spaced ¼ in. apart and cooked by immersing in boiling water for 30

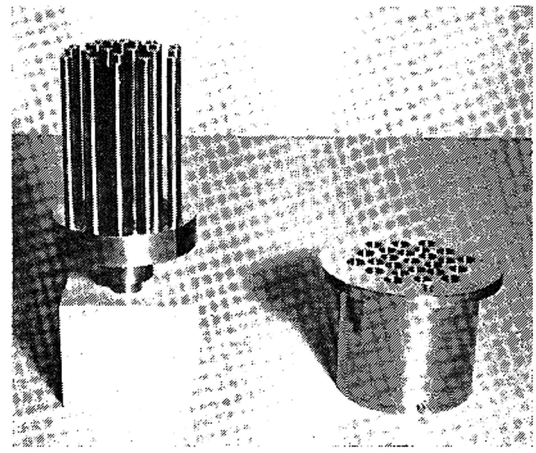

Fig. 1—Special head for measuring texture employed with the L.E.E. Kramer Apparatus (Vaughn, 1967).

min as described by Poole et al. (1959). The total number of muscles tested is indicated in Tables 2 and 3.

Since muscles of this size do not lend themselves to sampling by a coring device, a special procedure was devised. Circular samples 2⅛ in. in diameter were cut from the centers of the muscles, using a device similar to a cooky cutter (Fig. 2). Tenderness of samples was measured on a I..E.E. Kramer apparatus using special head shown in Fig. 1 and an automatic recorder. The head employed was adapted by Vaughn (1967) from the Christel texturometer and has been used in measuring texture of olives. It consists of 25 solid steel cylinders which are forced simultaneously through the sample. Photographs of cooked muscles and of samples used for measurement are shown in Fig. 2. The dystrophic muscles appear much darker in color due to a higher myoglobin content than occurs in the normal muscles.

RESULTS

THE WEIGHTS and percentage yields of pectoralis muscles are shown in Table 1. The relatively large size of the hypertrophic muscle and the small size of the atrophic muscle compared to normal pectoralis muscle are evident from the table.

Tenderness measurements are shown in Tables 2 and 3. Table 2 shows data on maximum shear values. Using this criterion, atrophic muscles were significantly more tender

Fig. 2—Method of sampling showing the three muscle types. Normal (200) left; Atrophic (307) center; Hypertrophic (308) right. The dark color of the mutant type is due to a higher myoglobin content.

Table 1—Average weights and percent eviscerated weight of muscles.

	Age (wks)	No. of muscles[1]	Ave. wt. (g)	Ave. % muscle wt. of eviscerated wt.[2]
Normal (200)	9	30	98	11.5 ± .2
	19	20	222	11.6 ± .4
Atrophic (307)	9	21	63	9.3 ± 1.0
	19	18	136	8.9 ± 1.0
Hypertrophic (308)	9	30	126	16.7 ± 1.0
	19	20	324	18.1 ± .4

[1] Left and right pectoralis from each bird was tested in all but one case.

[2] Values are mean and standard error. All lines are significantly different from each other ($p < .01$).

and hypertrophic muscles significantly tougher than normal muscles. It will be noted in Table 2 that the weights of the samples of hypertrophic muscles are greater than those of the other two types. In order to correct for this weight difference, the total shearing work was calculated by integrating the area under the curve for each sample; this value was then divided by the weight of the sample. The corrected data are shown in Table 3. Significant differences were still obtained with all comparisons in agreement with the data in Table 2 and with the results previously published comparing atrophic and normal muscle (Scholtyssek et al., 1967).

Data for loss of weight on cooking and for pH of the cooked samples are shown in Table 4. Both types of dystrophic muscles exhibit significantly greater weight loss on cooking than normal pectoralis muscles. In the data for the 19-week-muscles, a significant difference in cooking loss was also observed between the atrophic and the hypertrophic muscles.

DISCUSSION

THE COMPOSITIONS of the pectoralis muscles of each of the three genetic types employed in this experiment are both characteristic of the type and predictable. Since this has been discussed in considerable detail elsewhere (Peterson et al., 1968; Asmundson et al., 1966) only a brief summary is presented here. Typical analyses of the three types of muscle are shown in Table 5.

Atrophic (line 307) pectoralis muscle appears to differ in composition from hypertrophic pectoralis muscle (line 308) only in its content of lipid and collagen. The increased amount of lipid in 307 muscle consists mostly of

Table 2—Tenderness comparisons based on maximum shear force.[1]

Strain	Age (wks)	No. of muscles	Mean cut sample wt. (g)	Max. shear force (lb)	Significance of shear force differences compared with: Normal	Hypertrophic
Normal (200)	9	30	13.8 ± .6	54.7 ± 2.4	.....	***
Atrophic (307)	9	21	13.2 ± .2	42.8 ± 1.4	***	***
Hypertrophic (308)	9	30	15.3 ± .1	72.25 ± 1.9	***	
Normal (200)	19	20	15.2 ± .4	49.7 ± 1.5	.....	***
Atrophic (307)	19	18	14.9 ± .3	40.3 ± 1.6	***	***
Hypertrophic (308)	19	20	18.2 ± .3	107.8 ± 4.4	***	.....

[1] Values are mean and standard error.

*** $p < .001$.

Table 3—Tenderness comparisons based on shearing work/sample weight (same samples as in Table 2).

Strain	Age (wks)	No. of samples	Shearing work[1] (Joules/kg)	Significance of shear differences compared with	
				Normal	Hypertrophic
Normal (200)	9	30	57.1 ± 2.9	.....	**
Atrophic (307)	9	21	47.1 ± 1.7	**	***
Hypertrophic (308)	9	30	68.6 ± 1.9	**	
Normal (200)	19	20	46.2 ± 1.5	.....	***
Atrophic (307)	19	18	39.3 ± 1.6	**	***
Hypertrophic (308)	19	20	94.4 ± 4.0	***	

[1] Mean and standard error.
** $P < .01$.
*** $P < .001$.

Table 4—Weight loss on cooking.

Strain	Age (wks)	No. samples	Mean pH[1]	Cooking loss %	Significance level	
					vs Normal	vs Hypertrophic
Normal	9	30	6.09	34.6 ± .6	.....	***
Atrophic	9	24	6.28	48.8 ± .6	***	n.s.
Hypertrophic	9	30	6.05	49.2 ± .7	***	.....
Normal	19	20	6.18	33.8 ± 1.0	.....	***
Atrophic	19	18	6.22	49.2 ± 0.7	***	**
Hypertrophic	19	20	6.12	52.2 ± .6	***	.....

[1] Cooked sample.
n.s.—Not significant.
** $p < .01$.
*** $p < .001$.

Table 5—Typical composition of pectoralis muscle from 19-week-old chickens.

	H_2O %	Total N %	NPN %	0.1 *N* NaOH soluble protein N %	HO Proline %	Total lipid %
Normal (200)	73.8	4.02	0.66	3.22	0.14	2.43
Atrophic (307)	68.8	2.97	0.34	2.20	0.32	13.6
Hypertrophic (308)	76.7	3.16	0.44	2.54	0.18	4.14

triglyceride and therefore resembles adipose tissue (Jordan et al., 1964). Whether its collagen differs from that of line 308 has not been studied. If the compositions of the hypertrophic and atrophic muscles are compared on the *fat-free* basis, they differ in collagen content but are identical in content of water, non-protein nitrogen and protein nitrogen soluble in 0.1 *N* NaOH.

If the composition of the normal pectoralis which represents largely a "white" type muscle is compared on the *fat-free* basis with the dystrophic muscles, it is found to have a lower degree of hydration and a higher content of both 0.1 *N*-NaOH soluble protein and nonprotein nitrogen as well as different patterns of nonprotein constituents (Peterson et al., 1968) and enzyme activities than the dystrophic pectoralis (Cardinet, 1966). The dystrophic muscles, therefore, resemble in composition very closely "red" muscles such as chicken lateral adductor or "leg muscle" composite rather than "white" muscle as exemplified by the normal pectoralis (Peterson et al., 1968).

Considering these resemblances of dystrophic muscle to chicken leg muscle, one might expect that they would have some properties in common. This is indeed true in the case of the hypertrophic muscle. Chicken leg meat is less tender than breast meat (Van den Berg et al., 1963, 1964) and leg meat exhibits greater cooking loss than breast meat (Van den Berg et al., 1963, 1964; Goertz et al., 1962; Froning et al., 1966).

Tenderness of meat is considered to be closely associated with its waterholding capacity (WHC) (Wierbicki et al., 1956, 1957; Hamm, 1960). Loss of weight on cooking is ordinarily an indication of change in WHC produced by denaturation of the muscle proteins (Hamm, 1966). Since both the normal and hypertrophic muscles are low in fat, it may be assumed that the major component of their cooking-weight loss was water.

The properties of the atrophic muscle, however, are not explainable on any simple basis since it was both tenderer and suffered greater cooking loss than normal muscle. Fat content or marbling of meat has not been shown to have any consistent relationship to tenderness of meat (Szczesniak et al., 1965). However, the atrophic (307) muscles which have very high fat content (largely as triglyceride) were significantly more tender than the other two types. Such a result could be due to the physical dilution effect of the fat alone since the proportion of muscle fibers per unit weight is reduced in the atrophic

muscles. A previous study on the tenderness of atrophic muscles (Scholtyssek et al., 1967) suggested that there was no correlation between shear force values and fat content of samples taken from the muscles. Such a conclusion could be erroneous, however, since the fat content of the atrophic muscles has been found to vary with the location in the muscle (Jordan, 1959, unpublished data) and thus the fat content of a sample taken from one portion of the muscle may not be identical with that in the portion tested for tenderness.

The data obtained thus far indicate that further detailed study of the tenderness characteristics of these three types of muscle may provide useful information on factors which influence tenderness of meat. It is probable that the explanation of the differences in tenderness such as those between the normal and the hypertrophic muscle will eventually be explained in terms of differences in the proteins. At present only a limited amount of data is available on these (Barany et al., 1966; Cardinet, 1966).

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Smoke Flavor as Related to Phenol, Carbonyl and Acid Content of Bologna

SUMMARY—Phenol, carbonyl and acid determinations were made on layers of bologna that had been smoked during processing. Similar samples were evaluated for intensity of smoke flavor by taste panel. Correlation coefficients between phenols, carbonyls, acids and taste panel evaluation of smoke flavor intensity were 0.81, 0.37 and 0.32, respectively. Thus, phenols appear to be the principal contributor to smoke flavor and aroma.

INTRODUCTION

THE PRACTICE of smoking foods is an old process. It is thought to have developed as an adjunct to the drying method of preserving meat and fish by hanging the product over a fire or in a room in which a fire was maintained for warmth or cooking purposes. In addition to the preservation by dehydration, the bactericidal and antioxidant properties of the smoke also increased the storage stability of the treated meat or fish. Since controlled refrigeration is used to protect many foods from rapid deterioration and spoilage, meat products are generally smoked for the distinctive surface color development and the characteristic smoke flavor.

The components of smoke responsible for the distinctive flavor are found in the steam-distillable fraction and are mainly phenols, acids and carbonyls (Husaini et al., 1957). Tilgner et al. (1962b) reported that the main components of the steam-distillable fraction of wood-smoke condensate were carbonyls, 24.6%, acids (carboxylic), 39.9%, and phenols, 15.7%. Tucker (1942) and Kurko (1959) found that phenols were present in decreasing amounts from the outer to inner portions of smoked ham or sausage. Acid penetration of 10 to 12 mm into fresh ham was reported by Hamid et al. (1966).

The present study was undertaken to determine the relation of phenols, carbonyls and acids to smoke flavor as determined by taste panel.

METHODS AND MATERIALS

A BOLOGNA emulsion was stuffed into No. 5 Tee-Pak Clear Zip Fibrous casings (Tee-Pak, Inc.). The ingredients were as follows:

Pork trimmings	9.2 lb	Sodium nitrate	1.4 g
Beef trimmings	9.2 lb	Sodium nitrite	1.4 g
Nonfat dried milk	0.9 lb	Sodium ascorbate	4.0 g
Ice	5.9 lb	Sugar	32.6 g
		Salt (NaCl)	152.0 g

No spices were used because of the possible interference with taste panel evaluation. The bologna was processed in an air-conditioned smokehouse that had about 5 air changes per minute. Smoke was generated from smoldering, dampened hardwood sawdust. A temperature of 140°F and a relative humidity of 23% was maintained for the first 2 hr of processing. The temperature was then raised to 160°F with a relative humidity of 31% until the bologna reached an internal temperature of 128°F. The smokehouse temperature was then maintained at 160°F by injecting high pressure steam only, which resulted in 69% relative humidity. Total smoking and processing time was about 4 hr. The bologna was showered with cold water until the internal temperature was reduced to 90°F and then stored in a 36°F cooler.

Consecutive outside-to-inside layers were removed from the sticks of bologna for chemical analyses and taste-panel evaluation. They were 1.4 to 1.6 mm thick and were identified by the letters A-H (outer to inner). Samples for chemical analyses were ground 5 times through a 1.5-mm plate. Consumer-type panels varying from 18 to 30 members were used. Panelists were served samples that were 2.5 × 5.0 cm × 1.6 mm in size and were asked to score the samples from 1 to 7, where 1 = no smoke flavor and 7 = very strong smoke flavor.

Carbonyl determination

Carbonyls were determined by the method of Batzer et al. (1957). The carbonyl-free benzene, carbonyl-free ethanol, saturated 2,4-dinitrophenylhydrazine solution, 4% trichloroacetic acid solution, and the 4% KOH solution were prepared as outlined by Henick et al. (1954).

A 1- to 2-g sample of ground bologna was blended with 40 ml of carbonyl-free benzene and 40 ml of carbonyl-free ethanol in a Waring blender for 2 min. The slurry was filtered through Whatman No. 1 filter paper into a 100-ml volumetric flask. Carbonyl-free benzene was used to rinse the blender and filter paper slurry and to bring the filtrate to volume. A 2-ml aliquot of well-mixed filtrate, 3 ml of saturated 2,4-dinitrophenylhydrazine, and 3 ml of 4% trichloroacetic acid were added to a 25-ml volumetric flask. It was stoppered and placed in a 60 ± 5°C water bath for 30 min and then cooled to room temperature.

Color development was obtained by addition of 5 ml freshly filtered 4% KOH, and the solution was brought to volume with carbonyl-free ethanol. Absorbance values were read at 430 mμ with a Beckman spectrophotometer exactly 10 min after adding the KOH solution. The 2,4-dinitrophenylhydrazone of n-heptaldehyde in appropriate concentrations was used for the standard curve.

Acid determination

Milliequivalents of acid were determined by the method suggested by Hamid et al. (1966). An accurately

weighed 1- to 2-g sample of ground bologna was blended with 50 ml of distilled water in a Waring blender for 2 min. The blender was thoroughly rinsed with distilled water and the resultant slurry titrated to pH 8.3 with 0.2 *N* NaOH.

Phenol determination

The method of Tucker (1942) was used to estimate total phenols, which are reported as phenol, although they could have been based on 2,6-dimethoxyphenol or guaiacol.

A 20-g sample of the ground bologna was placed in a Waring blender with 100 ml of 50% ethyl alcohol and mixed at full speed for 5 min. The extract was filtered through S and S (Schleicher & Schuell) No. 560 hand-folded filter paper. The filtrate container was covered and allowed to stand for 12 to 16 hr at 2 to 4°C. It was then filtered through Whatman No. 2 filter paper.

The diluted or undiluted samples (based upon yellow color development) as well as the standard solutions were transferred to 15 × 180 mm test tubes. The standards contained varying amounts of phenol from 0.0 mg to 0.5 mg per 100 ml. Five ml of a 5% solution of sodium borate ($Na_2B_4O_7.10\ H_2O$) were added to a 5-ml sample of the diluted or undiluted extracts and the standard tubes. Color was developed by the addition of 1 ml of a *N*, 2,6-trichloro-p-benzoquinoneimine solution. This stock solution contained 0.25 g in 30 ml of ethyl alcohol. Color was allowed to develop 1½ hr at room temperature.

The samples and standards were added to separatory funnels containing 15 ml of N-butanol. After the solutions were shaken and allowed to separate, the water layers were drawn off and discarded. The butanol layers were transferred to graduated test tubes and brought to 21 ml by adding N-butanol. Finally, 2 ml of N-butanol saturated with NH_3 were added and the contents thoroughly mixed. The solutions were read in a Bausch and Lomb Spectronic 20 colorimeter at 635 mμ.

RESULTS AND DISCUSSION

THERE WAS a gradual decrease in the amount of phenols present in the outer to the inner layers of the bologna (Table 1). Tucker (1942) reported 1.36 mg phenols per 100 g of "lean surface tissue" and 0.32 mg per 100 g of "lean tissue ½ in. below lean surface" for ham smoked 24 hr. Sample size was 50 g but no indication was given as to sample thickness. In the present study, since the bologna emulsion contained no phenols, and the amount in the inner layer was very small, it is reasonable to assume that phenols are a good indicator of the amount of smoke deposition and also of smoke penetration.

Table 1. Phenol, carbonyl and acid values,[1] and panel smoke perception scores[2] from the emulsion and 1.6 mm layers A-H of a typical bologna stick.

Layer	Phenols mg/100 g	Carbonyls mg/100 g	Acids meq/100 g	Panel smoke perception scores
A (outer)	3.70	1.38	4.47	
B	2.04	1.17	5.48	5.8
C	1.41	1.23	5.51	5.1
D	1.02	1.06	6.58	4.4
E	0.78	1.09	7.01	3.9
F	0.43	1.22	6.86	3.5
G	0.26	1.24	6.37	3.1
H (inner)	0.12	1.05	8.72	2.9
Emulsion	0.00	1.21	5.35	....

[1] Fresh basis.
[2] 1 = no smoke flavor; 7 = very strong smoke flavor.

The amount of carbonyls in the bologna layers showed little difference from that in the emulsion before heating and smoking (Table 1). An increase was expected because carbonyls have been found to comprise 24.6% of the steam-distillable fraction of wood smoke (Tilgner et al., 1962b). In the present study the lack of a marked increase in carbonyl content due to smoking may have been due to the possible screening effect of the cellulose casing. However, Simon et al. (1966) found that water-filled cellulose casings were good absorbent systems for the phenols, acids and carbonyls in wood smoke.

The variability in the acid data (Table 1) may have been influenced to some extent by the processing (heating) of the meat component of the bologna. Hamid et al. (1966) found 0.05 meq of acid per gram for the lean of unsmoked fresh ham. They exposed pieces of the ham to known acid volatiles for 2 hr and obtained over 0.45 meq/g from the outer surface when isocaproic acid was used. Their results indicated a maximum acid penetration depth of 10 to 12 mm. A similar approximate penetration was observed in the present study based on the phenol data but the acid results were too variable to support the same conclusion.

Thirty-one samples of bologna were scored for smoke-flavor intensity and similar samples were analyzed for phenol, carbonyl and acid content. Different degrees of smoke flavor were obtained by presenting various layers to the panels. The outer, or A layer, was never used as it was dark in color and also dry in appearance. It was found that the panelists had difficulty in detecting differences in smoke-flavor intensity between adjacent layers. Therefore, the panelists were asked to score nonadjacent layers, with no more than 4 samples presented at a single session.

A correlation coefficient of 0.81 ($P < .01$) was determined when phenol content was compared with panel scores. This relationship agrees with the conclusions of researchers who studied the flavor characteristics of phenols as a group, or of phenolic components. Tilgner et al. (1962a) found that water solutions of smoke-phenolic fractions were characterized as "cured-smoky" by a sensory panel. They reported that threshold values ranged from 0.15 to 1.09 ppm of phenols expressed as resorcin.

Fiddler et al. (1966) stated that preliminary studies indicated the essential smoke odor was in the phenolic portion of their smoke condensate from hickory sawdust. Wasserman (1966) combined the three major phenolic components—guaiacol, 4-methylguaiacol, and 2-6 dimethoxyphenol—in the same proportion as they occurred in the smoke condensate analyzed by Fiddler et al. (1966), and observed the resulting mixture to have an odor only slightly similar to the smoke condensate.

Correlation coefficients of 0.37 ($P < .05$) and 0.32 were calculated when panel scores were compared with car-

bonyl and acid content, respectively. Carbonyl content accounted for about 14% of taste panel variation, whereas phenols accounted for 66% of panel variation. While the correlation (0.32) between panel scores and acid content approached statistical significance, it only accounted for 10% of the variation between panel scores and meq of acids present in the bologna.

Under the conditions of the present study, it can be concluded that phenols are a good indicator of smoke flavor intensity as evaluated by taste panel.

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A Study of the Sarcoplasmic Proteins of Porcine Muscle by Starch Gel Electrophoresis

SUMMARY—Extensive investigations were conducted on numerous variables in order to establish the most ideal conditions for the starch gel electrophoresis of the proteins of porcine skeletal muscle sarcoplasm. This technique was used to evaluate the characteristics of the sarcoplasm extracted from (a) muscles at various times post-mortem, (b) muscles which ultimately remained normal in color and gross morphology as well as muscles which became extremely PSE and (c) muscles which had altered color and gross morphology due to post mortem treatment (i.e., normal color and gross morphology due to surface treatment with L-N_2 and PSE characteristics due to post-mortem incubation at 37°C for 4 hr).

There were no discernible differences between the starch gel electrophoretograms of sarcoplasmic proteins extracted from pre- and post-rigor muscle. Likewise, there were no visually detectable differences between the starch gel electrophoretograms of sarcoplasmic proteins extracted from naturally occurring normal and PSE muscles. Furthermore, the preservation of normal color and gross morphology through L-N_2 treatment did not give rise to electrophoretic differences when compared with either naturally occurring normal or PSE muscle.

Although sarcoplasmic protein solubility diminished in naturally occurring PSE muscle, this loss in soluble protein did not necessarily manifest itself in the preferential denaturation of a specific sarcoplasmic protein. However when muscles were incubated at 37°C for 4 hr to induce the PSE condition they yielded sarcoplasmic extracts with marked electrophoretic reductions in creatine kinase, phosphoglucomutase, triosephosphate isomerase and F-protein (Scopes, 1965). Although there were no apparent differences in creatine kinase isozymes between samples of muscle which ultimately remained normal or became PSE, the minor (fastest moving) creatine kinase band, as well as the phosphoglucomutase bands, appeared more labile during incubation at 37°C than the major [illegible] creatine kinase band.

INTRODUCTION

A RAPID rate of glycolysis, with the onset of rigor mortis occurring at a low muscle pH and high muscle temperature, has been associated with the post-mortem development of pale, soft, exudative (PSE) musculature

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and the concomitant decrease in myofibrillar and sarcoplasmic protein solubility (Briskey, 1964; Briskey et al., 1961; Sayre et al., 1963).

Scopes (1964) and Scopes et al. (1963), through the use of starch gel electrophoresis (SGE), concluded that the electrophoretically detectable band of creatine kinase was absent in PSE muscle. These workers postulated that the creatine kinase had probably been denatured and precipitated onto the myofibrils and that this precipitated sarcoplasmic protein was responsible for the decrease in solubility of the myofibrillar proteins.

Borchert et al. (1964, 1965) demonstrated that the deterioration in tissue characteristics and protein solubility associated with the development of the PSE condition could be prevented by liquid nitrogen ($L\text{-}N_2$) treatment immediately post-mortem. Consequently $L\text{-}N_2$ treatment offered an ideal opportunity to study the sarcoplasmic protein in PSE (untreated) and normal ($L\text{-}N_2$ treated) muscles from the same carcass.

The purposes of this investigation were to: (a) establish the SGE conditions for resolving porcine sarcoplasmic proteins into distinct bands, (b) compare starch gel electrophoretograms of sarcoplasmic proteins extracted from muscle (held at 4°C) which ultimately became, through normal or artificial means, either severely PSE or normal in color and gross morphology.

METHODS

Materials

Muscle samples were obtained from the mid-portion of the longissimus dorsi of Poland China animals weighing approximately 90 kg. The muscle samples were excised at various post-mortem times from carcasses held at 4°C, 37°C or treated with $L\text{-}N_2$ (Borchert et al., 1965). A portion of each sample was used for pH determination (Beecher et al., 1965) and color and gross morphology estimations according to the subjective scale of Forrest et al. (1963). The remainder of each sample was frozen immediately in $L\text{-}N_2$ and stored at −18°C until required for study with starch gel electrophoresis.

Extraction of sarcoplasmic proteins

Scopes et al. (1963) and numerous other workers (Charpentier et al., 1963; Giles, 1962; Neelin, 1963; Neelin et al., 1964) have used various techniques for homogenizing and extracting unfrozen tissue. In the development of these studies, it became obvious that it would be advantageous if samples could be frozen for short periods of time to facilitate the comparison of samples from different post-mortem periods on the same starch gel. A study of freezing effect seemed further desirable because of previous success in uniform sample preparation with the $L\text{-}N_2$ muscle powdering procedures of Borchert et al. (1965).

Through extensive preliminary experimentation we found no marked qualitative differences between extracts of unfrozen samples and those frozen in $L\text{-}N_2$ and held at −18°C for periods up to and including 28 days of storage. Consequently all of the SGE work reported herein was accomplished on extracts of frozen muscle powder.

Frozen muscle was converted to frozen muscle powder by the procedure of Borchert et al. (1965). About 20 g of muscle powder were extracted with 40 ml of water and the mixture was centrifuged at 5000 × G. Six common extractants were evaluated (Helander, 1957; Neelin, 1963; and Scopes, 1964). Since there were no qualitative differences in electrophoretograms of a particular sample, due to kind of extractant, all data reported herein were obtained by water extraction, or 0.03M phosphate, pH 7.4, when a physiological buffer was required. The supernatant, containing between 20 to 25 mg protein/ml, was concentrated by adding Sephadex G-50. The protein solution was removed from the hydrated Sephadex G-50 by vacuum filtration.

Protein determination

The protein content of the extracts were determined by the biuret method of Layne (1957).

Starch gel electrophoresis

One-dimensional starch gel electrophoresis was carried out in the vertical apparatus described by Smithies (1959). Hydrolyzed starch for gel electrophoresis was obtained from Connaught Medical Research Laboratories, Toronto. A starch concentration of 11 g/100 ml of buffer was found to be suitable for effective protein resolution.

The buffer for the gel, in which protein separation occurred, was composed of 2 mM diethylenetriaminepentaacetic acid (DTPA) and 12 mM tris (pH 8.25). The outer gel buffer consisted of 100 mM boric acid and 60 mM tris; pH 8.6. The bridge solution was 0.3M boric acid −0.06M NaOH and 10% NaCl was used as the electrode solution. Electrophoresis was carried out with an initial field strength of about 1.5V/cm at 4°C.

Identification of enzymes in electrophoretograms

The identification of creatine kinase bands was carried out by the staining procedure of Fine et al. (1963) which was specific for creatine kinase. Some of the bands in each electrophoretogram were identified by orientation with the major and minor bands of creatine kinase and the identification standards presented by Scopes (1964).

RESULTS AND DISCUSSION

Sarcoplasmic proteins from muscle at exsanguination and 24-hr post-mortem

Fig. 1 shows the electrophoretograms of the sarcoplasmic proteins which were extracted from muscles excised immediately after exsanguination and at 24 hr post-mortem. These muscles (which are examples from the six pigs in this experiment) had an average pH of 5.5 at 30 min post-mortem and, with the exception of pig number P-3, they all became extremely PSE with color and gross morphology ratings of 1.0.

Muscles with color and gross morphology ratings of 1.0 have been previously shown to have marked reductions in solubility of sarcoplasmic and myofibrillar proteins (Bendall et al., 1962; Goldspink et al., 1964; Sayre et al., 1963). Scopes (1964) showed a denaturation of the sarcoplasmic protein creatine kinase in PSE muscle through SGE. However, it should be emphasized that this latter worker held muscles at 37°C for 4 hr in order to

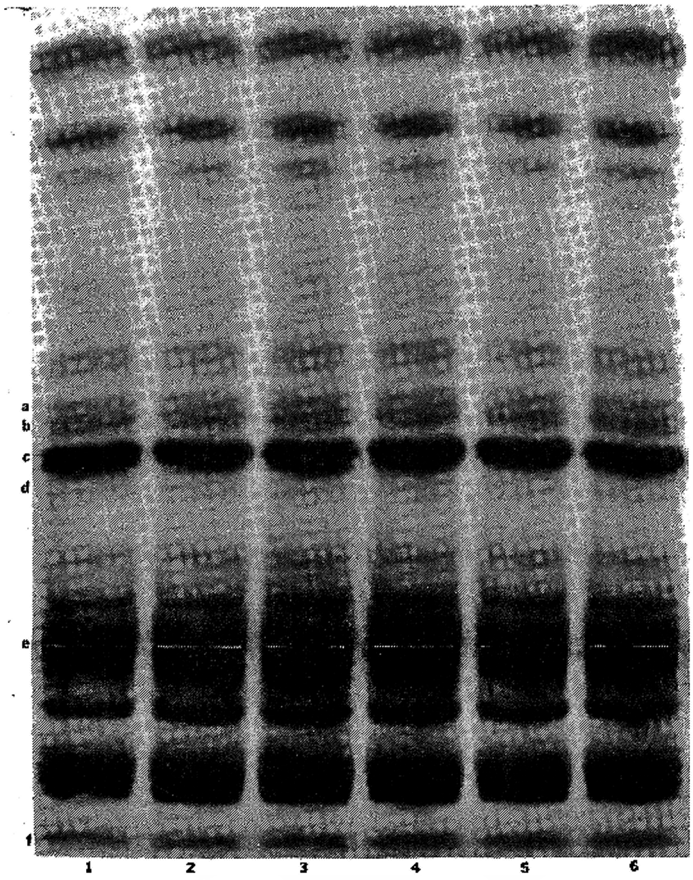

Fig. 1—Comparison of starch gel electrophoretograms of sarcoplasmic proteins extracted from muscle samples at 0 and 24 hr post-mortem. Conditions of SGE as indicated in text. Samples: (1 and 2) At 0 and 24 hr, respectively, from pig number P-1 with a 24 hr gross morphology and color rating of 1.0; (3 and 4). At 0 and 24 hr, respectively, from pig number P-2 with a 24 hr gross morphology and color rating of 1.0; (5 and 6) At 0 and 24 hr, respectively, from pig number P-3 with a 24 hr gross morphology and color rating of 1.5. Band identification (according to Scopes, 1966): a) creatine kinase (minor band-fastest migrating), b) phosphoglucomutase, c) creatine kinase (major band-slowest migrating), d) triosephosphate isomerase, e) sample slot, f) F-protein.

induce a rapid pH decline and a high degree of the PSE condition.

In an extensive study, Sayre et al. (1966) held entire carcass sides at this temeprature for 4 hr with the finding that temperature only exaggerated the pH decline and PSE development. Consequently, this treatment of entire sides only developed PSE when the control sides were already tending to he PSE. The disappearance of creatine kinase in the work of Scopes (1964), however, apparently involved muscles which were induced to become PSE by holding at 37°C for 4 hr.

As can he seen in Fig. 1, there were no major quantitative differences in the intensities of the bands of sarcoplasmic proteins from 0 and 24 hr samples, regardless of PSE development. The muscles at death had high pH values and were normal and firm in color and gross morphology, while the 24 hr samples varied from slightly to extremely PSE. The electrophoretograms from the PSE muscles showed prominent bands for creatine kinase, triosephosphate isomerase, phosphoglucomutase and F-protein (Scopes, 1966) which differed markedly from the Scopes (1964) work which showed a decrease in these bands. These findings, however, are in direct agreement with the work of Charpentier et al. (1963) who showed no differences in SGE of certain muscles, at death and 24 hr post-mortem, even though they exhibited a wide range in rate of pH decline and PSE development.

While it is widely accepted that the total solubility of the sarcoplasmic proteins may be reduced post-mortem by 50% or more (Sayre et al., 1963) it appears that the remaining soluble protein contains a consistent proportion of creatine kinase.

Sarcoplasmic proteins at various intervals post-mortem

Since it was recognized that many times muscles which become PSE may be improved at 24 hr post-mortem in relation to their transitional appearances (Briskey, 1964) an SGE study was made of extracts from muscle samples various times post-mortem. Fig. 2 shows representative starch gel electrophoretograms of samples at death, .25, .50, 1, 3 and 24 hr post-mortem from a muscle which became extremely PSE with a color and gross morphology rating of 0.5. Again these data support the earlier findings of

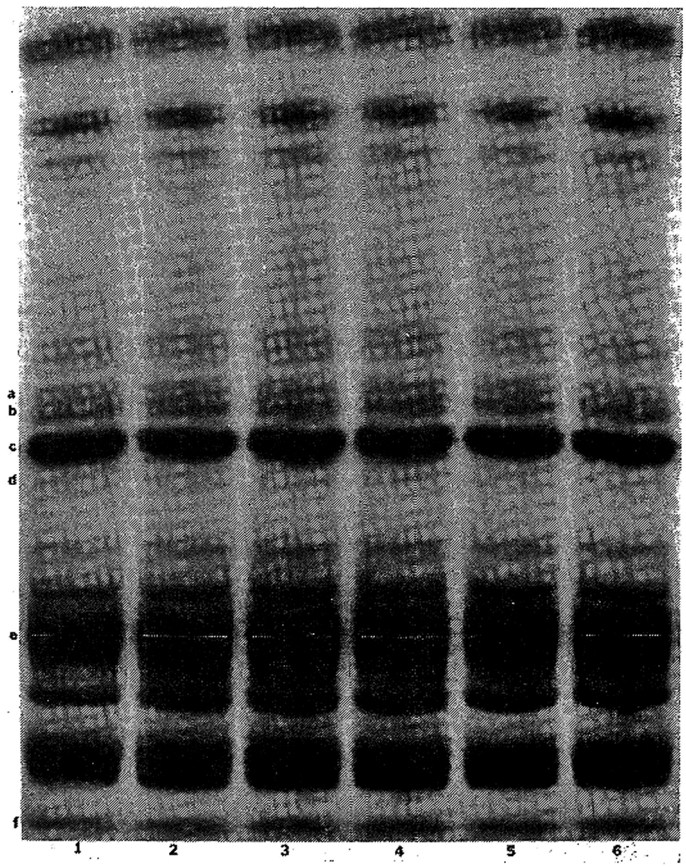

Fig. 2—Comparison of starch gel electrophoretograms of sarcoplasmic proteins extracted from muscle samples at various periods post-mortem. Conditions of SGE as reported in text. Samples: 1) 0 hr, 2) 15 min post-mortem, 3) 30 min post-mortem, 4) 1 hr post-mortem, 5) 3 hr post-mortem, 6) 24 hr post-mortem.

At 24 hr the muscle had a gross morphology and color rating of 0.5, which denotes an extreme PSE condition. Band identification (according to Scopes, 1966): a) creatine kinase (minor band-fastest migrating), b) phosphoglucomutase, c) creatine kinase (major band-slowest migrating), d) triosephosphate isomerase, e) sample slot, f) F-protein.

Charpentier et al. (1963) in that there were no readily detectable electrophoretic differences at any of the measured post-mortem periods.

Sarcoplasmic proteins from PSE and L-N_2 preserved muscle tissue

In the course of these investigations it seemed desirable to take advantage of the finding of Borchert et al. (1964) that a L-N_2 surface treatment and subsequent equilibration at 4°C would prevent PSE development. Consequently, through the use of this technique it was possible to have normal musculature in one carcass side while the other side became extremely PSE. SGE of these normal and L-N_2 treated muscles was of further interest because even though the L-N_2 treatment accelerated cooling slightly and retarded glycolytic rate slightly (Borchert et al., 1965) there was reason to believe that it may also have had an effect on nerve stimulation (Briskey et al., 1966) or lipid-protein, carbohydrate-protein, or protein-protein interactions, similar to the shock effect on other products.

The control sides in the six carcasses evaluated ranged in color and gross morphology ratings from 1.0 (extremely PSE) to 3.0 (normal) and 3.5 (slightly dark and firm). It is pertinent that there were no differences in SGE (Fig. 3) of sarcoplasmic proteins from control and L-N_2 treated sides regardless of the degree of PSE development under the control conditions.

While it was previously shown by Borchert et al. (1965) that L-N_2 treatment retained high solubility of sarcoplasmic protein, it does not appear from these starch gel electrophoretograms that this phenomena is due to preservation of any one particular protein of the sarcoplasm. Again, since there were no differences between the creatine kinase bands from the control and L-N_2 treated samples, it does not seem that L-N_2 maintains normal color and gross morphology by preserving creatine kinase solubility.

Sarcoplasmic proteins from muscle tissue with artificially induced PSE and normal characteristics

While it seems quite apparent that PSE muscle develops from or in association with a low pH and high temperature in the muscle at the onset of rigor mortis (Sayre et al., 1963), the state of contraction (Sink et al., 1965), and time-pH-temperature relationship (Briskey, 1964) may also be of paramount importance. It therefore seemed important to hold muscle samples at 37°C for 4 hr as was done in the work of Scopes (1964).

The appearance of representative samples from all three treatments can be noted in Fig. 4. The Poland China muscles were the only ones which consistently became PSE as a result of high temperature holding, although the holding samples from Chester White pigs demonstrated more pronounced effects than was previously noted by Sayre et al. (1966) when intact carcass sides were held at 37°C for the same time period.

It should be pointed out that Scopes (1964) held isolated muscles which were free to contract at 37°C, while in this particular experiment the muscles were somewhat restrained by their attachment to bone. The L-N_2-treated

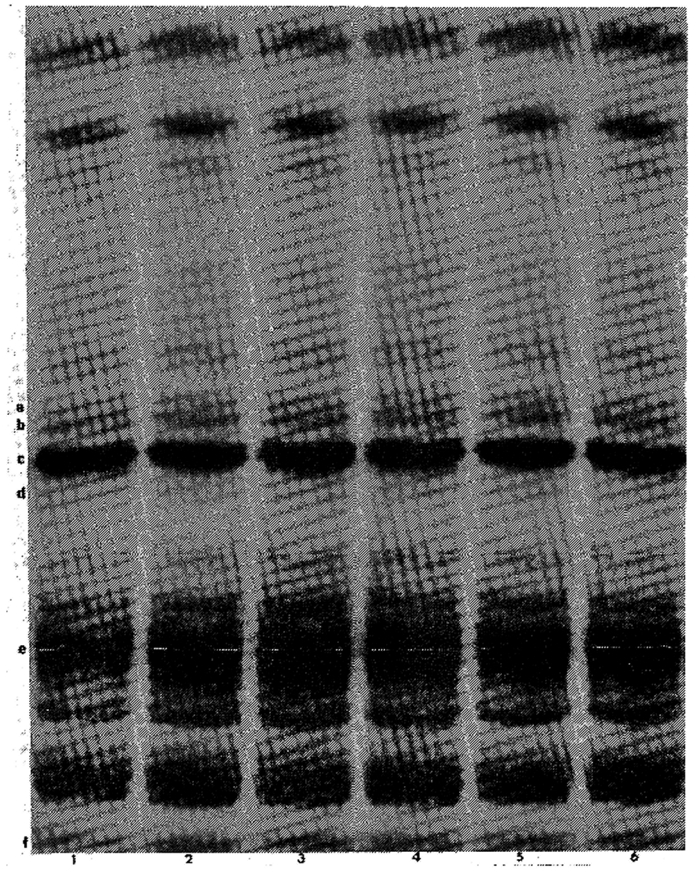

Fig. 3—Comparison of starch gel electrophoretograms of sarcoplasmic proteins extracted from muscle samples at 0 and 24 hr post-mortem and from samples 24 hr post-mortem which had been immersed in L-N_2 for periods of 10 sec/lb. Conditions of SGE as reported in text. Samples: (1, 2 and 3) 0, 24 hr control and 24 hr L-N_2, respectively, from pig number S-3 with a 24 hr gross morphology and color rating of the control sample 1.0 (extreme PSE condition) and of the L-N_2 sample 3.0 (normal condition); (4, 5 and 6) 0, 24 hr control and 24 hr L-N_2, respectively, from pig number S-2 with a 24 hr gross morphology and color rating of the control sample 3.0 (normal condition) and of the L-N_2 sample 3.0 (normal condition). Band identification (according to Scopes, 1966): a) creatine kinase (minor band-fastest migrating), b) phosphoglucomutase, c) creatine kinase (major band-slowest migrating), d) triosephosphate isomerase, e) sample slot, f) F-protein.

samples were slightly dark and firm with a color and gross morphology rating of 3.5. The control samples, in this case, were intermediate in color and gross morphology and had ratings of 1.5–2.0.

Fig. 5 shows starch gel electrophoretograms of samples at 0, 4 and 24 hr post-mortem from all three treatments. For these electrophoretograms, the concentration of the extract was accomplished by dialysis against 0.5M sucrose in 10 mM tris and 1 mM citric acid at 4°C and applied directly to the slot as described by Scopes (1964). The protein concentrations of each sample, prepared in this manner, are given as captions to Fig. 5.

By using these procedures it was unmistakably clear that holding at 37°C for 4 hr post-mortem drastically reduced the creatine kinase and F-protein bands as reported by Scopes (1964, 1966). In addition to markedly reducing the major (slowest migrating) creatine kinase band, this treatment almost entirely eliminated the minor

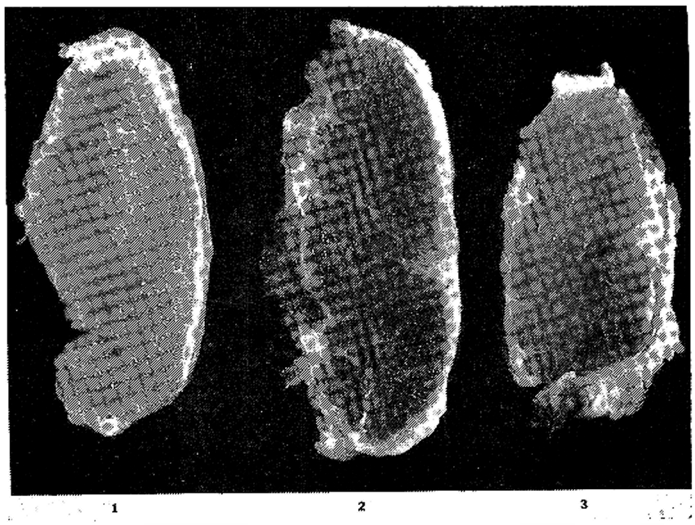

Fig. 4—Effect of post-mortem treatment with L-N_2 or heat upon the color and gross morphology of porcine longissimus dorsi. Samples: 1) Loin section (bone, muscle, fat and skin) held at 37°C for 4 hr immediately post-mortem, after which it was held at 4°C for an additional 20 hr. Sample was rated 0.5, which denoted an extreme PSE condition; 2) loin section treated immediately post-mortem with L-N_2 for a period of 10 sec/lb, after which it was allowed to equilibrate at 4°C for 24 hr. Sample was rated 3.5, which denoted a slightly DFD condition; 3) loin section held at 4°C for 24 hr. Sample was rated 1.5, which denoted a slightly PSE condition (all samples from the same carcass).

(fastest moving) creatine kinase band as well as the phosphoglucomutase and the triosephosphate isomerase bands. Additionally, while the F-protein band was markedly reduced when held at 37°C for 4 hr, it was nearly eliminated at the end of the 24 hr period (after subsequent holding for 20 hr at 4°C).

Fig. 6 shows the starch gel electrophoretograms of the same proteins under conditions whereby they were specifically adjusted to a uniform 40 mg/ml concentration before electrophoresis. Although this adjustment reduced the apparent effect on the major (slowest migrating) creatine kinase band, it was evident, in agreement with Scopes (1964), that this band was reduced by holding at 37°C for 4 hr post-mortem. Even after concentration adjustment, however, the F-protein (Scopes, 1965), minor (fastest migrating) creatine kinase and phosphoglucomutase bands were diminished to a greater extent than the major creatine kinase bands.

Complimentary gels to those in Fig. 6 were also stained specifically for creatine kinase. There were no discernible differences in forms or isozymes of creatine kinase between normal and PSE muscle, which was unlike the reported difference between normal and dystrophic muscle (Rosalki, 1965). These latter starch gel electrophoretograms further substantiated that the minor (fastest migrating) creatine kinase bands were probably the most labile of these band groups.

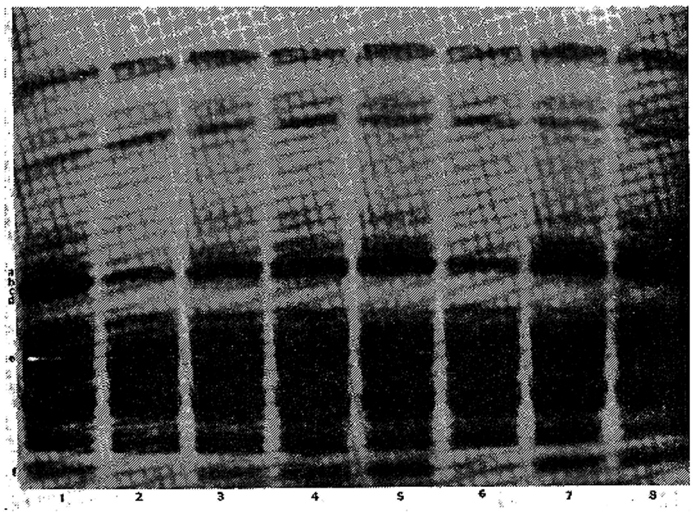

Fig. 5—Comparison of starch gel electrophoretograms of sarcoplasmic proteins extracted at various periods of post-mortem from heat or L-N_2 treated muscle samples. Conditions of SGE as described in text. Extract protein concentration of each sample is indicated below. Samples: (1-4) Extracted from muscles that were powdered 24 hr post-mortem; 1) loin section treated with L-N_2 for a period of 10 sec/lb, after which it was allowed to equilibrate at 4°C for 24 hr (27.9 mg/ml); 2) loin section held at 37°C for 4 hr, after which it was held 20 hr at 4°C (22.3 mg/ml), 3) loin section held 4°C for 24 hr post-mortem (25.0 mg/ml), 4) loin section removed from intact carcass side which had been held at 4°C for 24 hr (29.4 mg/ml); (5-7 Extracted from muscles that were powdered 4 hr post-mortem, 5) loin section treated with L-N_2 for a period of 10 sec/lb, after which it was allowed to equilibrate at 4°C for 4 hr (27.6 mg/ml), 6) loin section held at 37°C for 4 hr (24.0 mg/ml), 7) loin section held at 4°C for 4 hr (26.7 mg/ml), 8) loin section removed from carcass within 10 min (0 hr) post-mortem and powdered for immediate extraction (31.3 mg/ml).

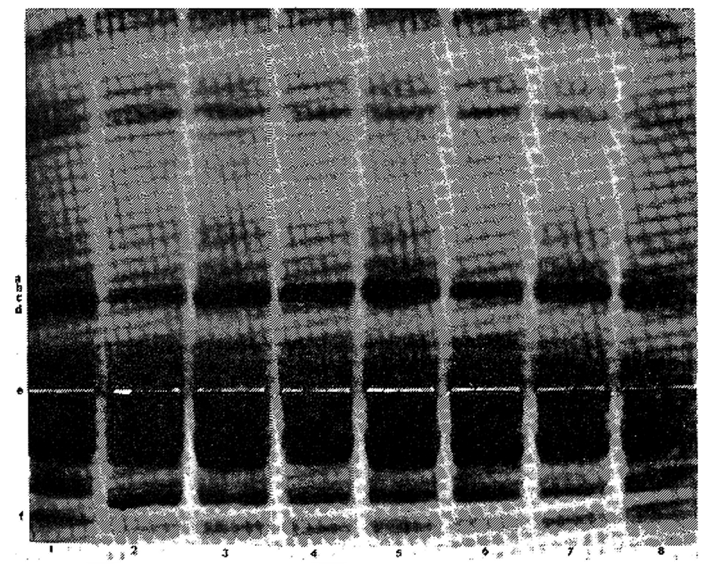

Fig. 6—Comparison of starch gel electrophoretograms of sarcoplasmic proteins extracted at various periods post-mortem from heat or L-N_2 treated muscle samples. Conditions of SGE as in Fig .5, after adjustment to a uniform protein concentration of 40 mg/ml. Samples: (1-4) Extracted from muscles that were powdered 24 hr post-mortem, 1) loin section treated with L-N_2 for a period of 10 sec/lb, after which it was allowed to equilibrate at 4°C for 24 hr, 2) loin section held at 37°C for 4 hr, after which is was held 20 hr at 4°C, 3) loin section held 4°C for 24 hr post-mortem, 4) loin section removed from intact carcass side which had been held at 4°C for 24 hr; (5-7) Extracted from muscles that were powdered 4 hr post-mortem, 5) loin section treated with L-N_2 for a period of 10 sec/lb, after which it was allowed to equilibrate at 4°C for 4 hr, 6) loin section held at 37°C for 4 hr, 7) loin section held at 4°C for 4 hr, 8) loin section removed from carcass within 10 min (0 hr) post-mortem and powdered for immediate extraction.

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Isolation of Strawberry Anthocyanin Pigments by Adsorption on Insoluble Polyvinylpyrrolidone

INTRODUCTION

ANTHOCYANIN PIGMENTS are readily extracted from plant materials with hydroxylic solvents such as water or methanol. Often it is desirable to further purify an extract before concentration and subsequent separation of the pigments by column, paper or thin-layer chromatography. This additional purification is particularly warranted when examining plant tissues which are low in anthocyanin content. Such extracts would require more concentration, thus providing more opportunity for anthocyanin degradation which is accelerated by sugars and other organic compounds.

Two commonly employed purification procedures are precipitation of anthocyanins as lead salts (by adding an excess of a saturated lead acetate solution) and by adsorption on cation-exchange resin with subsequent elution from the washed resin with acidified methanol. Both of these methods have disadvantages. Hayashi (1962) has reported that selective precipitation of anthocyanins occurs with the lead acetate procedures; anthocyanins containing adjacent free hydroxyl groups are precipitated quantitatively, while those not containing ortho hydroxyl groups are not completely precipitated.

In this laboratory we have applied the ion exchange procedure of Smith *et al.* (1965), but have experienced difficulty in eluting the pigment from the resin using 0.1–1% methanolic HCl. Increasing the acid concentration to 5% affords more complete recovery but also results in considerable hydrolysis of the pigments to the aglycones.

The oxygen in the peptide linkage of polyvinylpyrrolidone (PVP) will form unusually strong hydrogen bonds with the proton of phenolic hydroxyl groups; this property has been very useful in removing phenolic compounds during the isolation of plant enzymes (Loomis *et al.*, 1966). It was suggested by Loomis (private communication) that PVP might prove useful in isolating and purifying anthocyanin pigments. McFarlane (1961) has used insoluble PVP to determine the "anthocyanogens" (substances yielding anthocyanidins upon heating with HCl) in beer. The phenolic compounds were adsorbed onto the PVP and the other organic compounds removed by washing with water. After heating the PVP-adsorbate with *n*-methyl-2-pyrrolidone containing HCl and FeSO4, the anthocyanidins in the supernatant were assayed colorimetrically.

Sanderson *et al.* (1966) have developed a method using insoluble PVP to remove catechins and related polyphenols which interfere with carbohydrate determinations in plant tissues. The polyphenols in the plant extracts were adsorbed on the PVP, and the carbohydrates were removed by centrifugation.

Use of insoluble PVP to isolate anthocyanin pigments was investigated, and the method which was developed is reported in this paper.

EXPERIMENTAL

Materials

Strawberries were supplied by the OSU Department of Horticulture and the OSU North Willamette Experiment Station. Berries were washed and individually quick frozen at −34°. Frozen berries were packaged in polyethylene bags and stored at −26°.

Polyclar AT (insoluble PVP) was obtained from General Aniline and Film Corp., Dyestuff and Chemicals Division, 435 Hudson Street, New York, N.Y.

Isolation of pigments

Ten g of strawberry homogenate (Virtis homogenizer at setting 20 for 1 min) were extracted 3 times with 50 ml portions of water at 100°. The extracts were filtered through 2 layers of Whatman No. 1 filter paper on a Buchner funnel. The filtrate was shaken with 10 g of insoluble PVP on a wrist action shaker for 15 min. The mixture was centrifuged at 600 × G for 4 min; the supernatant was discarded. The pellet was washed with 175 ml of distilled water (enough to remove the sugars) and with the same volume of methanol to displace excess water.

The pigments were recovered from the PVP-pigment adsorbate by shaking the pellet with a 100 ml volume of 0.01% HCl in methanol for ½ min, centrifuging at 600 × G for 4 min and decanting the supernatant. This procedure was repeated 4 to 5 times. The extracts were combined and concentrated to approximately 1 ml on a Buchi rotary flash evaporator at 40°.

Determination of relative amounts of pigments

Pigment concentrates were spotted on 0.25 mm cellulose thin-layer plates and developed in acetic acid–water–HCl (15:82:3). After air drying the zones were scraped off the plate, extracted with spectro grade methanol containing 0.01% HCl and filtered through Whatman No. 1 filter paper. The filtrate volume was recorded and the absorbtivity determined at 510 nm with a Beckman DB Spectrophotometer.

RESULTS AND DISCUSSION

This procedure was developed in a study of the anthocyanin pigments of several strawberry varieties. Both

methanol and water were extracting solvents. Extracting the tissue three times effectively removed all the anthocyanins. Extracting with water caused difficulty, as unprecipitated pectic material interfered in filtration. Extracting with boiling water overcame this problem.

Extracts were shaken with 0.5 to 1.5 g of PVP per g of fresh tissue. PVP was added as a dry powder or as a slurry (1 g PVP/5 ml liquid) in methanol, water, or 0.1 *M* potassium phosphate buffer of pH 7.2. An all-methanol system was ineffective, fewer than 50% of the pigments were adsorbed on the PVP. Increased amounts were adsorbed when the methanol extracts were shaken with water or buffer slurries of PVP. Water extracts yielded the best results. Adding dry PVP to the extract was as effective as adding it as a slurry in buffer or water.

Methanol containing 0.01% HCl was effective in removing the adsorbed pigments from the PVP. Apparently the proton displaces the hydrogen of the phenolic group bonded to the PVP. McFarlane (1961) reported that *n*-methyl-2-pyrrolidone most effectively removed phenolics from PVP. This solvent was somewhat more efficient than the acidified methanol in removing the adsorbed pigment; however, its high boiling point made concentration of the extract impossible without applying considerable heat.

The fruit's total anthocyanin content had been measured by the procedure of Swain *et al.* (1959). Thus, recovery of anthocyanin pigments could be easily estimated by measuring the adsorbances of the pigment isolate before concentration. Recovery using water extracts and PVP was approximately 90%. Thin-layer cellulose chromatography of the pigment concentrate showed no evidence of pigment hydrolysis to the aglycones. Recovering anthocyanins by the ion exchange procedure was quantitatively as good if 5% HCl/methanol was used to elute the pigment from the resin. However, thin-layer chromatography showed 26% of the pelargonidin-3-glucoside had hydrolyzed to pelargonidin. Using 1% HCl/methanol recovery was 66% with 12% of the pelargonidin-3-glucoside undergoing hydrolysis.

Possible selective adsorption of anthocyanin pigments by PVP was checked by analyzing a strawberry variety which contains considerable amounts of cyanidin-3-glucoside, *Fragaria ananassa,* var. Marshall. Analysis with ion exchange resin showed 88% pelargonidin-3-glucoside and 12% cyanidin-3-glucoside. PVP analysis gave similar results: 84% and 16%.

Using this procedure, one would anticipate isolation of any phenolics present in the water extract. Yellow fluorescent spots on thin-layer chromatograms exposed to ammonia vapor and examined under ultra violet light suggested presence of flavones or flavonol glycosides. There was no further characterization or quantitative assay of non-anthocyanin phenolics.

McFarlane *et al.* (1962) reported that impurities were present in the PVP which oxidized the anthocyanogens and that PVP purification or inclusion of a reducing agent such as ascorbic acid was necessary to avoid this. Apparently there are no impurities in the PVP used in this study which can oxidize the anthocyanin pigments under experimental conditions. Neither PVP purification according to McFarlane's procedure nor inclusion of ascorbic acid gave increased pigment recovery.

Isolating anthocyanin pigments from strawberries using insoluble PVP is non-selective and gives high recovery without evidence of degradation. The procedure is not laborious and uses inexpensive materials. At present it has only been applied to different strawberry varieties in this laboratory. Modifications of the PVP quantity may be needed, depending on variables such as phenolic and/or water content. With other plant extracts, buffered solutions may be better than distilled water in the various isolation steps, because the pH should not be too high.

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Assessment of Green Tuna: Determining Trimethylamine Oxide and its Distribution in Tuna Muscles

SUMMARY—In order to assess green meat of tuna in preparation for canning, it is necessary to determine the trimethylamine oxide (TMAO) content of the raw meat.

A simple and rapid TMAO determination method combined with a reduction method, which converts TMAO to trimethylamine (TMA), was developed. Using this method, the distribution of TMAO content in the muscles of yellowfin tuna was investigated to determine the most suitable sampling portion of the fish.

A recommended practical method of sampling and determining TMAO for assessing green tuna is presented.

INTRODUCTION

"GREENING" is an industrial term given to an off-color condition seen in tuna of varying sepcies after they have been pre-cooked in canneries. There is no completely satisfactory means of predicting which raw fish will show this color after cooking. Koizumi et al. (1965), Nagaoka et al. (1962, 1964) and Sasano et al. (1962) reported that greening of tuna meat after cooking is very closely related to the TMAO content of raw meat. But greening action during precooking differed in various parts of fish bodies, generally being concentrated in the superficial layer near the head and tail-end of body muscles. It seemed that this phenomenon might depend upon the TMAO content of the fish itself.

Investigations on the distribution of TMAO content of the muscles of yellowfin tuna, and research on the relationship between TMAO content of raw meat and green color in cooked meat were carried out. The microdiffusion method, convenient for analysis of many samples, was applied for these purposes.

METHOD

Microdiffusion method

Where analyzation of many samples is required, any usual method, such as Dyer's (Dyer, 1945), is too complicated and needs a rather large amount of sample meat (10 g) to determine TMAO content.

Beatty et al. (1937) first reported application of the microdiffusion method of TMA determination. For the application of this method to TMAO determination, it is first necessary to reduce TMAO to TMA.

Bystedt et al. (1959) adapted a reducing method using titanous chloride (see Experimental), but two points should be noted in the utilization of this method.

First, a viscide and whitish precipitate forms when a 10% formaldehyde solution and saturated potassium carbonate is added to the titanous chloride solution in the outer ring of a Conway dish (Conway, 1950). The influence on diffusion of TMA due to this phenomenon must be considered.

Second, the sample solution containing titanous chloride must be left for 2 hr at room temperature according to the original report. A more rapid method of reducing TMAO by heating must be considered, confirming the accuracy of the procedure.

The influence of titanous chloride on the diffusion of TMA was determined in experiments using TMA solutions with and without titanous chloride. An arbitrary amount of TMA was dissolved in 4% trichloroacetic acid solution and 0.3% titanous chloride was added. The result is shown in Table 1. No difference between the two solutions was found, so, it was recognized that titanous chloride has no influence on the diffusion of TMA.

An experiment was also conducted concerning the influence of heating temperature and time on reduction of TMAO by titanous chloride. TMAO was dissolved in 4% trichloroacetic acid solution and 0.3% titanous chloride solution was added. The result is shown in Table 2. The recovery of TMAO-nitrogen was very good at every temperature, so the reduction temperature in the following experiment was set at 80°C in a water bath.

A comparison of the microdiffusion method for recovery of TMAO and Dyer's method with modification by Hashimoto et al. (1957) was conducted. Ten g of ordinary tuna muscles were extracted with 40 ml of 4% trichloracetic acid solution and a known amount of TMAO was added to it. The result is show in Table 3. Little difference in recovery between methods was noted, so the microdiffusion method, when available for determination of TMAO, should be to Dyer's method.

Based on these results, all TMAO in this report was determined by the microdiffusion method.

EXPERIMENTAL

Samples

Two pieces of frozen yellowfin tuna (*Thunnus albacores*) in which TMAO had been determined in the tail meat were chosen for samples. One fish (body length, 158 cm; weight, 71.6 kg) contained 19 mg %; the other

Table 1. Effect of titanous chloride on determination of TMA by microdiffusion method.

Test solution[1]	TMA-nitrogen found γ/ml			
TMA solution not containing titanous chloride	7.79	27.59	41.61	72.34
TMA solution containing titanous chloride[2]	7.64	27.36	42.06	72.11

[1] Arbitrary amounts of TMA were dissolved in 4% trichloroacetic acid solution.
[2] Titanous chloride was added in the concentration of 0.3%.

Table 2. Effect of heating on reduction of TMAO with titanous chloride.

Condition of heating		TMAO-nitrogen[1]					
Temperature of water bath	Time	Added	Found	Recovery	Added	Found	Recovery
°C	min.	mg	mg	%	mg	mg	%
37	90	0.020	0.0206	103.0	0.080	0.0784	98.1
50	30	0.020	0.0196	98.4	0.080	0.0801	100.2
70	3	0.020	0.0202	101.0	0.080	0.0796	99.5
80	1	0.020	0.0205	102.5	0.080	0.0796	99.5
100	0.5	0.020	0.0195	97.8	0.080	0.0789	98.7

[1] Determined by microdiffusion analysis of TMA formed from TMAO by reduction. See text for details of analytical method. TMAO was dissolved in 4% trichloroacetic acid solution. To the solution titanous chloride was added in the concentration of 0.3%.

Table 3. Comparison of recovery of TMAO between microdiffusion method and Dyer's method.

	TMAO-nitrogen			
	Microdiffusion method[1]		Dyer's method	
Added	Found	Recovery	Found	Recovery
mg	mg	%	mg	%
0	0.0364	.....	0.0357	.....
0.005	0.0415	102.0	0.0407	100.0
0.010	0.0468	104.0	0.0459	102.2
0.020	0.0569	102.5	0.0560	101.5
0.030	0.0761	99.3	0.0747	97.5

[1] See the text for details. Five g of the ordinary muscle of fish was extracted with 20 ml of 4% trichloroacetic acid. One ml of the extract was used for analysis.

(body length, 114 cm; weight, 28.4 kg) had 0.9 mg %. The samples were cut as shown in Fig. 1, and 5 g of muscle in each portion were numbered, weighed, minced and crushed, adding 20 ml of 4% trichloroacetic acid solution. After standing for 30 min, the filtrates were used as muscle sample solutions.

TMA determination method

The microdiffusion method of Beatty et al. (1937) was used to determine the TMA content. One ml of a muscle sample solution was placed in the outer ring of a Conway dish and 1 ml of standard acid solution was placed in the inner ring. One ml of 10% formaldehyde solution was added to the muscle sample solution in the outer ring of the Conway dish, after it was mixed to fix the ammonia and had stood for 3 min. One ml of saturated potassium carbonate was then added and mixed in the outer ring of the dish and left for 90 min in an incubator at 37°C. N/150 hydrochloric acid solution containing an indicator was used for the standard acid solution, and it was titrated with N/70 barium hydroxide solution.

TMAO determination method

To reduce TMAO to TMA, the Bystedt et al. (1959) method was used with some modification. Two ml of muscle sample solution in small test tube (8 × 80 mm) were mixed with 1 ml of 1% titanous chloride solution.

In the standard method, the mixed solution is left for 2 hr at room temperature and one drop of saturated potassium nitrate is added to remove excess titanous chloride.

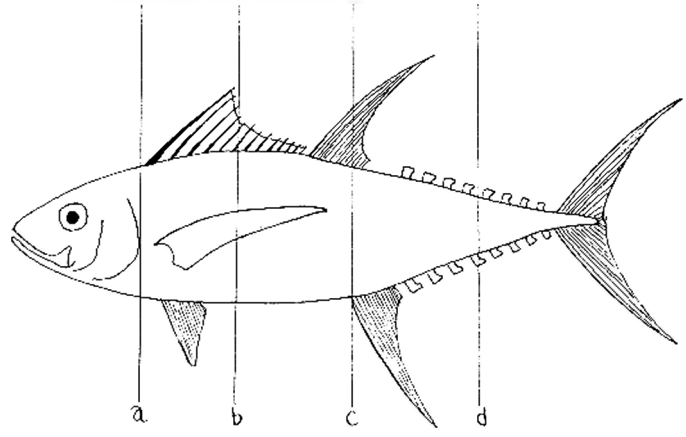
Instead, the mixed solution in the test tube was soaked in a water bath at about 80°C and heated for 1 to 1.5 min until the reddish-violet color of titanous chloride solution had faded away. After reduction was finished, the solution was quickly cooled under running water. One ml of each of the solutions derived from the two tuna muscle samples was used to determine the total TMA content.

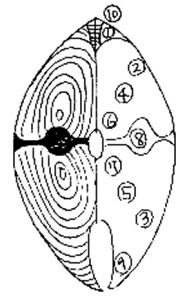
TMAO content is given in the following formula:

TMAO nitrogen mg % = Total TMA nitrogen mg % − TMA nitrogen mg %, where total TMA nitrogen mg % is the TMA nitrogen mg % obtained by heating the sample

Position of section

Position of section: a, Margin of gill cover; b, Seventh spine of the first dorsal fin; c, Anus; d, Fourth dorsal finlet.





Cross-section view

Cross-section view: 1, Upper dorsal muscle; 2, 3, Superficial ordinary muscle; 4, 5, Interior ordinary muscle; 6, 7, Deep ordinary muscle; 8, True red muscle; 9, Belly muscle; 10, Dorsal fin muscle.

Fig. 1—Sampled parts of the muscle of yellowfin tuna.

solution with titanous chloride solution, and TMA nitrogen mg % is the TMA nitrogen mg % in the sample solution not reduced.

Cooking operations

About 1.5 kg of the ordinary muscles of each section (Fig. 1, a, b, c and d) 500 g of the muscles of head, belly and 200 g of the muscles adjacent to fins were used for cooking operation.

These muscles were cooked at 105°C for 110 min in a retort. After cleaning, cooked meat was evaluated into A, B, C and D grades by our inspectors.

RESULTS AND DISCUSSION

Distribution of TMAO in yellowfin tuna muscle

The distribution of TMAO content in the epaxial or hypoxial ordinary muscles on either side of the fish body was determined. The TMAO content in each part of body muscles on the superficial, interior and deep muscles (Fig. 1, No. 2–7) at b section is shown in Table 4.

Table 4. TMAO content in the epaxial[1] and the hypoxial[2] ordinary muscle on both sides of the fish.

	(TMAO N mg %)			
	Right side		Left side	
Portion[3]	epaxial	hypoxial	epaxial	hypoxial
Superficial	17.1	17.0	16.9	17.0
Interior	16.5	15.4	16.4	16.4
Deep	10.3	9.7	10.0	10.4

[1] Dorsal meat.
[2] Ventral meat.
[3] Sampled at the section b indicated in Fig. 1.

The TMAO content of the superficial muscles was high while that of the deep muscles was low. There was no difference between epaxial and hypoxial ordinary muscles on both sides of the fish. The data in Table 5 shows the TMAO content in various portions of normal fish and green meat fish.

The TMAO content in the ordinary muscles such as superficial, interior and deep muscles was shown with average TMAO-nitrogen value of four portions, right side, left side and epaxial, hypoxial. But the TMAO content in the upper dorsal, belly, fin, true red muscles and other extraordinary parts was shown with average TMAO-nitrogen value of two portions in right side and the left side.

The TMA in the upper dorsal and belly muscles was determined only at section b, except for the true red muscles and other muscles, and TMA content is given in parentheses. The ordinary muscles for canning tuna fish are mainly loins, and the TMAO content in the loin muscles of green tuna was markedly high compared with those of normal tuna, especially in the superficial layer of body muscles near head and tail-end. The red muscles and the muscles adjacent to fins contained TMAO at high levels in both normal and green tuna.

According to Takada et al. (1958) and Kawabata (1953) TMAO content of red muscles was higher than

Table 5. TMAO content in various portions of fish muscle.

	Normal fish			TMAO-N mg %
Position of section[1]	a	b	c	d
Superficial muscle		1.4	0.8	0.4
Interior muscle	0.6(1.8)	0.3(0.6)	0.6(0.4)	0.8(2.2)
Deep muscle		0.1	0.4	0.8
Upper dorsal muscle	2.1	0.7(0.2)	1.6	1.8
Belly muscle	11.9	5.5(1.1)	2.1	2.0
True red muscle	7.4(6.6)	7.0(15.0)	11.2(14.1)	...
Muscle of head region 10 cm above the eye				6.5 (0.8)
Muscle of caudal fin				8.1 (0.6)
Muscle of dorsal fin				33.4 (1.2)
Muscle of anal fin				20.5 (2.4)
Muscle of pectoral fin				34.9 (2.1)

	Green-meat fish			
Position of section[1]	a	b	c	d
Superficial muscle		17.0	19.3	18.6
Interior muscle	15.9(0.4)	16.2(0.2)	13.3(0.4)	19.5(0.9)
Deep muscle		10.1	8.9	19.3
Upper dorsal muscle	17.4	11.4(0.4)	11.6	22.8
Belly muscle	45.0	21.3(1.2)	17.6	22.0
True red muscle	...	4.1(14.6)	5.0(14.2)	1.9(14.2)
Muscle of head region 10 cm above the eye				26.8 (0.7)
Muscle of caudal fin				25.5 (2.0)
Muscle of dorsal fin				39.9 (2.4)
Muscle of anal fin				59.0 (5.0)
Muscle of pectoral fin				46.0 (3.2)

TMA contents were also determined and are given in parentheses.
[1] See Fig. 1 for the positions of sections (a~d) and the sampled portions.

Table 6. Relation between color grades of cooked meat and TMAO content in raw meat.

Muscle portion[1]	Normal fish		Green-meat fish	
	TMAO content N mg %	Color[2] grade	TMAO content N mg %	Color[2] grade
Muscle of head region 10 cm above the eye	6.5	A	26.8	D
Interior ordinary muscle at the section a	0.6	A	15.9	C
muscle at the section b	0.3	A	16.2	C
muscle at the section c	0.6	A	13.3	D
muscle at the section d	0.8	A	19.5	D
Belly muscle	5.5	A	21.3	D
Muscle of caudal fin	8.1	B	25.5	D
Muscle of dorsal fin	33.4	D	39.9	D

[1] See Fig. 1.
[2] Color grade: A, normal; B, slightly green; C, moderately green; D, heavily green.

those of the ordinary muscles, and they said that the TMAO of red muscles is easily reduced to TMA. A high TMAO content in red muscles is considered normal. The TMAO content of the ordinary muscles of green meat tuna was almost identical to the normal state of the red muscles.

Based on this fact, it was concluded that the TMAO content of the ordinary muscles in the green meat tuna indicated an abnormal physiological property. There are a considerable number of unknowns in this physiological TMAO problem. The high TMAO content of the red muscles and those adjacent to the fins is thought to indicate some physiological relation to their exercise.

Relationship between the TMAO content and green meat

Results of the cooking operation are shown in Table 6. In the normal fish, normal cooked fish color appeared in every portion except in the muscles adjacent to the fins. In green meat fish, a green color appeared in all portions of the fish muscles.

The degree of coloration after cooking was closely related to the TMAO concentration in the corresponding portion of the raw meat. The color of the muscles adjacent to fins, head and belly was deeper than in the other muscles. In the ordinary muscles, the degree of greening was especially observed in superficial muscles and tail-end muscles.

Sample portion for assessment of green tuna

It is logical to take sample meat from a fish body to determine TMAO for assessment of green meat as there is no damage to the fish body. For this purpose, it was thought that tail-end muscles would be the most suitable portion. But Table 5 shows that the TMAO content of tail-end muscles was usually higher than that of the other ordinary muscles (loin meat) and that the TMAO in these muscles is not in a constant ratio to other ordinary muscles.

Based on these results, it is recommended that the interior portion of dorsal muscles (portion No. 4 or 5 of section b in Fig. 1) be taken for TMAO determination for the purpose of assessing green tuna before cooking.

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Oxidation Effects in a Freeze-Dried Gelatin-Methyl Linoleate System

SUMMARY—We studied oxidation of a freeze-dried model system consisting of methyl linoleate and gelatin by incubating the model system in air at 50°C for up to 10 days in the dry state or at controlled relative humidities. Incubation for 5–10 days caused a drop in the viscosity of gelatin solutions, an increase in the solubility of gelatin in ethanol-rich solvent mixtures, an increase in the retention time of gelatin on a Sephadex G-150 column, and a reduction in the melting point of a standard gelatin gel. There were no such changes in the viscosity and solubility properties of gelatin when incubation was at a relative humidity of approximately 60%. In some instances, incubation at high relative humidity led to partial insolubilization of gelatin in water or in acetate buffer. The oxidation effects in the dry state were consistent with the hypothesis that gelatin undergoes oxidative degradation. The effects of oxidation showed similarities to effects of ionizing radiations.

INTRODUCTION

OXIDATION OF LIPIDS during storage of freeze-dehydrated foods limits the shelf-life of food products. Damage caused to food by autoxidized lipids includes production of off-flavors, off-odors, and changes in texture and rehydratability. Changes in texture and rehydratability of products led to the speculation that interaction between lipid oxidation products and proteins takes place during the processing and storage of dried food (Koch, 1962). Mechanisms of such interactions have not been adequately explored, however.

Only a few research studies on model systems have investigated changes in protein after oxidation of protein-lipid systems (Narayan *et al.*, 1958, 1963, 1964; Nishida *et al.*, 1960; Desai *et al.*, 1963; Andrews *et al.*, 1965; Roubal *et al.*, 1966a,b). With the exception of the study of Andrews *et al.* (1965), the model systems in these studies had high moisture contents. The results indicate, in general, that oxidation of protein-lipid systems results in extensive browning accompanied by co-polymerization of oxidized lipids and proteins.

Aggregation of proteins, either through secondary or covalent bonds, has also resulted from oxidation. Decreases in the availability of ϵ-amino group of lysine and losses of amino acids, such as histidine, tyrosine, methionine, lysine and cysteine, result from oxidation of protein-lipid systems.

Andrews *et al.* (1965) studied changes in protein after oxidation of freeze-dried, gelatin-methyl linoleate and insulin-methyl linoleate systems. They report aggregation of proteins.

Further elucidation of oxidative changes in freeze-dried protein-lipid systems would be interesting since there have been severe problems of lipid oxidation in storage of dehydrated food and since only limited information is available in this field.

MATERIALS AND METHODS

Model system

We used the procedure of Andrews *et al.* (1965) with minor modifications. One milliliter of methyl linoleate (Hormel Institute, Auston, Minn.) was added to 5 g of gelatin (Atlantic Gelatin, Type B, Woburn, Mass.) dissolved in 100 ml of water. Vacuum distillation was used to reduce the initial oxidation level of linoleate to values not exceeding 0.5%. The mixture was emulsified for 15 min in a Sorvall Omni-Mixer mixing cup. The resulting viscous mixture was freeze-dried for 48 hr at ambient temperatures under pressure not exceeding 100 μ. We incubated freeze-dried samples for up to 10 days at 50°C in desiccators containing silica gel or over constant humidity solutions and then extracted samples at ambient temperatures by using a mixture of chloroform-methanol 1:1 for removal of linoleate.

Linoleate was extracted from samples immediately after freeze-drying. The samples were incubated and used as blanks. Care was taken to assure adequate oxygen supply during the incubation.

Viscosity

A Cannon-Fenske pipette viscometer size 50 (Cannon Instrument Co., State College, Pa.) was used for measuring flow times of solutions of gelatin in 0.15*M* acetate buffer (pH 4.8) at 37.5°C. Specific viscosity (η sp) was calculated using the following formula:

$$\eta \text{ sp} = \frac{(\text{flow time of solution}) - (\text{flow time of solvent})}{(\text{flow time of solvent})}$$

Reduced viscosity values were obtained by dividing specific viscosity values by the corresponding concentration.

Solubility

Solubility of gelatin in four mixtures of absolute ethanol-0.8*M* NaCl solution at 37°C was determined. The volume ratios of ethanol to 0.8*M* NaCl in the four mixtures tested were 4:1, 2.8:1, 2.16:1, and 1.34:1. Extraction was made by shaking the protein suspension in a controlled temperature, water-bath shaker for at least 3 hr at 37°C. We withdrew 0.5–1.0 ml portions of supernatant and determined protein concentration by the Folin-Ciocalteu method (Litwack, 1960).

Fractionation on Sephadex column

A jacketed column, 100 × 2.5 cm, was packed to a height of 85 cm with a mixture of 90% by weight of

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Sephadex G-150 gel particles (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.) and 10% cellulose powder (Whatman cc 41). The cellulose powder was added to improve packing characteristics of the column. The mixture was equilibrated prior to packing with 0.075*M* acetate buffer (pH 4.8). Fifty mg of gelatin contained in 2 ml of the buffer solution were applied to the top of the column. The protein was eluted by gravity with 430 ml of 0.075*M* acetate buffer (pH 4.8). We collected 2.2-ml fractions and determined the protein content by the Folin-Ciocalteu method.

Gelatin gel melting point

The method of Eldridge *et al.* (1954) was used.

Nondialyzable material

We dialyzed 100 ml of 0.1% (w/v) gelatin solutions against distilled water for 48 hr at 3.5°C. The dialyzed solutions were freeze-dried for 48 hr, and the weights of solid material were determined.

Free amino group content

The method of Block (1956) was used.

Nitrogen content

Kjeldahl method was employed.

Amide group content

The method of Bailey was used (Romani *et al.*, 1960).

Dye absorption

The method of Fraenkel-Conrat *et al.* (1944) was used for determining absorption of orange G and safranine by positively and negatively charged groups contained in gelatin, respectively.

Peroxide content

Determinations were made by the iodometric method with procedures identical to those of Karel *et al.* (1967).

C^{14} determination

Binding by gelatin of C^{14} from methyl linoleate labelled in the carboxy group (Tracerlab, Inc., Waltham, Mass.) was determined by the method of Mahin *et al.* (1966). This method consisted of degrading the gelatin by a mixture of perchloric acid and hydrogen peroxide and then counting the radioactivity with an ambient temperature scintillation counter (Nuclear Chicago Corp., Des Plaines, Ill.).

RESULTS AND DISCUSSION

Freeze-dried, gelatin-methyl linoleate mixtures were incubated aerobically at 50°C for periods of 5–10 days. When the incubation was carried out in the dry state, the following major physico-chemical changes in gelatin were found to be due to incubation in the presence of linoleate:

1. Viscosity of gelatin solutions dropped substantially (Fig. 1, Tables 1, 2).
2. Gelatin became more soluble in ethanol-rich solvent mixtures (Tables 1, 2).
3. Retention time on Sephadex G-150 column increased (Fig. 2, Table 3).
4. Melting point of a standard gelatin gel decreased (Table 3).

On the other hand, oxidation caused no substantial increase in nondialyzable material (Table 3). The changes described above are consistent with the hypothesis that during the incubation with methyl linoleate, gelatin undergoes oxidative degradation as a result of which its average molecular weight drops. Evaluation of the changes in viscosity with the usual correlation (Veis, 1964; Zirlin, 1968) indicates that the molecular weight fell by a factor of up to 4.

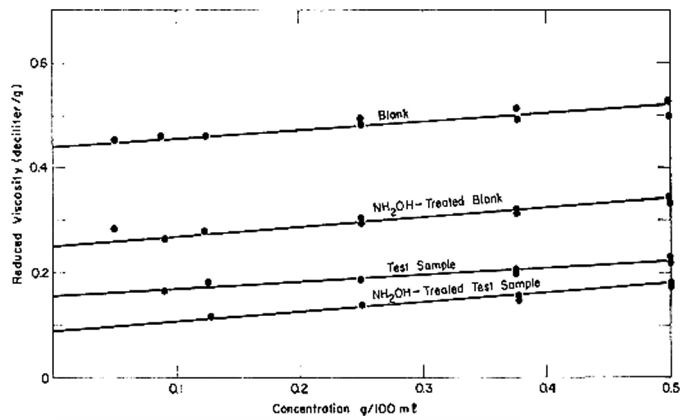


Fig. 1. Reduced viscosity vs. concentration curves for gelatin in 0.15M acetate buffer (pH 4.8) at 37.5°C (run 6).

Table 1. Changes in gelatin after aerobic incubation with linoleate at 50°C for 6 days (Run 8).

Sample description	Relative humidity during incub. (%)	Solubility in ethanol-0.8*M* NaCl solutions (%)				Specific viscosity	Free amino group (meq/g)	Amide group content (meq/100 g)	Peroxide content (meq/g linoleate)
		4.0:1[1]	2.8:1[1]	2.16:1[1]	1.34:1[1]				
Blank	~0	5.3	12.0	45.0	100	0.268	0.35	10.6	....
Oxidized sample	~0	35.0	57.0	87.0	100	0.070	0.32	23.9	0.89
Blank	31.5	4.7	10.4	39.2	100	0.268	0.35	10.4	....
Oxidized sample	31.5	17.5	40.7	78.8	100	0.114	0.315	16.0	0.40
Blank	59.5	4.0	11.5	40.0	90.0	0.285	0.335	10.4	....
Oxidized sample	59.5	5.0	11.1	32.5	91.1	0.285	0.31	10.9	0.93

[1] Volume ratio of ethanol to NaCl solution.

Table 2. Changes in gelatin after aerobic incubation with linoleate at 50°C.

Sample description	Relative humidity during incub. (%)	Solubility in ethanol-0.8M NaCl solutions (%) 4.0:1[1]	2.8:1[1]	2.16:1[1]	1.34:1[1]	Specific viscosity	Amide group content (meq/100 g)	Peroxide content (meq/g linoleate)
5 days of incubation								
Blank	0	4.9	11.8	44.8	100	0.274	10.8	0
Oxidized sample	0	29.0	58.3	81.5	100	0.110	26.7	0.80
6 days of incubation								
Blank	31.5	4.8	11.4	45.0	100	0.274	10.8	0
Oxidized sample	31.5	17.7	45.0	78.7	100	0.150	17.4	0.23
Blank	59.5	4.1	11.3	40.7	89.8	0.276	10.6	0
Oxidized sample	59.5	12.0	28.5	58.4	75.4	(0.218)[2]	13.6	0.37

[1] Volume ratio of ethanol to NaCl solution.
[2] Only 80% of the protein was soluble. The insoluble part was centrifuged out prior to measurement of viscosity.

However, incubation at high relative humidities caused smaller changes in viscosity and solubility characteristics of gelatin as compared with oxidative changes under dry conditions (Tables 1, 2). In one instance (Table 2) incubation at a relative humidity of 59.5% for 6 days caused partial insolubilization of gelatin in water and in acetate buffer (pH 4.8) and indicated aggregation of the protein. Data in Tables 1 and 2 show also that the magnitude of changes in viscosity and solubility at various relative humidities was not correlated with oxidation level of linoleate as measured by the peroxide value.

Experiments using C^{14}-labelled methyl linoleate showed that oxidation caused incorporation of linoleate-derived

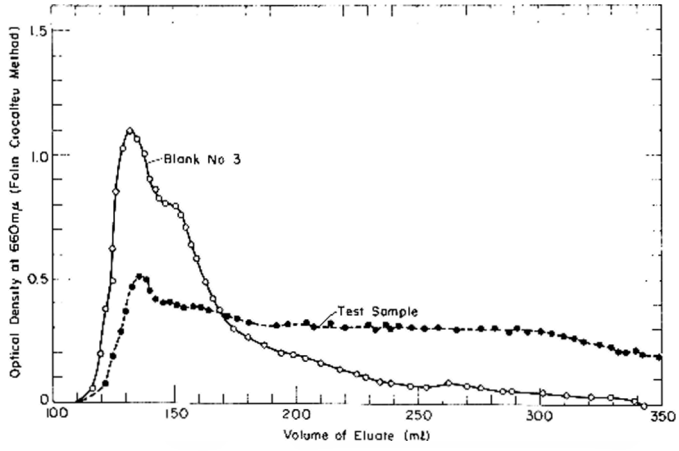


Fig. 2. Fractionation of gelatin on a Sephadex G-150 column (run 3).

Table 3. Oxidation-induced changes in gelatin gel melting point and in nondialyzable material.[1]

Sample description	Melting point of gelatin gel (°C)	Nondialyzable material (%)
Blank, Run No. 3	30.0	93.0
Blank, Run No. 6	30.0	93.9
Oxidized sample, Run No. 3	23.5	90.9
Oxidized sample, Run No. 6	23.0	90.0

[1] Samples were incubated 10 days in the dry state.

C^{14} in gelatin extracted from the model system. Table 4 shows the results obtained with carboxy group-labelled methyl linoleate. Limited experimentation with uniformly labelled methyl linoleate shows a similar trend (Zirlin, 1968).

We considered the possibility that incorporation of linoleate fragments in gelatin was responsible for the observed changes in physico-chemical properties. Incorporation of linoleate is likely to render the protein molecule more hydrophobic and cause it to assume a more contracted configuration in aqueous media. A contracted configuration would lower solution viscosity and could produce changes in solubility and other physico-chemical properties. However, the possibility that the changes observed in this study were due to incorporation of linoleate fragments is unlikely for the following reasons:

1. The viscosity changes seemed greater than could be attributed to changes in polarity, especially since studies on electrochemical properties of gelatin fail to show drastic changes in net charge (Zirlin, 1968).
2. Conditions which produced the most incorporation of the C^{14} label (high relative humidity) produced the least change in viscosity and solubility in ethanol-rich solvents.

Additional studies on changes in gelatin oxidized in the presence of methyl linoleate were undertaken in order to explore possible mechanisms of gelatin degradation.

Results on absorption of safranine and of orange G dyes by negatively and positively charged groups in gelatin, respectively, are in Table 5. These values indicate that oxidation under dry conditions did not cause significant changes in the negatively charged groups of protein. De-

Table 4. Incorporation of labelled linoleate in gelatin.

Relative humidity during incubation (%)	Duration of incubation (days)	C^{14} incorporated in gelatin (%)
	0	0.90
~0	3	1.30
59.5	3	1.30
~0	6	2.12
59.5	6	4.75

Table 5. Dye absorption by ionized groups in gelatin.[1]

Sample description	Absorption of orange G		Absorption of safranine	
	Optical density at 477 m	Decrease in optical density due to gelatin	Optical density at 50 m	Decrease in optical density due to gelatin
Protein-free sample	0.90		0.82	
Blank	0.74	0.16	0.69	0.13
Oxidized sample	0.81	0.09	0.69	0.13

[1] Samples were incubated for 10 days in the dry state.

creases in the positively charged group contents were probably caused by interactions between oxidation breakdown products of linoleate and the free amino groups of the protein. Decreases in free amino groups during incubation, observed by the ninhydrin method (Table 1), indicate that the apparent degradation of gelatin was not due to cleavage of peptide bonds because hydrolysis of peptide bonds would have increased the contents of charged groups.

In one experiment the amino acid pattern of samples oxidized in the dry state as compared with the control shows losses in methionine and tyrosine (Zirlin, 1968). Susceptibility to enzymatic hydrolysis of gelatin from samples oxidized in the dry state was also studied. There were definite decreases in hydrolysis, under the conditions studied, with trypsin, collagenase, pepsin and chymotrypsin (Zirlin, 1968).

Since degradation of gelatin through rupture of cross-links was a possibility, we determined hexoses, ester linkages and aldehyde contents of gelatin since these functional groups are believed to contribute to cross-linking in collagen (Bornstein *et al.*, 1966; Harding, 1965). However, because of interference of lipid fragments incorporated into gelatin, no definite conclusions could be made on the basis of the above experiments (Zirlin, 1968).

Data on amide group contents, presented in Tables 1 and 2, indicate increased amide concentration after oxidation. Increases were larger when incubation was carried out under dry conditions, but there were minimal changes when oxidation took place at relative humidity of 59.5%. The increases in amide group contents coincide with changes in the viscosity and solubility characteristics of gelatin (Tables 1, 2). Needles (1967) observed similar changes in the physico-chemical characteristics of gelatin and in amide group content after oxidation-induced degradation of gelatin by peroxydisulfate and by others after radiation-induced degradation of protein in the dry state (Jayko *et al.*, 1958); Bowes *et al.*, 1962; Garrison *et al.*, 1967). These authors suggest that increases in amide group contents during oxidation- or radiation-induced degradation of proteins are caused by cleavage of –N–C– links of the polypeptide chains.

The effects of incubation in the presence of linoleate had some interesting similarities with effects of ionizing radiation on gelatin. Both processes caused substantial degradation in the dry state and produced increases in amide group contents (Mateles, 1957; Bowes *et al.*, 1962). The similarities may be due to both processes causing their effects through production of free radicals of polypeptide (Roubal *et al.*, 1966a,b).

The studies on the effect of moisture content of a protein system on radiation-induced damage to proteins might therefore help in understanding some of the observations made in the present study. Mateles (1957) and Bailey *et al.* (1964) suggest that lack of chain mobility of gelatin molecules in the dry state prevents cross-linking of free radicals of polypeptide formed by radiation-induced scission of protein molecules; consequently, degradation of proteins takes place during irradiation in the dry state. However, high water contents may favor recombination of polypeptide radicals with the possibility that cross-linking predominates over oxidative scission.

Specifically we suggest that the interaction between proteins and radicals produced by lipid oxidation, such as the peroxy radical, may lead to hydrogen abstraction and formation of a protein radical:

$$LOO\cdot + PH \rightarrow P\cdot + LOOH,$$

where:

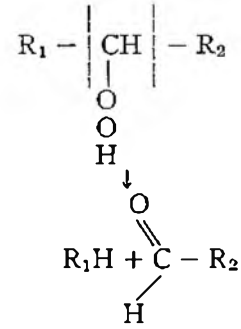
$LOO\cdot$ = lipid peroxide and PH = protein.

Under conditions in which chain mobility is high, i.e., at high water contents, the protein radicals may lead to cross-linking or may be quenched by reactions with water or water-soluble hydrogen-donors.

In the dry state it is likely that the protein radicals are attacked by molecular oxygen to give protein peroxides:

$$(P\cdot + O_2 \rightarrow POO\cdot)$$

If this peroxide formation were to occur on the α-carbon of the protein, then scission of the –N–C– bond may occur by a mechanism similar to scission of hydroperoxides of unsaturated fatty acids as shown below: (Evans, 1961)



In the case of proteins, if the original radical were on the α-carbon, R_1H would be an amide and would explain the increase in amide groups observed in this study. It is interesting to note in this connection that recent ESR studies on irradiated proteins have shown that the free radical formed at the α-carbon is one of 2 radicals observed to have significant life in solid irradiated proteins (Henriksen, 1966). The other stable radical was the cysteyl radical, which of course could not be present in gelatin.

The attack on amino groups by lipid oxidation may be responsible for some of the changes including C^{14} incorporation, loss of amino groups, and browning. Other changes, including the apparent oxidative degradation may be due to the free radical scheme suggested above.

Although other explanations of the effects of water on effects of radiation and of oxidation on linoleate are possible (Zirlin, 1968), the above hypothesis is consistent with the results obtained in the present study and seems to be consistent with the suggestion of Roubal *et al.* (1966a,b) that oxidation of lipid-protein mixtures results in the formation of protein radicals.

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NOTICE: Page Charges for Publication of Research Papers

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Sarcolemmae from Chicken Skeletal Muscle. 1. Preparation

SUMMARY—The outer sheath, or sarcolemma, of the muscle cell plays an important role in the contractile process and may, therefore, be significant in the changes which occur in muscle post-mortem. It has been suggested that a breakdown in a cytoskeleton of the cell is necessary before the contractile proteins become accessible to water and extractable.

One procedure for preparing sarcolemmae involves homogenization of the muscle in $CaCl_2$ solution, washing with NaCl-histidine, and an incubation for 30 min at 37°C. Several factors which govern the ability to prepare sarcolemmae from chicken breast muscle largely free of other cellular components have been studied in this report. These include $CaCl_2$ concentrations during homogenization, NaCl and histidine concentrations during the washes, the pH of the wash solutions, the number of washes required, the necessity of the incubation, and the pH of the extracting water.

The purpose of these experiments was to find a procedure which would allow variation of the preparatory conditions so that the association of enzymes with the sarcolemma could be studied. It is known that the association of enzymes with particulate fractions of the cell are sensitive to environmental conditions. A necessary step to allow restrictive conditions of preparation, such as low pH and ionic strengths, to be used was aging of the whole excised muscle for 4 hr at 0 4°C. The function of this aging process is not known. It does not appear to involve solubilization of protein nor major changes in several classes of phosphorus compounds.

INTRODUCTION

THE SARCOLEMMA, which forms the exterior boundary of the muscle cell, is composed of an outer network of collagen fibrils, a middle amorphous layer and the inner plasma membrane (Kono et al., 1964). Invaginations of the plasma membrane form the transverse (T) system (Franzini-Armstrong et al., 1964; Huxley, 1964). The tubules of this T system meet in a triadic joint with two terminal sacs of the sarcoplasmic reticulum; it is not clear whether there is a physical connection between the end of the transverse tubule and the terminal sacs of the sarcoplasmic reticulum (Walker et al., 1966). Apparently the T system has the function of extending the plasma membrane into the interior of the muscle, and it is this phenomenon which allows the muscle cell to respond as a unit with no lag period in the interior of the cell.

Depolarization of the plasma membrane and its intracellular extensions (T system) somehow affects the terminal sacs of the sarcoplasmic reticulum and causes the liberation of Ca^{+2} from the sarcoplasmic reticulum (Constantin et al., 1966). This liberation of Ca^{+2} activates the ATPase activity of the contractile proteins and allows for contraction to occur (Filo et al., 1965). Relaxation is achieved in part at least by a reversal of the process and the uptake again of the released Ca^{+2} by the sarcoplasmic reticulum (Hasselbach et al. 1961; Martonosi et al., 1964; Sreter et al., 1964).

The outer cell membrane along with its extensive invaginations plays, therefore, a prominent role in the contractile process. What role this membraneous system might play in the post-mortem conversion of muscle to meat is not certain, but many of the same processes, e.g., contraction or shortening, are operative in the early post-mortem stages as in muscle *in vivo*.

Although some membraneous systems, notably the mitochondria, have received considerable attention, the plasma membrane has not. This is no doubt due in large part to the lack of morphological distinguishing features which makes it difficult to identify the plasma membrane in a cellular homogenate.

The sarcolemma of the striated muscle cell, however, can be isolated with a remarkable retention of form and this, along with its importance in the contractile process, has led to its preparation in several laboratories (Kono et al., 1961; McCollester, 1962; Rosenthal et al., 1965; Abood et al., 1966; Westort et al., 1966).

In some procedures muscle cell segments are washed with strong salt solutions (Kono et al., 1961; Abood et al., 1966); and in others, moderate salt concentrations and an incubation at 37°C with a limited pH range are used (McCollester, 1962; Rosenthal et al., 1965).

It has been suggested that these treatments are necessary to break down the "cytoskeleton" of the muscle cell which allows the contractile proteins to become accessible to water and extractable (McCollester et al., 1964). This extraction of the contractile proteins removes other subcellular particles and leaves the sarcolemmae. Morphological appearance has been the principal criterion for establishing the purity of the isolated sarcolemmae.

In addition to establishing physical barriers inter- and intra-cellularly, membranes are now recognized as important because of their enzymic properties. The concept of intimate interplay between particulate structures and enzymic function has been most definitively studied in the case of the mitochondrion. However, other membraneous elements have also been shown to have associated with them certain enzymes, and it has been proposed that all major metabolic sequences are membrane-bound *in vivo* (Green et al., 1965). According to this concept, one does not always see this because the enzymes of any given sequence may be very, and variably, susceptible to the conditions of isolation. In the report just mentioned, it was possible by proper manipulation of the isolation conditions to prepare membranes from the bovine erythro-

cyte and the yeast cell that contained all enzymes of the glycolytic pathway, although the degree of retention varied with each enzyme.

The cellular localization of the glycolytic enzymes is undoubtedly an important factor in post-mortem glycolysis, and one may speculate that association of these enzymes with the sarcolemma is involved in controlling the rate and extent of post-mortem glycolysis. Glycolysis has been shown to be the principal cause of toughness in chicken breast muscle (de Fremery et al., 1963). The work of Green et al. (1965) showed the dependence of glycolytic enzyme retention on the conditions of isolation. Other reports (for example, Roodyn, 1956, 1957; Mansour et al., 1965; Hernandez et al., 1966; Hultin et al., 1966; Margreth et al., 1967) confirm this observation.

We wished to study the enzymic composition of the sarcolemma of chicken breast muscle and, therefore, wanted to be able to prepare it under a variety of conditions which could be changed as needed. We report here a detailed study of factors related to the phenomenon of emptying of muscle cell segments from chicken breast muscle. The emphasis is on using conditions which have in the past not allowed emptying, e.g., very low ionic strengths, since it is relatively simple to increase the ionic strengths to obtain emptying. A short report of a method utilizing low ionic strengths based on these data has been made (Westort et al., 1966). The following report describes some of the chemical and enzymic properties of the sarcolemmal preparation.

EXPERIMENTAL PROCEDURE

Preparation of empty cell segments

The breast muscle, *Pectoralis major*, of non-fasted domestic chickens was excised immediately after the bird was killed by air injection into the heart and placed in ice-cold water. Connective tissue was cut away, and 20 g of the muscle was cut into small pieces, blotted dry and placed into 200 ml of the homogenizing medium which was usually $CaCl_2$ solution; the concentration varied and is given in the text. It was then homogenized at 0 to 4° for 4 sec at high speed in an Osterizer blender fitted with a Polytron Model BEW cutter (Will Corporation) which consists of a cylindrical blade rotating within an external cylindrical cutting surface. We have found this type of cutter to be very efficient in producing muscle cell segments without causing excessive shredding of the tissue. The time of homogenization is also important to obtain sufficient cutting with a minimum of shredding. If the muscle is not homogenized enough, bundles of fiber segments will remain; we have found it impossible to empty these bound segments.

The homogenized muscle was then strained through cheesecloth. The basic procedure followed initially from this point on was that of McCollester (1962) with modifications as described. Briefly the procedure consisted of 3 preincubation washes, incubation in a water bath at 37° for 0.5 hr and 5-post-incubation washes. All of these were performed in a solution 25 m*M* in NaCl and 2.5 m*M* in histidine, adjusted to pH 7.4 with tris [tris(hydroxy)-aminomethane]; all the washes were carried out at room temperature. The salt-washed segments were then washed once in water adjusted to pH 7.4 with tris and finally placed in a large volume of the tris-buffered water where the intracellular contents are removed.

At this point the empty muscle segments can be distinguished in the light or phase contrast microscope from those which have not emptied or have only emptied in part. We have found that emptying bears no relationship to the length of the muscle segment. Therefore, we simply calculate the percentage of empty cell segments (number of empty cell segments divided by the number of empty plus full or partially empty segments × 100) regardless of size of the segments. At least 50 fields were counted for every preparation. Deviations from the standard procedure described above are specifically mentioned where applicable.

Analytical methods

Protein was determined by a modified Folin technique (Lowry et al., 1951). Whole homogenates were separated into acid-soluble, phospholipid, RNA plus protein and DNA phosphorus fractions by the procedure of Schmidt-Thannhauser-Schneider (Volkin et al., 1954). Phosphorus determinations were made on each fraction as well as the total homogenate by the procedure of Chen et al. (1956).

RESULTS AND DISCUSSION

Factors influencing emptying of the cell segments

Most of the preparations took about 0.5 day from the time they were homogenized until they were placed in the tris-buffered water for the second time, viz., extracted. We were interested in determining if muscle from the same bird we had used in the morning could be used for our experiments in the afternoon. During routine checks to make sure that the fresh and aged muscle behaved similarly, it became evident that muscle which had been held at 0 to 4°C for the morning and treated in the afternoon gave more extensive emptying than the fresh muscle under many conditions. Some of these conditions were critical for our goal of producing empty cell segments under conditions of low ionic strength and pH. Therefore, in most cases we examined factors on both fresh and "aged" muscle. Unless stated otherwise, the aging process was 4 hr and was carried out by holding the muscle at 0 to 4° in water or saline; it made no difference which was used.

Table 1. Effect of $CaCl_2$ concentration on emptying of muscle cell segments.

Concentration of $CaCl_2$, m*M*	Percentage of empty cell segments	
	Fresh muscle	Aged muscle
50	85	87
25	60	75
10	60	78
5	67	80
2	60	75
1	58	80
0.5	68	88
0	0	53

The procedure of McCollester (1962) was followed.

The first factor examined was the concentration of $CaCl_2$ in the homogenization medium. Results are listed in Table 1. Over the range of 0.5 to 50 m*M*, the concentration of $CaCl_2$ made little difference in the extent of emptying. The aged muscle was slightly better, but the effect was limited. Differences between fresh and aged muscle, and this included the effect of $CaCl_2$, became greater as the procedure became more restrictive with respect to number of washes, concentration of salts in the washing medium, etc. With rat muscle, McCollester et al. (1964) observed a much greater dependence of emptying on the concentration of $CaCl_2$. At the higher levels used (40 to 50 m*M*) the latter authors obtained greater emptying than we did with chicken muscle; however, it must be observed that they made their counts only after isolation of the segments was complete whereas, for the sake of convenience, we counted right after the intracellular contents were removed. No emptying was observed in the absence of $CaCl_2$ with fresh muscle. Even with aged muscle there was a decrease in the percentage emptying where $CaCl_2$ was omitted. Since 0.5 m*M* $CaCl_2$ gave good results and yet is a low concentration, it was used as the standard homogenizing medium.

Table 2 shows the results when muscle was aged for various times before homogenization in solutions at two levels of $CaCl_2$. The rest of the procedure was standard except that only one post-incubation wash was employed, which accounts for the low emptying in the fresh muscle as compared to the values shown in Table 1. Three to four hours are required to obtain maximal results.

In the next series of experiments, the concentration of NaCl and histidine buffer were varied in the wash and incubating media. In Table 3 the effect of changing one while holding the second constant is shown, using fresh muscle as the starting material. Decreasing either the concentration of NaCl or histidine resulted in a decrease in the percentage of empty cells. The very significant decrease that occurred when the histidine was lowered from 2.5 m*M* to 0.25 m*M* while maintaining the NaCl at 25 m*M* strongly indicates that something other than ionic strength is involved in the emptying since the decrease in histidine content contributes little to the overall ionic strength. Both the salt and buffer were required for good emptying. The concentration of $CaCl_2$ in the homogenizing medium was 0.5 m*M*.

In Table 4, the effect of varying the NaCl concentration on the emptying of segments prepared from fresh muscle was studied after homogenization of the muscle at two levels of $CaCl_2$, viz., 0.5 m*M* and 2 m*M*. The segments prepared by homogenization in the 2 m*M* $CaCl_2$ exhibited significantly more emptying than those prepared in 0.5 m*M* $CaCl_2$

Table 5 shows the effect of four combinations of salt and buffer when compared in fresh muscle and muscle aged for 4 hr. The improvement in emptying caused by the aging period is obvious at all levels.

In all results so far mentioned, the level of salt or buffer was varied through the whole procedure of pre-incubation washing, incubation and post-incubation washing.

In Table 6 we see the effect of decreasing the absolute amounts of both NaCl and histidine while maintaining a

Table 2. Effect of time of aging of muscle before homogenization on emptying of cell segments.

Time before homogenization, hr.	Percentage of empty cell segments	
	0.5 *mM* $CaCl_2$	2 *mM* $CaCl_2$
0	17	10
1	27	45
3	71	
4		82

One post-incubation wash was used. The muscle was homogenized at two levels of $CaCl_2$.

Table 3. Effect of salt and buffer concentrations on emptying of cell segments.

Concentration (*mM*)		Percentage of Empty cell segments
NaCl	Histidine, pH 7.4	
25	2.5	50
12.5	2.5	35
5	2.5	5
2.5	2.5	0
25	2.5	30
25	1.25	28
25	0.50	12
25	0.25	2

Fresh muscle was homogenized in 0.5 *mM* $CaCl_2$.

Table 4. Effect of salt concentration at two levels of $CaCl_2$ in the homogenizing media on emptying of cell segments from fresh muscle.

NaCl concentration	Percentage of empty cell segments	
	0.5 *mM* $CaCl_2$	2 *mM* $CaCl_2$
25	28	52
15	11	21
10	5	16
5	0	0

The concentration of the histidine, pH 7.4, buffer was 2.5 *mM*.

Table 5. Effect of salt and buffer concentrations on emptying of cell segments from fresh and aged muscle.

Concentration (*mM*)		Percentage empty cell segments	
NaCl	Histidine	Fresh	Aged
25	2.5	40	80
25	1.25	23	55
12.5	2.5	8	50
12.5	1.25	2	22

The muscle was homogenized in 0.5 *mM* $CaCl_2$.

Table 6. Effect of salt and buffer concentrations in the incubating medium on emptying of cell segments.

Concentration (*mM*)		Percentage empty cell segments	
NaCl	Histidine	Fresh	Aged
25	2.5	58	72
12.5	1.25	39	72
5	0.5	35	69
0	0	33	70

The muscle was homogenized in 0.5 *mM* $CaCl_2$ and had 2 pre- and 1 post-incubation washes at the normal levels of salt and buffer.

constant ratio between them in the incubating medium alone. The washes were carried out with the normal levels of salt and buffer: two pre-incubation washes and one post-incubation wash were used. Again the improvement with aging is readily seen.

We next examined the effect of the number of washes on the percentage of empty cells produced. In these experiments the muscle was homogenized in 0.5 m*M* $CaCl_2$; the regular salt and buffer concentrations were used, and the incubation was normal. The effect of the number of pre-incubation washes using both fresh and aged muscle is shown in Table 7.

There was some improvement in both the fresh and aged muscle as the number of washes in the salt-buffer solution was increased. The aged muscle showed considerably better emptying than the fresh muscle in all cases.

In Table 8, we see the effect of the number of post-incubation washes, using fresh and aged muscle. The same conditions held as in Table 7, with the muscle cell segments receiving only one pre-incubation wash. There was improvement in the fresh muscle sample as the number of washes was increased, but there was little effect on the aged muscle. The differential effect between fresh and aged muscle was greatest, therefore, at the lower number of washes.

pH is another important variable in the release or retention of enzymic activities. We studied the effect of pH in the wash and incubation solutions on the production of empty muscle cell segments. Three pH values were studied, and imidazole and histidine were compared at the normally used pH (7.4). Results are shown in Table 9. Although there was some advantage in using the higher pH with fresh muscle, the pH made little difference in the samples where the muscle had been aged. As before, the aged sample showed greater emptying at all values of pH. In these experiments, the muscle was homogenized in 0.5 m*M* $CaCl_2$ after a 4-hr aging period. Two pre- and one post-incubation washes were used.

The effect of varying the pH of the tris-buffered water in the extraction procedure from 6.5 to 8.0 is shown in Table 10. In fresh muscle a maximal effect was observed at pH 7.5 while in the case of aged muscle the percentage of emptying was equivalent, and high, at all values of pH studied.

Table 11 shows the effect of incubation on the tendency of the cell segments to empty. With samples prepared from fresh muscle using our procedure (Westort et al., 1966), the emptying was entirely dependent on the incubation. With aged muscle, emptying was independent of the incubation step. In addition, emptying of the aged muscle with or without incubation was superior to that of the fresh muscle with incubation.

In several experiments similar to those described above, K^+ was substituted for Na^+ in the washing and incubating solutions. In all cases, no differences were noted between the two cations.

The several steps appear to be additive, viz., there is no single part of the procedure on which the emptying depends, but rather it is necessary to follow many of the steps of the original procedure (McCollester, 1962) to achieve satisfactory results. An illustration of this is given in Table 12 which shows how small variations can

Table 7. Effect of the number of pre-incubation washes on emptying of the cell segments.

	Percentage of empty cell segments	
Number of washes	Fresh	Aged
0	9	45
1	26	67
2	33	71
3	41	76

The muscle was homogenized in 0.5 m*M* $CaCl_2$; normal salt and buffer concentrations were used throughout. The segments were washed 4 times after incubation.

Table 8. Effect of the number of post-incubation washes on emptying of the cell segments.

	Percentage of empty cell segments	
Number of washes	Fresh	Aged
1	0	54
2	30	63
3	38	63
5	48	58

The muscle was homogenized in 0.5 m*M* $CaCl_2$; salt and buffer concentrations were normal. The segments received one pre-incubation wash.

Table 9. Effect of pH and buffer in the wash and incubation media on emptying of cell segments from aged muscle.

	Percentage of empty cell segments	
	Fresh	Aged
Histidine, pH 7.4	46	85
Imidazole, pH 7.4	26	86
Imidazole, pH 7.0	13	89
Imidazole, pH 6.6	0	81

The muscle was homogenized in 0.5 m*M* $CaCl_2$ after a 4-hr aging period. Two pre- and one post-incubation washes were used. The NaCl concentration was 25 m*M* and the buffers were all 2.5 m*M*.

Table 10. Effect of pH of the extracting solution on emptying of cell segments from fresh and aged muscle.

	Percentage of empty cell segments	
pH of "tris-water"	Fresh	Aged
6.5	39	75
7.0	52	70
7.5	70	70
8.0	50	84

The fresh muscle sample was treated as per McCollester (1962) including homogenization in 50 m*M* $CaCl_2$. The aged muscle was homogenized in 0.5 m*M* $CaCl_2$. The sample had one pre- and 2 post-incubation washes.

Table 11. Effect of incubation on emptying of cell segments.

	Percentage of empty cell segments	
	Fresh	Aged
Incubation	26	59
No incubation	1	56

The procedure was ours (Westort et al., 1966) with and without an incubation period.

Table 12. Emptying of fresh and aged muscle under various conditions of $CaCl_2$ and water.

Sample	Percentage of empty cell segments	
	Fresh muscle	Aged muscle
A	60	70
B	0	53
C	0	0

All samples were prepared according to the method of McCollester (1962) with the following exceptions:
A. Homogenized in 0.5 *mM* $CaCl_2$.
B. Homogenized in distilled water.
C. Homogenized in 0.5 *mM* $CaCl_2$ and all washes (but not the incubation) done in distilled water.

drastically affect the results. It also illustrates again the greater emptying with muscle that has been aged.

In sample A, which was homogenized in 0.5 m*M* $CaCl_2$ but was otherwise subjected to the standard procedure, emptying of both the fresh and aged samples was good with the aged showing perhaps a slightly greater emptying. When distilled water was substituted for the 0.5 m*M* $CaCl_2$, emptying of the fresh muscle cell segments essentially disappeared; with the aged sample under similar conditions, a moderate reduction in the extent of emptying was observed.

When the muscle was homogenized in 0.5 m*M* $CaCl_2$ and all washes (but not the incubation) were done with water, emptying of cell segments from either fresh or aged muscle did not occur. Any procedure, therefore, designed to maintain a low ionic strength must be carefully chosen to give satisfactory emptying. Considerable variation in extent of emptying was noticed among preparations treated in a like manner. We ascribe this to a variability in the muscle samples, and we were not able to control it.

Standard procedure for preparing empty cell segments.

In all work described below and in the following paper we used a standard procedure for the preparation of the sarcolemma described by Westort et al. (1966) unless otherwise stated.

Briefly, it consisted of homogenizing in 0.5 m*M* $CaCl_2$ chicken breast muscle which had been aged for 4 hr, washing 4 times in a 25 m*M* NaCl-2.5 m*M* histidine buffer at pH 7.4 and extracting in tris-buffered water at pH 7.0. The empty cell segments were isolated by a differential centrifugation procedure. Fig. 1 shows a phase contrast photomicrograph of a typical empty cell segment. We ordinarily obtain 3 to 5 mg of sarcolemmal protein from 20 g of muscle (Westort et al., 1966).

Studies on possible mechanisms of emptying

The principal objective in the experimental work described in this paper was to devise a method for the preparation of muscle cell sarcolemma which would allow for variation in the conditions of preparation. Since emptying of muscle cells can be accomplished relatively easily at high ionic strengths and pH values, this meant essentially that conditions must be found that would allow the emptying reaction to occur at low ionic strengths and pH values. This was accomplished on the basis of the observation that aging of the excised whole muscle somehow

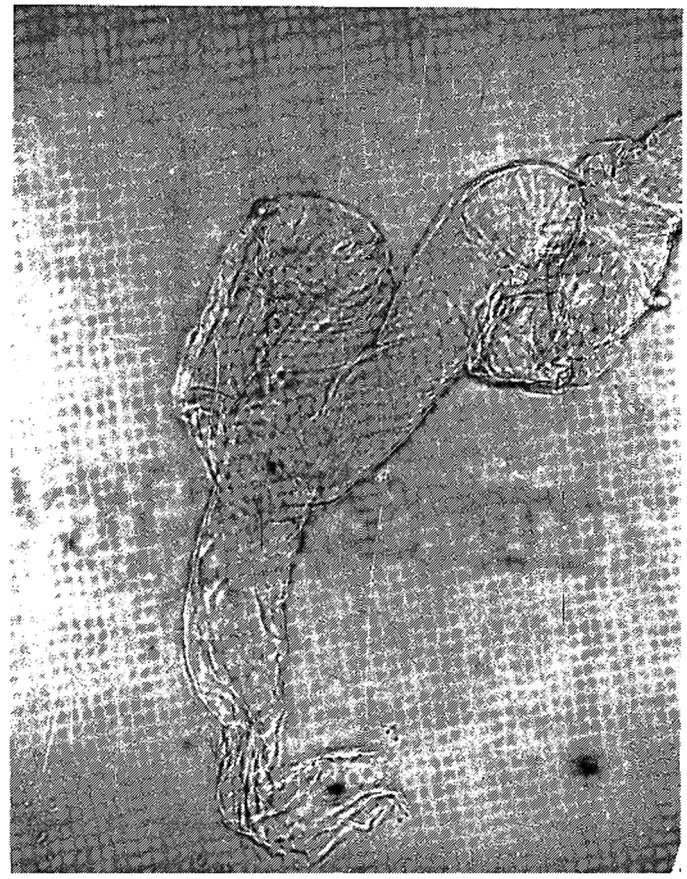

Fig. 1—Phase contrast photomicrograph of empty skeletal muscle cell segment (× 440).

facilitates the reaction. If the suggestion of McCollester et al. (1964) is correct that a cytoskeleton must be broken down before the contractile proteins become accessible to the extracting water, then it is reasonable to expect that the aging process somehow leads to this cytoskeletal breakdown. An understanding of this breakdown would be important not only as an aid in developing more effective procedures for preparing sarcolemmae but may have important implications in the post-mortem changes of muscle tissue.

There have been many studies of post-mortem changes in chicken breast muscle, although these have not in general been under the same conditions as used in our study, i.e., excision of the muscle and holding it in water at 0 to 4°C. It is reasonable to assume, nevertheless, that

Table 13. Distribution of phosphorus in fresh and aged muscle.

	μg P per mg of protein	
	Fresh	Aged
Total P	27.7	25.2
Acid-soluble P	19.6	20.8
Phospholipid P	1.8	2.5
RNA + protein P	0.6	0.5
DNA P	0.2	0.2
P not extracted	0.3	0.2

Muscle was homogenized for 1 min and the fractions obtained by the procedure of Schmidt-Thannhauser-Schneider (Volkin et al., 1954).

certain changes took place in our samples and that the samples with which we obtained good emptying were not pre-rigor. This latter was indicated by the pH of the homogenized muscle after aging which was usually around 6.0 to 6.2 (de Fremery, 1963). In addition it was probable that the ATP level in the muscle tissue had greatly decreased (de Fremery, 1963) and the muscle had lost most of its extensibility (May et al., 1962). Whether these changes are in any way related to the phenomenon of cytoskeleton breakdown is not known although it is tempting to speculate that similar mechanisms are involved in the changes accompanying rigor and aging and those involved in cytoskeletal breakdown.

It seemed logical that our low-temperature aging of whole muscle was equivalent to the 37° 30-min incubation of cell segments that McCollester (1962) used. To hasten the aging process we held our muscle samples at 37°C for various times. Very little decrease in holding time as measured by efficiency of emptying of cell segments could be attained by the increase in temperature. Therefore, there is no advantage to be gained by the increased temperature which may adversely affect enzyme systems of the sarcolemma.

Routine determination of protein distribution among various soluble and insoluble fractions prepared from chicken breast muscle by homogenization and differential centrifugation during a study on the subcellular distribution of lactate dehydrogenase showed that after 4 hr of aging at 0 to 4°C, a small but definite amount of protein was lost from the particulate to the soluble fraction (Hultin et al., 1967). This was usually 4 to 6% of the total muscle protein. We compared this solubilization of protein in whole, aged muscle with what happens during the 37°C incubation period in McCollester's procedure. This was done by carrying McCollester's procedure to the point of incubation. Here one-half of the sample of muscle cell segments was homogenized and centrifuged at 105,000 xG for 1 hr in the No. 40 rotor of a Spinco Model L ultracentrifuge, and protein determinations were performed on the residual and supernatant fractions. The other half of the sample was incubated at 37°C for ½ hr and then treated in a similar fashion. Contrary to an earlier report (Westort et al., 1966), no solubilization was found by incubation at 37°C similar to what occurs on aging whole muscle. The discrepancy was due to lack of control of pH and ionic strength in the earlier work. This then is another indication that there is a difference in the effects of aging whole muscle at 0°C and the muscle cell segments at 37°C.

Since we suspected that the ability to utilize lower $CaCl_2$ levels in the homogenizing medium on aging was due in part to depletion of oxidizable substrates and high energy compounds, e.g., ATP, we attempted to bring about "exhaustion" of the muscle by electrical stimulation in Ringer's solution containing Ca^{+2} (Sparrow et al., 1965). We have not been able to replace the aging time by means of this technique in our experiments to date.

Preparations of sarcolemmae made both by the procedure of McCollester (1962) and our own modification (Westort et al., 1966) are low in phosphorus (see following paper). Therefore, we examined the phosphorus content of several fractions in fresh and aged muscle to determine if there were any major differences in the two muscles. Analysis of phosphorus in the acid-soluble, phospholipid, RNA plus protein, and DNA fractions of fresh and aged whole muscle is shown in Table 13. The results show no major changes and indicate that major changes in these fractions may not be involved in changes in the muscle cell which allow the intracellular contents to be emptied. However, changes within each fraction, such as the conversion of ATP to ADP, may be crucial. This is currently under investigation.

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Sarcolemmae from Chicken Skeletal Muscle. 2. Properties

SUMMARY—Sarcolemmae are usually identified solely by morphological characteristics. We have determined several chemical and enzymic properties of sarcolemmae from chicken breast muscle prepared by homogenization of aged muscle in dilute $CaCl_2$ solution, washing 4 times in NaCl-histidine at pH 7.4, extraction with water buffered to pH 7 and isolation by differential centrifugation on a discontinuous sucrose gradient. The phospholipid content of the sarcolemmae was low, representing only 2 to 3% by weight compared with the 20 to 35% usually found in membraneous systems. This discrepancy may be due to the relatively small proportion of plasma membrane in the sarcolemma.

Analyses indicate little contamination by nuclei or mitochondria. The sarcolemmae have, like the microsomal fraction, high contents of RNA and glucose-6-phosphatase activity. The sarcolemma is either rich in these elements or is contaminated by other subcellular elements, such as the transverse tubules (T system), which are. The sarcolemmae display a Mg^{+2}-activated ATPase activity which is typical for membraneous systems. Lactate dehydrogenase was shown to be associated with the sarcolemmae. Whether this represents the situation *in vivo* or is an artifact of preparation is not clear. The sarcolemmae are capable of binding soluble LDH.

INTRODUCTION

IN OUR previous paper (Hultin et al., 1969) we presented a study of factors involved in the preparation of sarcolemmae from chicken breast muscle with a view to obtaining a method which would allow for as wide a variation as possible in the preparatory conditions. Aging of the whole, excised muscle for 3 to 4 hr at 0–4°C was a crucial step in the development of this technique. Here we report some chemical properties as well as some enzymic characteristics of the isolated sarcolemmae.

EXPERIMENTAL PROCEDURE

Preparation of empty cell segments

Empty cell segments (sarcolemmae) were prepared from aged chicken breast muscle as previously described (Westort et al., 1966). The background for the development of the emptying procedure is discussed in detail (Hultin et al., 1969). The empty segments are separated from unemptied segments and cellular debris by a differential centrifugation on a discontinuous sucrose gradient. The purity of the final preparations was routinely judged by counting the number of empty cell segments in a given volume in a Howard mold count chamber and dividing by the mg of protein in the same volume. The value obtained is called the specific purity. Since there was no preference for length of the cell segments as far as emptying was concerned, we simply took a total cell count, regardless of the length of the segments. Staining with Janus Green B was used to aid in evaluation of the preparation (McCollester, 1962).

Preparation of subcellular fractions

Mitochondrial and microsomal fractions were prepared as previously described (Hultin et al., 1967).

Analytical methods

Lactate dehydrogenase was assayed according to the procedure of Wu et al. (1959), ATPase in the presence of 1 m*M* $MgCl_2$, 5 m*M* KCl, and 56 m*M* NaCl and succinate oxidase as described by Umbreit et al. (1959), and glucose-6-phosphatase by the method of Hübscher et al. (1965). Protein was determined by a modified Folin technique (Lowry et al., 1951).

After fractionation of the preparation by the procedure of Schmidt-Thannhauser-Schneider (Volkin et al., 1954), RNA (Webb, 1956), and DNA and phospholipid phosphorus (Chen et al., 1956) were determined. Yeast RNA and fish roe DNA (Type VI), purchased from Sigma Chemical Company, were used as standards. Glucose-6-phosphate was also a product of Sigma. DPNH and ATP were purchased from P-L Biochemicals, Inc. Sodium pyruvate was obtained from Nutritional Biochemicals Corp. and sodium succinate from Fisher Scientific Co.

RESULTS

IN TABLE 1 are shown some chemical characteristics of our isolated preparations; these values are compared to those of other subcellular fractions. The amount of total phosphorus associated with our preparations was 1.34 μg of phosphorus per mg protein (12 samples); this compared with an average value of 0.9 μg of phosphorus per mg protein (8 samples) when the segments were prepared according to the procedure of McCollester (1962). The corresponding value of phospholipid phosphorus in our preparations was also low. These values are below what

Table 1—Some chemical characteristics of the isolated sarcolemmae.

Fraction	μg P/mg protein	μg phospholipid P/mg protein	μg DNA P/mg protein	μg RNA/mg protein
Sarcolemmae	1.34	1.10	0.08	8.6
Whole homogenate [1]	3.1	2.3	0.06	1.6
Mitochondria	12.3	11.9	0.08	3.8
Microsomal fraction	13.4	12.5	0.08	8.3

[1] The whole homogenate was the resuspended sediment after an initial centrifugation, viz., most of the soluble P compounds had been removed.

All figures represent a minimum of duplicate assays on duplicate samples.

one might expect to be contributed by the phosphorus of phospholipid associated with a membrane; e.g., see the values in Table 1 for mitochondria and microsomes of chicken breast muscle, as well as Fleischer et al. (1965).

If we assume that the average molecular weight of the phospholipid is 750, then the phospholipid content of the empty segments based on the determination of phospholipid phosphorus is of the order of 2 to 3% as compared to the 20 to 35% usually found in membranes. Part of this discrepancy may be due to the relatively small quantitative contribution which the actual plasma membrane makes to the total weight of the empty cell segment as compared to the basement membrane and collagen fibrils (Kono et al., 1964).

Although we have not examined our preparation in the electron microscope, collagen fibrils can be seen in the light or phase contrast microscope. However, we have obtained specific purities as high or higher with our preparations as with those prepared by a technique giving more normal phospholipid values (Kono et al., 1961). Since several factors that influence emptying (McCollester et al., 1964) also influence in the same way prospholipase C, it occurred to us that the mechanism of emptying might involve the action of this enzyme. If the phospho-base was removed from the phospholipid by the enzyme, it would result in the low phosphorus values observed. We extracted lipid from the membrane with chloroform: methanol, 2:1 and assayed the extract by thin-layer chromatography. We were looking for the presence of diglycerides, the product of the phospholipase C-catalyzed reaction, but found no evidence for them. We do not rule out the possibility of this mechanism at this time, nevertheless, since if diglyceride and soluble phosphate compounds are formed, they may be washed out during the preparative procedure.

More than 80% of the phosphorus of the sarcolemmal preparation is found in the phospholipid fraction. The DNA content as based on phosphorus determination is low and comparable to the mitochondrial and microsomal fractions. DNA was not determined in a nuclear fraction due to the difficulty of isolating nuclei from skeletal muscle (Edelman et al., 1965.) The content of RNA is high and comparable to that found in the microsomal fractions.

In Table 2 are given some enzymic properties of the sarcolemmal preparation and these are compared with those of other subcellular entities. Glucose-6-phosphatase was assayed in a medium containing EDTA and potassium fluoride to inhibit alkaline and acid phosphatase, respectively. This enzyme is considered to be predominantly associated with the microsomal fraction of the cell. As can be seen, the activity of this enzyme was almost as high in the empty cell segments as it was in the microsomal fraction. Its activity in the mitochondrial fraction was low. Succinic oxidase is considered specific for mitochondria. There was no detectable succinic oxidase activity associated with the sarcolemmal preparation.

Table 2—Some enzymic properties of the isolated sarcolemmae.

Fraction	ATPase[1]	Glucose-6-phosphatase[1]	Succinic oxidase[2]
Sarcolemmae	45	4.4	0
Whole homogenate	....	0.5	3.8
Mitochondria	....	0.2	49
Microsomal fraction	....	5.5	

[1] mμ *M* Pi released per min per mg protein.
[2] μl O_2 per min per mg protein.
All figures represent a minimum of duplicate assays on duplicate samples.

ATPase activity is a general property of most membraneous systems. Sarcolemmae from chicken breast muscle were no exception.

The activity of 45 mμ *M* of inorganic phosphate released per min per mg protein is of the same order of magnitude as that found by Kono et al. (1961), viz., 75 mμ *M* per min per mg protein. McCollester et al. (1961) reported that ATPase activity in their preparation of sarcolemmae constituted 16% of the total activity of the whole muscle homogenate. Although there may be some contamination with contractile protein ATPase, it appears that muscle sarcolemma, like other membranes, contains ATPase activity.

The enzymic activities of Table 2 are generally considered to be membraneous *in vivo*. We next examined our preparation of sarcolemmae for an enzyme generally considered to be sarcoplasmic, viz., lactate dehydrogenate (LDH). We are interested especially in the glycolytic activities of the sarcolemma and their role in post-mortem muscle. LDH was chosen as an average glycolytic enzyme in its degree of retention (Green et al., 1965).

Table 3 gives specific activities of LDH for several preparations of empty cell segments along with specific purities of each preparation. The specific activity of a whole muscle homogenate varied from 3 to 6 mμ *M* DPNH oxidized per min per mg protein. We see first of all a wide variability in the specific activity of LDH associated with the empty cell segments. This is perhaps not too surprising in view of the sensitivity of LDH binding to the environmental conditions (Hultin et al., 1966a), the length of time of preparation which with overnight storage may run to 36 hr, and the fact that much of the preparative procedure is performed at room temperature. What is clear is that empty muscle cell segments may be prepared which have associated with them significant LDH activities. There is little relationship between the specific purities and the LDH specific activities of the preparations. One could hope to find an increase in specific activity with an increase in specific purity of LDH were

Table 3—Specific LDH activities and specific purities of some typical preparations of sarcolemmae.

Sample	Specific activity of LDH mμ *M* DPNH/min/mg protein	Specific purity[1]
1	3.2	610,000
2	7.3	775,000
3	7.6	70,000
4	3.6	175,000
5	24.5	802,000
6	7.7	201,000
7	39.0	195,000

[1] Specific purity is defined as the number of empty muscle cell segments per mg protein.

associated with the membrane. However, the sensitivity of LDH association to the preparative conditions and the improbability that exact duplication of conditions can be achieved from one preparation to another precludes this condition from having any real significance.

The large number of washes that the preparation undergoes is strongly indicative that the LDH we measure is in fact associated with the particulate cell segment fraction whether from an association *in situ* or by adsorption. Further evidence for this which cannot be easily quantitized was obtained by picking out the pieces of cell segments for assay. This is easy to do since the segments have a tendency to clump, especially after standing for a while. These pieces have extremely high LDH activities, indicating that the enzyme is in fact associated with them.

It is sometimes difficult to obtain accurate determinations of the LDH activity because of difficulty in obtaining good dispersability of the segments due to clumping when one attempts to homogenize them. One way to overcome the problem is to treat the particulate fractions with salt prior to assay. This solubilizes the LDH, and accurate pipetting is then possible.

If the isolated cell segments are washed with salt solutions, the LDH can be solubilized. Essentially all of the enzyme may be removed by 2 washings in 0.15 *M* NaCl. If the segments are then washed with water and taken up in a solution low in ionic strength containing LDH (prepared by dialysis against water of a salt extract of muscle followed by centrifugation to remove insoluble protein and LDH), they will bind the enzyme. We have observed specific activities of rebound LDH in the cell segments of up to 50 mμ *M* DPNH oxidized per min per mg protein.

DISCUSSION

THE SARCOLEMMA has been defined as composed of three major layers: an outer network of collagen fibrils; a middle layer referred to as the basement or amorphous layer; and the inner plasma membrane (Kono et al., 1964). These authors have reported that the plasma membrane contains about 30% of the total protein in the sarcolemma and that, although the total lipid content of the plasma membrane is what one would expect (about 23%), only ⅓ of this lipid is phospholipid. This latter figure is low for a membraneous material (Fleischer et al., 1965).

These observations may account for the low phospholipid phosphorus content of our chicken muscle sarcolemmae. The fact that we could obtain no experimental evidence for the breakdown of phospholipid is consistent with the interpretation of low concentrations of phospholipid in the lipid fraction of the sarcolemma and that the lipid is associated mostly with the plasma membrane which in turn represents only a fraction of the sarcolemma. Other workers have also observed these low phosphorus values. The results of Kono *et al.* (1964) using rat skeletal muscle calculate to 1.35 μg of phospholipid phosphorus per mg of protein and those of Abood et al. (1966) with frog muscle to about 3.8. Neither of these reports give specific purities of the preparations. Other work has indicated that sarcolemmae contain levels of phospholipid phosphorus more to be expected for a membrane.

In an earlier report Kono (Kono et al, 1961) found 12.5 μg phospholipid phosphorus per mg protein in a preparation from rat muscle. Specific purities of these preparations were of the order of magnitude found by us. The discrepancy between this early work and that reported later is not explained.

Ashworth et al. (1966) determined phospholipid in sarcolemmae at levels which are equivalent to about 11 to 12 μg phospholipid phosphorus per mg protein. Reasons for these large differences among investigators are not known. All of the workers in these other reports utilized strong salt solutions to extract the muscle cell segments. We consistently found low phosphorus values in preparations made by our procedure (Westort et al., 1966) and that of McCollester (1962). The discrepancies in results will have to be explained before phospholipid content can be used as an effective marker to establish purity of the preparations.

The muscle cell segments as prepared are not free of contaminating subcellular components. A few nuclei can be observed but appear to be a very minor component quantitatively; the DNA data substantiate this. Rosenthal et al. (1965) observed mitochondria attached to a sarcolemmal preparation from rat muscle in both the phase contrast and electron microscopes. We could see relatively few in the phase contrast microscope and our succinic oxidase data suggest that such contamination must be small on a weight basis.

The high content of RNA and the high glucose-6-phosphatase activity in our preparation are striking. These are usually considered characteristic of microsomes and here are approximately the same as in the microsomal fraction. It is not likely that these results could be accounted for by a heavy contamination of microsomes, viz., the phospholipid phosphorus values are low and indicate that non-membraneous elements comprise a large part of the sarcolemmae. Microsomes are generally high in phospholipid (Fleischer et al., 1965) and, therefore, could represent only a small fraction of the preparation (see also Table 1).

It is probable that the data reflect either or both of two possibilities. One is that the outer muscle sheath is, like the microsomes, rich in glucose-6-phosphatase activity and RNA content. If our preparation has the usual structure of plasma membrane, basement membrane and collagen fibrils (the latter can be observed) and if the RNA and glucose-6-phosphatase activity are associated only with the plasma membrane, this would mean that the membrane proper (plasma membrane) is richer in these elements than the microsomes. Another possible explanation is that impurities not completely removed during preparation of the empty cell segments are particularly rich in these factors. It is conceivable that something like the transverse tubules (the T-system) may be more difficult to extricate than the sarcoplasmic reticulum. If the T-system were rich in the above-mentioned components, it would give the results obtained. In a preparation

of microsomes, the T-system membranes would be diluted out by sarcoplasmic reticulum.

In a preparation obtained from rat skeletal muscle by means of extraction with strong salt solutions, Kono et al. (1961) found no hexokinase, glucose mutarotase, glucose-6-phosphate dehydrogenase, fructose-diphosphate phosphatase, aldolase or triosephosphate dehydrogenase. They did, however, find phosphoglucoisomerase, as well as DPNH oxidase and ATPase. They correctly pointed out that the enzymes found might be contaminants and that the ones not found might have been destroyed or extracted during the preparative procedure. McCollester et al. (1961) examined their empty muscle-cell segments for several enzymes and found ATPase but not several glycolytic enzymes. They concluded that no glycolysis occurs in the membranes.

It is difficult to assess our data concerning LDH. It is clear that sarcolemmae can be prepared which have considerable LDH activity associated with them. It is also certain that the LDH is associated with these empty cell segments. An examination of the amount of contamination of the sarcolemmae by subcellular particles and the LDH content of these particles (Hultin et al., 1967) leads to the conclusion that there is not enough contaminating material to cause the observed effects. With what we know of the conditions leading to solubilization of LDH (Hultin et al., 1966a) and conditions of sarcolemmae preparation (Hultin et al., 1969), it should be possible to further increase the LDH associated with the empty segments. The principle question involved is whether what we see is a reflection of the situation *in situ* or represents an artifact of the preparative procedure caused by physical entrapment, isoelectric precipitation or adsorption. The question of entrapment and isoelectric precipitation offer no insurmountable problems and have been shown not to contribute to the activity of the preparation (Hultin et al., 1967).

Although we have studied the problem of adsorption in some detail (Hultin et al., 1966b), no clear-cut conclusions can be drawn on the basis of our results to date as to whether the LDH is associated with the subcellular structure *in situ* or is adsorbed during homogenization of the muscle. Several reports utilizing cytochemical techniques (Pette et al., 1962; Van-Wijhe et al., 1964; Fahimi et al., 1964) have indicated a particulate site for LDH in striated muscle. Amberson et al. (1965), using a different approach, have concluded that at least part of the LDH in rabbit muscle is attached to the ultrastructure. The strong adsorptive ability of the muscle particulates (Hultin et al., 1966b) may be an important factor in these observations, however.

Although we would urge caution in the acceptance of a particulate site for LDH in skeletal muscle based on the above experiments, we strongly feel that one should be just as hesitant in accepting a cytoplasmic location for this enzyme at the present time and with the information that is available. The acceptance of the classical soluble role of this enzyme is based principally on its being easily brought into solution on treatment with salt at ionic strengths comparable to those of physiological saline; therefore, the reasoning follows, in the cell it should be soluble.

It is possible to picture a cellular location which is shielded from the salinity of the cell and is only exposed when the cell is disintegrated. This could explain the data as completely as assigning LDH to the soluble phase of the muscle cell. Under certain conditions, e.g., nonaqueous environment or proper pH and low ionic strength, electrostatic interactions between molecules can be very stable. We cannot, for instance, remove LDH from our membrane fractions by repeated washing with water.

The effect of aging on the chemical composition and amount of enzymes associated with the empty cell segments is difficult to assess because we have no good control, i.e., we cannot prepare empty segments at such low ionic strengths and few washes unless we age the muscle. Nevertheless, we have studied the distribution of LDH in fresh and 4-hr aged muscle after homogenization and differential centrifugation (Hultin et al., 1967). No major differences were observed. We might expect, then, similar results to those found with the empty segments from aged muscle in fresh muscle if we could prepare the empty segments from the fresh tissue under the same conditions.

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Tenderization of Chicken Muscle: The Stability of Alkali-Insoluble Connective Tissue During Post-Mortem Aging

SUMMARY—The relation between tenderness of breast and thigh muscles from chicken broilers and stability of connective tissue therein was measured during post-mortem aging. Tenderness was determined with a Warner-Bratzler shear-force apparatus and with a trained taste panel. Maximum shear-resistance values occurred in breast muscles 3 to 4 hr post-mortem; minimum values were reached 12 hr post-mortem and did not change significantly during aging for 8 days. Maximum shear-resistance values occurred in thigh muscles 3 hr post-mortem; in these muscles tenderization continued during 8 days of aging.

In contrast, alkali-insoluble connective tissue determined in either raw or cooked muscle (as measured by alkali-insoluble hydroxyproline) did not change significantly as a function of post-mortem aging time (1 hr vs 24 hr for breast meat, 1 day vs 8 days for thigh meat). Cooking solubilized considerable amounts of the connective tissue. Post-mortem tenderization of chicken meat is not related to changes in connective tissue but must be ascribed to some other fraction (or fractions) of muscle tissue.

INTRODUCTION

THE DEVELOPMENT of tenderness during aging has important economic implications, and the mechanisms involved have intriguing biochemical considerations. It is now clearly recognized that many factors play a role in the establishment of ultimate tenderness, e.g. age of animal, adequacy of diet, conditions of processing, method of cooking. The factor of particular interest to us, however, is port-mortem aging, i.e. the holding of an animal carcass for some definite period of time with the intention of producing tenderization through one or more natural (but still unspecified) processes.

The muscle constituents most intimately related to meat texture are the proteins, since they comprise the structural components of muscle and account for about 80% of its total dry weight. They can conveniently be divided into three main fractions based on their solubility: the *sarcoplasmic* proteins, which are soluble in water and are composed primarily of the various soluble enzymes; the *myofibrillar* proteins, which are soluble in strong neutral salt solutions and are composed primarily of the contractile proteins, myosin, actin and tropomyosin; and the *stroma* proteins (connective tissue), which are insoluble in dilute alkali and are composed primarily of collagen and elastin. The muscle structure is due mainly to the myofibrillar and stroma proteins.

Many workers have studied the relation between connective tissue content and tenderness, particularly as it is affected by the age of the animal, the particular muscle that is studied and the conditions under which the muscle is cooked. Very few studies, however, have investigated possible degradative changes in connective tissue during post-mortem aging. In experiments on beef, Husaini et al.

(1950) and Hershberger et al. (1951) reported decreases of 10 to 30% in the alkali-insoluble protein during an aging period of 15 days, a period of time during which most of the post-mortem tenderization occurs. The significance of these results was obscured, however, by the large inter-animal variation that they encountered. Indeed, Wierbicki et al. (1954, 1955) and Bouton et al. (1958) reported that the alkali-insoluble protein content of beef remains unchanged during aging for 15 days. In studies of nitrogen distribution in chicken muscle, Khan et al. (1964) and Sayre (1968) indicate that there is no significant change in the stroma protein content during the time period required for maximum tenderization. The experiments reported here establish that the connective-tissue content of chicken muscle does not change during tenderization.

Raw leg and breast meat from chicken broilers was analyzed for alkali-insoluble connective tissue (as measured by the hydroxyproline content of the alkali-insoluble residue) at periods of time when the meat was known to be tough or tender. In other experiments, similar determinations were made on samples of breast meat which had been cooked at those same times. Our results show no decrease in alkali-insoluble connective tissue during post-mortem aging; the development of tenderness should thus be attributed to some other fraction (or fractions) of muscle tissue.

EXPERIMENTAL

Processing of chickens

The chickens used in these experiments were typical commercial broilers; their live weights ranged from 1.2 to 2.1 kg. For each individual experiment, birds were given unlimited access to feed and water and then were processed in the following manner: they were killed by slashing the throat following a brief electric shock, scalded either for 60 sec at 54°C or for 30 sec at 60°C, plucked by hand, eviscerated immediately and plunged into ice slush. The time required for these steps varied from 10 to 15 min. Unless the birds were sampled earlier, they were transferred, after chilling for 3 to 4 hr, to drained ice. In a few experiments, after chilling in ice slush, the birds were placed in plastic bags and aged in a cold room at 2°C. When a carcass was to be sampled, one entire Pectoralis major or an entire leg was excised from one side. The carcass was then placed in a plastic bag and held in a cold room at 2°C for the remainder of the aging period.

Tenderness evaluation

Tenderness of the cooked meat was determined either by the use of a Warner-Bratzler shear-force apparatus or by a trained taste panel. Samples that were to be sheared were cooked in boiling water following the technique of de Fremery et al. (1960). The excised muscle was clamped between metal plates that were maintained a fixed distance apart, immersed in a vigorously boiling water bath for 10 min and then cooled in running tap water. Strips were cut out parallel to the fibers, and the shear resistance was determined on these strips. Pectoralis major (breast) muscles were cooked between plates maintained 0.63 cm apart; strips from these muscles were 1.9 cm wide. The mixed muscles from the entire thigh were cooked between plates maintained 0.95 cm apart; strips from these muscles were 2.5 cm wide.

When the tenderness of breast meat was to be judged by a taste panel, Pectoralis major muscles were cooked by boiling as described above. Panelists were asked to rate the strips from the cooled muscles on a scale of 1 (very tender) to 6 (very tough). Leg meat (drumstick) was evaluated by a taste panel after roasting at 163°C to an internal temperature of 82°C.

Analysis for alkali-insoluble hydroxyproline

In preliminary experiments, we encountered large variation in the connective tissue content of replicate samples from the same raw muscle. To reduce this variation as much as possible, we dried and powdered the entire muscle before sampling. In the case of the Pectoralis major, the dorsal and anterior regions of the muscle were trimmed off and discarded since they contain relatively large amounts of tendinous material. The rest of the muscle was diced into 6-mm cubes, frozen, freeze-dried to about 2% moisture, suspended briefly in boiling petroleum ether (42°C) to remove fat, ground in a Wiley mill to pass through a 20-mesh screen and stored over silica gel until analyzed. The mixed thigh muscles were treated similarly except that the dried samples were extracted with petroleum ether for 24 hr in a Soxhlet extraction apparatus before grinding.

Alkali-insoluble connective tissue was isolated by a modification of the procedure of Lowry et al. (1941). Samples of the raw muscle powders (0.2 to 0.4 g) were weighed into plastic centrifuge tubes, 35 ml of 0.1 *N* NaOH was added, and the mixtures were stirred occasionally for about 8 hr at room temperature. After standing overnight, the tubes were centrifuged at room temperature for 20 min at 27,000 X G, and the supernatants were discarded. The insoluble pellets were refluxed in 6 *N* HCl for 24 hr, the hydrolysates were dried on a steam bath under a stream of air, and hydroxyproline was determined in the redissolved residues by Method I of Woessner (1961). Results are reported as mg alkali-insoluble hydroxyproline per g dry muscle. Assuming that raw muscle contains 75% water and that collagen (the major hydroxyproline-containing protein in muscle) contains 14% hydroxyproline (Leach, 1957), these values can be converted to "% collagen" by dividing by 5.6.

Heat-solubilized connective tissue could not be extracted completely from cooked muscle powders unless the extraction with alkali included two or more washing operations. For convenience, two 4-hr extractions, followed by one 16-hr extraction, were carried out; the supernatants were replaced with fresh alkali after each centrifugation. Otherwise, the procedures were identical. Hydroxyproline was determined by Method II of Woessner (1961) in which interfering colored material is removed by benzene extraction, thus increasing the sensitivity of the test when large amounts of other amino acids are present.

RESULTS AND DISCUSSION

Tenderness changes during post-mortem aging

Tenderness changes in the Pectoralis major were followed by cooking and shearing breast muscles after aging on the carcass for time periods ranging from 1 to 24 hr. Since the tenderness variation between individual birds is much larger than that between paired muscles from the same bird, the shear resistance of a muscle aged for a given time was always compared to the shear resistance of its pair aged for a reference time. The reference times selected were 1 hr and 24 hr; the comparison aging times were 2, 3, 4, 5 and 6 hr (compared to 1 hr) and 9, 12 and 18 hr (compared to 24 hr). For each of the eight comparison times, a group of 6 to 9 birds was processed, aged, cooked and sheared as described above. The shear resistance was determined for the comparison and reference muscles of each bird in the group; the *mean shear difference* from the group reference time could then be calculated for each comparison time. These mean differences were then added to (or subtracted from) the mean shear resistance of *all* muscles aged for the reference time (43 observations for the 1-hr reference time; 19 observations for the 24-hr reference time). In this way, a calculated shear resistance was obtained for each comparison time. The data are shown in Fig. 1. The relative values are similar to those reported by others (Koonz et al., 1954; Stadelman et al., 1966) and establish that our samples exhibit a typical tenderness pattern.

Although the shortest aging period studied in these experiments was 1 hr, data from our earlier report indicate that the shear resistance at 2 min post-mortem (essentially zero time) would be 72% of that at 1 hr, or about 3.5 kg (de Fremery et al., 1963). The pattern of tenderization in chicken breast muscle thus follows a rapid rise to maximum shear resistance at 3 to 4 hr, a rapid decrease to low shear resistance at about 9 hr and very little change thereafter. In additional aging experiments conducted similarly, no changes in tenderness were observed beyond 24 hr, even though aging was carried on for 8 days.

To determine the reaction of a trained taste panel to breast meat of widely varying shear resistance, 40 chicken broilers were processed and then aged for various times (3 to 24 hr) before cooking. One muscle from each bird was tested on the Warner-Bratzler shear-force apparatus; its pair, cooked simultaneously, was presented to one member of a taste panel and rated for tenderness. The mean shear-resistance values were calculated for each panel rating and are presented in Fig. 2. Each point represents the mean of 5 to 10 samples; the rating of 3 (slightly tender) is excluded since this rating was given only twice during this experiment The correlation coefficient between panel ratings and shear-resistance values, determined over all samples, was 0.85 ($P < 0.001$). In general, the panelists felt that meat with a shear resistance greater than 4 kg was tough, whereas meat with a shear resistance less than 3 kg was tender. By reference to Fig. 1, it can be seen that broilers aged for 5 hr or less before cooking have breast meat that would be rated as tough by this panel; conversely, if they are aged somewhat more than 6 hr, their breast muscles would be rated as tender. Obviously, mean values obscure the variation that is encountered.

In the experiment reported in Fig. 1, some birds aged for 5 hr or less (3 out of 36) had shear resistance values less than 3 kg. Birds aged 9 hr or more were less variable; all shear-resistance values in these groups were less than 2 kg. These results are similar to those reported for experiments conducted with consumer-type panels (Palmer et al., 1965).

The tenderization pattern of thigh muscles was determined in a similar manner. For this experiment, only one reference time (24 hr) was used. The comparison aging times were 3, 6, 12, 48, 96 and 192 hr. The results were summarized similarly to the experiment on breast muscles and are presented in Fig. 3. The point for the reference

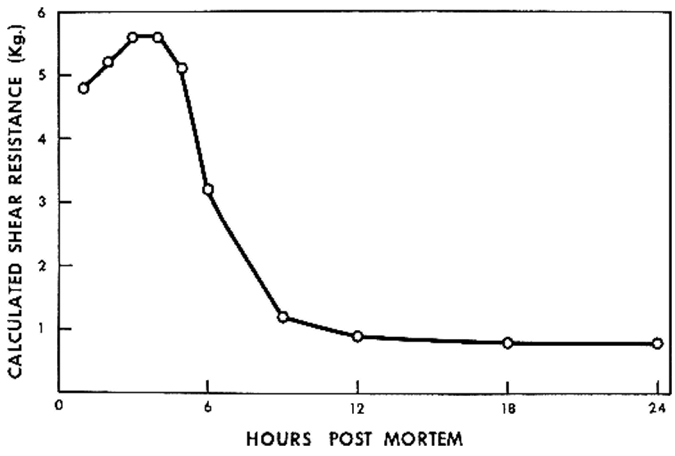

Fig. 1—Calculated shear resistance of cooked Pectoralis major at various aging times (shearing cross section = 1.2 cm²). See text for method of calculation.

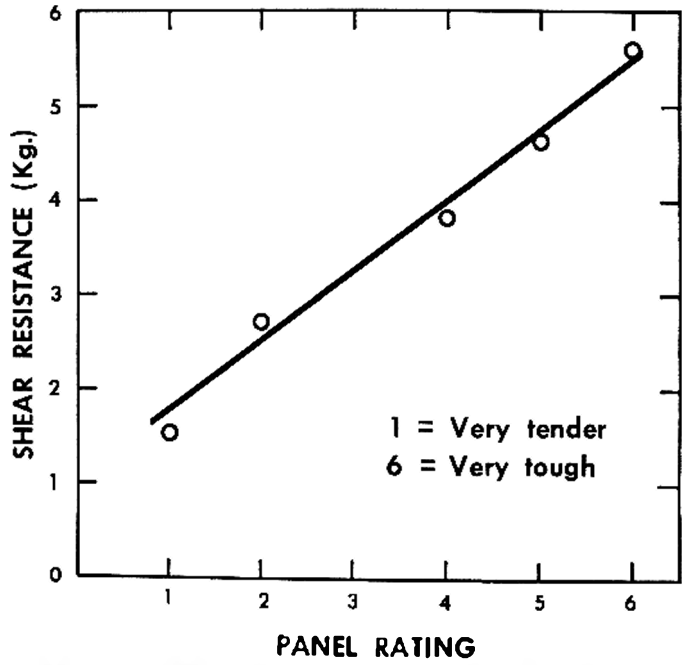

Fig. 2.—Relation between panel rating and shear resistance of cooked Pectoralis major (shearing cross section = 1.2 cm²).

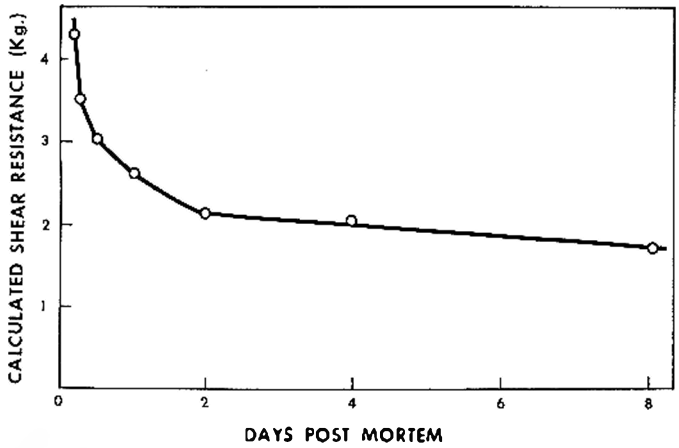

Fig. 3—Calculated shear resistance of cooked thigh muscles at various aging times (shearing cross section = 2.4 cm²). See text for method of calculation.

time is the mean of 36 observations; other points are means of 6 observations each. The tenderization pattern of these muscles differs from that of breast muscles in that tenderization proceeds well beyond an aging time of 12 hr.

Additional experiments (not shown in Fig. 3) showed that thigh meat from birds aged for 4 days was significantly more tender than that from birds aged for 2 days; similarly, 8-day thighs were more tender than 4-day thighs (*t*-tested on paired muscles; $P < 0.01$). These results agree with those of van den Berg et al. (1964), who reported that chicken and turkey thigh meat tenderized more slowly than breast meat.

The tenderization of leg meat (drumstick) apparently follows a time course that is similar to that of thigh meat. Twelve broilers were aged for one day before one leg was excised, sealed in a plastic bag and stored at −34°C. The carcass was then aged for an additional 7 days before the second leg was excised, frozen and stored. The samples were thawed for 24 hr at 2°C, then roasted as previously described and served in pairs to a trained taste panel. The panelists were asked to state which leg of the pair (always from the same carcass) was the more tender. Although the panelists commented that there was not much difference between members of the pairs, 10 of the 12 judgments indicated that the 8-day legs were the more tender. This difference is significant at the 5% level of probability.

Connective tissue stability during post-mortem aging

The experiments reported in the preceding section are in agreement with general experience that poultry muscle, like mammalian muscle, is relatively tough shortly after slaughter but becomes tender thereafter. Poultry muscle, however, becomes tender more rapidly than mammalian muscle. If tenderization is the result of a breakdown or dissolution of the connective tissue proteins, the content of alkali-insoluble connective tissue should decrease during the period of tenderization. To investigate possible changes in these proteins during aging, we determined alkali-insoluble hydroxyproline in raw muscles that had been aged for various periods of time post-mortem. Paired breast muscles were aged on the carcass either for 1 hr or for 24 hr; paired thighs were aged either for 1 day or for 8 days. During these respective aging periods, a significant amount of tenderization has occurred (see Figs. 1 and 3). To eliminate inter-animal variation in connective tissue content, we always compared unaged muscles to aged muscles from the same carcass. Aging treatments were alternated between right and left sides to minimize positional effects. The results are summarized and presented in Table 1. No statistically significant differences were observed. The complete analyses of variance are presented in Tables 2 and 3.

Although no post-mortem breakdown of connective tissue can be detected in raw muscle by means of solubility in 0.1 *N* NaOH, we wished to eliminate the possibility that aging might labilize the connective tissue in some way. This labilization, while having no effect on alkali solubility

Table 1—Alkali-insoluble hydroxyproline during aging of chicken muscle.

Muscle	Hydroxyproline (mg/g dry muscle)	
	Unaged[1]	Aged[1]
Breast, raw (12)[2]	2.01	1.97
Thigh, raw (12)	7.20	7.09
Breast, cooked 5 min (6)	0.474	0.480
Breast, cooked 10 min (6)	0.359	0.341

[1] Breast muscles aged 1 or 24 hr; thigh muscles aged 1 or 8 days.
[2] Numbers in parentheses refer to number of broilers per experiment.

Table 3—Analysis of variance of raw thigh muscle hydroxyproline during post-mortem aging.

Source of variation	Degrees of freedom	Sum of squares	Mean square	F	P
Aging time	1	0.2211	0.2211	2.52	n.s.
Birds	11	23.9907	2.1810	102.39	< 0.001
Interaction	11	0.9649	0.0877	4.12	< 0.001
Error	48	1.0238	0.0213	.....	.....
Total	71	26.2005	.....	.....	.....

Table 2—Analysis of variance of raw breast muscle hydroxyproline during post-mortem aging.

Source of variation	Degrees of freedom	Sum of squares	Mean square	F	P
Aging time	1	0.038042	0.038042	4.62	n.s.
Birds	11	6.030291	0.548208	431.66	< 0.001
Interaction	11	0.090482	0.008226	6.48	< 0.001
Error	48	0.060952	0.001270	.....	.....
Total	71	6.219767	.....	.....	.....

Table 4—Analysis of variance of 5-min-cooked breast muscle hydroxyproline during post-mortem aging.

Source of variation	Degrees of freedom	Sum of squares	Mean square	F	P
Aging time	1	0.000261	0.000261	0.31	n.s.
Birds	5	0.158001	0.031600	237.59	< 0.001
Interaction	5	0.004157	0.000831	6.25	< 0.001
Error	24	0.003187	0.000133	.....	.....
Total	35	0.165606	.....	.....	.....

Table 5—Analysis of variance of 10-min-cooked breast muscle hydroxyproline post-mortem aging.

Source of variation	Degrees of freedom	Sum of squares	Mean square	F	P
Aging time	1	0.002704	0.002704	1.77	n.s.
Birds	5	0.051071	0.010214	638.38	< 0.001
Interaction	5	0.007629	0.001526	95.38	< 0.001
Error	24	0.000386	0.000016	.....	.....
Total	35	0.061790	.....	.....	.....

in the raw state, might manifest itself by an increased rate of gelatinization, or solubilization, during cooking. Aged muscles that had been cooked for a short time would then have a lower alkali-insoluble connective-tissue content than unaged muscles cooked identically. To study this possibility, we repeated the preceding experiment on breast meat with the exception that the muscles were cooked either for 5 min or for 10 min immediately after the aging period. Analysis for alkali-insoluble hydroxyproline was performed by the modified procedure described in the Experimental section. The results are summarized and presented in Table 1. Although the alkali-insoluble connective tissue content decreased about 80% as a result of cooking, there was no statistically significant change attributable to post-mortem aging. The complete analyses of variance are presented in Tables 4 and 5.

The data presented in this paper furnish conclusive evidence that the changes in tenderness that occur during post-mortem aging of chicken meat are not caused by the breakdown or dissolution of the connective tissue proteins, nor by their increased lability to gelatinization, or solubilization, during cooking. For the present, then, the biochemical mechanism of post-mortem tenderization must remain obscure.

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The Use of Cryoscopy in Enzymatic Studies

SUMMARY—The feasibility of cryoscopy for measuring changes in water-soluble components of five enzyme-substrate mixtures was investigated. Cellulase, pectinase, pectin esterase, alpha-amylase and beta-amylase were studied. Viscosity measurements were run simultaneously with freezing points as a conventional method for determining enzymatic activity. Measurements taken at periodic intervals revealed changes in both viscosity and freezing-point values. A comparison of the two sets of values can be useful in assaying the purity of commercial enzyme preparations.

INTRODUCTION

IN RECENT years thermister-type cryoscopes have been developed which greatly facilitate the determination of freezing points. Since the freezing point of an aqueous system depends on the concentration of water-soluble components, experiments were conducted to determine if cryoscopy would provide a practical means for measuring the activity of enzymes that alter the concentration of these components.

EXPERIMENTAL

Enzyme-substrate preparations

Five mg of a commercial cellulase, "Cellase 1000" (Wallerstein Co. 1967), is dissolved in 5 ml of water and added to 200 g of a solution of carboxymethyl cellulose (Hercules, Type 7HO). The carboxymethyl cellulose is buffered at pH 5.0 with a sodium acetate buffer, and the viscosity is adjusted to approximately 30 centistrokes to facilitate the freezing-point measurements. Forty mg of both pectinase (General Biochemicals) and purified pectin esterase (Nutritional Biochemicals Corp.) are suspended separately in 1 ml of distilled water and made up to 50 ml with a 34% solution of commercial pectin ("Certo"). The pH of these mixtures is 3.1. Both enzyme-substrate mixtures are immediately transferred to 50-ml Erlenmeyer flasks and placed in a 35°C waterbath. Alpha and beta-amylase (Nutritional Biochemicals Corp.) suspensions (5 mg/5 ml water) are added to 200 ml of a 2% cornstarch solution.

General procedure

All enzymes are added to their respective substrates immediately before the initial freezing point, and viscosity readings are measured. Enzyme-substrate mixtures are incubated at 35°C during a 5-hr experimental period with aliquots tested at hourly intervals. Controls are run at the beginning and end of the 5-hr period.

Freezing-point measurements are made according to the procedure outlined by the manufacturer of the cryoscope (Advanced Instruments, Inc., 45 Kenneth St., Newton Highlands, Mass. 02161). A 2-ml aliquot is required for the test which takes about 3 min to complete. Freezing points are read to the nearest thousandth of a degree C. The temperature range of the scale is from 0 to −1.0°C and is normally calibrated against either 7 and 10% sucrose solutions or their salt equivalents (Shipe, 1961). The freezing points of the 7 and 10% sucrose solutions are −0.422 and −0.621°C, respectively.

Viscosity is measured simultaneously with the freezing points. Triplicate readings expressed in centistrokes (CS) are obtained, using a Cannon Fenske (size 350) viscometer. Viscosity measurements are made primarily to establish the activity of the enzymes being tested.

RESULTS AND DISCUSSION

THE EFFECT of "Cellase 1000" on carboxymethylcellulose is shown in Fig. 1. As will be noted, the rate of change in viscosity is rapid at first and then slows down, whereas the rate of change of freezing point is slow at first and then increases slightly with time. The differences

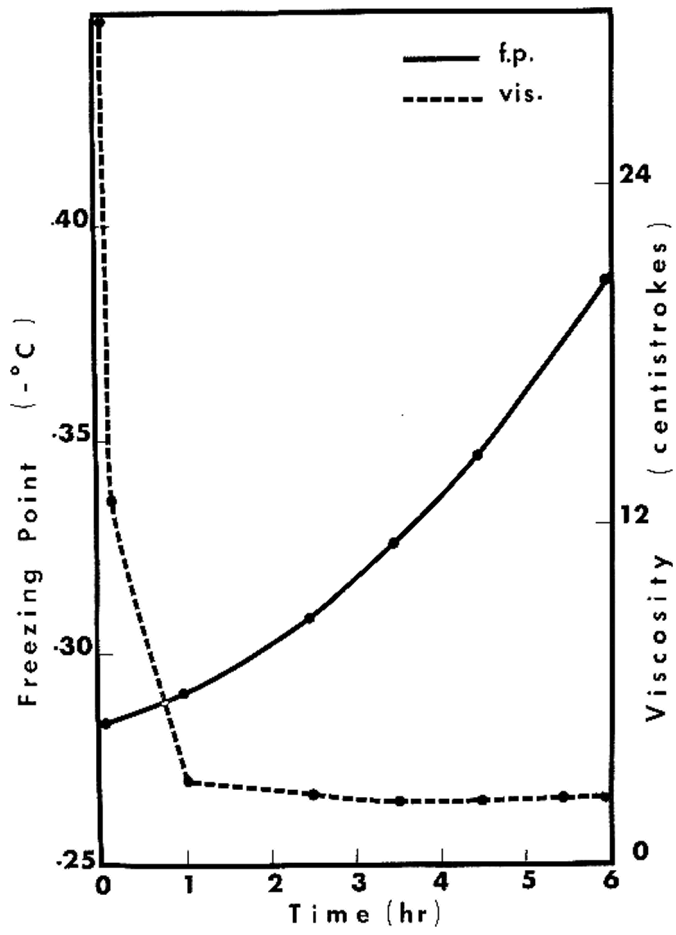

Fig. 1—Measurement of cellulase activity.

in rates are interpreted as reflecting two types of changes. The rapid drop in viscosity, primarily, reflects a reduction in cross-linkages in the cellulose preparation. The changes in freezing points reflect a degradation of cellulose molecules to give water-soluble fragments. Therefore, the results indicate that the reduction in cross-linkages is a more rapid process than the molecular degradation.

As is shown in Fig. 2, pectinase produced greater changes in both freezing point and viscosity values than pectin esterase. The apparent contrast between these two enzymes may be due in part to the type of substrate. The effect of pectin esterase would differ if there are major differences in the degree of methylation of the polygalacturonic acid polymers. When a crude pectin esterase preparation was tested, it gave values which are similar to those obtained with pectinase. We assumed that this is due to contamination with pectinase since both enzymes are frequently isolated from the same source.

The freezing point and viscosity values (Fig. 3) for the two amylases illustrate the difference in mode of action for the two enzymes. Alpha-amylase, the so-called "liquefying" amylase, reduces the viscosity more rapidly than beta-amylase. On the other hand, beta-amylase produces water-soluble components more rapidly as is demonstrated by the faster increase in freezing-point depressions. Mixing the two amylases increased the rate of change of both freezing points and viscosity.

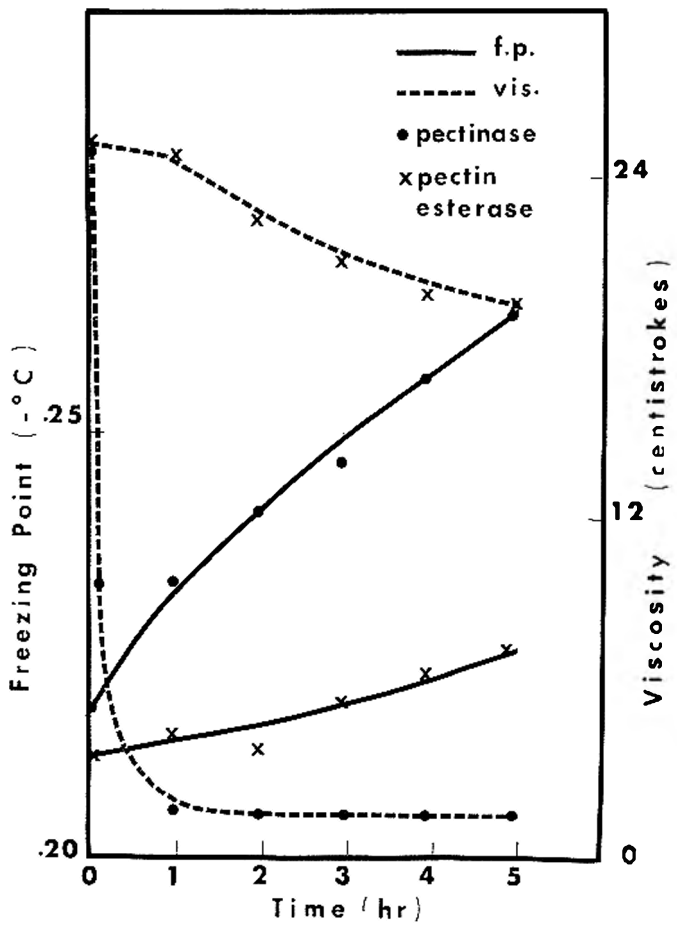

Fig. 2—Enzymatic hydrolysis of pectin.

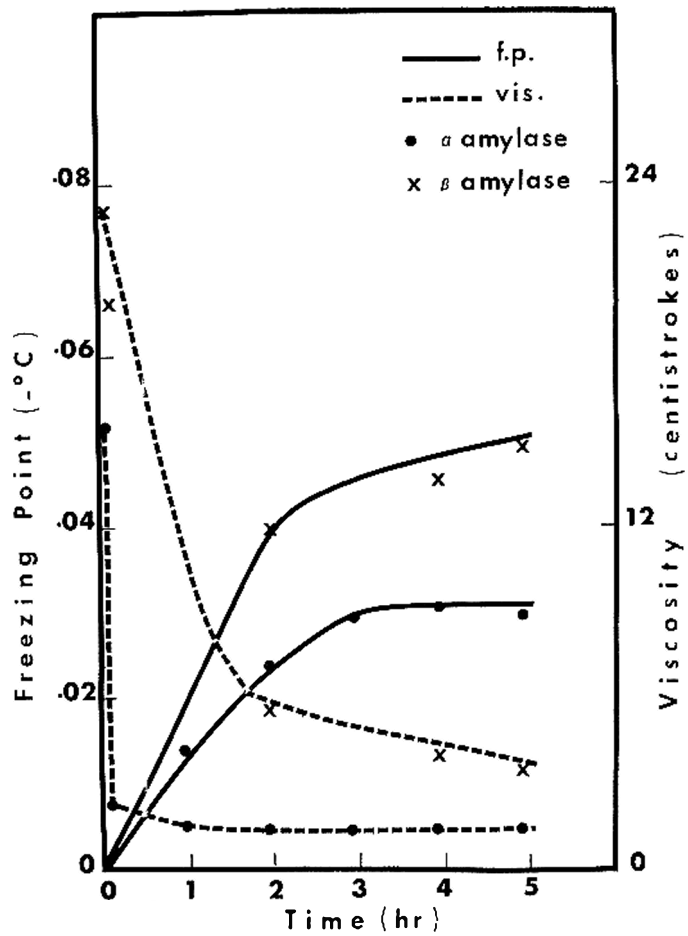

Fig. 3—Measurement of amylase activity.

CONCLUSIONS

THE RESULTS obtained in this study illustrate that cryoscopy can be used as a rapid, easy method for measuring the activity of a variety of enzymes. Furthermore, by using cryoscopy in conjunction with viscosity measurements, it would be possible in some instances to determine the relative rates of molecular rearrangements and molecular degradations. A comparison of these rates could provide an index of the purity of the enzyme preparation. For example, a study of the effect of an amylase preparation on the viscosity and freezing point of a starch solution would be useful in determining the relative alpha- and beta-amylase activities of the preparation.

Cryoscopy can also be used to measure enzymatic changes that do not involve significant changes in viscosity. Cryoscopy provides a useful tool for measuring changes in the water-soluble solids in foods. It has an advantage over refractometry for such measurements since water-insoluble components do not interfere with cryoscopic measurements. Cryoscopy should be useful in studying the kinetics of enzymes in model systems.

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Molecular Properties of Post-Mortem Muscle. 6. Effect of Temperature on Protein Solubility of Rabbit and Bovine Muscle

SUMMARY—Studies were conducted to investigate the effect of temperature on the actin-myosin interaction of rabbit and bovine muscle during rigor and post-rigor shortening. Muscle was stored at four different temperatures (2°, 16°, 25° and 37°), corresponding to three types of post-mortem muscle shortening: cold, minimal and high temperature. These three types of shortening are presumably related to different states of the actin-myosin interaction in post-mortem muscle. Post-mortem tenderization may be the result of either actin-myosin dissociation or F-actin depolymerization.

To detect the occurrence of either of these possible changes, two salt solutions, differing widely in their myofibrillar protein extracting abilities, were used to compare post-mortem myofibrillar protein solubility after different times of post-mortem storage and to provide information about the actin-myosin complex. Myofibrillar protein solubility of both rabbit and beef muscle in 0.5M KCl, 0.1M phosphate, pH 7.4, increased markedly with increasing post-mortem storage at temperatures up to 25°. Similar solubility changes were obtained with 1.1M KI, 0.1M K phosphate, pH 7.4, but these changes were much smaller in magnitude. Solubility in both salt solutions, in general, decreased for muscle stored at 37°.

Although time and temperature of post-mortem storage caused appreciable alterations in protein solubility, these alterations could not be directly related to changes in tenderness or sarcomere length or to species differences in the effects of temperature on post-mortem shortening. Viscosity, analytical ultracentrifugation, and ATPase assays all indicated the absence of "normal" actomyosin in all myofibrillar protein extracts in this study. It was suggested that the 1.1 M KI extracts contained G-actomyosin, but the available evidence indicated the presence of only myosin in 3-hr, 0.5 M KCl extracts.

INTRODUCTION

MANY of the physical and chemical changes in post-mortem muscle have been characterized, but the molecular nature of post-mortem alterations in the myofibrillar proteins and how these alterations are related to rigor mortis, muscle contraction and meat tenderization remain an enigma. Temperature has been shown to have a significant effect on shortening and isometric tension development of post-mortem muscle (Busch et al., 1967; Galloway et al., 1967; Locker et al., 1963) and on *in vitro* contractile properties of actomyosin (Levy et al., 1962; Maruyama et al., 1964). Busch et al. (1967) and Marsh et al. (1966) demonstrated that temperature also markedly affects post-mortem tenderness changes in beef.

[a] ATP: adenosine triphosphate; Tris: tris-(hydroxymethyl)-aminomethane.

Although Bendall (1960) maintains that resolution of rigor does not occur in the absence of bacterial action, Goll et al. (1964) found significant increases in tenderness of excised beef muscles after 24 hr post-mortem. These findings, together with the continued difficulty in demonstrating post-mortem proteolysis of myofibrillar proteins (Davy et al., 1966; Locker, 1960; Sharp, 1963), particularly during the first 72 hr post-mortem when the largest changes in tenderness occur, suggest that post-mortem tenderization of meat may be due in part to temperature-dependent nonproteolytic alterations in the contractile apparatus.

Wierbicki et al. (1954) first suggested that post-mortem tenderization may originate from a dissociation of the actin-myosin complex, but these same authors subsequently reported that they could not find any evidence for this hypothesis (Wierbicki et al., 1956). Weinberg et al. (1960) indicated that post-mortem tenderization may result from a breakdown of F-actin structure, this breakdown presumably originating from loss of forces that bind G-actin subunits together. King's (1966) recent report, suggesting the appearance of a G-actomyosin complex during post-mortem aging of muscle, provides some evidence supporting the hypothesis of Weinberg et al.

On the other hand, Partmann (1963) has reiterated the view that post-mortem tenderization originates from either a dissociation or an inhibition of the actin-myosin interaction. This view is strengthened by the recent finding (Fujimaki et al., 1965) that the actin-myosin complex in post-mortem muscle could be dissociated by ATP[a] levels about 1/6 to 1/3 of those required for at-death muscle, suggesting that the actin-myosin association is weakened after 24-48 hr post-mortem. Work in our laboratory has demonstrated that post-mortem muscle loses the ability to maintain isometric tension after 24–48 hr post-mortem (Busch et al., 1967; Jungk et al., 1967). This loss of tension development could result from either "depolymerization" of F-actin or a weakening of the actin-myosin interaction. Using superprecipitation and ATPase measurements, we (Galloway et al., 1967; Goll et al., 1967) have found evidence for some significant post-mortem changes in the actin-myosin interaction, but the exact nature of these changes has not been elucidated.

Either actin-myosin dissociation or F-actin "depolymerization" might be expected to cause appreciable changes in myofibrillar protein solubility. Since strong KI solutions have the ability to dissociate the actin-myosin complex and depolymerize F-actin to G-actin (Szent-

Gyorgyi, 1951) whereas strong KCl solutions lack these abilities, it seemed likely that a careful comparison of myofibrillar protein solubility in these two solutions could provide significant information on the state of the actomyosin system during rigor mortis and post-rigor aging. Because temperature has a profound effect on post-mortem muscle shortening of rabbit and beef (Busch, 1966; Locker et al., 1963) and therefore presumably also on post-mortem actin-myosin interactions, experiments were done using these two species at four different temperatures corresponding to three types of post-mortem shortening, i.e., cold shortening, minimal shortening and high-temperature (thermal) shortening. The results indicate that time and temperature of post-mortem storage have sizable effects on protein solubility. However, it was difficult to relate these effects to changes in the actin-myosin interaction because: 1) in the presence of 0.1 *M* phosphate, even strong KCl solutions extracted only myosin, and 2) there was an inability to adequately characterize the protein in the 1.1 *M* KI extracts.

MATERIALS AND METHODS

Sampling and extraction

The first part of this study was conducted by using leg muscles from 29 rabbits. Immediately after stunning, exsanguinating and skinning, the right leg muscles were excised for at-death sarcoplasmic and myofibrillar protein solubility analyses. Extraction procedures were essentially those as outlined by Helander (1957). Quadruplicate 4 g samples were weighed into 50 ml plastic centrifuge tubes and suspended in cold 0.03 *M* potassium buffer, pH 7.4, (1:10 w/v). After setting for 2 hr at 2°C, the tubes were centrifuged in a Lourdes Model A Betafuge at 28,500 x g for 15 min and the supernatant decanted and stored at 2°C. This extraction procedure was repeated two more times on the residue with extraction intervals of 2 and 1 hr.

Following extraction of sarcoplasmic proteins, the four samples were divided into two groups of duplicate tubes. Ten volumes (based on original fresh sample weight) of 0.5 *M* KCl in 0.1 *M* K-phosphate buffer, pH 7.4, was added to one pair of samples and 10 volumes of 1.1 *M* KI in 0.1 *M* K-phosphate buffer, pH 7.4, was added to the second pair; the samples were suspended in these solutions by gentle stirring. After a 3 hr extraction at 2°C, the tubes were centrifuged at 28,500 x g for 15 min and each residue resuspended in 10 more volumes of its respective extraction solution. Two more extractions of 3 and 2 hr were conducted on each myofibril residue.

The first series of experiments indicated that virtually no sarcoplasmic or myofibrillar protein was extracted during the third extraction, and therefore, the third extraction period for both sarcoplasmic and myofibrillar protein was discontinued during the last half of this study.

The left leg muscles were also excised immediately after death, but were stored in plastic bags at 2°, 25°, 28°, 31° and 37°C. After various post-mortem times, samples were removed and subjected to the same extraction procedure as that described for the at-death muscle. All extractions were carried out at 2°C using pre-cooled solutions made up in deionized, distilled water that had been redistilled in glass.

In the second part of this study, semitendinosus and psoas muscles from both the right and left sides of five Angus heifers weighing approximately 410–450 kg were excised as soon as possible after stunning and exsanguination. Initial extraction, using the procedure just described for rabbit muscle, commenced approximately 20 min post-mortem. At-death samples were removed from the right muscles and the remainder of these muscles were then stored at 2° for subsequent sampling. Left muscles were used for samples stored at 16° and 37°. Samples were removed after 6, 12, 24, 48 and 312 hr post-mortem. Protein concentration was determined by the biuret procedure (Gornall et al., 1949).

Viscosity

Viscosity measurements on the extracted protein were done at 25° using Ostwald viscometers with flow times of approximately 90 sec for water. No kinetic energy corrections were made.

Ultracentrifugation

Analytical ultracentrifugal studies were done using a Spinco model E ultracentrifuge equipped with a phase plate and RTIC temperature control unit. All ultracentrifuge runs were done at 20.0°, and plates were measured using a Nikon comparator.

Statistical analysis

Results were analyzed by the least squares procedure as outlined by Snedecor (1956).

RESULTS

Sarcoplasmic protein extractability

The effects of temperature and post-mortem storage on sarcoplasmic protein extractability are shown in Tables 1 and 2. In spite of efforts to carefully reproduce extraction conditions, considerable variation, both among different animals and among different samples from the same animal, was encountered in these experiments. Thus, at post-mortem storage temperatures of 2°, sarcoplasmic protein extractability, particularly of bovine muscle, fluctuated erratically without exhibiting any trend with increasing time of post-mortem storage.

There are, however, two post-mortem trends in sarcoplasmic protein solubility worthy of mention. First, in rabbit muscle at 25°, sarcoplasmic protein solubility increased after 16 hr post-mortem, with 25–30% more sarcoplasmic protein being extracted after 72 hr post-mortem than immediately after death. This same trend was noted at 28° and 31° although the data are not as extensive at these two temperatures. Second, in both rabbit and bovine muscle at 37°, sarcoplasmic protein solubility decreased with increasing time of post-mortem storage, this decrease evidently occurring earlier in bovine muscle (between 6 and 12 hr post-mortem) than in rabbit muscle (between 12 and 24 hr post-mortem).

Storage at 37° for 24 hr post-mortem caused an 18–25% decrease in sarcoplasmic protein extractability.

Sarcoplasmic protein extractability of a tender muscle (psoas) was almost identical to that of a less tender muscle (semitendinosus).

Myofibrillar protein extractability

The effects of temperature and post-mortem storage on myofibrillar protein extractability are shown in Tables 3 and 4, with some of the important trends being summarized in Figs. 1 and 2. Again, substantial variation, as indicated by large standard errors, is evident particularly in bovine muscle after 48 or 312 hr of post-mortem storage. The most noticeable result is that solubility in 0.5 *M* KCl, 0.1 *M* potassium phosphate, pH 7.4, increased markedly with increasing time of post-mortem storage at temperatures up to 25°. In bovine muscle the 0.5 *M* KCl solution extracted three to four times more protein after 312 hr

Table 1—Effect of temperature on sarcoplasmic protein solubility of rabbit leg muscle.[1]

Storage temperature (°C)	Time post mortem (hr)							
	0	3	6	12	16	24	72	120
2°	69.0 ± 1.1[2]	69.8 ± 1.8	63.4 ± 1.8	....	61.3 ± 0.4	65.2 ± 1.0	67.3 ± 1.6	58.6 ± 0.3
25°	66.5 ± 4.0	69.4 ± 0.9	67.3 ± 2.1	....	62.6 ± 1.6	75.5 ± 2.5	87.4 ± 1.4	....
28°	56.1 ± 1.2	....	....	....	....	61.5 ± 0.4	....	....
31°	56.0 ± 1.2	....	....	....	....	63.9 ± 0.8	....	....
37°	65.0 ± 1.3	....	66.2 ± 1.1	61.2 ± 1.6	....	53.4 ± 0.7	....	....

[1] Soluble in 0.03 M potassium phosphate buffer, pH 7.4.
[2] Means plus or minus standard errors.

Table 2—Effect of temperature on sarcoplasmic protein solubility of bovine muscle.[1]

Storage temperature (°C)	Muscle	Time post-mortem (hr)					
		0	6	12	24	48	312
2°	Semitendinosus	57.1 ± 2.7[2]	53.8 ± 0.1	63.0 ± 3.9	51.6 ± 0.8	63.9 ± 2.5	57.2 ± 0.4
	Psoas	52.7 ± 2.5	55.5 ± 0.7	64.9 ± 2.8	53.7 ± 0.4	64.3 ± 3.2	57.0 ± 3.5
16°	Semitendinosus	57.1 ± 2.7	55.8 ± 0.7	67.0 ± 3.3	54.1 ± 0.4	63.6 ± 2.6	58.8 ± 1.2
	Psoas	52.7 ± 2.5	54.7 ± 1.0	62.1 ± 2.9	55.4 ± 1.9	65.5 ± 3.0	48.8 ± 4.0
37°	Semitendinosus	58.0 ± 2.1	52.8 ± 0.8	43.0 ± 3.1	52.7 ± 0.4	....	....
	Psoas	65.6 ± 2.4	59.3 ± 0.7	46.8 ± 1.5	48.6 ± 1.3	....	....

[1] Soluble in 0.03 M potassium phosphate buffer, pH 7.4.
[2] Means plus or minus standard error.

Table 3—Effect of temperature on myofibrillar protein solubility of rabbit leg muscle.

Storage temperature (°C)	Extracting solution	Time post-mortem (hr)							
		0	3	6	12	16	24	72	120
2°	0.5 *M* KCl	24.3 ± 1.0[1]	22.5 ± 1.6	22.3 ± 1.2	....	27.4 ± 3.5	48.8 ± 4.0	51.7 ± 3.9	53.0 ± 4.6
	1.1 *M* KI	23.4 ± 0.8	28.0 ± 0.9	38.5 ± 1.9	....	107.5 ± 1.4	118.6 ± 2.5	115.9 ± 4.4	101.5 ± 0.8
25°	0.5 *M* KCl	112.8 ± 1.7	116.7 ± 3.6	111.8 ± 2.2	....	37.2 ± 4.2	75.8 ± 4.6	107.6 ± 3.4	....
	1.1 *M* KI	109.8 ± 1.9	116.4 ± 3.1	118.8 ± 2.7	....	115.5 ± 2.9	126.6 ± 3.8	144.6 ± 4.2	....
28°	1.1 *M* KI	110.6 ± 1.7	....	....	....	....	97.4 ± 2.9	....	....
31°	1.1 *M* KI	110.6 ± 1.7	....	....	....	....	99.4 ± 1.7	....	....
37°	0.5 *M* KCl	32.5 ± 1.6	....	48.8 ± 6.1	32.7 ± 3.5	....	27.8 ± 1.4	....	....
	1.1 *M* KI	112.7 ± 2.2	....	105.9 ± 2.4	96.3 ± 4.5	....	94.2 ± 10.0	....	....

[1] Means plus or minus standard error.

Table 4—Effect of temperature on myofibrillar protein solubility of bovine muscle.

Storage temperature (°C)	Extracting solution	Muscle	Time post-mortem (hr)					
			0	6	12	24	48	312
2°	0.5 *M* KCl	Semitendinosus	19.0 ± 1.8	17.8 ± 0.1	19.1 ± 1.6	25.6 ± 2.7	52.7 ± 7.6	69.0 ± 18.4
		Psoas	18.2 ± 1.2	17.5 ± 0.8	22.9 ± 1.4	31.8 ± 3.5	42.2 ± 5.9	86.8 ± 3.8
	1.1 *M* KI	Semitendinosus	96.0 ± 5.7	91.4 ± 3.5	118.0 ± 2.2	101.5 ± 7.4	120.6 ± 7.2	135.3 ± 9.4
		Psoas	97.6 ± 6.5	99.2 ± 1.8	128.4 ± 8.1	107.0 ± 1.4	130.2 ± 7.0	137.5 ± 10.6
16°	0.5 *M* KCl	Semitendinosus	19.0 ± 1.8	17.2 ± 0.9	23.7 ± 2.2	32.8 ± 3.4	63.5 ± 9.5	78.0 ± 14.6
		Psoas	18.2 ± 1.2	18.1 ± 0.9	20.6 ± 1.4	36.2 ± 4.8	75.5 ± 10.0	73.6 ± 15.2
	1.1 *M* KI	Semitendinosus	96.0 ± 5.7	95.3 ± 1.4	113.8 ± 7.3	105.2 ± 1.7	123.8 ± 11.8	134.9 ± 12.0
		Psoas	97.6 ± 6.5	100.8 ± 1.2	123.3 ± 10.0	113.1 ± 1.7	123.2 ± 7.6	102.9 ± 7.5
37°	0.5 *M* KCl	Semitendinosus	20.9 ± 2.2	18.0 ± 1.1	19.0 ± 0.8	28.9 ± 1.4	....	....
		Psoas	17.1 ± 1.2	17.8 ± 1.0	15.8 ± 0.4	22.5 ± 1.0	....	....
	1.1 *M* KI	Semitendinosus	103.4 ± 1.6	85.0 ± 1.0	85.0 ± 4.6	59.6 ± 1.3	....	....
		Psoas	82.2 ± 4.7	83.8 ± 2.6	59.5 ± 1.3	64.5 ± 2.2	....	....

post-mortem immediately after death. Rabbit muscle also exhibited this same trend, with 0.5 *M* KCl extracting five times more protein after 72 hr post-mortem at 25° than immediately after death.

The increased extractability in 0.5 *M* KCl began to appear about 16–24 hr post-mortem for both rabbit and bovine muscle at 2°, about 12 hr post-mortem for bovine muscles at 16°, and about 3–6 hr post-mortem for rabbit muscle at 25°. This suggests that a common phenomenon, accelerated by increasing temperature, is responsible for the increased myofibrillar protein solubility in 0.5 *M* KCl. At 37°, however, solubility of rabbit myofibrillar protein in 0.5 *M* KCl first increases slightly and then decreases. Bovine muscle at 37° exhibits only a slight increase in 0.5 *M* KCl solubility, this increase occurring between 12 and 24 hr post-mortem.

Assuming that the same phenomenon which enhanced solubility in 0.5 *M* KCl at storage temperatures of 25° or below is still effective at 37°, these results suggest that at 37° a second phenomenon, most likely an unfolding and subsequent aggregation of the myofibrillar proteins (Galloway et al., 1967), is now occurring to decrease solubility in 0.5 *M* KCl. The observed solubility thus is a resultant of the relative rates and intensities of these two phenomena.

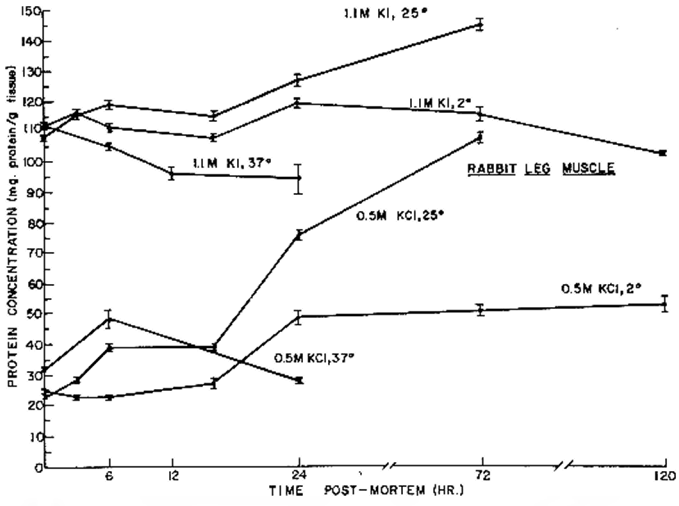

Fig. 1—Effect of temperature on post-mortem myofibrillar protein solubility of rabbit leg muscle (means plus or minus standard error).

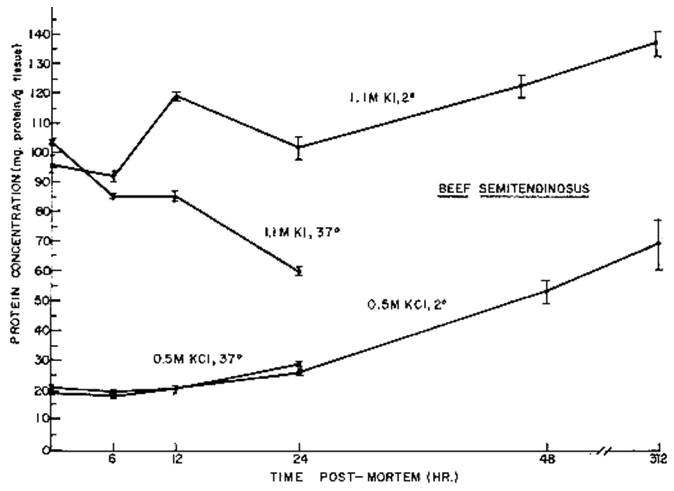

Fig. 2—Effect of temperature on post-mortem myofibrillar protein solubility of bovine semitendinosus muscle (means plus or minus standard error).

When 1.1 *M* KI, 0.1 *M* potassium phosphate, pH 7.4, is the extracting solution, myofibrillar protein solubility changes much smaller in magnitude, but similar in nature to those already discussed for 0.5 *M* KCl, are observed at post-mortem storage temperatures of 25° or below. One exception to this is rabbit leg muscle at 2° in which solubility in 1.1 *M* KI remains almost constant for 120 hr post-mortem. In addition, no change in rabbit myofibrillar protein extractability by 1.1 *M* KI is observed at post-mortem storage temperatures of either 28° or 31° although the data at these two temperatures are too limited to permit any final conclusions.

At storage temperatures of 37°, solubility in 1.1 *M* KI decreases after approximately 6–12 hr post-mortem, with the decrease being much larger for bovine muscle than for rabbit muscle. It is obvious that at all times and temperatures of post-mortem storage, but particularly below 30°, 1.1 *M* KI solubilizes considerably more myofibrillar protein than 0.5 *M* KCl. Furthermore, it is obvious that bovine psoas and semitendinosus muscles display similar myofibrillar protein extractability at all post-mortem times and temperatures.

Viscosity

Tables 5 and 6 show the results of viscosity measurements made on sarcoplasmic and myofibrillar protein extracts of rabbit and bovine muscle, respectively. The measurements on the three types of extracts, sarcoplasmic, 0.5 *M* KCl and 1.1 *M* KI, were not done at the same protein concentrations, so it is not possible to compare relative viscosities among these three types of extracts. However, measurements for any one type of extract were done at the same protein concentration for all times and temperatures of post-mortem storage. Thus, the results presented do permit the conclusion that, with only one exception, viscosity of the extracts was not markedly affected by either time or temperature of post-mortem storage. The exception to this generalization is the 1.1 *M* KI extracts of both rabbit and bovine muscle stored at 37°, where viscosity of the extract decreased with increasing post-mortem time.

Appreciable variation was experienced among duplicate viscosity measurements on the same sample. This may be attributable, in part, to difficulty in eliminating small lipid globules from the protein solutions. Even high-speed (17,000 rpm) centrifugation followed by filtration through glass wool did not completely alleviate this problem.

Analytical ultracentrifugation

Fig. 3 shows some representative schlieren diagrams from analytical ultracentrifugal studies of the 0.5 *M* KCl and 1.1 *M* KI extracts of bovine muscle. Extracts of rabbit muscle gave almost identical sedimentation patterns. In some extracts, particularly those at greater post-mortem times or higher storage temperatures, considerable

Table 5—Relative viscosity of sarcoplasmic and myofibrillar protein extracts of rabbit leg muscle.[1]

	Storage temperature (°C)	Time post-mortem (hr)						
		0	3	6	12	24	72	120
Sarcoplasmic [2]	2°	1.12	....	....	....	1.15	1.16	1.05
	25°	1.12	....	....	....	1.16	1.15	....
	37°	1.12	1.16	1.15	1.08	....	....	....
0.5 *M* KCl [3]	2°	1.04	....	....	....	1.02	1.08	1.20
	25°	1.04	....	....	....	1.08	1.10	....
	37°	1.04	1.05	1.01	1.01	....	....	....
1.1 *M* KI [4]	2°	1.42	....	....	....	1.36	1.44	1.38
	25°	1.42	....	....	....	1.39	1.33	....
	37°	1.42	1.36	1.27	1.08	....	....	....

[1] Figures are relative viscosities calculated as means of 6 determinations at 25°.
[2] Measurements done at protein concentrations of 1.0 mg/ml in 0.03 *M* potassium phosphate, pH 7.4.
[3] Measurements done at protein concentrations of 0.5 mg/ml in 0.5 *M* KCl, 0.1 *M* potassium phosphate, pH 7.4.
[4] Measurements done at protein concentrations of 2.5 mg/ml in 1.1 *M* KI, 0.1 *M* potassium phosphate, pH 7.4.

Table 6—Relative viscosity of sarcoplasmic and myofibrillar protein extracts of bovine semitendinosus muscle.[1]

Extracting solution	Storage temperature (°C)	Time post-mortem (hr)					
		0	6	12	48	96	312
Sarcoplasmic [2]	2°	1.01	....	....	1.02	1.03	1.03
	16°	1.01	....	....	1.02	1.03	1.03
	37°	1.01	1.03	1.04	....	....	....
0.5 *M* KCl [3]	2°	1.06	....	....	1.06	1.09	1.05
	16°	1.06	....	....	1.08	1.12	1.10
	37°	1.05	1.06	1.05	....	....	....
1.1 *M* KI [4]	2°	1.46	....	....	1.40	1.56	1.59
	16°	1.46	....	....	1.51	1.55	1.54
	37°	1.46	1.25	1.11	....	....	....

[1] Figures are relative viscosities calculated as means of determinations at 27°.
[2] Measurements done at protein concentrations of 1.0 mg/ml in 0.03 *M* potassium phosphate, pH 7.4.
[3] Measurements done at protein concentrations of 0.5 mg/ml in 0.5 *M* KCl, 0.1 *M* potassium phosphate, pH 7.4.
[4] Measurements done at protein concentrations of 2.5 mg/ml in 1.1 *M* KI, 0.1 *M* potassium phosphate, pH 7.4.

heterogeneity was evident in the ultracentrifugal patterns, probably indicative of both some aggregation and some degradation of myofibrillar proteins in these extracts.

The sedimentation diagrams for both the 0.5 *M* KCl and the 1.1 *M* KI extracts of at-death muscle exhibited a single major peak, which possessed a sedimentation coefficient of 4.8 S (average of 3 determinations with a range of 4.5 to 5.0 S) in the case of the KCl extracts and 5.2 S (a single determination) in the case of the KI extracts.

After 24 hr of post-mortem storage, the sedimentation diagrams of both types of extracts exhibited two classes of material migrating with sufficiently sharp boundaries to permit measurement of a sedimentation coefficient. In the 0.5 *M* KCl extracts, these two classes possessed sedimentation coefficients of 5.0 S (average of 4 determinations with a range of 4.5 to 5.2 S) and 10.8 S (average of 7 determinations with a range of 9.9 to 11.1 S), whereas in 1.1 *M* KI, the two classes had sedimentation coefficients of 4.6 S (average of 3 determinations with a range of 4.3 to 4.8 S) and 5.9 S (average of 4 determinations with a range of 5.7 to 6.1 S). The sedimentation diagrams of the extracts of muscle stored for 24 hr post-mortem exhibited either both classes of sedimenting material, or in about half of the post-mortem extracts, only the more rapidly sedimenting boundaries (i.e., the 10.8 or 5.9 S) were distinguishable.

Since the molecular nature of these extracts is, at best, poorly understood and since there was not any obvious uniformity in aggregation or degradation products among different extracts, no further analysis of the sedimentation patterns was attempted. However, it is obvious that none of the sedimentation patterns exhibit any peaks that might be identified as actomyosin. Because of their very heterogeneous character, the sarcoplasmic protein extracts in this study were not examined in the analytical centrifuge.

Other studies

Two other types of experiments were done in an effort to obtain more information on the nature of the myofibril-

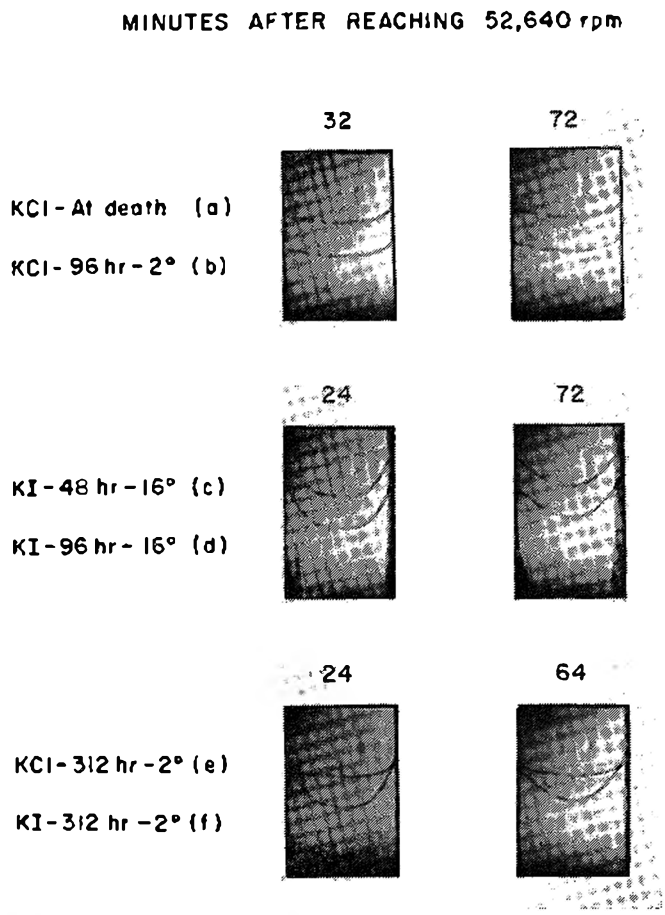

Fig. 3—Analytical ultracentrifugation patterns of myofibrillar proteins from bovine semitendinosus muscle stored at 2° and 16°. Protein concentrations were: a) 2.7 mg/ml; b) 1.8 mg/ml; c) 4.2 mg/ml; d) 4.7 mg/ml; e) 2.5 mg/ml; f) 4.8 mg/ml. Schlieren analyzer angle = 65°. Temperature = 20°.

lar protein extracts. The first of these involved dilution of the 0.5 *M* KCl or 1.1 *M* KI extracts to a final ionic strength of 0.20. At this ionic strength, myosin is soluble but actomyosin is precipitated. Dilution was done using deionized, redistilled water at 2°; the tubes were allowed to set at 2° for 120 min and then centrifuged at 27,500 x g for 15 min. None of the protein in the 1.1 *M* KI extracts was ever precipitated by this treatment, indicating the complete absence of actomyosin, at least in its usual form, in these extracts. Furthermore, this dilution did not precipitate any protein from the 0.5 *M* KCl extracts of either rabbit or bovine muscle stored at 37°. The percentage of protein precipitated from 0.5 *M* KCl extracts of muscle stored at 2°, 16°, or 25° is shown in Table 7.

Dilution of 0.5 *M* KCl extracts of at-death muscle did not precipitate any protein whatsoever, but with increasing time of post-mortem storage, some protein, precipitable at an ionic strength of 0.20, began to appear in the extracts. This precipitable protein appeared sooner post-mortem at higher storage temperatures. The precipitation test is quite indiscriminate and does not necessarily indicate the presence of actomyosin. Since a typical actomyosin peak was never observed in the analytical centrifuge, it seems most likely that the precipitated protein in this experiment was an aggregated form of myosin or possibly aggregates of myosin containing small amounts of actin.

The other type of experiment done to characterize the myofibrillar protein extracts in this study involved determination of Mg^{++}- and Ca^{++}-modified ATPase activities possessed by the extracts. To remove the inorganic phosphate or KI in them, the extracts were dialyzed exhaustively (4 days) at 2° against five or six changes of 0.4 *M* KCl. After dialysis, the extracts were diluted to a KCl concentration of 0.1 *M* and ATPase assays conducted according to the methods described by Goll et al. (1967), but using 1 m*M* ATP and 1 m*M* Mg^{++} or Ca^{++} instead of 5 m*M*. The results indicated the complete absence of Mg^{++}-modified ATPase activity in all extracts and the absence of Ca^{++}-modified activity as well in the 1.1 *M* KI extracts. Moreover, Ca^{++}-modified activity could not be consistently detected even in the 0.5 *M* KCl extracts. It therefore appears at this stage that 1.1 *M* KI extraction, the prolonged dialysis period, or both, are inactivating the myosin ATPase enzyme.

DISCUSSION

ONE OF THE MOST noteworthy aspects of this investigation was the total absence of a typical actomyosin peak in analytical ultracentrifugal diagrams of any of the myofibrillar protein extracts. In the case of 1.1 *M* KI extracts, this was not totally unexpected since it is known that 1.1 *M* KI dissociates F-actin to G-actin (Szent-Gyorgyi, 1951). It seems possible, therefore, that the material observed in schlieren diagrams of the 1.1 *M* KI extracts is G-actomyosin.

The problem of identification of G-actomyosin has been considered in detail by Johnson et al. (1964) and will not

Table 7—Percent of protein in 0.5 *M* KCl extracts precipitated by dilution to an ionic strength of 0.2.[1]

Species	Storage temperature (°C)	Time post-mortem (hr)						
		0	24	48	72	96	120	312
Rabbit	2°	0.0	0.0		46.4	....	45.3	....
	25°	0.0	37.4		44.8	....	....	....
Beef	2°	0.0	....	9.0	....	30.5	....	43.6
	16°	0.0	....	41.7	....	59.8	....	42.9

[1] Myofibrils were extracted with 0.5 *M* KCl, 0.1 *M* potassium phosphate as described in MATERIALS AND METHODS. The 0.5 *M* KCl extracts were diluted to a final ionic strength of 0.20 and then centrifuged. The amount of precipitated protein was divided by the amount of protein originally in the extract to give the percent of protein precipitated by the dilution.

be discussed further here. However, from the known composition of muscle, it can easily be calculated that the 1.1 *M* KI solutions in our study are extracting 75–100% of total myofibrillar protein present in our samples. Therefore, some actin must be present in these extracts even though actomyosin itself does not appear in the sedimentation diagrams. Our study thus provides strong additional evidence for occurrence of a substance such as G-actomyosin, whose existence has heretofore remained hypothetical.

Even more unexpected was the finding that the 0.5 *M* KCl extracts evidently did not contain actomyosin, regardless of species, and time and temperature of post-mortem storage. The 4.9 S component in sedimentation diagrams of the 0.5 *M* KCl extracts may tentatively be identified as myosin. The 10.8 S component has been observed before in actomyosin extracts by Haga et al. (1965, 1966). These workers suggested that this component may represent either an aggregated form of myosin (Haga et al., 1965) or a substance related to the solubilization of F-actin (Haga et al., 1966).

From the known composition of striated muscle, it can be calculated that a gram of muscle tissue should contain 55–65 mg of myosin. With the exception of the 25° for 24 hr and 25° for 72 hr rabbit muscle samples and the 2° and 16° for 312 hr bovine samples, 0.5 *M* KCl did not extract more than 65 mg of protein/g of tissue from any of the samples in this study. Thus, from a strictly quantitative point of view, it is quite possible that the 0.5 *M* KCl solution principally extracts myosin.

These results are quite surprising in view of the presently accepted concepts of actomyosin extraction. Several reports (Banga et al., 1942; Haga et al., 1966) have suggested that when minced muscle is suspended in a high ionic strength salt solution, myosin is first extracted into solution. After the ATP present in muscle at death has fallen to a level so low that it cannot effectively dissociate the actin-myosin interaction, the affinity between myosin in solution and actin in the thin filaments breaks the bond between the Z-disk and the thin filament, pulling actin into solution to form actomyosin. In terms of this concept, therefore, actomyosin extraction would start immediately upon suspension of myofibrils, which do not contain any ATP, in the 0.5 *M* KCl solution.

There are, however, three reports in the literature (Barber et al., 1966; Mihalyi et al., 1966; Perry, 1955) which indicate that actomyosin extraction may be a more complex phenomenon than suggested by the preceding scheme. Of these, the report of Mihalyi et al. has direct implications to our findings. These workers found that the presence of 0.2 *M* phosphate inhibits actomyosin extraction from freshly minced muscle at ionic strengths of 0.6 or above even though the splitting of ATP in the extraction suspensions was not affected. They suggest that ATP, pyrophosphate and phosphate anions do not affect actomyosin extraction by dissociation of the actin-myosin interaction, as is commonly thought, but that these anions act to preserve myofibrillar structure, possibly by strengthening the bond between thin filaments and the Z-line and thereby preventing liberation of the thin filaments from the myofibril residue. Haga et al. (1966) have shown that actomyosin extraction by Weber-Edsall solution is preceded by a break between the Z-disk and the thin filaments. It is perhaps well to reiterate at this point that, for the sake of brevity, we have been referring to 1.1 *M* KI and 0.5 *M* KCl extracting solutions, whereas in fact both of these solutions also contain 0.1 *M* K phosphate buffer at pH 7.4. As indicated by the preceding discussion, this K phosphate may have important effects on the solution's extracting properties in addition to making a sizeable contribution to the ionic strength.

These findings have fundamental importance to other studies of post-mortem changes in myofibrillar protein solubility. Our study appears to be one of the first to consider in some detail the nature of the myofibrillar protein extracts of post-mortem bovine muscle although King (1966) has published a similar ultracentrifugal analysis of extracts of post-mortem fish muscle. King also used phosphate buffer to extract myofibrillar proteins and his ultracentrifugal patterns clearly show the presence of actomyosin in his myofibrillar protein extracts.

At the present time, we do not have any satisfactory explanation for the absence of atcomyosin in our 0.5 *M* KCl extracts, but it is most likely related to the relatively short extraction times of 3 hr used in our study. Our experience has been that myofibrillar protein solubility may vary considerably depending on the time and conditions of extraction and whether extraction is done on myofibrils after removal by a low ionic strength solution of the sarcoplasmic proteins, inorganic ions and ATP, or on minced whole muscle followed by precipitation by dilution to remove the sarcoplasmic proteins. Indeed, it now appears possible that only myosin was being extracted in some of the earlier studies on myofibrillar protein solubility of bovine muscle, and the conclusions of these studies may now need to be re-examined.

Although total myofibrillar protein extractable with 0.5 *M* KCl, 0.1 *M* potassium phosphate increases markedly with increasing time of post-mortem storage at temperatures of 2°, 16° or 25°, the absence of either a viscosity increase of an actomyosin peak in the ultracentrifugal sedimentation diagrams both argue against this increase being due to extraction of F-actomyosin. It is possible, however, that post-mortem storage does lead to some degradation of F-actin structure, as originally suggested by Weinberg et al. (1960), and that degraded F-actin, being extracted in the form of G-actin monomers or dimers, is responsible for the increase in 0.5 *M* KCl-extractable protein. The resulting G-actomyosin may very well be indistinguishable from myosin in either its sedimentation diagram or its viscosity, thereby preventing its identification in the 0.5 *M* KCl extracts.

Fragmentation of F-actin may be caused either by pH-induced alterations in conformation of G-actin or by catheptic proteolysis of a protein which structurally supports the F-actin filament. Extraction of fragmented actin would account for the protein that cannot be quantitatively accounted for as myosin in these 0.5 *M* KCl extracts.

The decrease of myofibrillar protein solubility in 1.1 *M* KI during storage at 37° is also enigmatic. This decrease cannot be simply ascribed to a lowered myosin solubility,

as described in the hypothetical scheme of Galloway et al. (1967), since solubility in 0.5 *M* KCl, which extracts only myosin, does not change appreciably or even increase slightly during post-mortem storage at 37°. Nonetheless, a lowered myosin solubility in 1.1 *M* KI after 12 to 24 hr at 37° would explain both the decrease in extractable protein and the lowered viscosity of these extracts, and it is possible that during post-mortem storage at 37°, myosin is altered in a way which lowers its solubility in 1.1 *M* KI but does not change its solubility in 0.5 *M* KCl. Indeed, since 1.1 *M* KI has such strong dissociating effects on both F-actin structure and the actin-myosin interaction, it would appear that structural alterations in myosin are the only phenomena which could cause a marked decrease of myofibrillar protein solubility in this solution.

The results of our study do clearly indicate that myofibrillar protein solubility is not directly related to tenderness. Bovine muscle stored at 37° is considerably more tender at 12 hr post-mortem than muscle stored at either 2° or 16° for a similar period (Busch et al., 1967), but this increased tenderness is not accompanied by an increase in myofibrillar protein solubility at 37°; indeed solubility in 1.1 *M* KI actually decreases at 37°.

Although it may be possible under a certain set of restricted conditions to demonstrate a relationship between myofibrillar protein solubility and tenderness at storage temperatures of 2° (Aberle et al., 1966; Hegarty et al., 1963), this too is probably only an indirect correlation originating from the relationship of both tenderness and solubility to some common third variable (Goll et al., 1964). Moreover, the similar myofibrillar protein solubility of a very tender muscle (psoas) and a less tender muscle (semitendinosus) supports the lack of any real relationship between tenderness and myofibrillar protein solubility.

Similarly, since post-mortem shortening of bovine muscle is greatest at 2° and least at 16° (Busch et al., 1967) and since myofibrillar protein solubility of bovine muscle at these two post-mortem storage temperatures is almost identical, it would appear that there also is no direct relationship between sarcomere length and myofibrillar protein solubility. Again, this conclusion is substantiated by the similar myofibrillar protein solubility of the psoas (average sarcomere length of 3.8 μ) and the semitendinosus (average sarcomere length of 2.4 μ) (Herring et al., 1965).

It is obvious from this study that protein extractability of rabbit muscle is very similar to that of bovine muscle regardless of time or temperature of post-mortem storage. These two species differ markedly in their post-mortem response to temperature with bovine muscle undergoing substantial shortening at post-mortem storage temperatures of 2° while rabbit muscle shortens very little, if any, under these conditions. It is therefore evident that the events responsible for post-mortem shortening at 2° in bovine muscle do not cause any change in either sarcoplasmic or myofibrillar protein solubility.

Although the apparent absence of actin in our 0.5 *M* KCl extracts makes it difficult to interpret our results in terms of post-mortem changes in actin-myosin interaction, this absence has at the same time indicated several new possibilities concerning subtle post-mortem alterations in myosin that may affect its solubility. Additional studies on the extraction of actomyosin from myofibrils and more complete characterization of these extracts may eventually permit some conclusions regarding our original objective, i.e., possible post-mortem changes in the actin-myosin interaction and in structural integrity of the sarcomere.

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Changes in Molecular Weight of Pectin During Methylation with Diazomethane

SUMMARY—Acid and enzymatic demethylation procedures suggest that the change in molecular weight during methylation is a result of depolymerization and that a change in the percent methoxyl groups probably has very little effect on the change in molecular weight during methylation or demethylation with pectins having an ester content between 60% and 95%.

Samples of a commercially prepared citrus pectin were acid washed, dissolved and neutralized to different levels with dilute NaOH. These samples were treated with isopropanol to yield an open gel-like precipitate, the alcohol was replaced with ether and the pectin treated with diazomethane at $< -30°C$. The samples methylated to 70%, 85% and 95% had molecular weight values which decreased with increasing degrees of esterification. Molecular weight was determined by measuring the viscosity of a 0.1% sol in 1% Calgon.

INTRODUCTION AND LITERATURE

DURING pectin manufacture the methoxyl content and the molecular weight are subject to change and these changes are not always desirable. Setting times and jelly grades are dependent on the methoxyl content, and controlled demethylation or methylation are often carried out during commercial pectin production to arrive at a desired optimum level. It is generally accepted that a better grade is obtained at a high molecular weight. However, depolymerization occurs during extraction and manufacture of pectin and this limits the molecular weight of the finished product.

Numerous studies have been carried out in the past to determine the effect of methoxyl content and molecular weight levels on jelly grades. Such studies have often been difficult to interpret because different testing methods are frequently used by different investigators, samples from various sources are compared and prepared samples often differed with respect to more than one variable.

In a recent attempt to determine the influence of the methoxyl content on jelly grade (Smit et al., 1968) it was claimed that molecular weights of the samples, with different methoxyl levels, were the same. In this study, molecular weights were determined by using a viscosity measurement of a 0.1% sol in 1% sodium hexametaphosphate (Christensen, 1954). There was no evidence presented to show that the viscosities obtained were not dependent on the methoxyl content of the sample. Further investigation of this aspect appeared necessary, particularly in the light of earlier work published by Deuel et al. (1950) and Vollmert (1950a, 1950b) on the low temperature methylation of pectin with diazomethane. Vollmert found a decrease in viscosity after methylation and ascribed it to the

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effect of methoxyl groups and not to a possible depolymerization of the pectin. These viscosities were determined in water and also in acetone after nitration. He supported his conclusion by the observation that careful acid demethylation of his methylated pectin gave a substantial increase in viscosity.

Deuel et al. (1950) subjected their methylated pectin to alkaline saponification and compared the viscosity in an aqueous medium of the sodium pectate obtained in this manner with a sodium pectate prepared from the original pectin before methylation. These two pectates had the same viscosity and this led them to the conclusion that no depolymerization occurs during low temperature methylation with diazomethane.

Heim et al. (1962) indicated that some depolymerization always occurs during low-temperature methylation with diazomethane. This made earlier conclusions on the effect of methoxyl groups on viscosity questionable. Viscosity measurements were commonly used for the determination of molecular weight of pectins and it seemed warranted to do some experiments in an attempt to establish how it is influenced by a change in the methoxyl content. This paper presents the data obtained.

EXPERIMENTAL

Preparation of methylated samples

Three 20-g portions of an unstandardized slow-set citrus pectin were acid-washed by stirring for 10 min in 400 ml 60% isopropanol (I.P.A.) containing 20 ml concentrated HCl. The liquid was removed by suction and the pectin washed 6 times with 50-ml quantities of acid-alcohol. The pectin was washed with 60% I.P.A. until free of chlorides and made into a 1% sol. To one portion of pectin were added 215 ml 0.1 *N* NaOH, to another 108 ml 0.1 *N* NaOH while the third did not receive any alkali. The pectin was then precipitated with two volumes of I.P.A. during vigorous stirring.

As much as possible of the liquid portion was removed from the gel-like precipitate by applying suction on a Büchner funnel without pressing. The precipitate was resuspended and liquid was removed as before. This was continued until an alcohol concentration of 98% was reached. The alcohol was replaced with ether and the suspension cooled to $< -30°C$. An ether solution of 3.0 g of diazomethane at $< -30°C$ was added to each batch and the temperature was held at $< -30°C$ by cooling in an acetone-dry ice bath and by addition of dry ice directly to the reaction mixture.

After 30 min the solvent was removed with suction, the precipitate washed with cold ether and dried in air.

The three methylated samples, as well as a sample of the original starting material, were made into 0.5% sols, filtered through nylon cloth, precipitated with I.P.A., filtered again, washed with I.P.A. and ether, and dried. When dry, these samples were ground to pass through a 40-mesh screen, acid washed as described earlier and dried at room temperature.

Demethylation of pectin

Some methylated samples were demethylated using the following three procedures:

1. HCl in I.P.A. suspension. A 3-g quantity of methylated pectin was suspended in a mixture of 81 ml I.P.A., 33 ml H_2O and 11 ml conc. HCl at 38°C. The suspension was filled into a thermos flask and agitated for 6 hr during which time the temperature decreased to 25°C. The pectin was then filtered off, washed with 60% I.P.A. until free of chlorides and rinsed with I.P.A. and ether before drying.

2. H_2SO_4 in an aqueous medium. A 4-g quantity of methylated pectin was wetted with I.P.A. and dissolved in 200 ml 2% H_2SO_4 (Vollmert, 1950a). The sol was left in a stoppered flask at 40°C for 22 hr. The pectin was then precipitated with 2 volumes of I.P.A. The precipitate was recovered, washed free of sulphates with 60% I.P.A. and washed with I.P.A. and ether. After drying, the sample was ground to pass a 40-mesh screen.

3. Enzymatic demethylation. A 10-g quantity of methylated pectin was dissolved in one liter of 0.05 *M* $CaCl_2$. One g of orange flavedo pectinesterase (obtained from the Western Regional Research Division of the U. S. Department of Agriculture) was suspended in the sol and the pH was adjusted to 5.5. The reaction was allowed to proceed overnight at 25°C. By the next morning the sol had gelled. Two volumes of I.P.A. were added and the kernel-like precipitate was recovered. This precipitate was acid-washed, as described earlier, before finally washing with 60% I.P.A. and ether. After drying, this material was ground to pass through a 40-mesh screen.

Analytical procedures

Moisture, ash, percent methoxyl, degree of esterification, percent galacturonic acid, equivalent weight, molecular weight and jelly grade were determined as described by Smit et al. (1967).

DATA AND DISCUSSION

THE DATA obtained on analysis of the methylated samples are given in Table 1. Addition of a limited amount of dilute sodium hydroxide to an acid-washed pectin sol probably caused a random neutralization of acid groups. The remaining carboxyl groups were then methylated with diazomethane at $< -30°C$. With increasing degrees of esterification the apparent molecular weight, as determined by a viscosity measurement in 1% sodium hexametaphosphate, decreased markedly. According to Vollmert (1950a, 1950b) this change should be primarily due

Table 1. Analyses of prepared pectin samples.

	Sample number			
	1	2	3	4
NaOH (0.1 *N*) used (ml)		215	108	0
Moisture (%)	5.7	10.3	7.8	13.3
Methoxyl (%)[1]	8.3	10.6	11.9	13.5
Esterification (%)	61.5	72.5	85.0	95.8
Eqv. wt[1]	596	770	1,480	5,630
Galacturonic acid (%)[1]	84.6	91.1	87.4	87.9
Mol. weight ÷ 1,000[2]	173	153	146	127
Jelly grade[2]	313	252	228	...

[1] Expressed on ash- and moisture-free base.
[2] Expressed on a galacturonic acid base.

Table 2. Change in pectin during demethylation.

		OCH_3[1] %	Ester %	Gal. acid[1] %	M.W. ÷ 1000[2]	Jelly Grade[2]
HCl in I.P.A. suspension	Before	10.2	72	88	147	255
	After	9.0	62	90	142	299
HCl in I.P.A. suspension	Before	13.0	95	85	114	.....
	After	12.1	84	90	109	198
H_2SO_4 in sol	Before	11.9	85	87	146	228
	After	10.8	75	90	136	227
H_2SO_4 in sol	Before	13.5	96	88	127	.....
	After	11.9	82	91	119	215
Enzyme	Before	12.4	83	93	130	299
	After	10.0	69	90	129	243

[1] Expressed on ash- and moisture-free base.
[2] Expressed on galacturonic acid base.

to the increased methoxyl content. If this were the case, it should be possible to demonstrate an increase in the apparent molecular weight during careful demethylation.

Several samples which had been methylated to various ester levels were therefore treated with HCl in I.P.A., H_2SO_4 in a sol (Vollmert, 1950a, 1950b) or with purified pectin methylesterase. An acid treatment which resulted in a decrease in the ester level of 10 to 15% caused apparent molecular weight losses of 5,000 to 10,000 (Table 2). Orange pectin methylesterase gives a nonrandom demethylation of pectin (Doesburg, 1965) and viscosity measurements on pectin treated with this enzyme are not directly comparable with the acid-treated samples. In this case, the apparent molecular weight did not change with a decrease in the ester level from 84 to 69%.

The small change in the apparent molecular weight during demethylation with acid may be expected to be a result of depolymerization. However, if the methoxyl content also played an important role in the apparent molecular weight obtained, one may have expected an increase in apparent molecular weight during careful acid demethylation. This was not the case. Further, enzymatic demethylation also should have given some increase in apparent molecular weight if methoxyl content had any effect on viscosity in 1% sodium hexametaphosphate.

It seems sound to conclude that a change in the methoxyl level of a pectin will have no serious effect on the viscosity in 1% sodium hexametaphosphate; further, that the experimental conditions used during methylation resulted in considerable depolymerization and that the subsequent acid demethylation was responsible for an additional decrease in apparent molecular weight. This latter change could be avoided by using pectin methylesterase.

Because both the molecular weight and the methoxyl content changed during acid demethylation, the reported jelly grades are not of much interest. Normally there is an increase in grade as determined by the ridgelimeter when the ester level is decreased (Smit et al., 1968) during acid treatment. Enough data were not available to demonstrate this point. During enzymic demethylation there was a marked decrease in grade. This was no doubt due to the nonrandom demethylation which results in a very heterogeneous pectin.

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Semiautomated Thiocyanate Method for Determining Peroxide Values of Lipids

SUMMARY—A liquid-flow semiautomated ferrous thiocyanate procedure, designed for use in monitoring peroxide values of extracted oils, is presented. Twenty samples per hour can be measured by means of a system using conventional manifold tubing and AutoAnalyzer components.

INTRODUCTION

THE RANCIDITY of extracted oils is commonly measured by using either the thiobarbituric acid test (TBA test) or a peroxide test. Both tests have been adapted to semimicro investigations (Heaton *et al.*, 1958; Yu *et al.*, 1966). However, these tests are not amenable to automation and remain unsuitable for making a large number of multiple analyses.

The purpose of the present study was to devise a sensitive, semiautomated procedure for determining peroxide values (PV; milliequivalents peroxide/kilogram sample) of fish oils and for measuring low levels of pure hydroperoxides. The procedure described is a semiautomated version, with numerous modifications, of the ferrous thiocyanate procedure of Wagner *et al.* (1947).

Although earlier investigators reported a nonlinear relation between absorbancy and concentration, we have been able, by careful control of solvent and sample dilution, to achieve linearity when measuring very low levels of pure hydroperoxide ($\sim 2 \times 10^{-6}$ to 40×10^{-6} m.e.q.). In the case of extracted oils, the Lambert-Beer relation extends from PV = 1 to PV = 100+. However, since the thiocyanate test is somewhat dependent on the nature of the oil being tested and since color formation bears no direct relation to results obtained by the use of pure compounds in model systems, the method used for oils requires that an initial calibration be made using a conventional titration procedure. Once this calibration has been made, only small samples of oil are required (0.02 to 0.05 ml), and the method is not limited to oils with low peroxide values. The calibration curve is prepared by progressively oxidizing a sample of oil, periodically titrating an aliquot, and using some of each aliquot, monitoring color formation of the diluted material in the automated system.

MATERIALS AND METHODS

Apparatus

A suitable bench assembly is shown in Fig. 1. A VOM 5-Spectronic 20 combination (Bausch and Lomb Inc., Rochester, N. Y.) was used in conjunction with a water-cooled flow cuvette (Roubal, 1968) for recording absorbancies. The manifold pump, pump tubing, mixing-holding coil, debubbler tee and transmission tubing, all standard Technicon AutoAnalyzer components (Technicon Chromatography Corp., Ardsley, N. Y.), were employed for dipping samples and pumping reagents. An optimum sampling time of 8 sec separated by about 180 sec of rinsing was achieved by using a special timing cam for the Technicon Sampler II. The cam, easily constructed from aluminum, is 1-11/16 in. in diameter and has two 1/8-in. wide projections spaced 180° apart.

Ferrous thiocyanate reagent

Careful attention must be given to the preparation of the ferrous thiocyanate solution; in the presence of traces of oxidant, appreciable darkening occurs due to facile oxidation of ferrous ion; the solution should be prepared in the reservoir used in the analyses (Fig. 1). Dissolve 2 g of reagent-grade ammonium thiocyanate in 355 ml of deaerated spectro-grade methanol. To this solution, add 40 ml of deaerated, distilled, and deionized water and 5 ml of 25% (by wt) sulfuric acid in deionized water, bubbling the solution all the while with prepurified nitrogen. Deaerate the mixture with nitrogen for an additional 10 min. Add 0.4 g of reagent-grade ferrous ammonium sulfate (material less than one year old) and continue bubbling with stirring, for 10 more min. The solution, stored under prepurified nitrogen and away from light, is then ready for use. A fresh solution is prepared daily.

Calibration and sample preparation

Fresh oil with a PV of 0.1 or less is oxidized at room temperature with oxygen bubbling under ultraviolet light;

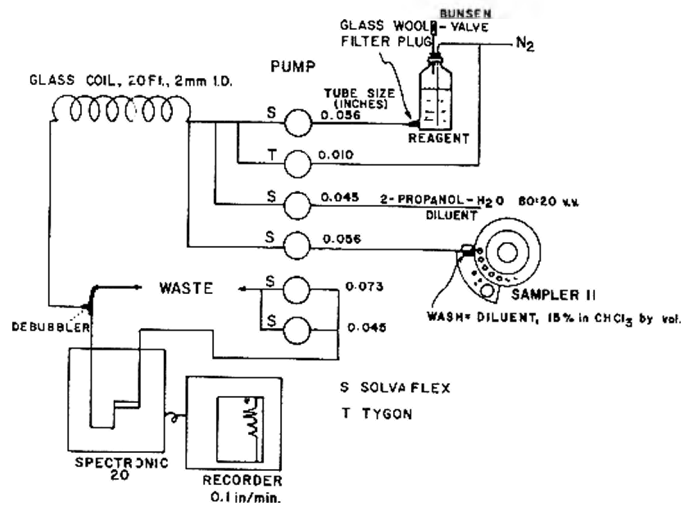

Fig. 1. Flow diagram for semiautomated PV determination. Standard AutoAnalyzer components are used except where noted.

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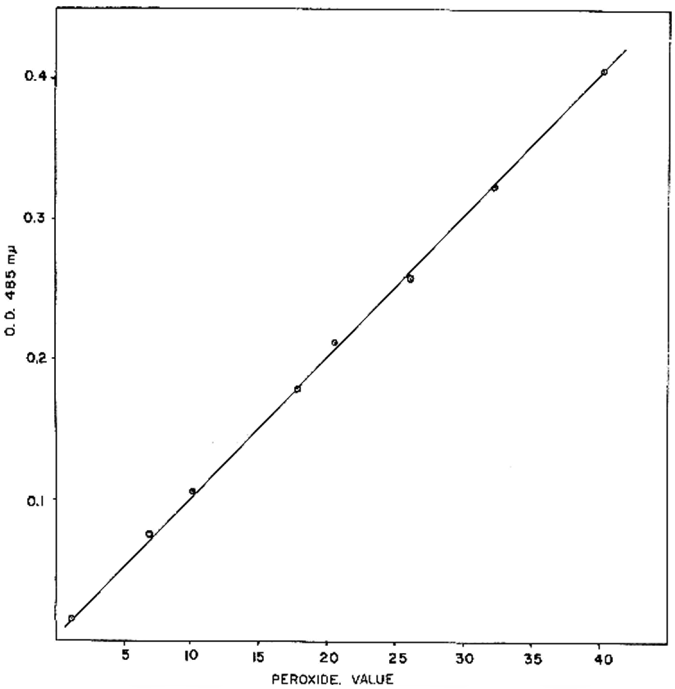

Fig. 2. Relationship between PV and O.D. for whole salmon oil.

samples are removed hourly and titrated using the AOCS procedure. An 0.05-ml portion of each is dissolved in separate 10-ml volumetric flasks, each containing 1.5 ml of reagent-grade chloroform. The mixtures are diluted to volume with 2-propanol (80:20, reagent-grade 2-propanol: deionized water by volume). Duplicate samples are then transferred to Sampler II cups and analyzed in the flow system; a plot of O.D. vs PV is prepared. Calibration is rechecked daily; diluted calibration mixtures (oxidized oils) are usable for a period of about a week provided that they are stored at −25°C or lower.

RESULTS AND DISCUSSION

FIG. 2 SHOWS a typical plot of data using whole salmon oil. Similar plots were obtained for oil from the dark flesh of salmon and oil from whole menhaden. The method differs markedly in sensitivity when used with pure hydroperoxide and when used with oxidized oil, as is apparent from the data in Fig. 3. Sensitivity in the case of oils with low PV could be enhanced by increasing the concentration of oil in the solutions sampled. However, some oils (0.02 to 0.05 ml) dissolve incompletely in propanol-water-chloroform when the chloroform is below the 15% level.

The chloroform should be maintained at the 15% level or below, however, since concentrations of chloroform above this level frequently lead to erratic results. Although chloroform itself does not absorb light in the region of 485 mμ, its presence apparently enhances the absorbance of the ferric thiocyanate complex in such areas that small differences in concentration of chloroform above 15% lead to large differences in absorbancies. Therefore, it

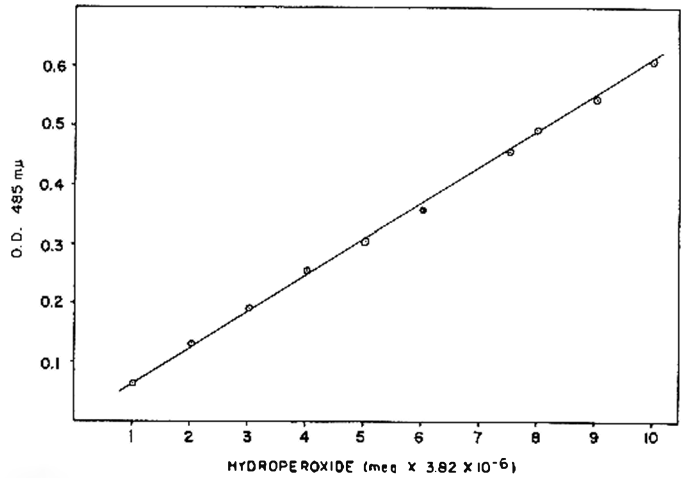

Fig. 3. Relationship between t-butyl hydroperoxide concentration and O.D.

is important that each flask, as well as the rinse mixture, contain the same quantity of chloroform. Also the attack on manifold tubes by the solvent increases markedly with increases in the concentration of chloroform; accordingly, 15% chloroform appears to be optimal.

Although small-bore silicone rubber tubing that resists chloroform is available for use as manifold tubing, this material was not investigated as a solveflex substitute in the present system. Silicone tubing loses its resiliency during continued usage; reliability falls off, necessitating frequent replacement of pump tubing.

Generally, an 0.05-ml sample is used for each test, but lesser amounts could be used with oils of high PV. It is important, however, that the amount of sample used both for the preparation of the calibration plot and for the test be the same. It has been mentioned that the thiocyanate method exhibits a certain nonlinearity; a plot of data prepared using 0.02-ml samples does not parallel that obtained using 0.04-ml samples. In this respect, the semiautomated method exhibits the same empirical relationship, although not as marked as that observed in the case of the AOCS titration procedure.

The 0.05-ml level is of little consequence with esters and other oil derivatives readily soluble in chloroform-propanol solution; larger samples can therefore be used to enhance sensitivity.

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Molecular Properties of Post-Mortem Muscle. 7. Changes in Nonprotein Nitrogen and Free Amino Acids of Bovine Muscle

SUMMARY—Proteolysis and its relationship to tenderness were studied by measuring nonprotein nitrogen (NPN), free amino groups, and shear resistance during post-mortem aging of bovine muscle. Both NPN and free amino groups increased during post-mortem aging, indicating some degradation of proteins and/or peptides. However, neither the increase in NPN nor free amino groups was related to post-mortem tenderization since these quantities increased only after most of the improvement in tenderness had occurred. Much of the increase in NPN or free amino groups may originate from degradation of sarcoplasmic proteins or peptides. It is suggested that weakening or breaks at crucial points in the sarcomere, such as at the junction of the Z-line with the thin filaments, occur within the first 48–72 hr post-mortem and that this weakening or cleavage is responsible for tenderization. Cathepsin D may be responsible for this weakening but most of the available evidence is against proteolysis as the primary cause of post-mortem tenderization.

INTRODUCTION

PROTEOLYSIS and its relationship to post-mortem tenderization have received considerable interest and emphasis. Since the early work of Hoagland *et al.* (1917) which indicated that the increase in nonprotein nitrogen and tenderness that occurred during post-mortem storage was due to proteolysis, substantial effort has been devoted to analysis of protein breakdown products, post-mortem tenderness changes, and the mechanism(s) responsible for these changes. It has been shown that during post-mortem storage free amino acids and nonprotein nitrogen increased in beef muscle (Niewiarowicz, 1956; Locker, 1960; Thompson *et al.*, 1961; Davey *et al.*, 1966; Gardner *et al.*, 1966), poultry muscle (Khan *et al.*, 1964; Miller *et al.*, 1965), rabbit muscle (Suzuki *et al.*, 1967), and fish muscle (Hodgkiss *et al.*, 1955). Other evidence indicating the occurrence of autolysis in skeletal muscle under aseptic conditions has been reported by Zender *et al.* (1958) and Sharp (1963).

In many instances post-mortem autolysis and tenderness have been attributed to catheptic activity. Cathepsins, principally cathepsin D (Parrish *et al.*, 1966, 1967; Iodice *et al.*, 1966), are very likely responsible for any post-mortem proteolysis that may occur because they are present in muscle and, obviously, they are abundantly supplied with substrate. Further evidence by Iodice *et al.* (1966) suggested that cathepsin D was the major protease present in muscle and that cathepsin A acted as a peptidase to further digest breakdown products of cathepsin D in skeletal muscle. Even with this information, the exact nature and function of cathepsins in post-mortem muscle remain an enigma. Despite the impressive amount of research concerning proteolysis, little relationship has been found between extent of proteolysis and tenderness in either beef (Husaini *et al.*, 1950a,b; Wierbicki *et al.*, 1956; Locker, 1960; Davey *et al.*, 1966) or poultry (Miller *et al.*, 1965), although Khan *et al.* (1964) suggested that proteolysis affects tenderness.

Storage at 2° and 16° has a profound effect on post-mortem muscle shortening (Locker *et al.*, 1963; Busch *et al.*, 1967) and post-mortem tenderization (Busch *et al.*, 1967). However, post-mortem storage at these two temperatures apparently does not affect the rate of ATP degradation and pH decline (Cassens *et al.*, 1966; Busch *et al.*, 1967), protein solubility (Chaudhry *et al.*, 1969), or ATPase activity of myofibrils prepared from post-mortem muscle (Goll *et al.*, 1967; Robson *et al.*, 1967). The effects of different storage temperatures on proteolysis have been investigated by Locker (1960), Gardner *et al.* (1966), and Suzuki et al. (1967). Results from these studies indicated that muscle tissue stored at higher temperatures had larger quantities of nonprotein nitrogen.

Recent efforts have also been made to quantitate changes in the individual amino acids during post-mortem storage. Locker (1960) and Ma *et al.* (1961) used paper chromatography to detect specific amino acids in beef muscle and Miller *et al.* (1965) and Suzuki *et al.* (1967) used column chromatography to identify amino acids in poultry and rabbit muscle. Satterlee *et al.* (1967) adapted the gas chromatography procedures of Lamkin *et al.* (1965) and Stalling *et al.* (1966) for the analysis of free amino acids in sausage products. They found that gas chromatography was a very capable method of quantitating individual amino acids of meat products.

The purposes of this study were to investigate the: effect of storage temperature on changes in nonprotein nitrogen; and quantitative changes in total and individual free amino acids and their relationship to tenderness during post-mortem storage at 2°.

MATERIALS AND METHODS

Experiment 1. Effect of temperature on nonprotein nitrogen

Semitendinosus and psoas muscles from five bovine animals were sampled immediately after exsanguination and after various times of post-mortem storage at 2°, 16°, or 37°. Muscles stored at either 2° or 16° were sampled after 6, 12, 24, 72 and 312 hr post-mortem, whereas muscles stored at 37° were sampled after 3, 6, 12 and 24 hr post-mortem. Not every muscle was sampled at each post-

mortem time, hut every muscle was sampled at death to give some indication of animal variation. After initial sampling, individual muscles were stored in cryovac bags, which were opened at the appropriate time post-mortem, and sampled. Sarcoplasmic proteins were extracted with 10 volumes (w/v) of 0.03 M potassium phosphate buffer, pH 7.4, by the procedure described by Chaudhry *et al.* (1968). NPN was determined on trichloroacetic acid (TCA) filtrates by adding one volume of 10% TCA to an equal quantity of sarcoplasmic protein extract.

After setting at room temperature for 30 min, the TCA-insoluble fraction was sedimented in an International centrifuge at 2000 × G for 15 min. The TCA-soluble fraction was carefully decanted and an aliquot was removed for NPN determination. Measurement of NPN was done by a modified method of Lang (1958). In brief, the procedure involved digesting 1 ml of TCA filtrate with 0.4 ml of digestion mixture on a micro-Kjeldahl digestion apparatus. Tubes containing ammonium sulfate standards accompanied the unknowns. After digestion, the samples were diluted to appropriate volumes and analyzed in duplicate by adding Nessler's reagent. Absorbance was read at 500 mμ.

Experiment 2. Quantitative changes in free amino acids and shear resistance

Wholesale ribs were removed from the left and right sides of 12 USDA Choice grade carcasses 24 hr post-mortem. The longissimus dorsi muscle was excised from the ribs, sampled, and the samples analyzed to give 24-hr Warner-Bratzler shear force and total free amino acid values. The remaining portion of the longissimus dorsi was vacuum packaged in a cryovac bag and stored at 2°. After 3 days at 2°, some samples were moved to a 30° environment for 24 hr and then returned to 2° storage until sampled. Steaks were periodically removed for Warner-Bratzler shear and chemical analyses. Warner-Bratzler shear and pH measurements were carried out by the procedure described by Goll *et al.* (1964a).

Fat and moisture were analyzed on both cooked and raw samples by AOAC (1955) procedures. Water soluble proteins were extracted with 10 volumes (v/w) of water for 2 hr at room temperature and the extracts centrifuged at 1600 × G for 20 min. The supernatant was decanted and the residue re-extracted with water for another 20 min period. After centrifugation at 1600 × G for 20 min, the two supernatant solutions containing the sarcoplasmic proteins were combined. An aliquot of the sarcoplasmic protein was taken and enough 30% TCA added to give a final concentration of 10% TCA. After setting at 25° for 30–60 min, the precipitated protein was sedimented at 600 × G for 20 min and the supernatant solution analyzed for free amino acids by Rosen's (1957) ninhydrin procedure. Leucine served as a standard. The sarcoplasmic protein precipitated by TCA was dissolved overnight in 0.1 N NaOH at 25°, filtered, and protein concentration of the filtrate determined by the modified (Goll *et al.*, 1964b) Folin-Lowry method (Lowry *et al.*, 1951).

Experiment 3. Quantitative changes of individual free amino acids by gas chromatography (GC)

Samples for GC were obtained from the longissimus dorsi of six USDA Low Choice beef carcasses after storage for 3, 7, 14 and 21 days post-mortem at 2°. The extraction, purification and formation of n-butyl-N-trifluoroacetyl derivatives of free amino acids were carried out by the procedure of Satterlee *et al.* (1967) with the exception that the desalting step was omitted. Amino acids were analyzed with an F & M Model 810 gas chromatograph equipped with dual hydrogen flame detectors. The column consisted of 1% neopentyl glycosuccinate on 60–80 mesh chromosorb W. The conditions of gas chromatographic analysis and quantitation of amino acids were those outlined by Satterlee *et al.* (1967). Amino acids were identified by comparing their retention times with those of pure standards.

Analysis of variance was conducted according to Snedecor (1956); Duncan's (1955) multiple range test was used to analyze for treatment differences.

RESULTS AND DISCUSSION

THE EFFECT of post-mortem storage at 2°, 16° and 37° on changes in NPN can be observed in Fig. 1. At 2°, NPN increased after 12 hr and then decreased after 24 hr post-mortem for both semitendinosus and psoas. Locker (1960) observed a similar trend in NPN during post-mortem storage and suggested this occurred because the muscle was in rigor. A marked increase of NPN in semitendinosus stored at 2° occurred after 72 hr and only a slight additional increase occurred after 312 hr. NPN values for muscle stored at 16° followed the same general trend as those for muscle stored at 2°. The anomalously high NPN content of semitendinosus stored at 16° for 312 hr was possibly a result of bacterial contamination since no particular measures were taken to prevent bacterial growth. Thus, there was essentially no difference between NPN values for a tender muscle, (psoas) and a less tender muscle (semitendinosus) stored at either 2° or 16°.

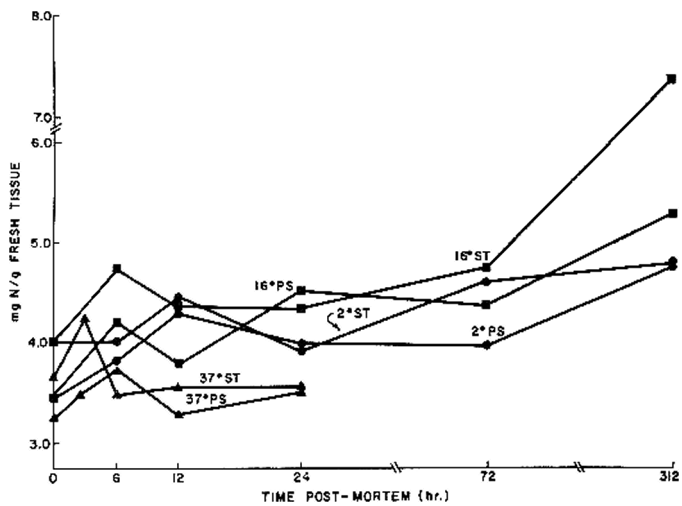

Fig. 1. The effect of post-mortem storage temperature on changes in nonprotein nitrogen of bovine semitendinosus (ST) and psoas (PS) muscle.

With the exception of degree of post-mortem shortening (Busch *et al.*, 1967), it appears that bovine muscles stored at 2° and 16° are similar in a variety of ways. For example, Cassens *et al.* (1966) and Busch *et al.* (1967) observed that pH drop and phosphorus degradation proceeded at about the same rate, Goll *et al.* (1967) and Robson *et al.* (1967) found similar ATPase activities in myofibrils or myosin B prepared after various times of post-mortem storage, and Chaudhry *et al.* (1969) obtained similar quantities of extractable myofibrillar proteins from muscle stored at these temperatures. This similarity of post-mortem muscle stored at 2° and 16° argues against the primary involvement of cathepsins, which are very temperature dependent, in post-mortem changes of muscle (Parrish *et al.*, 1967).

The low quality of NPN observed in muscle stored at 37° substantiates this view since proteolytic activity should be accelerated at 37° and result in considerable quantities of protein breakdown products, whereas smaller quantities of NPN was obtained from muscle stored at 37° than from muscle stored at either 2° or 16°. It is possible that the rapid drop in pH during storage at 37° caused aggregation of the myofibrillar proteins and some binding of NPN. However, the results of Suzuki *et al.* (1967) argue against this since they found an increase in NPN at 37° in rabbit muscle. It is also possible that some NPN was lost in the large exudation that occurs on storage at 37° and during mincing of the samples but such a loss could probably not by itself account for our failure to find an increase in NPN during post-mortem storage at 37°.

Since only small amounts of NPN were obtained from muscle stored at 37° and since bovine muscle becomes tender quite rapidly at this temperature (Busch *et al.*, 1967), our study indicates that there is no direct relationship between amount of NPN and tenderness. Data from muscle stored at 16° clearly indicates lack of relationship between NPN content and tenderness since tenderness measurements of muscle stored at this temperature are not confounded by the presence of post-mortem shortening which greatly affects tenderness (Herring *et al.*, 1965; Marsh *et al.*, 1966; Busch *et al.*, 1967).

On the other hand, excised muscle stored at 2° shortens considerably, exhibits a loss of tension corresponding to a resolution of rigor mortis, and requires a much longer post-mortem aging time to become tender than does muscle stored at 16° and 37° (Busch *et al.*, 1967) although muscle attached to the carcass stored at 2° essentially becomes tender during the first 24 hr of post-mortem aging (Goll *et al.*, 1964a). Hence, an experiment was designed to follow concomittantly the changes in free amino acids and tenderness of bovine longissimus dorsi muscle stored at 2°. The results of this experiment are presented in Tables 1 and 2 and Fig. 2. Tenderness increased from 1 to 7 days, but remained about the same thereafter. Statistically, the only significant difference ($P < .05$) in tenderness occurred between 1 day of storage and all other storage times except 3 days.

There was no difference in tenderness between 1 and 3 days of post-mortem storage (Table 1). Content of water-soluble amino groups of uncooked muscle during this time decreased after three days and then increased throughout the remainder of the aging period. The amino acid decrease at 3 days was not statistically different from all other aging times ($P < .05$). There was not any statistical difference between 21 and 28 day amino acid content (Table 2). Storing muscle at a temperature of 30°C for 24 hr, after 3 days of 2° post-mortem storage had no significant effect on free amino acid production or tenderness.

A significant increase in free amino acids has occurred, but only a small proportion of this increase occurs during the period of maximum change in tenderness. It is also not entirely clear that this increase in free amino acids is due to enzymic proteolysis although such proteolysis is one likely cause. Even at 2°, it appears that there is no direct relationship between tenderness and free amino acid content since the largest quantities of amino acids are

Table 1. Mean values for Warner-Bratzler shear force values on cooked muscle.[1]

Post-mortem time (days)	1	3	7	14	21	28
Shear force (Kg/cm²)	1.95	1.61	1.29	1.27	1.29	1.26

[1] Means not underlined by the same line are significantly different at $P < .05$.

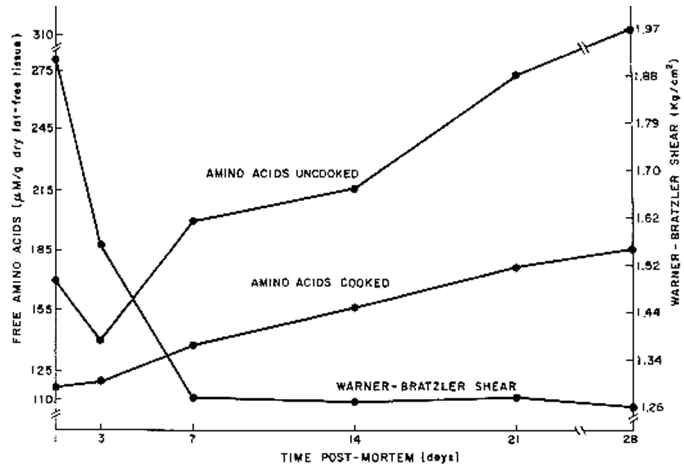

Fig. 2. The effect of post-mortem aging at 2° upon the relationship between tenderness of cooked and free amino acids of cooked and uncooked bovine longissimus dorsi muscle.

Table 2. Mean values for amino acids on uncooked and cooked samples.[1]

Post-mortem time (days)	3	1	7	14	21	28
Uncooked	140.2[2]	169.9	198.6	216.9	272.7	312.2
Cooked	120.3	117.7	138.0	157.3	176.8	186.6

[1] Means not underlined by the same line are significantly different at $P < .05$.
[2] Means are expressed as mg of free amino acids per gram dry fat-free tissue.

liberated during the post-mortem time of 7-28 days, whereas tenderness remains essentially unchanged during this period.

There is a gradual increase in amino acids from cooked meat with increasing time of post-mortem storage although considerably less ninhydrin positive material was extracted from cooked muscle than from uncooked muscle (Table 2 and Fig. 2). Since cathepsin activity is accelerated at temperatures up to 45°C (Parrish *et al.*, 1967), an increase in extractable amino acids would have been expected if cathepsin or other proteases were involved in production of extractable amino acids. Paul *et al.* (1966) found increased sarcoplasmic protein solubility of rabbit muscle during cooking between 45° and 60° and suggested that this increase may be the result of proteolytic activity. Several possibilities exist which may explain the extraction of smaller amounts of amino acids in cooked muscle.

One possibility is that enzymic proteolysis does not occur in significant amounts in post-mortem muscle and that our observed increase in extractable amino acids is due to some unknown factor whose activity is not strictly proportional to heat. Another possibility is that extensive binding of free amino groups to sites on the denatured protein occurs during cooking, and a third possibility is that amino acids are leached out of the tissue during cooking. The third possibility is probably partially responsible for our findings since Ginger *et al.* (1954) found that the amount of NPN was always greater in cooked steak plus drippings than in raw meat. It is also difficult to eliminate the possibility that some binding of water-soluble amino groups occurs to sites on proteins denatured during cooking.

Another means of detecting possible proteolytic activity has been to measure changes in water-soluble protein in the anticipation that proteolysis of myofibrillar proteins would release some water-soluble fragments causing an increase in total water-soluble protein. Furthermore, investigation of changes in water-soluble proteins of cooked meats could be a measure of proteolysis since temperature up to 45° (Parrish *et al.*, 1966) accelerates protease activity. If cathepsins are important contributors to post-mortem proteolysis of myofibrillar proteins, more water-soluble protein could be expected during cookery because of accelerated catheptic activity. As can be observed in Table 3, water-soluble protein extractable from either raw or cooked tissue did not change significantly during storage. It is obvious that considerably less water-soluble protein was extracted from the cooked sample than from the uncooked sample. The low water-soluble protein content of cooked muscle agrees with the results of Ginger *et al.* (1954) and Paul *et al.* (1966).

Table 3. Water-soluble protein of uncooked and cooked samples during aging (mg protein/g dry fat-free tissue).

Time post-mortem (days)	Uncooked	Cooked
1	144.8	7.0
3	139.6	3.5
7	138.6	8.1
14	152.8	13.2
21	138.1	17.1
28	139.1	13.6

Table 4. pH values for uncooked and cooked samples during aging.

Time post-mortem (days)	Uncooked	Cooked
1	5.44	5.67
3	5.35	5.52
7	5.48	5.68
14	5.53	5.68
21	5.54	5.70
28	5.56	5.70

Both aging and cookery have a significant effect on pH (Table 4). In every case, pH of the cooked sample was higher than pH of the raw sample taken adjacent to the cooked sample. Very little change in pH of the cooked sample occurred after 7 days.

Since the myofibrillar proteins contain very high proportions of the charged amino acids (Kominz *et al.*, 1957), quantitative analysis of the individual amino acids appearing during post-mortem storage might indicate whether these amino acids had originated from the myofibrillar proteins. Fig. 3 depicts a typical gas chromatogram of amino acids obtained from muscle stored at 2°. Generally, 12 peaks were obtained which included the internal standard, norleucine and 3 unresolved peaks consisting of threonine-leucine, proline-serine and aspartic acid-phenylalanine.

The peaks identified in increasing order of retention time were: alanine, valine, isoleucine, glycine, threonine-leucine, norleucine, proline-serine, methionine, aspartic acid-phenylalanine, glutamic acid, lysine and tryptophan. In general, free amino acids increased during storage (Table 5). Of these, isoleucine, tryptophan, valine,

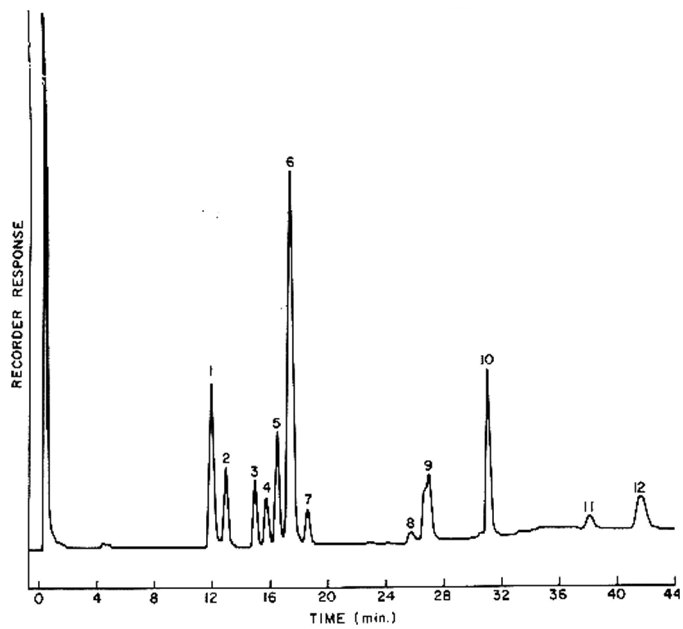

Fig. 3. A typical gas chromatogram of amino acids from bovine longissimus dorsi muscle. Number peaks are: 1 (ala), 2 (val), 3 (ileu), 4 (gly), 5 (thre-leu), 6 (norleu), 7 (pro-ser), 8 (met), 9 (asp-phe), 10 (glu), 11 (lys), and 12 (try).

threonine-leucine and aspartic acid-phenylalanine increased as much as four- to six-fold during storage. These results confirm those of Suzuki *et al.* (1967) who found marked increases in isoleucine, leucine and phenylalanine as well as in arginine and tyrosine during storage of rabbit muscle at both high and low temperatures.

In our study, glutamic acid gave an anomalously high value at 14 days, but this may have been the result of bacterial contamination. Gardner *et al.* (1966) also found a large increase in glutamic acid, but they attributed this increase to bacterial metabolism of glutamine. They also obtained very large increases in tryptophan and suggested that this increase was due to autolysis. Although our results show an increase in tryptophan and other amino acids, they do not permit any definite conclusions regarding the source of the increased free amino acids occurring during post-mortem storage. In view of the relatively low increase in glutamic acid, however, it seems likely that much of the increase in free amino acids does not originate from the myofibrillar proteins.

CONCLUSIONS

1) There is an increase in free amino acids and NPN in post-mortem bovine muscle, this increase indicating some breakdown of protein and/or peptides during post-mortem storage; and 2) this increase in free amino acids or NPN is not related to the post-mortem increase in tenderness; in fact, the amino acid increase is observed only after most of the improvement in tenderness has already occurred.

The lack of a direct relationship between the release of free amino acids and post-mortem tenderization may be explained in several ways. Most investigators would agree that the sarcoplasmic proteins are not directly involved in tenderness and that improvements in tenderness during post-mortem aging must come from changes in the myofibrillar or connective tissue proteins. Thus, if the additional amino acids observed during post-mortem storage originate from the sarcoplasmic protein fraction, their appearance would not be expected to bear any relationship to post-mortem tenderization.

On the other hand, even the release of free amino acids from the myofibrillar or connective tissue proteins would not necessarily be directly related to post-mortem tenderization. Indeed, it is easy to see that a very few selective cleavages at crucial sites in the myofibril, such as the junction of the thin filaments with the Z-line, would result in a substantial improvement in tenderness, whereas extensive removal of amino acids from the N- or C-terminus of the myosin peptide chain would have very little effect of tenderness. Such limited and selective cleavages would be extremely difficult to detect by the classical methods of estimating protein breakdown, such as precipitation of protein and determination of TCA-soluble amino acids or nitrogen. In fact, if a molecule such as myosin were broken near its center, both pieces would still be precipitable by TCA and the cleavage would not be detectable by measurements on the TCA-supernatant.

Although proteolysis has been classically considered as a possible mechanism responsible for increase in free amino acids and post-mortem tenderization, the lack of this relationship, and the increase in free amino acids in our study, require a closer examination. It cannot be unequivocally stated that this increase originates totally from the enzymic hydrolysis of protein and it may be instructive at this point to evaluate the evidence concerning proteolysis in post-mortem muscle.

Based on their known vulnerability to trypsin, there are three myofibrillar sites that might be expected to be affected by cathepsins. The first of these is myosin, which is very quickly cleaved into two macromolecular fragments, light and heavy meromyosin, by either trypsin (Mihalyi *et al.*, 1953) or chymotrypsin (Gergely *et al.*, 1955). The work of Bodwell *et al.* (1964) and Martins *et al.* (1968) show that neither myosin nor actomyosin is affected by cathepsins partially purified from muscle, and Goll *et al.* (1967) and Robson *et al.* (1967) have shown biochemically on the basis of ATPase activity that there is no appreciable change in the myosin molecule *in situ* for as long as 13 days post-mortem at either 2° or 16°. Together, these two lines of evidence strongly suggest that myosin itself is not proteolytically cleaved during post-mortem aging.

Table 5. Quantitative post-mortem changes in free amino acids of bovine longissimus dorsi muscle.[1]

Peak No.	Amino acid	Time post-mortem (days) 3[2]	7[2]	14[3]	21[2]
1	Ala	5.02 ± .23[4]	6.95 ± .24	7.81 ± .52	9.25 ± .16
2	Val	0.92 ± .05	2.46 ± .14	2.12 ± .14	4.71 ± .11
3	Ileu	0.60 ± .04	0.60 ± .02	1.49 ± .13	3.50 ± .10
4	Gly	1.20 ± .07	1.27 ± .05	1.66 ± .13	2.76 ± .07
5	Thr-Leu	1.44 ± .08	1.71 ± .05	3.80 ± .30	6.44 ± .21
7	Pro-Ser	1.12 ± .04	0.81 ± .14	1.42 ± .08	2.79 ± .05
8	Met	1.15 ± .06	0.86 ± .07	1.20 ± .30	1.73 ± .07
9	Asp-Phe	1.31 ± .05	1.48 ± .07	3.60 ± .26	5.76 ± .13
10	Glu	7.23 ± .32	8.78 ± .31	15.99 ± 1.16	8.29 ± .31
11	Lys	3.84 ± .75	5.29 ± .30	11.01 ± .70	10.60 ± .31
12	Try	0.58 ± .10	0.73 ± .18		3.93 ± .98

[1] Mg amino acids/100 g fresh tissue.
[2] Average of three replicates.
[3] Average of two replicates.
[4] means plus or minus standard errors.

The second proteolytically-vulnerable site in the myofibril is the tropomyosin-troponin complex, which has been shown to be rapidly degraded to small peptides by trypsin (Laki, 1957; Ebashi *et al.*, 1966). Biochemical evidence on Ca^{++}-sensitivity of the ATPase activity of post-mortem myofibrils has shown that neither troponin nor tropomyosin are proteolytically destroyed, even after 13 days post-mortem at 16° (Galloway *et al.*, 1967; Goll *et al.*, 1967).

A third possible site for post-mortem proteolysis of myofibrils is at or near the Z-line. Stromer *et al.* (1967a) have shown that trypsin very quickly removes the Z-line from myofibrils and there is some available evidence suggesting that there may be alteration of Z-lines during post-mortem storage. Using phase microscopy, Stromer (1967) have shown that after 13 days of post-mortem storage at either 2° or 16°, bovine myofibrils are more subject to shearing by homogenization into pieces only three to four sarcomeres in length than immediately after death; this shearing occurs at the level of the Z-line.

Takahashi *et al.* (1967) have also reported increased shearing of myofibrils after 4 days of post-mortem storage of chicken muscle at 2°. Moreover, Fukazawa *et al.* (1967) have reported complete loss of Z-line structure in chicken muscle after 24 hr at 2°. These findings suggest that there may be some catheptic destruction of the Z-line in post-mortem muscle, but Fukazawa *et al.* (1967) have found that incubation of at-death myofibrils, having intact Z-lines, with cathepsins does not produce any loss of Z-line structure. Furthermore, Stromer *et al.* (1967b) have shown that it is possible to completely remove Z-lines from myofibrils by simple extraction with low ionic strength solutions. It is therefore possible that a combination of post-mortem events, including pH changes, may cause loss of Z-line structure in post-mortem myofibrils rather than cathepsins alone.

Most of the available evidence suggests that the myofibril itself is not proteolytically cleaved during post-mortem storage and that most of the increase in free amino acids observed in post-mortem muscle may originate from the sarcoplasmic proteins (Sharp, 1963; Bodwell *et al.*, 1964). Post-mortem proteolysis of the myofibril must be extremely limited and perhaps even synergistic with the effects of pH or other variables, and it most likely occurs at or near the Z-line. The proof of such proteolysis awaits the demonstration that purified lysosomal enzymes or cathepsins can alter the Z-line or the I-Z junction of at-death myofibrils. Such experiments are now underway in our laboratory.

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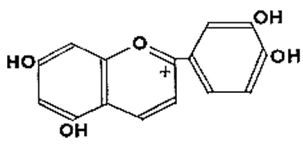
Tannins of Grain Sorghum: Luteoforol (Leucoluteolinidin), 3',4,4',5,7-Pentahydroxyflavan

SUMMARY—The principal tannin of sorghum is a leucoanthocyanin yielding luteolinidin (3',4',5,7-tetrahydroxyflavylium) when heated with mineral acid. The precursor, luteoforol, has most of the properties of 3',4,4',5,7-pentahydroxyflavan prepared by reduction of eriodictyol. Luteoforol, when treated with concentrated mineral acid in the cold, gives a purple color with λ_{max}550nm. A method for the determination of luteoforol in sorghum, based on this property, is described. The results with a number of varieties of sorghum are compared with those obtained by the AOAC Folin-Denis method. The contribution of luteoforol to the "tannin" so determined varies from 1 to $\leq$ 25%. Except for one sample of Kaffir corn, which contained leucocyanidin as well as luteoforol, no other tannins were detected. The "tannin" content varied widely, (from 0.05 to 0.67% as tannic acid), a white-skinned variety having the least. The uniformity of commercial samples can be rapidly evaluated by single-grain determinations of luteoforol.

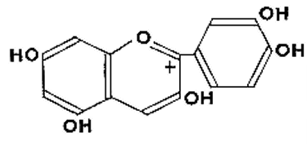
INTRODUCTION

ALTHOUGH almost unknown in England, sorghum is an important cereal crop for animal feeding in the United States and an important food grain in many tropical countries. As a cereal crop it is especially well adapted to hot, semiarid areas. Its use as food could be greatly exanded in much of the world if need arose, but to do so it would be necessary to improve the grain quality and acceptability of present varieties (Kramer, 1959). The low palatability of most varieties is largely due to a high tannin content. The presence of tannins in a cereal grain is most unusual, with barley the only other cereal in which they are known to occur. Those in sorghum are only found in grasses of the tribe Andropogoneae (Bate-Smith et al., 1967), to which sorghum belongs. These, when heated with mineral acid, yield the very uncommon anthocyanidin, luteolinidin.

It is interesting that this pigment is formed in the hypocotyls of seedlings of the sorghum variety "Wheatland" when grown in the dark (Stafford, 1965). Luteolinidin differs from cyanidin, the most commonly occurring of all anthocyanidins, in the absence of a hydroxyl group from the 3-position, having its absorption maximum at 496 nm compared with that of cyanidin at 535 nm:



luteolinidin



cyanidin

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A further distinctive feature of this new type of leucoanthocyanin is the formation of a blue color, with an absorption peak at 550 nm, when treated with concentrated mineral acid *in the cold*. Most samples of sorghum grain so treated behave in a similar way, rapidly giving a deep blue-purple color with concentrated HCl or H_2SO_4, which intensifies and becomes redder on standing. Spectrometric examination shows that two reactions are, in fact, taking place, one producing a blue-purple color with an absorption peak at 550 nm, and a second, slower than the first, producing an orange-yellow color with an absorption peak at 465 nm. It seemed possible that one, or perhaps both, of these reactions might provide a practical method of evaluating the tannin content of sorghum. The present paper describes work which has been done to investigate this possibility, to extract the chromogens from the grain, and to consider their contribution to the tannin content as determined by the AOAC method.

COLOR DEVELOPMENT IN SITU

THE FIRST objective was to determine the acid treatment conditions favorable to pigment absorption at 550 nm, while minimizing pigment(s) absorption at lower wavelengths. The variables considered were the composition of the medium, the nature and concentration of the acid, temperature, the presence or absence of oxygen and the state of subdivision of the experimental material. It was established that absolute alcohol (methanol) was preferable to alcohol-water mixtures, and that the substitution of nitrogen for air had no effect on the course of the reactions. Both concentration of acid and temperature were, however, important. Because of its freedom from water, sulphuric acid was preferred to hydrochloric acid; no other acids have so far been tested, but phosphoric and toluene *p*-sulphonic acids have been considered. The effect of temperature was studied at room temperature, 25°C, and in cold rooms at 1–2°C.

Material

Unless otherwise stated, the sorghum used for this work was a sample of Kaffir corn supplied by British Oil and Cake Mills through the Poultry Department, Unilever Research Laboratories, Colworth House, Sharnbrook, Bedford.

General procedure

Grain samples (0.5 g) were shaken gently with 50 ml of the acid reagent in 250 ml stoppered, round-bottomed flasks at specified temperatures for periods of 1–3 days. Absorption curves of the colored extracts were recorded

at room temperature between 650 and 420 nm using a Unicam SP 800 recording spectrophotometer with a 1 cm cell. Absorbancy was measured between the steady baseline at 650 nm and that at 550, 465, or (in particular instances) 496 nm. Typical absorption curves are given in Figs. 1 to 4. Dilution, if necessary, of the extract was carried out quantitatively with methanolic H_2SO_4 of the same concentration.

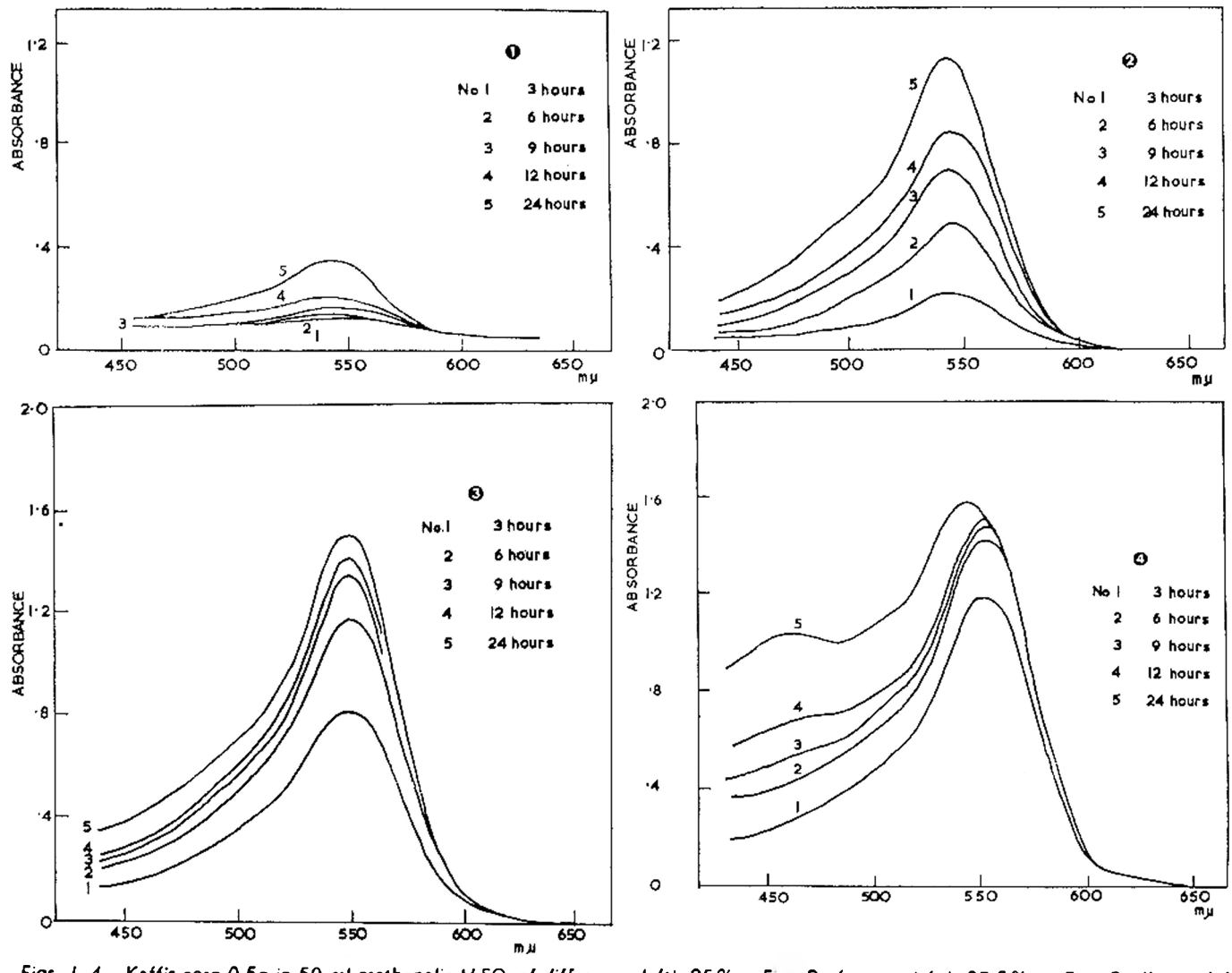
Effect of concentration of acid

Preliminary experiments with concentrated H_2SO_4 added dropwise with rigorous cooling to grains immersed in methanol showed that an increase in acid concentration beyond 50% by volume did not increase either the rate of development or the ultimate depth of color but would ultimately result in charring. Mixtures of methanol and sulphuric acid were therefore made up at suitable intervals between 25 and 50% by volume. After 24 hr treatment in a cold room the 550 nm absorption of the 25% H_2SO_4 extract was less than a quarter that of the 50% H_2SO_4 extract (Fig. 1), that of a 43% H_2SO_4 extract being almost equal to the latter.

With increasing acid concentration the 465 nm absorption progressively increased, the absorption curve of the 50% H_2SO_4 extract showing a marked peak at this wavelength (Fig. 4). The optimum concentration of acid, combining the maximum absorption at 550 nm with relatively little at 465 nm was 43%. At all concentrations of acid except 25% no further development of absorption at 550 nm occurred beyond 24 hr; in fact, beyond this time absorption tended to decrease. For the purposes of routine analysis of whole grain, extraction at approximately 0°C for 24 hr with 43% (by volume) methanolic H_2SO_4 has therefore been adopted.

Effect of temperature

The effect of temperature was studied in detail with methanolic extracts of the grain. In general, both with whole grain, milled grain and extracts, development of absorption below 550 nm was more marked and stability of the 550 nm absorption lower at room temperature and 25°C than at 0°C. For these reasons, no attempt was made to work with whole grain at these more elevated temperatures.



Figs. 1–4—Kaffir corn 0.5g in 50 ml methanolic H_2SO_4 of different concentrations, shaken in cold room (1–2°C). Fig. 1. (upper left) 25%. Fig. 2. (upper right) 37.5%. Fig. 3. (lower left) 43%. Fig. 4. (lower right) 50%.

Effect of milling

Three kinds of laboratory mill have been tested: (1) a Brabender mill, which divides the product into three streams roughly corresponding with bran, a middle fraction and flour; (2) a Wiley mill, which enables varying degrees of subdivision to be achieved, but is difficult to work with quantitatively; and (3) a Moulinex domestic coffee mill. The last of these was by far the most convenient and was used for the tests described here.

With the flour, as would be expected, color development was much more rapid than with whole grain (Fig. 5) and led to a higher 550 nm absorption at 24 hr. The form of the absorption curve suggests, however, that absorption at 550 nm is increased by irrelevant absorption due to other constituents having peaks at lower wavelengths (cf. curve 3 in Fig. 5 with curve 5 in Fig. 3). While, therefore, results can be obtained much more quickly by milling the grain, this advantage is offset by their lower reliability.

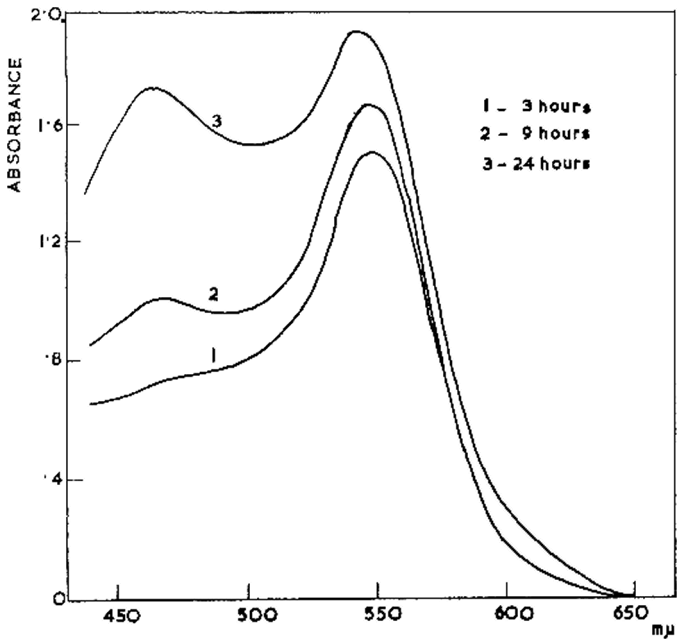


Fig. 5—0.5g milled Kaffir corn in 50 ml 43% methanolic H_2SO_4, shaken in cold room (1-2°C).

EXTRACTION OF THE CHROMOGENS

IN A PRELIMINARY study of the effects of concentration of acid and temperature on the development of color in methanolic extracts, a stock solution was prepared by extracting, with continuous shaking, 1 g of Kaffir corn with 50 ml methanol for 24 hr in the cold room. 2 ml aliquots of this extract were then acidified with H_2SO_4, with rigorous cooling, in such proportions that the resulting concentrations of H_2SO_4 in the individual solutions were 25, 37.5, 43 and 50% by volume. The solutions were then kept up to 72 hr at 0° and 25°C. The increase in absorption with time at 465 and 550 nm is shown in Tables 1 and 2, and the form of the absorption curves after 3 hr in Figs. 6 and 7.

At both temperatures the rate of development of 550 nm absorption was nearly maximal at 37.5% H_2SO_4, but at 25° absorption at 465 and 500 nm was also strongly in evidence at this and even lower concentrations of acid. At 50% H_2SO_4 absorption at these lower wavelengths was so marked that the peak at 550 nm was little more than a shoulder. It is clear that at 25° 43% H_2SO_4 is the upper limit of concentration of acid for measurements of absorption at 550 nm to be meaningful.

The absorption at 465 nm, moreover, continued to increase steadily with time, whereas that at 550 nm increased very little after 3 hr. In extracts, therefore, a much shorter period of color development is indicated than in the case of intact grains.

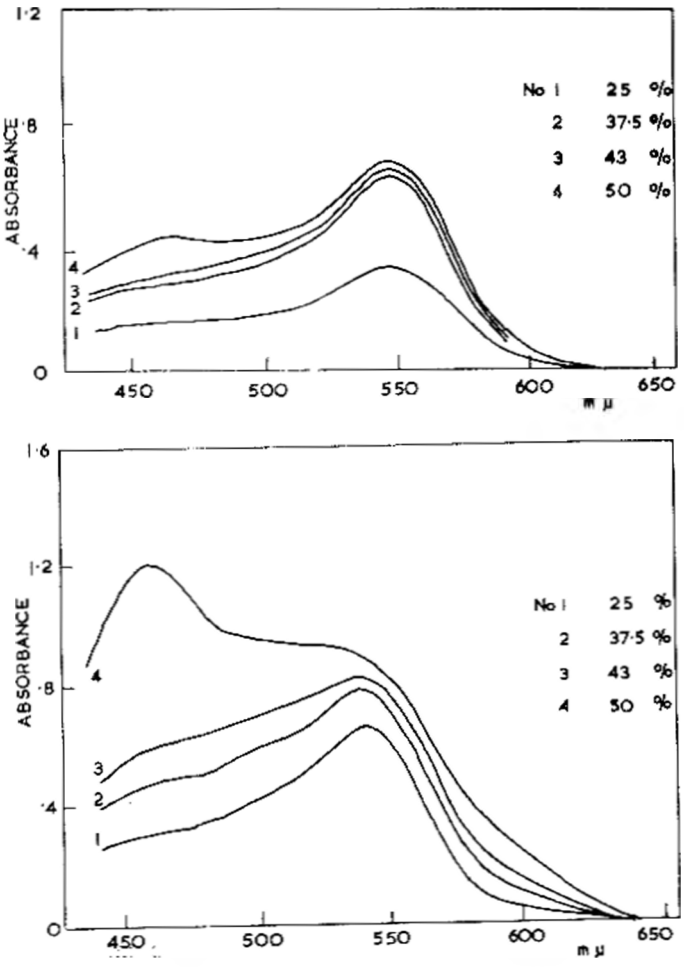
A further observation was that absorbance values of the extract were only one-half those of the intact grain. This clearly required that the extractability of the chromogens from the grain should be studied. Experiments were therefore carried out both with longer times of extraction with

Table 2. Extraction of the same sample of Kaffir corn at 0° with successive volumes of methanol.

Extract	Time of extraction (hr)	Absorption at 550 nm of extract
1st	24	0.64
2nd	24	0.355
3rd	24	0.125
4th	48	0.265
	Total	1.385

Table 1. Extraction of replicate samples of Kaffir corn with methanol for different times.

Temperature	Time of extraction (hr)	Absorption of extraction at 465 nm	Absorption at 550 nm of extract of residue		
			A	B	A & B
0°	24	0.29	0.42	1.01	1.43
	48	0.44	0.645	0.94	1.585
	72	0.63	0.915	0.95	1.865
	96	0.68	0.885	1.04	1.925
25°	0	.....	.....	1.50	1.50
	3	0.22	0.355	1.09	1.44
	6	0.29	0.39	0.91	1.30
	9	0.40	0.455	1.09	1.54
	14	0.51	0.52	1.04	1.56
	24	0.59	0.57	0.96	1.53
	48	0.78	0.67	0.86	1.53
	72	0.72	0.64	0.96	1.60



Figs. 6 and 7—Kaffir corn extracted with methanol at 1–2°C for 24 hr, the extracts adjusted to the concentration of H_2SO_4 as shown, and allowed to stand for 3 hr at 1–2°C (Fig. 6, top) and 25°C (Fig. 7, bottom).

a single volume of solvent and with repeated change of solvent, attention being especially directed to the 550 nm component. As a measure of completeness of extraction, residual color development in the grain after extraction was determined (at 0°) as described earlier. Results of experiments at 0° are set out in Tables 1 and 2.

The final residue gave an absorption of 0.95 so that even after exhaustive extraction of the grain with methanol there was still an appreciable amount of unextracted chromogen. It is obvious, therefore, that it is better to estimate the chromogen by direct development with methanolic H_2SO_4 in the intact grain, rather than to attempt the quantitative extraction of the chromogen with methanol.

Methanolic extraction of flour

Since extraction of intact grain for long periods and with repeated change of solvent did not succeed in completely extracting the chromogens, the attempt was repeated with milled grain ("flour"). The flour was prepared in a Moulinex mill and 0.5 g samples were extracted with 50 ml methanol at 25° for periods between 15 min and 72 hr. Both the extract and the residue were developed at 0° with 43% methanolic H_2SO_4. Maximum absorption at 550 nm (1.36 units) was developed after 30 min. Extraction of the residue remained fairly constant (average 0.65) for 48 hr, but absorption at 465 increased progressively from 1.02 at 30 min to 1.34 at 72 hr. While extraction of 550 nm chromogen was more rapid and was higher with flour than with whole grain, it was still not complete, and any advantage of using milled grain was offset by the much greater extraction of 465 nm chromogen.

A question to be decided was whether water might be a more effective extractant than methanol, but since development with mineral acid in presence of water had been shown to be unsatisfactory, the answer to this question had to be obtained indirectly, as described in the next section.

TANNIC ACID EQUIVALENT OF SORGHUM EXTRACTS AND OF LUTEOFOROL

THE TANNIN content of grain sorghum has been shown to be important in relation to their feeding value (Chang et al., 1964; Fuller et al., 1966). Tannin content is determined by the Folin-Denis method (A.O.A.C. Official Methods of Analysis) in the hot aqueous extract of the grain and is expressed as equivalents of tannic acid percent of the grain as fed.

Bate-Smith et al. (1967) suggested that, as a leucoanthocyanin, the 550 nm chromogen would have the properties of a tannin. On the reasonable assumption that the chromogen was, in fact, 3′,4′,4,5,7-pentahydroxyflavan, this substance has been prepared by borohydride reduction of eriodictyol, the corresponding flavanone, and its tannic acid equivalent determined by the Folin-Denis method.

For the sake of brevity, this substance will, from now on, be referred to as luteoforol.

Preparation of luteoforol

Three hundred mg of eriodictyol were suspended in 5 ml H_2O, cooled in ice, and solid sodium borohydride added with stirring until vigorous effervescence ceased. At this point all the suspended solid had dissolved. The pale yellow solution was kept for 1 hr in the cold room and was then acidified with *N* acetic acid. An equal volume of *n*-propanol was added, the solution saturated with NaCl, the upper layer so formed evaporated to dryness in a stream of cold air, and the solid redissolved in methanol. The UV absorption of this solution, and the shift on addition of KOH (Fig. 8) showed only a very slight trace of unreduced eriodictyol (peak in KOH at 327 nm). Reduction to the flavan-4-ol seems, therefore, to have been very nearly complete. The solution was, however, brown in color, deepening with every manipulation, and this color could not be removed by selective solvent extraction of the dry solid. The latter was, therefore, used without further purification for a preliminary study of the properties of luteoforol and to determine the approximate stoichiometric equivalent of its 550 nm absorption and its tannic acid equivalent in the standard Folin-Denis determination.

Properties of luteoforol

The outstanding property is the formation of a blue-rose pigment when acted upon in the cold by mineral acid. This reaction took place even with 2*N* HCl in presence of amyl

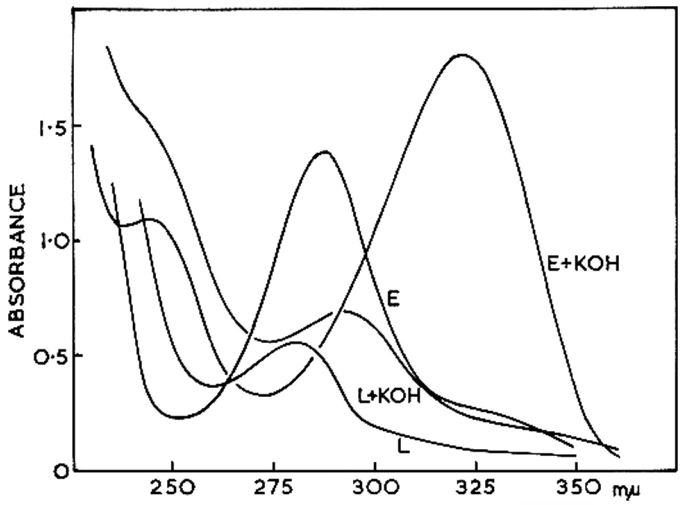


Fig. 8—UV absorption curves of eriodictyol and its reduction product (luteoforol).

alcohol, the alcoholic layer almost immediately becoming rose and eventually deep crimson purple. On keeping at room temperature this gradually became orange brown, the solution absorbing over a wide range between 420 and 500 nm.

When heated with 2*N* HCl the blue-rose color first formed disappears, the solution becoming colorless and then slowly yellow. The pigment then formed has the spectral and chromatographic properties of luteolinidin, viz. an absorption peak at 496 nm and R_f 0.63 in Forestal solvent. It was suggested by Blessin et al. (1963) that this pigment might be fisetinidin and by Yasumatsu et al. (1965) that it might be pelargonidin.

The borohydride reduction product of eriodictyol, which appears to consist almost entirely of luteoforol, has a single peak in the UV at 281 nm in methanol, shifting to 287 on addition of KOH.

When freshly prepared, luteoforol is colorless, or almost so, but slowly becomes orange-brown at room temperature both in solution and in the solid state.

It is strongly adsorbed on hide powder. When tested by the official chrome-tanned hide powder method of the Society of Leather Trades Chemists (1965, SL 2/3), the 550 nm absorption of an aqueous solution was decreased by 90% and that of a methanolic solution by 78%.

It is also adsorbed by cellulose. When chromatographed in Forestal solvent it reacted with the HCl in the solvent forming a mauve-colored streak from the start-line as far as R_f 0.6.

It reacts with Folin-Denis reagent, 1.0 mg of the reduction product having a tannic acid equivalent to 1.5 mg. As the major Folin-Denis reacting constituent of some varieties of sorghum, it is probably responsible for the decreasing feed value of grain with increasing percent of tannin (Chang et al., 1964; Fuller et al., 1966).

In general, these properties all suggest that luteoforol as prepared, and as it exists in the grain, is in a polymeric form. In this respect it resembles the "classical" leucoanthocyanins, the flavan-3,4-diols (Bate-Smith et al., 1956). In the grain, it is not ruled out that the chromogen may be combined with, for instance, sugar groups, or in other complex forms. The fact that it has not been possible to extract it completely with methanol indicates that part of it at least is strongly bound within the tissues. The methanol extracted chromogen does *not* react immediately with 2*N* HCl in presence of amyl alcohol, as does the borohydride reduction product, probably indicating a more complex physical or chemical state in the naturally-occurring tannin.

Finally, the eriodictyol reduction product is markedly astringent to the taste. This is probably the most significant of all the properties enumerated in relation to the use of sorghum as food or feed, and is certainly the one which most qualifies luteoforol, in the context of food, to be described as a tannin (Bate-Smith, 1954).

Luteoforol equivalent of 550 nm absorption

The absorbance developed in methanolic H_2SO_4 solutions depends on the way in which the reduction product is acidified. Values of absorbances at 550 nm as high as 450 per g per 100 ml in a 1 cm cell have been obtained by adding concentrated acid rapidly to the methanolic solution. When the addition is made in circumstances as near as possible to those prevailing during the treatment of intact grain, viz., by adding the methanolic solution of the reduction product slowly, with stirring, to ice-cold 43% methanolic H_2SO_4, the absorbance lay between 300 and 340 (4 separate determinations). When kept in the cold room for 24 hr the absorbance gradually decreased to between 250 and 270. For the purposes of calculating the luteoforol equivalent of the absorption developed in the complicated situation of intact grain being shaken for 24 hr in the cold room with 43% methanolic H_2SO_4 the round value of 300 units per g per 100 ml, based on these data, is provisionally assumed. This is likely to give a minimum value for the actual luteoforol content of the grain.

The tannic acid equivalent of the eriodictyol reduction product

The determination was carried out according to the A.O.A.C. Official Method. In this method the transmission of the blue solution obtained when the Folin-Denis reagent is allowed to react with known amounts of tannic acid is used to construct a calibration curve, from which the "tannin" content of the unknown solution can be read off in terms of tannic acid equivalent. With eriodictyol itself (which is not, of course, a tannin) the average value of the tannic acid equivalent per 1.0 mg was 0.70 mg. With the solid reduction product (luteoforol) the corresponding value was 1.50 mg. The flavan-4-ol has, therefore, almost exactly twice the reducing power of the original flavanone.

These data for the conversion of 550 nm absorption units and Folin-Denis tannic acid equivalent can now be applied to the results obtained with various samples of sorghum.

VARIATION IN THE TANNIN CONTENT OF SORGHUM

THE VARIETIES used by Fuller et al. (1966) in their study of the feeding value of grain sorghums ranged in tannin content (% of tannic acid equivalent) from 0.2

(Kaffir) to 2.0 (Sargrain and NK 230). The tannin content showed some correlation with seed-coat color, those with the lowest having white or yellowish seeds, while the highest had red-brown or dark brown seeds. The sample of Kaffir corn used in the present experiments had an overall tannin content of 0.45, but it was not a uniform one, the grains varying from light brown to red-brown, and also varying in important respects anatomically (Miss J. Rest, personal communication).

Variation in pigmentation can occur in three distinct layers of the seed-coat: in two outer layers, the epicarp and the hypocarp which together make up the pericarp, and an inner layer, the testa, which is separated from the pericarp by a colorless layer, the mesocarp. The pigmented layers may, or may not, also contain tannin, and any one of them may be completely absent, all these situations being genetically determined (Quinby et al., 1954). If the pericarp is absent the grain is white, but may still possess an underlying, colored testa. Several experimental genotypes have been made available, and the results obtained with them, and a number of commercial varieties, which are given in Table 3, show how complex the situation is.

Sample 1, a white Jowar, was a variety produced in the course of the breeding program sponsored by the Rockefeller Institute at the Institute of Agricultural Research, New Delhi, the only pigment present being a minute area at the hilum. This sample shows that genotypes can be produced almost completely free from both tannin and pigment.

Sample 7 and Kaffir corn were the only samples in which a testa was present, but whereas the latter had pigment and tannin present both in the epicarp cell contents and the testa, the former had none. Pigment was present, however, in the cell-wall of the epicarp, so that the grains were similar in appearance. Samples 2 and 3 were of American origin, samples 4 and 5 African, sample 4 being described as a high-tannin, "bird-proof" variety.

Kaffir corn differed from all other samples in producing cyanidin as well as luteolinidin when heated with 2*N* hydrochloric acid. This proved to he due to the presence in the sample of about 20% of grains differing both in appearance and anatomical structure from the remaining grains. The difference consisted in the color being rose-brown ("bronze") rather than red, and also appearing very dark under the UV laamp. The bronze grains, but not the red ones, had a testa, which stained black with ferric chloride, and it was these grains, and only these, which produced cyanidin when heated with HCl. The cyanidin must be present in the testa as leucocyanidin, since no cyanidin was extracted from the grains into cold aqueous HCl. Apart from the occurrence of leucocyanidin in barley, where it is present only in the endosperm, this is the first recorded occurrence of leucocyanidin in the Gramineae. Cyanidin is, however, invariably the anthocyanidin present in the stems, leaves and glumes of all the pigmented grasses which have so far been examined.

The question now arises as to how to account for the "tannin" determined by the AOAC method, but not accounted for by luteoforol. Much of this must be attributed to the cinnamic acid derivatives present. These were shown by chromatography in Forestal solvent and toluene-acetic acid to be *p*-coumaric, ferulic and caffeic acids in varying amounts. The presence of caffeic acid is especially notable, since this is not so regular a constituent of the Gramineae as are the other two acids. Although determined as "tannin" by the Folin-Denis reagent, the combinations in which they occur in plants are not regarded as tannins in the sense of possessing astringency and combining with hide powder. It has yet to be shown whether any of the other extractives, for instance those responsible for the absorption below 550 nm and the deep orange color of the hydrolysates, have these properties, or, indeed, whether they are phenolic in character and contribute to the Folin-Denis titre. These should not be difficult questions to answer.

Within-sample variation

Variation within the sample of Kaffir corn has been studied by examination of the two fractions separable into "bronze" and "red", respectively, by color, and checked by appearance under the UV lamp (*vide supra*). The luteoforol content of each sub-sample was determined by measuring the 550 nm absorption of 0.5 g of grain when shaken for 24 hr with 50 ml of 43% methanolic H_2SO_4 in the cold room. The results were:

Bronze: absorbance 1.33 units/g/100 ml = 0.045% luteoforol

Red: absorbance 0.77 units/g/100 ml = 0.026% luteoforol

The luteoforol content of single grains was also measured by shaking for 24 hr with 5 ml of 43% methanolic H_2SO_4 in the cold room. Individual grains varied in weight from 0.025 to 0.045 g, so that the depth of color

Table 3. Tannin and luteoforol content of different samples of sorghum.

Sample	Tannin % as tannic acid	Luteoforol % (minimum)	Tannic acid % equivalent of luteoforol (minimum)	% of tannin accounted for by luteoforol (minimum)
1	0.05	0.0	0.0	0.0
2	0.14	0.18	0.012	9
3	0.20	0.06	0.04	18.5
4	0.24	0.09	0.06	25
5	0.26	0.045	0.03	12
6 (Kaffir corn)	0.45	0.045	0.03	7
7	0.67	0.004	0.003	0.45

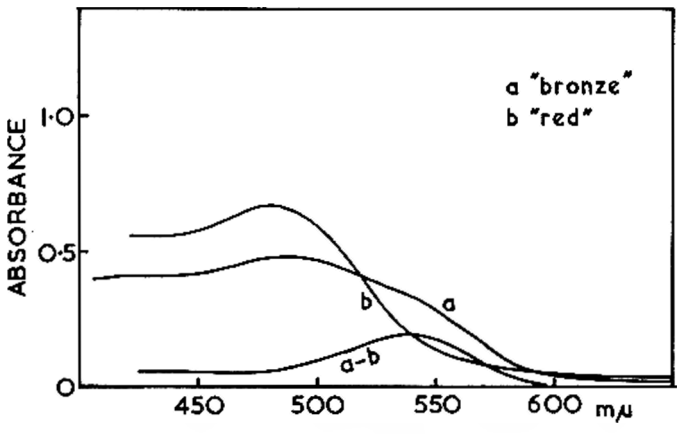


Fig. 9—Absorption curves of hydrolysates of "bronze" and "red" grains of Kaffir corn, and the difference spectrum between them, showing absorption due to cyanidin in the "bronze" grain hydrolysate.

produced was comparable with that of the 0.5 g samples treated with 50 ml of the developing reagent. The results for 5 grains of each color were:

Bronze: 0.475, 1.39, 1.90, 1.82, 1.12 units/g/100 ml
Red: 1.58, 0.305, 0.60, 0.81, 0.28 units/g/100 ml

The sum of the values for individual grains corresponded with values of 1.34 and 0.62 units/g/100 ml, respectively, which agree reasonably well with the values for the 0.5 g samples.

Within-sample variation was also studied by observing the color developed when single grains were heated for 20 min with 2*N* HCl and extracted with amyl alcohol. In every case the extract of the red grains was deep orange in color, that of the bronze grains rose or rose-apricot.

The absorption spectra of the amyl alcoholic extracts from the two types of grains are shown in Fig. 9. That of the bronze grains shows a definite "hump" in the 500–550 nm region compared with that of the red grains, and a difference spectrum (obtained by diluting the more strongly-absorbing red-grain extract until the absorptions at 450 nm were approximately equal) shows that this hump corresponds with a peak at 540 nm. The absorption of cyanidin being 535 nm, it can be concluded that this represents the absorption of the cyanidin formed from leucocyanidin present in the testa of the bronze variety, but absent from the red variety. The greater height of the peak at 480 nm in the spectrum of the red variety may also account for the greater depth of orange color in its hydrolysate, but the nature of the components responsible is at present unknown.

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Destruction of Trichina Larvae in Cooked Pork Roasts

SUMMARY—The effect of heat on trichina larvae was studied in pork roasts cut from trichinae infected loins. Roasts were cooked to end-point temperatures of 120, 125, 130, 135, 140, 145, 150 or 160°F in electric household ranges at oven temperatures of 200, 325 or 350°F. Samples of the cooked roasts were artificially digested; the recovered larvae were examined for viability. Also samples were fed to rats; 4 weeks later the rats were killed and examined for presence of trichinae by digesting aliquots of ground rat tissue. All larvae were destroyed in roasts cooked to 140° or higher; viable larvae were found in some of the roasts cooked to 135°F and in all of the roasts cooked at 130°F or lower. Thus the thermal death point of trichinae is between 130° and 140°F.

INTRODUCTION

UNTIL RECENTLY the general practice has been to cook pork to 185°F to assure complete destruction of *Trichinella spiralis*. Carlin *et al.* (1965) recommended that pork rib and loin roasts be roasted to 170°F for optimum eating quality, lower cooking losses, and less cooking time.

Early studies on the effect of heat on trichinae were conducted either on the decapsuled larvae on a heated microscope stage or with ground pieces of infected animal tissue cooked in a test tube in a boiling water bath (Ranson *et al.*, 1919; Otto *et al.*, 1939). Results of these experiments indicated that the thermal death point of trichinae was 131°F. The United States Department of Agriculture, Meat Inspection Division (1960) has regulations that include heating to 137°F any pork products which are considered ready to eat and are produced under Federal Meat Inspection.

Observations are needed under conditions that more closely represent the household situation. This study was conducted to determine the effect of final internal temperature on the viability of trichina larvae in pork roasts cooked in household ovens.

EXPERIMENTAL PROCEDURE

THE TRICHINAE infected pork loins were supplied by the Veterinary Medical Research Institute at Iowa State University for the study that was conducted in three parts. In part I, eight loins from four infected hogs were cut into rib, loin, and blade roasts for a total of 24 roasts, (average weight 8.4 lb.). Using household electric ranges at an oven temperature of 325°F, nine roasts were cooked to 140°F, seven to 150°F, and eight to 160°F. In part II, eight loins from five trichinae infected hogs were cut into eight rib and eight loin roasts (average weight 5.0 lb). The 16 roasts were cooked at 350°F and were randomly assigned to final internal temperatures of 120, 125, 130, 135, 140, 145, 150, or 160°F. In part III, 10 loins from five trichinae infected hogs were cut into 10 rib and 10 loin roasts, (average weight 7.9 lb). Paired roasts were cooked at oven temperatures of 200 or 350°F to final internal temperatures of 120, 125, 130, 135, or 140°F (Table 1). The household electric oven used for the 200°F treatment was one having two settings. When the meal minder was set at 1½ and the tendermatic at 5, the oven temperature was 350°F for the first 1 hr of cooking and then was reduced automatically to 200°F for the remainder of the cooking time.

Roasts were removed from the oven when the iron-constantan thermocouple placed in the goemetric center of the roast reached the predetermined end point. In parts I and II each roast was cut immediately after removal from the oven and samples of lean for testing were removed from the areas of the roast exhibiting the least degree of doneness as determined by visual color observation. In

Table 1. Summary of experimental treatment of trichinae infected pork roasts, parts I, II and III.

	Part I	Part II	Part III
No. of loins	8	8	10
No. of roasts	24	16	20
Roast cut	Rib, loin, blade	Rib, loin	Rib, loin
Average wt (lb)	8.4	5.0	7.9
Oven type	Household electric	Household electric	Household electric, Tendermatic
Oven temp (°F)	325	350	350 200
Final internal temp (°F)	140, 150, 160	120, 125, 130, 135, 140, 145, 150, 160	120, 125, 130, 135, 140
Samples removed	Immediately after cooking	Immediately after cooking	10 min after cooking
Tests	Digestion, bio-assay	Digestion, bio-assay	Digestion, bioassay

part III after roasts were removed from the oven, they were allowed to stand for 10 min before samples were cut and stored in the refrigerator overnight.

On the day after cooking, samples were taken to the Veterinary Medical Research Institute for testing. Two examinations were performed on each sample. In the first test, cooked meat samples were artificially digested using the artificial digestion-Baermann technique—as described by Zimmermann *et al.* (1961). The recovered trichina larvae were examined to determine viability. In the second test (bioassay), a 15-g sample of the cooked meat was fed to rats each day for 3 consecutive days; and 4 weeks after the final feeding, each rat was killed and examined for presence of larvae by artificially digesting a 100-g portion of the ground rat tissue. Rats that died before the end of the 4-week period were assumed to have been infected by trichinae at lethal levels; no digestion tests were performed.

Table 2. Results of the artificial digestion and bioassay tests for samples from trichinae infected roasts cooked at 350°F, part II.

Hog diaphragm[1]	Final internal temp.	Digestion test[2]		Bioassay	
		1	2	1	2
Trichinae/gram	°F			Trichinae/gram[3]	
2540	120	+	+	0	0
2540	125	+	+	Died	8073
1800	125	+	+	2800	630
1800	125	+	+	5010	290
1800	130	+	+	Died	Died
1800	130	+	+	Died	1140
1420	135	+	—	0	0
1420	135	—	—	0	0
980	140	—	—	0	0
980	140	—	—	0	0
980	145	—	—	0	0
980	145	—	—	0	0
640	150	—	—	0	0
640	150	—	—	0	0
640	160	—	—	0	0
640	160	—	—	0	0

[1] Trichinae/gram indicates degree of infection of the hog.
[2] +, trichinae living; —, trichinae dead.
[3] Trichinae/gram of rat tissue.

RESULTS AND DISCUSSION

SAMPLES FROM ROASTS cooked at 325°F to end-point temperatures of 140, 150 or 160°F (part I) were negative in both the digestion and the bioassay tests. The digestion tests on the meat samples cooked at 350°F to final internal temperatures of 120, 125 or 130°F (part II) indicated that living trichinae were present (Table 2). The bioassay on samples cooked to 125 or 130°F also indicated that the larvae were not destroyed at these temperatures. The negative results on the samples cooked to 120°F were unexpected and could not be explained. One roast cooked to 135°F had a positive digestion test; the other was negative. Bioassay tests indicated no trichinae present in the tissues of either rat; i.e. the sample that was positive in the digestion test did not infect the rat. For all roasts cooked to 140°F or above, in part II, meat digestion and bioassay tests indicated all larvae had been destroyed. This confirmed the results found in part I.

In part III the oven temperatures used were 350°F for 1 hr and 200°F for the remainder of the cooking period. Roasts cooked in this manner took from 1½ to 2 times longer to reach the desired temperature than paired roasts cooked for the entire period at 350°F.

During the 10 min period after removal from the oven, roasts cooked at 350°F rose an average of 7°F while those cooked at 200°F rose only 1°F. At final internal temperatures of 120 and 125°F, bioassay results indicated that the longer application of heat at the lower oven temperature was not as effective as the shorter time at the higher oven temperature (Table 3). The reverse was true for the rib roasts cooked to 130°F. Rats fed the loin roast samples showed no signs of infection. In roasts cooked at 200°F to 135°F, the larvae were destroyed and at 350°F to 135°F, the rat tissues were infected with a few trichinae. Roasts cooked to 140°F at both oven temperatures in part III showed no signs of having viable trichinae.

Table 3. Results of the artificial digestion and bioassay tests for samples from trichinae infected roasts cooked at 350 or 200°F, part III.

Hog diaphragm[1]	Final internal temp.	Cut	Digestion test[2]		Bioassay	
			350°F	200°F	350°F	200°F
Trichinae/gram	°F				Trichinae/gram[3]	
3760	120	Rib	[5]	+	2240	9880
		Loin	[5]	[4]	500	[4]
3000	125	Rib	[5]	+	0.1	480
		Loin	[5]	+	0	3510
2840	130	Rib	+	—	4920	120
		Loin	+	—	0	0
2180	135	Rib	+	—	4	0
		Loin	+	—	15	0
1840	140	Rib	—	—	0	0
		Loin	—	—	0	0

[1] Trichinae/gram indicates degree of infection of the hog.
[2] +, trichinae living; —, trichinae dead.
[3] Trichinae/gram of rat tissue.
[4] Roast was discarded because of abscess.
[5] No test performed on these samples.

CONCLUSIONS

AN INTERNAL temperature of 135°F in pork roasts is very near to the thermal death point of trichina larvae. This agrees closely with the findings of Ransom *et al.* (1919) and Otto *et al.* (1939). All roasts cooked to 140°F or above show no signs of infection. The final internal temperature of 170°F recommended for pork rib and loin roasts (Carlin *et al.*, 1965) and for fresh pork hams (Carlin *et al.*, 1968) is well above the thermal death point of *Trichinella spiralis.*

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The Detection and Enumeration of Clostridium perfringens in Foods

SUMMARY—A method that uses SPS agar and incorporates an improved egg yolk agar and nitrate motility medium has been developed for the enumeration and confirmation of vegetative cells and/or spores of *Clostridium perfringens* in foods. The method is based upon several diagnostic criteria and can be completed within 48 hr.

INTRODUCTION

DURING the course of studies to standardize the *Clostridium perfringens* detection methods of Angelotti et al. (1962) for use by our microbiology control laboratories, we had difficulty in demonstrating consistent nitrate reduction and sporulation by *Cl. perfringens* strains. To overcome these obstacles to routine quality control testing, we investigated techniques for enhancing nitrate reduction and criteria for *Cl. perfringens* identification that do not rely on spore production.

We have developed a method using the SPS agar of Angelotti et al. (1962), which can provide confirmation of the number of *Cl. perfringens* spores and/or vegetative cells within 48 hr. The procedure incorporates a lactose egg yolk agar medium modified after those of Willis et al. (1958, 1959) and McClung et al. (1947).

EXPERIMENTAL

Cultures

Fourteen cultures of clostridia, listed in Table 1, including three strains of *Cl. perfringens,* were purchased from the American Type Culture Collection. Two additional strains of *Cl. perfringens,* also listed in Table 1, were obtained from the stock culture collection of General Foods Corp. laboratories. Seven samples of a sausage product suspected of contamination with *Cl. perfringens* were also used in this study.

Media

Clostridia were isolated and enumerated on BBL sulfite-polymyxin sulfadiazine agar (SPS-Lot No. 801605). Confirmatory media for *Cl. perfringens* included thioglycollate gelatin medium (Difco), a modified nitrate-motility medium and an improved lactose egg yolk agar (LEY). The modified nitrate-motility medium was prepared from nitrate broth (Difco) supplemented with 0.5% galactose, 0.5% glycerol (Prevot, 1966) and 0.3% agar. The medium was tested for nitrites by adding a few drops of sulfanilic acid and alpha naphthylamine reagent solutions. LEY agar prepared by Microbiological Sciences, Inc., Saw Mill River Road, Yonkers, New York, had the following formulation per liter: trypticase (BBL), 15.0 g; lactose, 10.0 g; egg yolk, 10%; NaCl, 5.0 g; $CaCl_2$, 0.1 g; sodium thioglycollate, 1.0 g; K_2HPO_4, 2.5 g; phenol red, 0.02 g; and agar, 20.0 g. The final pH of the modified nitrate-motility medium and the LEY agar was 7.2.

Culture preparation

Pure cultures of clostridia were grown anaerobically in cooked meat medium for 24 hr at 35°C and serial 10-fold dilutions prepared in 0.1% peptone water.

Table 1—Differential characteristics of laboratory strains of clostridia.

Culture (ATCC[1] No.)	H_2S Production (SPS agar)	Gelatin liquefaction	Motility	Nitrate reduction	Lecithinase production	Lactose fermentation
Cl. perfringens Type B (3626)	Pos.	Pos.	Neg.	Pos.	Pos.	Pos.
Cl. perfringens (8009)	Pos.	Pos.	Neg.	Pos.	Pos.	Pos.
Cl. perfringens Type A (3624) G.F. Stock	Pos.	Pos.	Neg.	Pos.	Pos.	Pos.
Cl. periringens Culture CP-101 G.F. Stock	Pos.	Pos.	Neg.	Pos.	Pos.	Pos.
Cl. perfringens Culture CP-102	Pos.	Pos.	Neg.	Pos.	Pos.	Pos.
Cl. sporogenes (11437)	Pos.	Pos.	Pos.	Neg.	Pos.	Neg.
Cl. sporogenes (7955)	Pos.	Pos.	Pos.	Neg.	Pos.	Neg.
Cl. sporogenes (319)	Variable	Pos.	Pos.	Neg.	Pos.	Neg.
Cl. bifermentans (9714)	Pos.	Pos.	Pos.	Neg.	Pos.	Neg.
Cl. haemolyticum (9652)	Variable	Pos.	Pos.	Neg.	Pos.	Neg.
Cl. histolyticum (8034)	Variable	Pos.	Neg.	Neg.	Neg.	Pos.
Cl. acetobutylicum (824)	Variable	Neg.	Pos.	Neg.	Pos.	Neg.
Cl. nigrificans (7946)	Variable	Pos.	Pos.	Neg.	Pos.	Neg.
Cl. botulinum Type A (7948)	Pos.	Pos.	Pos.	Neg.	Pos.	Neg.
Cl. botulinum Type B (7949)	Pos.	Pos.	Pos.	Neg.	Pos.	Neg.
Cl. botulinum Type E (9564)	No growth	Neg.	Pos.	Pos.	Pos.	Neg.

[1] American Type Culture Collection.

Sample preparation

A 10-fold dilution of the sausage product was prepared by weighing 50 g of the sample into a Waring Blendor containing 450 ml of 0.1% peptone water and blending at high speed for 90 sec. Subsequent 10-fold serial dilutions were prepared by aseptically transferring 10 ml of the previous dilution into 90 ml of sterile peptone water.

Procedure

SPS agar pour plates were prepared with inocula from dilutions of the clostridial cultures or dilutions of suspect sausage samples. After solidification, each plate was overlaid with 3–5 ml of SPS agar and incubated anaerobically at 35°C for 24 hr. The number of cells per milliliter was calculated by counting the number of black colonies produced in the medium and multiplying by the dilution factor. To enumerate spores, pure culture and sausage sample dilutions were also heat shocked at 80°C for 20 min followed by immediate cooling in a 5°C bath for 10 min. SPS agar pour plates were prepared as outlined above.

Growth from 10 black colonies shown to consist of gram positive rods was inoculated into thioglycollate-gelatin medium and incubated for 4 hr in a 46°C water bath or until visible growth was evident. The thioglycollate gelatin tubes were then used as an inoculum source for stabbing the nitrate-motility tubes and for streaking lactose egg yolk agar plates. Growth was transferred to LEY agar by streaking two loopfuls of inocula across the center of the plate. All media including the thioglycollate gelatin were incubated anaerobically for 24 hr at 35°C.

RESULTS

MORPHOLOGY was determined by gram staining. Motility was recorded by noting whether growth spread away from the line of stab in the nitrate-motility medium. Nitrate reduction was determined with alpha naphthylamine and sulfanilic acid test reagents. Gelatin liquefaction was determined by the fluidity of thioglycollate gelatin medium after 30 min refrigeration at 10°C. LEY agar plates were observed for evidence of lecithinase production and lactose fermentation. Lecithinase activity alone was observed as a brick red zone of opacity surrounding the line of growth. Lactose fermentation was indicated by a yellow halo around the line of growth. The simultaneous presence of lecithinase production and lactose fermentation was indicated by a yellow zone of opacity surrounding the line of growth.

The number of clostridial spores and/or vegetative cells was estimated by multiplying the initially recorded counts by the percentage of the 10 colonies that were confirmed as *Cl. perfringens*.

Of the seven sausage sample products analyzed, five were found to contain the vegetative cells of *Cl. perfringens* in the range of 10–20 g. No spores were detected as evidenced by the absence of colony growth on SPS agar plates prepared from the heat-shocked dilutions.

DISCUSSION

WHILE EMPLOYING the method of Angelotti et al. (1962), we found that in some instances not all and, occasionally none, of the clostridial colonies obtained on anaerobically incubated SPS agar produced H_2S. Difficulty was also encountered in demonstrating sporulation of *Cl. perfringens* in several sugar free sporulation media. In addition, the evidence of nitrate to nitrite reduction was often variable using the recommended nitrate motility medium of Angelotti et al. (1962).

We observed that production of H_2S and colony development was improved when an overlay was used during the initial plating with SPS agar. The addition of 0.5% glycerol and galactose (Prevot, 1966) to the nitrate-motility medium more consistently enhanced the reduction of nitrate to nitrite. Thioglycollate gelatin medium was used as an alternative to thioglycollate medium in order to obtain the additional evidence of gelatin liquefaction.

Sporulation was eliminated as a criterion and the two alternative biochemical characteristics of lactose fermentation and lecithinase production were investigated in several modifications of the egg yolk media of McClung et al. (1947) and Willis et al. (1958, 1959). The most consistently luxurious growth was obtained with a trypticase soy agar base. Since it has been shown that the lecithinase reaction is Ca^{++} dependent (MacFarlane et al., 1941a,b), 0.01% $CaCl_2$ was added to the medium to enhance lecithinase activity. Phenol red provided a more distinct indication of lactose fermentation than the neutral red indicator employed in the egg yolk medium of Willis et al. (1958, 1959).

Evidence of *Cl. perfringens* is strongly supported by detection of lecithinase activity, lactose fermentation and confirmation of non-motility from H_2S producing colonies. To our knowledge, of the clostridia that produce lecithinase only *Cl. perfringens* is non-motile and ferments lactose (Collins, 1967; Willis et al., 1958; Breed et al., 1962). The additional tests described were included to provide supplemental diagnostic results. Because demonstration of gelatin liquefaction or nitrate reduction may vary, confirmatory emphasis was not placed on these tests.

The diagnostic criteria employed here for confirmatory evidence of *Cl. perfringens* include the following characteristics:

1. Morphology	gram positive rod
2. H_2S production	positive
3. Lecithinase production	positive
4. Lactose fermentation	positive
5. Anaerobiosis	positive
6. Gelatin liquefaction	positive
7. Motility	negative
8. Nitrate reduction	nitrites produced

It may be noted from Table 1 that growth and H_2S production was variable with *Cl. sporogenes* (319), *Cl. hemolyticum*, *Cl. histolyticum* and *Cl. acetobutylicum* on SPS agar. Similar results have been obtained by other workers (Gibbs et al., 1965). It has also been found that quantitation may vary from lot to lot of commercially prepared SPS medium, but Hauschild et al. (1967) and Gibbs et al. (1965) have both concluded that, at present, this is the best medium for enumeration of *Cl. perfringens* as well as other clostridia. In any case, all strains of *Cl. perfringens* tested and isolated always grew well and produced H_2S on SPS agar.

The method reported here is useful in obtaining consistent biochemical confirmation of the number of *Cl. perfringens* spores and/or vegetative cells in food products within 48 hr.

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Measurement of the Relative Sweetness of Selected Sweeteners and Sweetener Mixtures

SUMMARY—The relative sweetness of sugars and sugar mixtures was studied. In addition to the simple sugars (sucrose, dextrose and fructose), the amino acids, glycine and D,L-alanine, and the synthetic sweeteners, calcium cyclamate and sodium saccharin, were studied. Using the method of magnitude estimation, considerable data were obtained about relative sweetness over a reasonably wide concentration range. Only two sessions per subject were required to obtain meaningful results. Relative sweetness of the sugars was found to increase with increasing concentration—a pattern quite similar for all the sugars. Changing the reference or reference concentration resulted in shifts in the relative sweetness values for a particular sugar; however, these changes were consistent at all concentrations tested. Slope values for the individual sugars were in good agreement with previously reported results. The individual subjects responses showed a consistent pattern throughout the 10-month period.

Synergistic effects, as much as 20 to 30%, were noted in several sugar mixture combinations but not all concentrations. The data support the concept that there are optimal mixture combinations. The potential applications of these observations are discussed.

INTRODUCTION

BECAUSE of the importance of sugars in foods and their role in food acceptance, the relative sweetness of sugars has been of great interest to psychologists and food technologists for many years (Dahlberg et al., 1941; Cameron, 1947; Lichtenstein, 1948; Schutz et al., 1957; Nieman, 1960; and Pangborn, 1963). Relevant studies have been reviewed by several investigators (Pfaffmann, 1959; Nieman, 1960 and Amerine et al., 1965).

The use of many different kinds of sugars and sugar mixtures has complicated the picture because sugars contribute more than sweetness to a food, and replacement of one sugar by another is not very satisfactory. Sugars differ not only in sweetness but also in chemical properties. Thus in a food system, the added sugar (or that already present in the food) is capable of undergoing reaction with itself or with other chemicals, thus yielding final products having more- or less-sweet taste and/or flavor properties.

Early investigators noted that on a weight basis, fructose was sweeter than most all polyhydroxylated sugars. It was subsequently demonstrated that calcium and sodium salts of saccharin and cyclamates, as well as dulcin, were considerably sweeter than either sucrose or fructose. However, relative sweetness varies with concentration of the sugars. The classic examples were the comparisons of sucrose with dextrose and fructose; dextrose was cited as being about 65 to 75% as sweet as sucrose, and fructose as being 120% as sweet as sucrose. These values—especially the ones for dextrose—were significantly affected by concentration. Thus at about 40% (by weight), dextrose was equi-sweet with a similar concentration of sucrose, but at 5% the sucrose was much sweeter.

More recent research by Schutz et al. (1957), Nieman (1960), and Pangborn (1963) has drawn attention to the problems associated with measuring the relative sweetness of sugars: the psychophysical techniques, individual taste sensitivities and the extrapolation to food products. Using a single-sample presentation with a nine-point category scale of intensity to establish the relative sweetness of 16 sugars, Schutz et al. (1957) found that the technique was useful in establishing a relationship between subjective sweetness and stimulus concentration without recourse to the more traditional and more time-consuming paired-comparison procedure. On the other hand, Pangborn (1963) utilized paired-comparison for establishing the sweetness of dextrose, fructose and lactose relative to sucrose at threshold and suprathreshold concentrations.

Of the methods that have been used, paired-comparison is the most exact in that responses are the result of direct comparison between two stimuli. One disadvantage of the method is the need for a large number of comparisons and considerable amounts of test solution. Pangborn's results were in good agreement with some of the earlier studies; of particular interest was the observation that fructose in a food product (pear nectar) was less sweet than sucrose in the same product. Pangborn noted that one should exercise caution in extrapolating from an aqueous model system to a complex food. Dahlberg et al. (1941) and Nieman (1960) also noted that for some sugar mixtures a synergistic effect in an aqueous system was reduced when applied to a food. This reduction in sweetness may be explained on the basis of taste interactions; i.e., as the food is a more complex testing system, it generates a variety of sensory inputs that would mask or inhibit perception of the increased sweetness. How important this is to flavor is a more complex question which cannot be answered simply by making a product more or less sweet. It is beyond the scope of the present investigation.

In the food industry considerable attention has been given to the use of dextrose in partial replacement of sucrose. Historically, sucrose has maintained an advantage because of its greater sweetening property and the federal standards of identity which have limited the amount of dextrose that can be added.

One can conclude from a review of existing literature that there are a variety of polyhydroxylated, "simple"

sugars that are more or less sweet than each other, depending on concentrations and the technique for establishment of relative sweetness. The mixture of two or more of these sugars at the proper concentration can result in a solution possessing a sweetness greater than the simple addition of the two alone (synergism). Not all sugars possess synergistic properties. There are a variety of other "synthetic" or nonhydroxylated sugars, all of which are considerably sweeter than the simple sugars and can produce synergistic effects when mixed in appropriate concentrations (with both kinds of sugars). Finally, the sweetness of these sugars (singly or mixed) is altered (usually decreased) when added to foods because of taste interactions and masking effects. The measurement of relative sweetness is a complex, at times contradictory, and time-consuming process.

The results presented here were obtained by the technique of magnitude estimation (Stevens, 1961), utilizing an aqueous model system.

EXPERIMENTAL METHODS

Test stimuli

The test materials were sucrose (Mann Assayed, lots R1591 and 3089), dextrose (highest purity, Corn Products Company), calcium cyclamate (Abbott Laboratories, USP grade, lot 5114), sodium saccharin (Monsanto Company, NF and FCC soluble powder, lot QII-201), glycine (Mann Assayed, lot R3561) and D,L-alpha-alanine (Mann Assayed, lot P2041). All compounds had a purity in excess of 99%.

Solution preparation

Glass-distilled, charcoal-filtered water was used to prepare the solutions and was available to the subjects for rinsing purposes. All solutions were prepared 24 hr before testing and no solution was retained for more than 48 hr. If additional tests were required, fresh solutions were prepared. Distilled water was usually prepared within 48 hr of a test.

Each solution was prepared on a wt/wt basis after establishing the dry weight of each compound. Solutions were stored and presented at room temperature (21° ± 2°C). For each single-sugar and sugar-mixture experiment, a series of five solutions ($\log_2$) were prepared. This reasonably wide range of concentrations enabled us to generalize our results.

Sensory panel

The panel consisted of six highly trained subjects, selected from a larger group (15) because of their performance during a preliminary test period and their ability to detect low concentrations of a bitter stimulus, n-propyl thiouracil. The bitter stimulus was included to enable us to learn more about each subject's sensitivity to other kinds of taste stimuli. The subjects (three males and three females, aged 21 to 30) all had 3 to 12 months' previous experience with psychophysical investigations. There were three alternates; however, data from these subjects were not interchanged with data from the regular panelists. Unless otherwise noted, the regular panelists made up all missed experiments.

Each subject was tested separately, at a specific time which was maintained as closely as possible throughout the experiment. In one 20-min session it was possible to obtain 6 responses at each of the 5 test concentrations. For any one stimulus there were as few as 6 and as many as 12 responses per subject at each concentration. Subjects were informed of the correctness of their decisions at the conclusion of each test.

All tests were accomplished in individual taste booths, which were air-conditioned and equipped with constant illumination and sinks for expectoration.

Threshold measurement

Prior to measuring relative sweetness, baseline data were obtained from each subject in the form of recognition thresholds for the test sugars (sucrose, dextrose, fructose, sodium saccharin, and sodium and calcium cyclamates). In addition, the threshold was established for the bitter stimulus, n-propyl thiouracil, to differentiate between "tasters" and "nontasters." All our subjects were "tasters."

Determination of the recognition thresholds was accomplished in the following manner. We used a single-sample, randomized, forced-choice procedure (Guilford, 1954) in which the subject was presented with many stimuli close to his threshold (referred to as a "tracking" procedure). The subject was instructed to taste the stimulus (but not swallow it) and to indicate, by a "yes" or "no" response, whether he recognized the stimulus as sweet (or bitter in the case of n-propyl thiouracil). First, a descending series at decreasing concentrations of solutions was presented, one at a time, until the subject could no longer recognize the stimulus. At this point he was presented with blanks (distilled water) and random test concentrations in the immediate vicinity of those at which the response was no longer perceived. Sufficient observations, interspersed with blank-trials, were taken until approximately 50% correct responses occurred. The series was then terminated. This procedure was repeated on the next three days and the threshold was estimated from the data obtained during the four test sessions. Previous experiments had provided sufficient background data on the usefulness of this technique (Stone et al., 1967).

Relative sweetness measurement

Measurement of the relative sweetness of the sugars and sugar mixtures was accomplished using the method of magnitude estimation (Stevens, 1956, 1961), which yields a direct quantitative measure of the subjective intensity of sweetness. The subject was informed that he would be presented with a reference sample with a designated intensity of 10, followed by a random series of solutions with intensities both less than and greater than the reference intensity. His task was to estimate the sweetness intensity of the unknowns relative to the reference.

He was instructed to use whatever integers seemed appropriate (but not zero) and to judge each solution separately; i.e., "if Sample 2 was five times sweeter than the reference, he would assign it a sweetness intensity of 50, etc." He was also informed that the reference would

be presented to him periodically. The technique makes no distinction as to use of a reference or its position in the test series. In our experiment, we placed the reference at the geometric mean of the series.

When the reference concentration was changed, it was necessary to adjust the test concentrations up or down a sufficient number of steps to maintain a balance of the test solutions around the reference. Preliminary testing revealed that when the reference was at either extreme of a series, it resulted in a skewness to the line when depicted graphically; i.e., the variance increased significantly for the samples farthest from the reference. This observation has led some investigators to eliminate the reference entirely and adjust subjective responses at the conclusion of the experiment prior to data analysis (Engen et al., 1963; Luce et al., 1963). We used a reference because we were interested in the effect of changing reference on relative sweetness and in being able to compare relative sweetness values of sugars.

For the mixture experiments, we used dextrose and sucrose as the standard sugars and added a second component to each of them. First it was necessary to establish the relative sweetness of the individual components and then to decide upon concentration levels. Usually, three concentration levels of the second component were used, although in some tests only two were studied. Sugar mixtures included dextrose-fructose, sucrose-fructose, dextrose-glycine, dextrose-D,L-alanine, dextrose-calcium cyclamate and dextrose-sodium saccharin. The test concentrations are listed in Results.

Data analysis

Analysis of the data was accomplished by means of the Burroughs 5500 computer (Fortran compilation), using a program specifically written for magnitude estimation experiments. With minor changes, the program can be run on the IBM S/360 or 7090/94; however, we adapted it to the B5500 (available at our facility). Input information included individual responses from each experiment, stimulus concentrations, and—where appropriate—reference estimations. Data output included pooled geometric mean responses for each stimulus concentration, the variance, standard deviation, medians, first and third quartile values, equations of the line through the geometric mean, least square line through the medians (for normalized and non-normalized data) and Pearson R and R^2.

RESULTS AND DISCUSSION

THE RECOGNITION THRESHOLD experiments are presented in Table 1. For comparative purposes we have included the published values for some of the sugars. Our data (column 2) are in good agreement with previously reported results, although one might question our low value for fructose (0.134 *M* vs 0.052 *M*).

We took considerable care to instruct subjects to respond only when the stimulus was recognized as sweet and not merely because it seemed different. Each subject was tested separately, with several days of pretraining, to minimize test bias or confusion over instructions.

The tracking method for threshold determination is a rapid technique suitable for more widespread usage. It requires less time than other methods and appears to be as sensitive. However, some precautions must be taken to obtain reliable threshold measures. Since the technique involves first bracketing the threshold (i.e., that concentration range at which the threshold is lost), the experimenter must be careful not to go directly to that same set of concentrations on the second or third (or more) replications. Such a practice conceivably could bias the subject's responses to the stimuli so that the data, while seemingly consistent, would be invalid. We know that a subject's sensitivity will vary, even within a one-hour test session (Stone, 1967).

Table 1—Recognition thresholds for six taste stimuli.[1]

Stimulus	Molar concentration	
	Mean ± SD[2]	Reported range[3]
Sucrose	0.0101 ± .0064	0.012–0.037
Dextrose	0.0366 ± .0166	0.04–0.09
Fructose	0.0134 ± .0081	0.052
Sodium saccharin	0.0000316 ± .0000196	0.00002–0.00004
Sodium cyclamate	0.000295 ± .000275	
Calcium cyclamate	0.000295 ± .000162	

[1] Purity of each compound was in excess of 99%.
[2] Each entry is the pooled mean value ± standard deviation (SD) for the 9 subjects determined on 4 successive days. All subjects were tasters of bitterness; the mean ± SD threshold for n-propyl thiouracil was 0.000021 ± .0000152 M which is in good agreement with previously reported results from our laboratory.
[3] Pfaffmann (1959); Amerine et al. (1965).

The other important requirement is the use of a large number of blank samples (possibly as many as the test samples). In the recognition test, we were especially concerned with the subject's pattern of response; i.e., whether trends were evident. No trends were observed. With the tracking technique, we obtained a recognition threshold value in a relatively brief test session of 10 to 20 min. We believe that the technique is most useful in this respect and, therefore, that the reported recognition thresholds are valid.

The results of the magnitude-estimation experiments for the individual sugars (sucrose, dextrose, fructose) are summarized in Tables 2 and 3. In Table 2 are the geometric means of the estimates of sweetness intensity for each of the test stimuli as a function of changing the reference concentration. As expected, increasing the reference concentration decreased the relative sweetness intensity of the test stimuli. The changes (decrease or increase) were consistent from concentration to concentration for any stimulus. The data in Table 3 show this effect most clearly. The slopes of the lines for the same sugar but with different references and reference concentrations are not significantly different, although a pattern is suggested. By increasing reference concentration, the slope value increases; i.e., the line tends to flatten and the Y-intercept decreases. One might consider using the Y-intercept for comparison purposes.

Since the analysis provided us with results for the individual subjects, we also examined these data and found the same general pattern. The slopes of the individual lines for all the experiments reported in Table 3 ranged

Table 2—Relative sweetness of dextrose, sucrose and fructose as a function of changing reference and reference concentration.[1]

Reference (molar conc.)	Sugar (molar concentration) 0.065	0.125	0.25	0.50	1.0	1.5	2.0	2.25
Dextrose				Dextrose				
0.25	1.02	1.85	8.16	23.85	51.96	....	....	....
0.50	....	1.78	4.09	9.02	20.20	....	37.34	....
		(1.19–2.75)	(3.11–5.47)	(7.78–10.21)	(16.68–24.52)		(27.21–48.33)	
Sucrose								
0.149	....	1.78	3.24	9.68	19.37	....	35.53	....
0.25	....	1.30	2.44	5.98	14.16	....	26.07	....
0.46	....	....	2.52[2]	4.21[3]	9.53	18.4		32.9
			(1.26–4.14)	(3.83–4.58)	(8.86–11.33)	(15.11–22.19)		(29.69–39.61)
Dextrose				Sucrose				
0.25	5.81	13.90	24.80	47.16	64.91			
0.50	2.71	6.09	13.51	22.17	33.47			
	(2.74–3.29)	(5.09–7.42)	(12.40–13.96)	(20.14–25.29)	(31.17–37.50)			
Sucrose								
0.25	1.82	4.39	10.04	20.01	34.42			
0.46[4]	....	3.39	6.70	12.40	21.13	36.07	....	....
		(2.76–4.64)	(5.92–7.60)	(10.50–13.97)	(19.00–23.67)	(33.04–37.95)	....	....
Dextrose				Fructose				
0.50	1.86	4.47	11.33	19.55	32.21	....		
	(2.16–2.76)	(4.09–4.94)	(9.62–12.16)	(17.19–18.30)	(25.45–34.87)			
Sucrose								
0.149	1.95	3.87	10.36	19.37	35.19	....	....	....
0.25	1.17	2.16	6.47	13.48	28.31	....	....	
0.46[4]	....	1.85	3.64	8.04	14.20	25.10	....	....
		(1.47–2.77)	(3.32–3.71)	(7.09–8.62)	(12.12–17.00)	(20.87–30.50)		

[1] Each entry is the geometric mean of 60 responses (a few tests were based on 36 or 72 responses). Values in parentheses are the first and third quartile values for the data depicted in Fig. 1. In all experiments, the reference was designated as 10 to the subjects.
[2] The actual molar concentration was 0.44.
[3] The actual molar concentration was 0.67.
[4] Molar concentrations for these entries were 0.18, 0.30, 0.50, 0.833 and 1.39, respectively.

Table 3—Slope values, variances, standard deviations, Y-intercepts and correlation coefficients (R) for the test stimuli reported in Table 2.

Reference (molar conc.)	Slope	Variance	Std. dev.	Y-intercepts	R[1]
			Dextrose		
Dextrose					
0.25	1.5024	0.2006	0.4479	0.7608	.9857
0.50	1.1086	0.0552	0.2350	0.2726	.9905
Sucrose					
0.149	1.1215	0.1280	0.3578	0.2546	.9914
0.25	1.1174	0.0698	0.2638	0.1058	.9882
0.46	1.6257	0.1783	0.4222	−0.0415	.9734
			Sucrose		
Dextrose					
0.25	0.8724	0.0249	0.1578	0.8828	.9767
0.50	0.9116	0.0087	0.0933	0.5926	.9800
Sucrose					
0.25	1.0674	0.1019	0.3192	0.5911	.9931
0.46	1.1506	0.0274	0.1654	0.4126	.9865
			Fructose		
Dextrose					
0.50	1.0361	0.0983	0.3135	0.5783	.9838
Sucrose					
0.149	1.0664	0.0840	0.2898	0.5876	.9911
0.25	1.1833	0.2156	0.4643	0.4716	.9835
0.46	1.2891	0.0990	0.3146	0.2458	.9885

[1] R refers to Pearson R, the correlation coefficient.

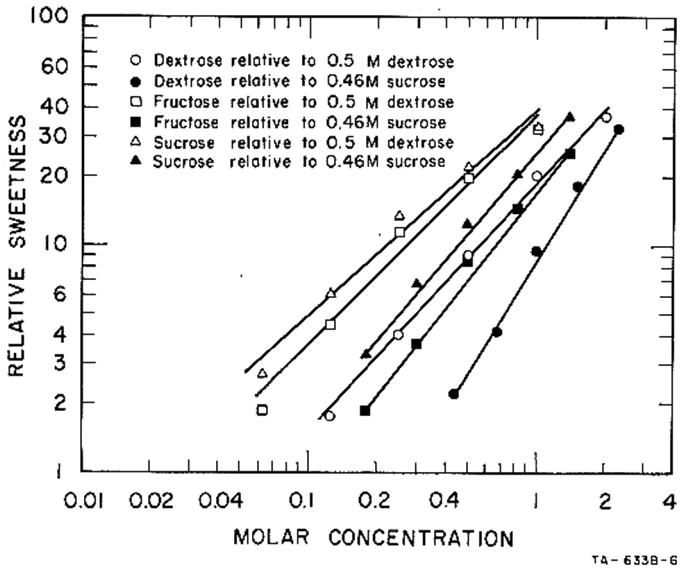

Fig. 1—Scales of relative sweetness for the individual sugars as a function of the different reference stimuli. The equations of the individual lines are available from the data in Table 3. For purposes of clarity we have excluded the range of values for each point. These data are available on request.

Table 4—Relative sweetness of mixtures of dextrose, fructose and sucrose.[1]

Reference (molar conc.)	Sugar (molar concentration) 0.0625	0.125	0.25	0.50	1.0
Dextrose		Dextrose + 0.0625 *M* Fructose[2]			
0.50	.....	3.66	7.36	14.08	27.69
		Dextrose + 0.125 *M* Fructose			
0.50	.....	6.04	10.59	19.05	31.05
		Dextrose + 0.25 *M* Fructose			
0.50	.....	12.86	15.56	24.18	32.57
		Dextrose + 0.625 *M* Sucrose			
0.50	.....	4.89	9.04	16.69	28.92
Sucrose		Dextrose + 0.0625 *M* Fructose			
0.25	.....	2.03	3.94	9.10	20.59
		Dextrose + 0.125 *M* Fructose			
0.25	.....	3.51	5.78	11.37	21.62
		Sucrose + 0.0625 *M* Fructose			
0.25	2.92	6.17	12.25	20.08	.....
		Sucrose + 0.125 *M* Fructose			
0.25	4.09	7.51	12.97	23.59	.....

[1] Each entry is the geometric mean of 60 responses. In all experiments the reference was designated as 10 to the subjects.
[2] The relative sweetness values for the individual sugars are obtained by referring to the appropriate reference and reference concentration in Table 2.

from 0.6246 to 2.2021 (almost a fourfold difference) and averaged 1.16 for sweetness. This is reasonably close to the power function, or β value, for sucrose of 1.3 reported by Stevens (1961). Some of these data are depicted graphically in Fig. 1. The correlation coefficients were greater than 0.97 for all the lines.

It might be argued that the method of paired-comparison represents the only realistic means of comparing the sweetness of sugars. In reviewing these results we have attempted to establish some means of comparing sweetness values of the different sugars; however, it was first necessary to consider how to make these comparisons. One method is to compare the concentration of a particular sugar whose sweetness value is equal to that of the reference. Thus, dextrose at 0.5 *M* is equal in sweetness to sucrose at 0.146 *M;* 0.75 *M* dextrose is equal to 0.25 *M* sucrose; and 1.0 *M* dextrose is equal to 0.46 *M* sucrose. Similar comparisons can be made with dextrose as the reference for sucrose and for fructose. One must also take into account the weight of sucrose, which is approximately twice that of dextrose (and fructose). After adjusting for weight, the relationship between sugars remains about the same.

We present these comparisons as an alternative means of comparing the sweetness of various sugars. They are intended to serve only as guidelines and to show agreement with previously reported investigations.

The results of the mixture experiments with the simple sugars are presented in Tables 4 and 5. With dextrose as the reference, the mixtures exhibited a synergistic effect of about 20 to 30%. This was especially evident at the two highest dextrose concentrations, 0.50 and 1.0 *M*, with the added fructose at 0.125 and 0.25 *M*. It appears that there is an optimal fructose level at which the synergistic effect is greatest; e.g., 1.0 *M* dextrose with 0.125 *M* fructose had a sweetness intensity of 31.05, which was not significantly increased if the fructose concentration was doubled to 0.25 *M* (32.57). In fact, at this higher fructose level (0.25 *M*), there was a synergistic effect only with 0.50 *M* dextrose; at all other levels the relative sweetness values were additive. A synergistic effect of about 20 to 25% was found with the dextrose-sucrose mixture as well. These data are included because of previously reported results that showed these mixtures to be synergistic (Dahlberg et al., 1941). In that study, the roles were reversed; i.e., sucrose was the major component and dextrose the additive. With 0.25 *M* sucrose as the reference, the synergistic effect was about the same order of magnitude as in the mixtures with dextrose as the reference. In the sucrose-fructose experiments, only a very slight synergistic

Table 5—Slope values, variances, standard deviations, Y-intercepts and correlation coefficients (R) for the test stimuli reported in Table 4.

Reference (molar conc.)	Slope	Variance	Std. dev.	Y-Intercepts	R[1]
Dextrose		Dextrose + 0.0625 *M* Fructose			
0.50	0.9690	0.2070	0.1643	0.4430	.9861
		Dextrose + 0.125 *M* Fructose			
0.50	0.7934	0.0131	0.1145	0.5027	.9863
		Dextrose + 0.25 *M* Fructose			
0.50	0.4657	0.0158	0.1255	0.5097	.9715
		Dextrose + 0.0625 *M* Sucrose			
0.50	0.8581	0.0063	0.0796	0.4696	.9898
Sucrose		Dextrose + 0.0625 *M* Fructose			
0.25	1.1232	0.3307	0.5751	0.3012	.9831
		Dextrose + 0.125 *M* Fructose			
0.25	0.8839	0.0648	0.2545	0.3238	.9866
		Sucrose + 0.625 *M* Fructose			
0.25	0.9346	0.0504	0.2246	0.6148	.9866
		Sucrose + 0.125 *M* Fructose			
0.25	0.8364	0.0129	0.1136	0.6229	.9875

[1] Refers to Pearson R, the correlation coefficient.

Table 6—Relative sweetness of dextrose, glycine, D,L-alanine and mixtures of dextrose and the two amino acids as a function of dextrose references of 0.25 and 0.50 M.[1]

Dextrose (molar conc.)	Stimulus (molar concentration)					
	0.0625	0.125	0.25	0.50	1.0	2.0
				Dextrose		
0.25	1.02	1.85	8.16	23.85	51.96	
0.50		1.78	4.09	9.02	20.20	37.34
				D,L-Alanine		
0.25	2.68	5.46	11.17	22.31		
0.50	1.02	2.04	3.96	8.76	19.67	
				Dextrose + 0.0625 D, L-Alanine		
0.50		2.41	4.84	11.91	26.62	
				Dextrose + 0.125 D,L-Alanine		
0.50		2.76	6.84	14.67	26.51	
				Dextrose + 0.25 D,L-Alanine		
0.50		4.06	8.86	16.39	31.05	
				Glycine		
0.25	1.86	3.66	8.68	14.82		
0.50	1.01	1.48	2.62	7.59	14.51	
				Dextrose + 0.125 *M* Glycine		
0.25		4.76	8.60	17.24	33.58	
				Dextrose + 0.0625 *M* Glycine		
0.50		2.27	4.03	10.20	23.33	
				Dextrose + 0.125 *M* Glycine		
0.50		2.26	5.01	12.12	24.12	
				Dextrose + 0.25 *M* Glycine		
0.50		4.71	7.32	15.98	31.15	

[1] In all experiments, the reference was designated as 10 to the subjects. Each entry is the geometric mean of 60 responses; for the mixtures, there were 36 responses.

effect was observed in some of the mixtures. Since we felt that synergism is evident in mixtures of sugars that are quite dissimilar in relative sweetness (e.g., dextrose-sucrose and dextrose-fructose) and sucrose and fructose have quite similar sweetening properties, we would not expect mixtures of these sugars to exhibit synergistic properties.

Results of the amino acid (glycine and D,L-alanine) mixture experiments are shown in Tables 6 and 7. Only the highest dextrose levels produced any synergistic effects. For these experiments, the panel was instructed to respond only to sweetness and disregard other taste sensations. Both of these amino acids have bitter as well as sweet taste properties that are concentration-dependent (Stone, 1967). Therefore we used only the lower amino acid concentrations, although some bitterness is perceived at the 0.25-*M* level in both glycine and D,L-alanine. While we cannot be completely sure that our subjects responded only to sweetness, the data are consistent and do not exhibit any greater variance than that found in previous experiments.

Tables 8 and 9 show the results of the mixture experiments with calcium cyclamate and sodium saccharin. Only the dextrose-calcium cyclamate mixtures yielded a synergistic effect. The sweetness of the saccharin-containing mixtures was additive at all test levels. Synergistic effects have been reported for sucrose-calcium cyclamate (Kamen, 1959) and for saccharin-cyclamate (Vincent et al., 1955). It was not possible to test these same mixtures in the present experiments, so we cannot draw any conclusions or make any comparisons at this time.

Magnitude estimation for measuring the relative sweet-

Table 7—Slope values, variances, standard deviations, Y-intercepts and correlation coefficients for the test stimuli reported in Table 6.

Dextrose (molar conc.)	Slope	Variance	Std. dev.	Y-Intercepts	R[1]
			Dextrose		
0.25	1.5024	0.2006	0.4479	0.7608	.9857
0.50	1.1086	0.0552	0.2350	0.2726	.9905
			D,L-Alanine		
0.25	1.0197	0.0483	0.2199	0.6581	.9802
0.50	1.0632	0.1023	0.3199	0.2711	.9876
			Dextrose + 0.0625 D,L-Alanine		
0.50	1.1689	0.0521	0.2283	0.4201	.9717
			Dextrose + 0.125 D,L-Alanine		
0.50	1.0895	0.0575	0.2397	0.4584	.9884
			Dextrose + 0.25 D,L-Alanine		
0.50	0.9688	0.0217	0.1473	0.5033	.9907
			Glycine		
0.25	1.0221	0.0593	0.2435	0.5050	.9807
0.50	1.0060	0.2689	0.5186	0.1322	.9634
			Dextrose + 0.125 *M* Glycine		
0.25	0.9459	0.0155	0.1247	0.5208	.9929
			Dextrose + 0.0625 *M* Glycine		
0.50	1.1418	0.0276	0.1660	0.3502	.9775
			Dextrose + 0.125 *M* Glycine		
0.50	1.1523	0.0526	0.2293	0.4004	.9852
			Dextrose + 0.25 *M* Glycine		
0.50	0.9300	0.0259	0.1610	0.4787	.9804

[1] R refers to Pearson R, the correlation coefficient.

Table 8—Relative sweetness of dextrose, calcium cyclamate, sodium saccharin and mixtures of these as a function of dextrose reference of 0.50 *M*.[1]

Dextrose (molar conc.)	Stimulus (molar concentration)				
	0.125	0.25	0.50	1.0	2.0
			Dextrose		
0.50	1.78	4.09	9.02	20.20	37.34
	0.00125	0.0025	0.0050	0.010	
			Calcium cyclamate		
0.50	1.66	4.29	12.06	24.95	
			Dextrose + 0.00135 *M* Calcium cyclamate		
0.50	3.12	7.31	14.54	26.83	
			Dextrose + 0.0025 *M* Calcium cyclamate		
0.50	6.59	10.17	18.49	30.57	
	0.00035	0.00053	0.0008	0.0012	0.0018
			Sodium saccharin		
0.50	3.81	5.80	8.95	13.56	17.90
			Dextrose + 0.00035 Sodium saccharin		
0.50	5.34	7.82	13.58	25.01	
			Dextrose + 0.00053 Sodium saccharin		
0.50	7.31	10.21	16.03	26.02	

[1] In all experiments, the reference was designated as 10 to the subjects. Each entry is the geometric mean of 60 responses.

Table 9—Slope values, variances, standard deviations, Y-intercepts and correlation coefficients for the test stimuli reported in Table 8.

Dextrose (molar conc.)	Slope	Variance	Std. dev.	Y-Intercepts	R[1]
			Dextrose		
0.50	1.1086	0.0552	0.2350	0.2726	.9905
			Calcium cyclamate		
0.50	1.3215	0.1850	0.4301	3.0726	.9885
			Dextrose + 0.0025 *M* Calcium cyclamate		
0.50	0.7505	0.0129	0.1137	0.4834	.9802
			Dextrose + 0.0013 *M* Calcium cyclamate		
0.50	1.0298	0.0463	0.2151	0.4525	.9861
			Sodium saccharin		
0.50	0.9640	0.0080	0.0893	2.9235	.9792
			Dextrose + 0.00035 *M* Sodium saccharin		
0.50	0.7483	0.0251	0.1583	0.3757	.9852
			Dextrose + 0.00353 *M* Sodium saccharin		
0.50	0.6145	0.0180	0.1341	0.4008	.9720

[1] R refers to Pearson R, the correlation coefficient.

ness of sugars represents, in our estimation, an improvement because it is a rapid, reliable method appropriate for the task. Not only does it provide direct numerical estimates of the apparent intensity of a stimulus, but minimal amounts of test solution are required, and computer programs are available to facilitate analysis. Even though many investigators favor the method of paired-comparison, it would seem that considerable time and effort could be saved by first using the method of magnitude estimation, if only as a means of reducing experimental variables.

The exponents found in the present study were in good agreement with those reported by Stevens (1961). He showed 1.3 for sucrose, versus 0.62 to 1.75 (on an individual subject basis) in our study. For all three sugars (dextrose, fructose and sucrose) the exponent range (for all subjects) was 0.62 to 2.2, which yielded an average exponent value of 1.16. For saccharin, Stevens listed a value of 0.8 versus 0.96 (0.81 to 1.07) in the present study. Whether these differences are important and/or tell something about the underlying mechanism of taste perception remains to be ascertained. The degree to which the present data are compatible with the psychophysical law is evident from Fig. 1. Data analysis enabled us to observe the pattern of individual responses across all the experiments. In more than 60% of the experiments, the subjects generated exponent values that were consistent from experiment to experiment; i.e., if a subject's exponent was greater (or less) than the panel average, it would be greater (or less) throughout the remaining experiments. Two subjects had three or fewer reversals (out of 38 experiments) and the remaining four had 13 to 15 reversals.

With respect to the synergistic effects of the sugar mixtures (dextrose-fructose and sucrose-fructose), the fact that relative sweetness can be significantly enhanced with the appropriate mixtures of simple sugars should have

considerable food application. Although the present studies were carried out in an aqueous system, some of the most promising mixtures warrant further study in a variety of foods. This is especially important because sugars, especially sucrose, undergo irreversible changes as a function of pH (or processing temperature, etc.), and by the time the consumer samples the product its sweetening properties may have changed. For example, a high-acid, carbonated beverage made from 100% sucrose as the sweetener would probably undergo reactions in storage leading to a loss in sucrose and appearance of dextrose and fructose. So the consumer's response would be based on the mixture of dextrose and fructose and the many reactions that these sugars had undergone.

This report presents evidence on synergistic effects of sugar mixtures as measured by magnitude estimation. Testing of these effects in foods remains to be accomplished. It is possible that the replacement process may not be a simple substitution. It would be naive to assume that the sucrose (or dextrose) adds only sweetness to a food. There are many chemical reactions taking place that may or may not be reversible. Factors such as viscosity, temperature and pH must be considered (Pangborn, 1963; Stone et al., 1966), so that the replacement (partially or entirely) of one sugar with another probably would involve other changes as well.

Additional experiments (in model systems as well as in a variety of food products) are being planned to evaluate the effects of pH, temperature and viscosity on the relative sweetness of dextrose-fructose mixtures.

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