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COLLAGEN AND ELASTIN IN DIFFERENT CUTS OF VEAL AND BEEF. I. VOGNAROVÁ, Z. DVORÁK & R. BÖHM. *J. Food Sci.* **33**, 339–343 (1968)—The collagen and elastin content was determined in different cuts of veal and beef. Increases in the amount of total connective tissue are primarily due to increased amounts of collagen, whereas the elastin content is relatively constant. More collagen was found in veal than in beef. The amount of collagen solubilized by heating in one-fourth strength Ringer's solution was twice as high for veal as for beef. Results indicate that the amount of connective tissue proteins in meat is dependent upon the anatomical location and the physiological function of the muscles. Results of chemical and histological analysis are discussed from the standpoint of their nutritional significance.

FROZEN GEESE QUALITY AS AFFECTED BY SEX AND BREED. ELIZABETH LARMOND, A. PETRASOVITS, E. S. MERRITT & N. W. TAPE. *J. Food Sci.* **33**, 349–352 (1968)—Eating quality of Pilgrim, Hungarian and Chinese geese and their crosses was examined in 88 geese, equal numbers of males and females, in two experimental plans. Flavor and tenderness were ranked by an experienced taste panel. A modified Warner-Bratzler shearing device was used for tenderness determinations. Data were obtained for cooking rates, yield, color and moisture content. Variance components were estimated for tenderness measurements on the Warner-Bratzler Shear.

A difference among the genotypes was observed only for percent fat. Small, but statistically significant, differences between sexes were observed for cooking rate, percent meat, moisture of breast meat and color of breast and thigh meat.

POLYPHENOLIC COMPOUNDS IN CANNED TOMATO PASTES. N. RIVAS & B. S. LUH. *J. Food Sci.* **33**, 358–363 (1968)—Polyphenolic compounds in canned pastes made from VF-145 tomatoes (*Lycopersicum esculentum*, Mill) were extracted with methanol and ethyl acetate. The compounds were separated by two-dimensional paper chromatography with n-butanol-acetic acid-water (4:1:5 v/v) and 2% acetic acid as solvents. Twelve spots were found when the chromogenic reagent was $FeCl_3$-$K_3Fe(CN)_6$. The individual compounds were identified by their R_f values and color reactions with $FeCl_3$-$K_3Fe(CN)_6$, diazotized paranitroaniline, Hoepfner, sodium borohydride, and vanillin-HCl reagents, fluorescent behavior, and absorption spectra. Present in the extracts of tomato pastes were two chlorogenic acid isomers, two caffeic acid derivatives, rutin, naringenin, caffeic acid, and ferulic acid. Naringenin and trans-chlorogenic acid were present in larger amounts than the other polyphenolic compounds.

GEL STRENGTH OF KAPPA CARRAGEENAN AS AFFECTED BY CATIONS. M. E. ZABIK & P. J. ALDRICH. *J. Food Sci.* **33**, 371–377 (1968)—Ion-exchange with Al, Fe^{+2}, Fe^{+3} and Sn prevented gel formation when either the exchanging cation or KCl was used as the gelling salt. In both these instances Mg- and Ca-exchanged carrageenan had greater gel strengths than the NH_4- and Na-exchanged ones. For non-exchanged carrageenan the rank order of effect of cation was K, Ca, Mg, Fe^{+2}, NH_4, Sn, Fe^{+3} and Al. Using increasing concentrations of NH_4Cl, NaCl, KCl, and $SnCl_2$ resulted in increased gel strength whereas, increasing concentrations of $CaCl_2$, $FeCl_3$, and $MgCl_2$ decreased gel strength.

STUDIES IN MEAT TENDERNESS. 6. The Nature of Myofibrillar Protein Extracted from Meat During Aging. C. L. DAVEY & K. V. GILBERT *J. Food Sci.* **33**, 343–348 (1968)—Myosin, which constitutes 50–52 pe cent of the myofibrillar protein, can be wholly extracted throughou aging, whereas actin can be extracted in increasing amounts as agin proceeds. In contrast, tropomyosin cannot be extracted and remain firmly held within the myofibrillar structures throughout aging. A comple mixture of extra protein, soluble at low ionic strength, is also release in increasing quantity during aging.

SUBSTRATES AND INTERMEDIATES IN THE ENZYMATIC REDUCTION O METMYOGLOBIN IN GROUND BEEF. B. SALEH & B. WATTS. *J. Foo Sci.* **33**, 353–358 (1968)—Intermediates of the glycolytic pathway whic proved to be effective were glyceraldehyde-3-phosphate and fructose-1,6 diphosphate. Other substrates, oxidized by NAD-linked dehydrogenase known to be present in meat are α-glycerophosphate, malate and glut mate. These also increased metmyoglobin reduction when added to mea The pathway of electron transport from NADH to metmyoglobin is be lieved to be mainly by way of DT diaphorase (menadione reductase and quinones, since dicumarol, a specific inhibitor of this enzyme, pa tially blocked the reaction in meat.

FORMATION OF NITRIC OXIDE MYOGLOBIN: Mechanisms of the Rea tion with Various Reductants. J. B. Fox, Jr. & S. A. Ackerman. *J. Foo Sci.* **33**, 364–370 (1968)—The thermodynamics and kinetics of the form tion of nitric oxide myoglobin have been determined for the reductic of both metmyoglobin nitrite and nitric oxide methemoglobin for th reductants ascorbic acid, cysteine, NADH, hydroquinone and glyce aldehyde. The thermodynamic, kinetic, pH and concentration dependenc data have been used to determine the mechanisms of the vario reactions.

THE ISOLATION AND CHARACTERIZATION OF AN α 1,4 (4,5 dehydr galacturonosyl) GALACTURONATE HYDROLASE. C. W. NAGEL & HASEGAWA. *J. Food Sci.* **33**, 378–382 (1968)—An unsaturated olig galacturonate hydrolase was isolated from the cell extracts of a *Bacill* sp. This enzyme attacked only the α 1,4 glycosidic bond adjacent the terminal 4,5-dehydrogalacturonate of unsaturated oligogalacturo ides and preferentially attacked short chain unsaturated uronides. Th rate of activity was maximal with unsaturated dimer followed by trim (65% that of dimer) tetramer (47%) and pentamer (35%). The p optimum was 6.3 to 6.6 and the enzyme did not require calcium io for its activity. The enzyme was relatively stable below 30°C but lo 90% of its activity after 10 minutes at 40°C.

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SARCOPLASMIC AND MYOFIBRILLAR PROTEIN IN SKELETAL MUSCLE OF TWO BREEDS OF PIG. J. V. McLOUGHLIN. *J. Food Sci.* **33**, 383–85 (1968)—Sarcoplasmic and myofibrillar proteins were extracted from two muscles of Landrace and Large White pigs using solutions of low and high ionic strengths. Breed did not affect the extractability of the proteins. It was concluded that the genetic background did not influence the inherent extractability of the sarcoplasmic and myofibrillar proteins, i.e., the extractability of the muscle proteins before differences in rate of post-mortem glycolysis (considered to exist between the types of pig studied) could induce changes in protein solubility.

QUANTITATIVE DETERMINATION OF CARRAGEENAN IN MILK AND MILK PRODUCTS USING PAPAIN AND CETYL PYRIDINIUM CHLORIDE. H. D. GRAHAM. *J. Food Sci.* **33**, 390–394 (1968)—Cetyl pyridinium chloride (C.P.C.) was used to precipitate carrageenan added to milk, chocolate milk, ice cream and evaporated milk at levels of 0.01 to 0.2% of the weight of the product. Precipitation was carried out in the presence of 0.5–1.0 *M* KCl and Celite. Prior to precipitation, papain was used to digest the proteins at 70°C. The precipitate was freed of adsorbed carbohydrates by washing with 0.1% C.P.C.–0.05 *M* KCl until the washings were negative to the Benedict's test. Then, the precipitate was dissolved in 30% H_2SO_4 and the carbohydrate content determined by the phenol-H_2SO_4 method. Recoveries of carrageenan of 90 to 102%, depending on the product, were obtained.

PEANUT ALCOHOL DEHYDROGENASE. 2. Physico-Chemical and Kinetic Properties. H. E. SWAISGOOD & H. E. PATTEE. *J. Food Sci.* **33**, 400–405 (1968)—Some of the physical, chemical, and kinetic properties of peanut alcohol dehydrogenase have been investigated. A molecular weight of 112,000 and a sedimentation coefficient of 5.46 S were obtained in aqueous salt solutions. The enzyme preparation contained 1.5 g-atoms of Zn per mole of enzyme and was inactivated by 1,10-phenanthroline, EDTA, 8-hydroxyquinoline-5-sulfonic acid, and iodoacetate. Comparison of the peanut enzyme kinetic properties with those of yeast and liver alcohol dehydrogenases indicated generally a greater similarity to the yeast enzyme.

THERMAL RESISTANCE OF SPORES OF FIVE STRAINS OF *Clostridium botulinum* TYPE E IN GROUND WHITEFISH CHUBS. F. D. CRISLEY, R. T. PEELER, R. ANGELOTTI & H. E. HALL. *J. Food Sci.* **33**, 411–416 (1968)—Thermal death-time determinations in the temperature range of 165° F (73.9°C) to 185°F (85°C) were made of spores of five strain Alaska, 4.3 min; Beluga, 2.1 min; 8E, 1.8 min; Iwanai, 1.6 min; and Tenno, 1.6 min. F_{176} (80°C) values for the destruction of approximately 1×10^6 spores per gram of fish paste were: strain Alaska, 34.2 min; Beluga, 17.0 min; 8E, 14.0 min; Iwanai, 12.5 min; and Tenno, 13.2 min. D_{176} values were: strain Alaska, 4.3 min; Beluga, 2.1 min; 8E, 1.8 min; Iwanai 1.6 min; and Tenno, 1.6 min. Z_F values were: strain Alaska, 13.2; Beluga, 13.3; 8E, 10.3; Iwanai, 13.6; and Tenno, 13.1.

MAJOR VOLATILE COMPONENTS OF THE JUICE OF AMERICAN CRANBERRY. R. J. CROTEAU & I. S. FAGERSON. *J. Food Sci.* **33**, 386–389 (1968)—Volatiles from American cranberry (*Vaccinium macrocarpon* Ait.) juice were investigated using gas chromatography, mass spectrometry and infrared spectrophotometry. Forty-two compounds comprising over 95% of the aroma complex were identified: 14 aromatic compounds, 7 terpenes, 9 aliphatic alcohols, 6 aliphatic aldehydes and 6 other compounds including benzoic and 2-methylbutyric acids. Aromatic compounds and terpenes appear to be the major contributors to juice aroma. The remaining 5% of the aroma complex contains over 200 components. Although occurring in very small concentrations, they appear to be important in the total aroma.

WHISKEY COMPOSITION: Identification of Components by Single-pass Gas Chromatography-Mass Spectrometry. J. H. Kahn, E. G. LaRoe & H. A. Conner. *J. Food Sci.* **33**, 395–400 (1968)—A single-pass gas-liquid chromatography method is described for identifying compounds not reported previously in distilled alcoholic liquors. Water-free concentrates of ether-pentane extracts of the distillates were injected into a gas chromatographic column train and the column effluent transported to a mass spectrometer. Several hydrocarbons, and compounds apparently resulting from the reaction of acrolein with ethyl alcohol, were identified in the samples.

GROWTH OF SALMONELLAE ON IRRADIATED AND NON-IRRADIATED SEAFOODS. JACK R. MATCHES & J. LISTON. *J. Food Sci.* **33**, 406–410 (1968)—*Salmonella give* grew competitively in crabmeat at 22°C but not at 11°, 8°, or 5°C. At 22°C the cells grew rapidly reaching high numbers in all samples; but decreased in numbers at all lower temperatures. On English sole tissue, *S. heidelberg*, *S. typhimurium* and *S. derby* all grew rapidly at temperatures as low as 8°C, from inocula as low as 10^1 cells/g and even in the presence of 10 to 100-fold higher numbers of competing saprophytes. Ionizing radiation at relatively low levels, by reducing the numbers of competitive saprophytes, enhances the growth of salmonellae on fish fillets. No growth was obtained under any condition when the temperature was held below 6°C.

SALT, MOISTURE AND AGING TIME EFFECTS ON THE VIABILITY OF *Trichinella spiralis* IN PORK HAMS AND SHOULDERS. D. L. GAMMON, JAMES D. KEMP, J. M. EDNEY & W. Y. VARNEY. *J. Food Sci.* **33**, 417–419 (1968)—Weanling pigs were infested with *Trichinella spiralis*, raised to market weight and sacrificed. Lean samples from hams and shoulders contained respectively an average of 192 and 175 larvae per gram. Hams and shoulders were dry-cured, hung 30 days for salt equalization, smoked and aged at 75°F. Cores from selected hams and shoulders were obtained at weekly intervals and examined for trichinae and analyzed for salt and moisture. The number of live trichinae decreased after smoking until no live ones were present after one month of aging. Percent moisture decreased and percent salt increased during this time.

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CHEMICAL INDUCTION OF MUTATION OR VARIATION IN AFLOTOXIN-PRODUCING CULTURES OF *Aspergillus flavus*. E. G.-H. LEE & P. M. TOWNSLEY. *J. Food Sci.* **33**, 420–423 (1968)—The mutation of a strain of aflatoxin-producing *Aspergillus flavus* was induced chemically after six successive generations of exposure to barium ions. The new characteristics exhibited by the mutant of *A. flavus* are the inability to produce aflatoxins and yellow pigment, accompanied by the loss of fluorescence in the culture under ultraviolet light. These changes did not revert after eight successive passages in a barium-free medium. Chemical-induced mutation was indicated by the mutation of colonies arising from single spores. The mutation characteristics are permanent and irreversible.

NUTRITIVE QUALITY OF SIMULATED MILK MIXTURES PREPARED FROM TROPICAL PLANTS. B. R. STANDAL & H. G. KIAN. *J. Food Sci.* **33**, 426–431 (1968)—Using tropical plant products—taro, soybeans, and coconut—for carbohydrate, protein and fat, respectively, mixtures to simulate milk were prepared and tested on rats for the quality of protein, utilization of calcium, the effect of vitamin B_{12} and the composition of blood and liver. Vitamin B_{12} raised the NPU of Poi-II from 40.4 to 50.0 by raising both nitrogen intake and storage. The NPU of mixtures Poi-II ranged from 50.0 to 51.1, compared to 54.4 for Sobee, 76.6 for Similac and 80.8 for milk. Blood hemoglobin and liver lipid and moisture of rats fed the mixtures Poi-II and milk were similar and within the normal range. Poi-II is similar to Sobee for protein quality, similar to milk for hemoglobin formation and deposition of lipid and moisture in the liver, and poorer than milk for retention of calcium.

LEMON OIL ANALYSIS. 3. Rapid, Capillary Gas Chromatography with Combined Flow and Temperature Programming. W. D. MacLEOD JR. *J. Food Sci.* **33**, 436–437 (1968)—Without preliminary treatment, cold-pressed lemon oil was gas chromatographed on a 100-ft temperature and flow programmed capillary in 10 min. Twenty important volatile flavor constituents were identified and approximately 40 more were detected. The speed and convenience of this procedure suggest use in general survey applications.

HYDROCHLORIC ACID IN ISOLATING ANTHOCYANIN PIGMENTS FROM MONTMORENCY CHERRIES. J. H. von ELBE & D. R. SCHALLER. *J. Food Sci.* **33**, 439–440 (1968)—During isolation of cherry anthocyanin pigments, an increase in the amount of cyanidin-3-monoglucoside and the appearance of cyanidin were noted during drying in the presence of hydrochloric acid; in addition, a cyanidin-diglycoside was found not attributable to partial hydrolysis.

THE MINOR PIGMENT COMPONENT OF MONTMORENCY CHERRIES. D. R. SCHALLER & J. H. von ELBE. *J. Food Sci.* **33**, 442–443 (1968)—The minor pigment component of Montmorency cherries was identified as cyanidin-3-monoglucoside.

***Clostridium perfringens* IN DEHYDRATED SOUPS AND SAUCES.** M. NAKAMURA & K. D. KELLY. *J. Food Sci.* **33**, 424–426 (1968)—The organism was found in 18.2% of the samples. Spaghetti sauce mixes had the highest incidence of *C. perfringens* and the soup mixes had the lowest incidence. One strain possessed heat-resistant spores that were able to withstand boiling at 97.4°C for one hour prior to isolation. The presence of preservatives in the food products did not influence the presence of *C. perfringens* in these food preparations. No common ingredient was detected as the source of contamination. The general presence of this organism in dehydrated soups and sauces may have epidemiological significance in *C. perfringens* food poisoning.

DILUTION OF COW'S MILK AND EGG PROTEINS WITH GLUTAMIC ACID AND THE EFFECT ON THE PROTEIN EFFICIENCY RATIO. V. A. DANIEL, B. L. M. DESAI, S. VENKAT RAO, M. SWAMINATHAN & H. A. B. PARPIA. *J. Food Sci.* **33**, 432–435 (1968)—Addition of glutamic acid to diets containing 8.5% to 5.0% egg proteins to maintain the nitrogen content of the diet constant at 1.6% (equal to 10% protein) did not cause any increase in the growth rate of rats as compared to that on corresponding diets without added glutamic acid. The protein efficiency ratios progressively decreased from 4.74 for 10% egg protein diet to 2.88 for 5% egg protein + 8.4% glutamic acid diet. Addition of glutamic acid to diets containing 8.5 to 5.0% milk proteins to maintain the nitrogen content of the diet constant at 1.6% level caused a significant decrease in the growth rate. The protein efficiency ratios also progressively decreased from 3.48 for 10% milk proteins to 1.46 for a mixture of 5% milk proteins + 8.4% glutamic acid.

PECTIC SUBSTANCES OF DRY BEANS AND THEIR POSSIBLE CORRELATION WITH COOKING TIME. S. KON. *J. Food Sci.* **33**, 437–438 (1968)—Only about 16% of the total pectin extracted from the marc were water soluble. This fractionation was done both without and with prior heating and incubation of marc with α-amylase. More total pectin are extracted with the α-amylase treatment, and a higher percentage of the extracted pectin is water soluble after such a treatment. No significant difference was found between the pectin fractions from high moisture beans and low moisture beans after prolonged storage, even though there was a significant difference in their cooking quality.

QUANTITATIVE DETERMINATION OF DIACETYL BY ELECTRON CAPTURE. R. A. SCANLAN & R. C. LINDSAY. *J. Food Sci.* **33**, 440–441 (1968)—A gas chromatographic procedure utilizing an electron capture detector is described for the quantitative determination of diacetyl. The method involves a gas entrainment, on-column trapping technique, and is applicable to concentrations in the ppb range.

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Collagen and Elastin in Different Cuts of Veal and Beef

SUMMARY—The collagen and elastin content was determined in different cuts of veal and beef. Increases in the amount of total connective tissue are primarily due to increased amounts of collagen, whereas the elastin content is relatively constant. More collagen was found in veal than in beef. The amount of collagen solubilized by heating in one-fourth strength Ringer's solution was twice as high for veal as for beef. Results indicate that the amount of connective tissue proteins in meat is dependent upon the anatomical location and the physiological function of the muscles. Results of chemical and histological analysis are discussed from the standpoint of their nutritional significance.

INTRODUCTION

THERE ARE REASONS TO EXPECT differences in the composition and structure of connective tissue between veal and beef. Wilson *et al.* (1954), Lawrie (1961) and Goll *et al.* (1963) have found more collagen in veal than in beef.

Nevertheless, Wilson *et al.* (1954) did not ascribe differences in the organoleptic properties of veal and beef to a low content of collagen in muscle of beef, but to the greater chronological age of the animal. Goll *et al.* (1964b, 1964c) indicated that increased toughness of beef may be caused by formation of more and stronger cross-links in the collagen molecule.

Mature collagen in meat is also less susceptible to enzymic hydrolysis (Goll *et al.*, 1964a). According to Harding (1965), the changes in collagen are due to the gradual introduction of intramolecular and intermolecular cross-links. Differences between the amount of cross-links in collagen as well as differences in digestibility can be evaluated to some degree on the basis of its solubilization by collagenase (Goll *et al.*, 1964a), by solubilization in buffer medium at 100°C (Goll *et al.*, 1964c) or at 77°C in ¼-strength Ringer's solution (Hill, 1966).

Although there is considerable data on the content of collagen in veal and beef, little is known about the elastin content of meat (Wilson *et al.*, 1954). The amount of both proteins can be estimated on the basis of the content of hydroxyproline, which is absent in other meat proteins.

However, it is not possible to ascertain the ratio of collagen to elastin from hydroxyproline content, since both proteins contain varying amounts of this amino acid.

From a nutritional aspect, collagen and elastin are of lower biological value than other meat proteins. Thus, variation in the content of collagen and elastin influences the biological value of meat proteins. Dvorák *et al.* (1965) have found a linear relationship between the content of a particular available essential amino acid and logarithmus of the concentration of hydroxyproline in proteins of different cuts of beef. The authors ascertained that similar relationships also are valid for the proteins of veal. This relationship permits determination of the biological value of meat proteins from the known content of nitrogen and hydroxyproline in meat either by the Chemical Score method (Mitchell *et al.*, 1946) or as an Index of Essential Amino Acids (Oser, 1951).

Considering the unequal content of hydroxyproline in collagen and elastin, this relation is valid, when at a given amount of hydroxyproline the ratio between collagen and elastin is constant or when the amount of elastin in meat is negligible. The digestibility of the proteins of connective tissue is also important, very low in the case of elastin and in the case of collagen decreases with the chronological age of the animal.

This paper reports a measure of the ratio between collagen and elastin in different cuts of veal and beef. The content of solubilized collagen by the method of Hill (1966) was also studied as an indication of digestibility. To gain information on the changes in the ratio of collagen and elastin in relationship to other meat proteins, the meaty part and the tendrillar part of the biceps femoris were analyzed independently. Chemical analysis was further verified by histological studies.

EXPERIMENTAL

VEAL AND BEEF SAMPLES were taken in the slaughterhouse from animals of red-speckled breeding. Beef samples were taken from heifers about 2 years old, using first or second quality carcasses. Veal samples were from animals of the same breeding, approximately 3 weeks old and weighing 50 to 70 kg.

Particular anatomical cuts were chosen so that the following muscles were predominantly present in the samples: Fillet: psoas major; round: biceps femoris, glutaeobiceps; loin: longissimus dorsi, spinalis et semispinalis dorsi; flank: transversus abdominis, obliquus abdominis; ribs: mostly intercostales interni et externi; neck: sternocleidomastoideus, trapezius; shank: all muscles.

Samples of the same weight from 3 heifers or 3 calves were taken from a particular anatomical cut. They were ground together and mixed. This composite sample was used for analysis.

The biceps femoris was dissected from 5 heifers or 5 calves. For analysis, the meat was chosen from the broadest position of the front branch of its distal part, where it grows together with fascie lata femoris and fascie cruris. The tendrillar end of this muscle was also analyzed. Standard samples of this muscle were prepared, as described before, for particular anatomical cuts.

For determination of collagen and elastin, the ground sample of tissue was homogenized with a solution of 0.2 *M* NaCl. An aliquot of the homogenate was taken for nitrogen determination using the semimicro-Kjeldahl analysis

method of Block (1960). Other aliquots were diluted tenfold with a solution of 0.2 *M* NaCl.

After correcting the pH to 7.4, the mixture was extracted 24 hr at + 5°C and centrifugated. The insoluble residue was extracted once more in the same manner.

Then followed two extractions of the insoluble residue with a tenfold volume of 0.1 *N* NaOH.

After centrifugation and discarding of the supernatant, the residue was washed three times with water. The residue, representing the insoluble collagen and elastin, was autoclaved twice at 121°C with water 1 hr. The hot solutions of gelatin were filtered. The insoluble residue was washed with boiling water three times. The combined solutions of gelatin were diluted to a known volume with water and represent the insoluble collagen. The insoluble residue on the filter represents the elastin. Aliquots of the gelatin solution as well as the total amount of elastin were hydrolyzed with 6 *N* HCl in sealed tubes at 110°C for 24 hr.

The digests were used for the determination of hydroxyproline by the method of Serafini-Cessi *et al.* (1964). Factors of 7.46 or 52.3 were used for converting hydroxyproline to collagen or elastin, resp. (Neuman *et al.*, 1950). Hydroxyproline, as well as collagen and elastin, are reported in g/16 g N.

Soluble collagen was determined in the meat by solubilization in ¼-strength Ringer's solution at 77°C as described by Hill (1966) followed by analysis for hydroxyproline. For the histological study, cuts were made from meaty and tendrillar parts of the biceps femoris. Collagen was stained in the preparations of van Gieson and Mallory's stain, while elastin was stained with Weigert's resorcin fuchsin.

RESULTS

HYDROXYPROLINE, COLLAGEN AND ELASTIN in the different cuts of veal and beef, are given in Table 1. Results are expressed in g/16 g N, or a fat-free, moisture-free basis. The amount of connective tissue proteins was calculated from the hydroxyproline content of corresponding fractions of collagen and elastin, multiplied by the respective factors. The hydroxyproline content of collagen increased gradually from fillet to shank. With exception of the neck, more hydroxyproline was found in veal than in beef. The hydroxyproline content of elastin represented only 0.69 to 2.05% of the total hydroxyproline and was varying in both veal and beef within these limits.

Collagen increased from 4.125 to 23.618 g/16 g N in veal and from 3.088 to 22.783 in beef. Elastin values varied between 0.418 and 2.144 g/16 g N for veal and between 0.209 to 2.510 in beef. Elastin represented from 4.7 to 9.6% and 5.4 to 15.2% of all connective tissue proteins in veal and beef, respectively. The percentage of elastin in the connective tissue did not increase parallel with the total amount of collagen and elastin.

The distribution of hydroxyproline and proteins of the connective tissue in the biceps femoris is indicated in Table 2. It is remarkable that in the meaty part of this muscle, collagen represented approximately half the amount of that found in the round, although the biceps femoris is a component of the latter. Nevertheless, the content of elastin is greater, and represents 37.4% and 27.9% of the total amount of collagen and elastin in the samples of veal and beef, respectively. Tendrillar part of the biceps femoris consisted largely of collagen, the amount of which was 59.5 and 81.8 g/16 g N in veal and beef, respectively. The percentage of elastin in this cut decreased to 5.8 and 3.4 g/16 g N in veal and beef, respectively.

Histologically, the meaty part of the biceps femoris is covered by a connective tissue layer, with an average thickness of 0.3 mm. The connective tissue septa, which penetrate the inside of muscle, are relatively thin. In tendrillar cuts, aponeurose combines connective tissue layers of the outside surface and weaker connective tissue layer of the inside surface of this muscle. Here muscle fibers disappear. Both connective tissue layers of the surface of muscle represent mostly collagen, through which thinner layers only of elastin fibers penetrate.

Table 1. Hydroxyproline, collagen and elastin in different cuts of veal and beef.

		Hydroxyproline			Connective tissue proteins		
		In collagen g/16g N	In elastin g/16g N	Hypro elast. from Hypro total. %	Collagen g/16g N	Elastin g/16g N	Elastin from collagen + elastin %
Fillet:	Veal	0.553	0.008	1.43	4.125	0.418	9.23
	Beef	0.414	0.004	0.96	3.088	0.209	6.35
Round:	Veal	1.296	0.015	1.14	9.668	0.785	7.51
	Beef	0.845	0.020	2.31	6.304	1.046	14.23
Loin:	Veal	1.398	0.015	1.06	10.429	0.785	7.00
	Beef	1.219	0.010	0.82	9.094	0.523	5.44
Flank:	Veal	2.509	0.036	1.41	18.717	1.883	9.14
	Beef	1.874	0.048	2.50	13.980	2.510	15.24
Ribs:	Veal	2.706	0.041	1.49	20.187	2.144	9.61
	Beef	2.607	0.056	2.10	19.448	2.929	13.10
Neck:	Veal	1.967	0.029	1.45	14.674	1.517	9.38
	Beef	2.144	0.026	1.20	15.994	1.360	7.84
Shank:	Veal	3.166	0.022	0.69	23.618	1.151	4.65
	Beef	3.054	0.034	1.10	22.783	1.778	7.88

Table 2. Hydroxyproline, collagen and elastin in meaty and tendrillar part of the biceps femoris of veal and beef.

	Hydroxyproline			Connective tissue proteins		
	In collagen g/16g N	In elastin g/16g N	Hypro from elast. Hypro total. %	Collagen g/16g N	Elastin g/16g N	Elastin from collagen + elastin %
Meaty part:						
Veal	0.562	0.048	7.82	4.193	2.510	37.44
Beef	0.490	0.027	5.22	3.655	1.412	27.87
Tendrillar part:						
Veal	7.972	0.070	0.87	59.471	3.661	5.79
Beef	10.970	0.055	0.50	81.836	2.877	3.39

Muscle in the meaty part is distributed into bundles, separated by connective tissue septa. The latter are thicker in veal than in beef. In veal they are predominantly composed of elastin fibers, which are relatively rare in beef. Primary bundles of muscle fibers are a little thicker in veal than in beef. Results are indicated in Table 3 and Fig. 1.

Solubilization of collagen was ascertained in particular

Table 3. Quantitative comparison of morphological proportions in the biceps femoris of veal and beef.

	Veal	Beef
Dimensions of muscle bundles, μ	1,157 × 1,817	2,789 × 3,258
Thickness of connective tissue septa between muscle bundles, μ	50 × 100	$\leqq$ 50
Dimensions of primary bundles, μ	544 × 399	452 × 336
Number of fibers in a primary bundle	128	76
Thickness of muscle fiber, μ	30.4	48.3

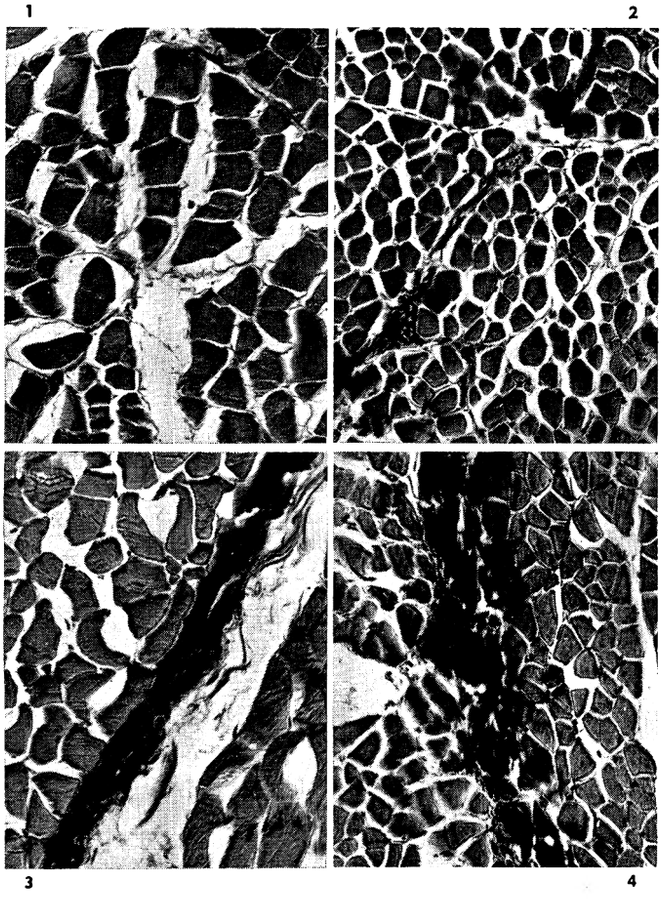

Fig. 1. Comparison of the thickness of muscle fibers and connective tissue septa in the biceps femoris of beef and veal. Cross-section, 175 × enlarged. Van Gieson staining.
1. Muscles of beef. Connective tissue endomysium between thick muscle fibers.
2. Muscle of veal. Connective tissue endomysium between thin muscle fibers. Primary bundle contain more fibers.
3.-4. Connective tissue septum between bundles of muscle fibers in beef and veal, respectively.

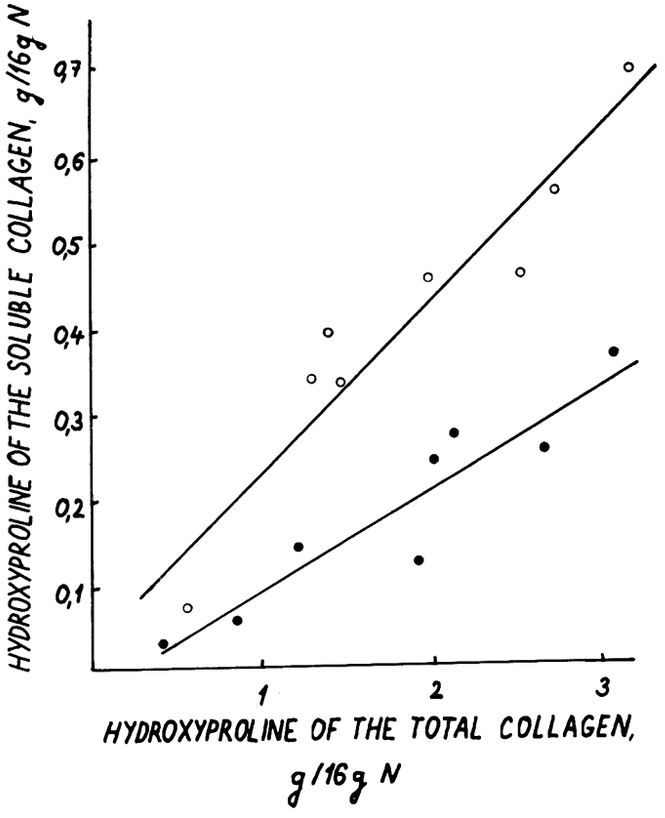

Fig. 2. Relationship between the content of the total collagen and the soluble collagen in veal and beef. (○—veal, ●—beef).

anatomical cuts of veal and beef. The amount of soluble collagen contains roportionately more soluble collagen. pressed in Fig. 2.

Regression straight lines indicate linear dependence. This indicates that meat with greater amounts of total collagen contains proportionately more soluble collagen. The latter represents approximately 23% and 10% of total collagen in veal and beef, respectively.

DISCUSSION

THE PRESENT PAPER DESCRIBES the relationship between collagen and elastin in different cuts of meat. Both proteins were evaluated on the basis of hydroxyproline content, of which elastin contains a small amount in comparison to collagen. Values of the amount of hydroxyproline for collagen and elastin indicate that there are negligible changes in hydroxyproline of elastin in comparison with total hydroxyproline. The relationship between total hydroxyproline and the content of a particular available amino acid is important in evaluating the meat proteins. This relationship enables one to enumerate the biological value of meat proteins.

Results indicate that the amount of the connective tissue proteins in meat increases according to anatomical location and physiological function of particular muscles. In that sense, only collagen increases. The presence of the tendrillar ends of muscles specifically influences the total evaluation, because 59% and 82% of the collagen from all proteins of veal and beef is present in this part. Conversely, elastin with its content 0.2 to 3.7% of all meat proteins is relatively constant.

Goll *et al.* (1963) cite the ratio of collagen to elastin as 3:1, on the basis of the work of Wilson *et al.* (1954). Nevertheless, results here cited indicate that this ratio changes in different cuts of meat. In the biceps femoris muscle, 37.4 and 27.9% of elastin from the total amount of the connective tissue proteins was found for veal and beef, respectively. These values approach the ratio cited by Goll *et al.* (1963).

In the meaty part of the biceps femoris of veal, three times more elastin was found than in veal round. Also in other anatomical cuts of meat, the percentage of elastin to the total amount of the connective tissue proteins was not found to be constant and varied between 4.65 and 15.24%. These low values can be explained by the fact that elastin penetrates muscle tissue directly but is found in only small amounts in the surface layers of muscles, which are richer in collagen.

The histological study confirms the relatively high amount of elastin in the biceps femoris of veal, showing that connective tissue septa between muscle bundles are composed mostly of elastin fibers, which are more abundant than in beef muscle.

Larger amounts of collagen in veal than in beef confirm results in the literature. On the other hand, elastin content was higher in some cuts of beef than in veal. Gersh *et al.* (1949) explained greater amount of collagen and elastin in veal on the basis of a constant content of connective tissue in the muscle throughout the life of the animal. With increasing age, only the thickness of connective fibers increased. On the other hand, Hiner *et al.* (1953) reported that fibers of muscle tissues increase directly with age. Thus, the proportion of connective tissue to total muscle proteins decreases.

Neseni *et al.* (1955) and Böhm (1963) have also reached the same conclusion, which has been verified by the results of the histological study. The number of fibers in primary bundles decreases with increasing age because connective tissue septa of perimysium become thicker while new, thinner primary bundles of muscle fibers arise by dividing. Therefore, primary bundles are thinner in beef than in veal. Böhm (1963) found similar changes in muscles of rabbit. Differences between the thickness of the muscle bundles of veal and beef and strength of the connective tissue septa between them indicate increased amounts of myofibrillar proteins in beef.

Structural changes in the collagen molecule also occur with increasing age of the animal. According to Goll *et al.* (1964b, 1964c) cross-links in the molecule are formed. These changes in collagen during maturation cause its decreased solubilization in ¼-strength Ringer's solution at 77° (Hill, 1966). Solubilization of collagen is to some degree an indicator of the number of cross-links.

Solubilization made in different parts of veal and beef indicates that the amount of solubilized collagen increases with increasing amounts of the connective tissue in constant proportion. There was no differentation of collagen due to location of muscle but only due to the age of the animal. Collagen in veal contains about twice as many soluble compounds as beef. The amount of soluble collagen may be considered also as an indicator of decreased digestibility of meat. A lower digestibility of beef collagen in comparison to veal can be attributed to the structural differences of collagen. These changes may also influence the biological value of meat proteins.

REFERENCES

Block, R. J. 1960. "Analytical Methods of Protein Chemistry," Vol. 2, p. 3. Pergamon Press, Oxford.

Böhm, R. 1963. Príspěvek k mikroskopické anatomii některych svalu králíka (Contribution to the microscopic anatomy of some muscles in rabbit.) *Acta univ. agriculturae,* Brno. B, **11**, 41.

Dvorák, Z. and Vognarová, I. 1965. The relationship between the content of the connective tissue and the biological value in meat. 11th Europ. Meeting of Meat Res. Workers. Belgrade.

Gersh, I. and Catchpole, H. R. 1949. The organization of ground substance and basement membrane. *Amer. J. Anat.* **85**, 457.

Goll, D. E., Bray, R. W. and Hoekstra, W. G. 1963. Age-associated changes in muscle composition. The isolation and properties of a collagenous residue from bovine muscle. *J. Food Sci.* **28**, 503.

Goll, D. E., Hoekstra, W. G. and Bray, R. W. 1964a. Age-associated changes in bovine muscle connective tissue. I. Rate of hydrolysis by collagenase. *J. Food Sci.* **29**, 608.

Goll, D. E., Hoekstra, W. G. and Bray, R. W. 1964b. Age-associated changes in bovine muscle connective tissue. II. Exposure to increasing temperature. *J. Food Sci.* **29**, 615.

Goll, D. E., Bray, R. W. and Hoekstra, W. G. 1964c. Age-associated changes in bovine muscle connective tissue. III. Rate of solubilization at 100°C. *J. Food Sci.* **29**, 662.

Harding, J. J. 1965. Unusual links and cross-links of collagen. *Advances in Protein Chem.* **20**, 109.

Hill, F. 1966. The solubility of intramuscular collagen in meat animals of various ages. *J. Food Sci.* **31**, 161.

Hiner, R. L., Hankins, O. G., Sloane, H. S., Fellers, C. R. and Anderson, E. E. 1953. Fiber diameter in relation to tenderness of beef muscles. *Food Res.* **18**, 364.

Lawrie, R. A. 1961. Studies on the muscles of meat animals. I. Differences in composition of beef longissimus dorsi muscles determined by age and anatomical location. *J. Agric. Sci.* **56**, 249.

Mitchell, H. H. and Block, R. J. 1946. Some relationships between the amino acid contents of proteins and their nutritive values for the rat. *J. Biol. Chem.* **163**, 599.
Neuman, R. E. and Logan, M. A. 1950. The determination of collagen and elastin in tissues. *J. Biol. Chem.* **186**, 549.
Neseni, R. and Müller, Ch. 1955. Ueber die Muskelfaserstärke bei Klein- und Grosstieren. *Z.f. Tierzüchtung u.Züchtungsbiologie.* **65**, 333.
Oser, B. L. 1951. Method for integrating essential amino acid content in the nutritional evaluation of protein. *J. Amer. Dietet. Assoc.* **27**, 396.
Serafini-Cessi, F. and Cessi, C. 1964. An improved method for determination of hydroxyproline in protein hydrolysates. *Anal. Biochem.* **8**, 527.
Wilson, G. D., Bray, R. W. and Phillips, P. H. 1954. The effect of age and grade on the collagen and elastin content of beef and veal. *J. Animal Sci.* **13**, 826.
Ms. accepted 4/3/68.

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Studies in Meat Tenderness. 6. The Nature of Myofibrillar Proteins Extracted from Meat During Aging

SUMMARY—A study has been made of the myofibrillar proteins extracted from beef and rabbit meat by a buffer that dissociates the actomyosin complex of the muscle cell. Myosin, which constitutes 50–52 per cent of the myofibrillar protein, can be wholly extracted throughout aging, whereas actin can be extracted in increasing amounts as aging proceeds. In contrast, tropomyosin cannot be extracted and remains firmly held within the myofibrillar structures throughout aging. A complex mixture of extra protein, soluble at low ionic strength, is also released in increasing quantity during aging. It is proposed that in meat aging there is a progressive loss of the tensile strength of the myofibrillar component of muscle brought about by the weakening and final dissolution of the Z-band structures. Such a disintegration would lead to the observed changes in the extractabilities of the myofibrillar proteins.

INTRODUCTION

WHEN MEAT IS STORED at above freezing temperature it becomes progressively more tender with time (so-called aging), a phenomenon of considerable importance to the meat-processing industry. Before it is possible to determine optimal time-temperature conditions for aging it is necessary to understand the mechanism of the process. Only then can the effect on aging of such factors as the species, age, and pre-slaughter condition of the animal be expected to be understood and fully controlled.

Meat aging has usually been considered to be due to changes within the fibrous components of muscle, so that most studies of the aging mechanism have been concerned with alterations in the properties either of connective tissue or of myofibrils. However, significant and consistent biochemical changes have been observed in the myofibrillar components only, so that meat aging is now thought to be due to morphological changes in these structures (Davey *et al.*, 1968). The sarcomere—the fundamental repeating unit of the myofibril—has a clearly-defined structure with a precise organization of its protein components (Huxley *et al.*, 1957). Biochemical studies involving the selective extraction of these components have located myosin in the A band of the sarcomere, and actin, probably in association with tropomyosin, in the I-band region (Hasselbach *et al.*, 1951; Corsi *et al.*, 1958). It is to be expected that similar biochemical studies of changes during aging in the pattern of protein extractability from myofibrils would be a useful first step in elucidating alterations in sarcomere morphology. In this respect a number of workers have demonstrated clearly that an increase in extractability occurs when extracting buffers of relatively high ionic strength ($I > 0.5$) are used (Weinberg *et al.*, 1960; Khan *et al.*, 1964; Aberle *et al.*, 1966). However, other workers have failed to show a change in extractability during aging (Scharpf *et al.*, 1964; Goll *et al.*, 1964; Fujimaki *et al.*, 1965). These conflicting views have been reconciled in large part in a previous communication of this series (Davey *et al.*, 1968), where it was shown that the extent of the extractability changes is determined by the ultimate pH value of the meat. Although these changes in extractability were consistently observed, they were small and therefore rather difficult to determine at low ultimate pH values.

In this paper, the major components of the protein extracted from the myofibrils during aging have been identified. On the basis of these extractability studies, a theory of meat aging is proposed.

METHODS

Meat sampling

Meat was obtained from beef animals slaughtered at the local meat works, and from mature domestic rabbits. Sections of longissimus dorsi (LD) muscles adjacent to the 10th–14th ribs from a variety of beef animals were removed after the halved carcasses had been held for 24 hr at chilled temperature (2–4°C). Rabbit LD muscles, excised as soon as possible after decapitation, were stored at 2°C for 16 hr in a moist nitrogen atmosphere. Zero

times of aging for the muscles from beef and rabbit carcasses were taken to be 24 hr and 16 hr post-mortem, respectively. In these times the ultimate pH values of the samples had been reached. Only those muscles having ultimate pH values below 5.7 were chosen for further study.

Bacterial control

In order to limit bacterial spoilage, meat samples were prepared under aseptic conditions (Davey *et al.*, 1966), stored in a nitrogen atmosphere and sprayed with a mixture of aureomycin (100 ppm) and chloroamphenicol (100 ppm) at intervals during the storage period. Myofibrils were isolated and washed also under aseptic conditions, using buffers that had been boiled for 20 min. The method for determining the extent of bacterial growth both on samples of meat and in compacted myofibrils has been described previously (Davey *et al.*, 1968). Strict adherence to these procedures ensured that bacterial numbers were maintained at the low level of $< 10^2$ organisms/g for meat aged for 30 days at 2°C and for myofibrillar preparations aged for 21 days at 2°C.

Composition of extracting buffers

Buffers used for extracting proteins selectively from the myofibrillar preparations had the following compositions: HS buffer, 0.6*M* KCl, 0.1*M* potassium phosphate, 1m*M* magnesium chloride, 10m*M* sodium pyrophosphate, pH 6.4 (Hasselbach *et al.*, 1951); pyrophosphate (P-P) buffer of high ionic strength (I = 0.98), 0.9*M* KCl, 0.02*M* potassium phosphate, 1m*M* magnesium chloride, 10m*M* sodium pyrophosphate, pH 6.80; phosphate-KCl buffer of relatively low ionic strength (I = 0.19), 0.1*M* KCl, 0.05*M* potassium phosphate, pH 7.0.

Preparation of myofibrils

The method for preparing myofibrils from bovine and rabbit LD muscles, separated as far as possible from other cell components and debris, has been described (Davey *et al.*, 1968).

Extraction of myofibrils

All manipulations used in the extraction of myofibrils were carried out at 2°C. A sample of the compacted myofibrils (8 g) containing approximately 10% protein was made into a thick slurry, free of lumps, with 0.16*M* KCl (10 ml) and 80 ml of the selected buffer (HS, P-P or phosphate-KCl) were added. A portion of the gently-stirred suspension was then removed for total-protein analysis. The suspensions, after 40 min stirring, were centrifuged for 30 min at 10,000 × G, and the concentration of extracted protein in the supernatant fraction obtained was expressed as a percentage of the total protein concentration in the suspension.

Electrophoresis

Moving-boundary electrophoresis was carried out in the buffer 0.25*M* NaCl, 0.036*M* potassium phosphate, pH 7.6 using the Perkin-Elmer electrophoresis apparatus (model 38). At the pH value (7.6) and ionic strength (I = 0.34) of this buffer, no precipitation of the extracted proteins occurred during dialysis. Myosin, actomyosin and tropomyosin were identified in the myofibrillar extracts from their electrophoretic mobilities previously determined for the purified proteins. If the identity of a protein was in doubt an additional electrophoresis run was performed on the extract to which the purified protein had been added. A single symmetrical peak after prolonged dialysis indicated that the two proteins were electrophoretically identical.

Estimation of proteins

Determination of the amounts of myosin and actin in HS-buffer extracts is made possible by using the fact that the concentration of a protein is proportional to the area of the peak it gives in a moving-boundary electrophoresis diagram (Longsworth, 1939). In this study, myosin and actomyosin were the two major components in the protein solution prepared for electrophoresis. The latter protein is produced by a combination of F-actin with four times its weight of myosin (Spicer *et al.*, 1951; Maruyama *et al.*, 1962), during the removal of pyrophosphate and magnesium ions by dialysis against 0.04*M* KCl. The actomyosin formed, together with remaining myosin, are wholly precipitated at the low ionic strength (I = 0.04) of the dialysate and can be separated by centrifugation essentially free from protein that remains soluble. Thus the amount of myosin in the precipitates (redissolved by the electrophoresis buffer) is proportional to the area of the myosin peak plus 80% of the area of the actomyosin peak, whereas the concentration of F-actin is proportional to 20% of the area of the actomyosin peak alone.

The amount of protein that remains soluble on dialysis can be detrmined readily so that the extraction of both myosin and actin from myofibrils during aging can be expressed as a percentage of the total myofibrillar protein. In practice, it was impossible to determine the area of the super-sharp actomyosin peak accurately. The area was thus estimated as the difference between the total area of the protein mixture undergoing electrophoresis (determined on the broad less-resolved diagram from the descending limb of the electrophoresis cell) and the summated areas of myosin (peak B) and the small peak C (Fig. 1).

To ensure the accuracy of the method, planimeter readings of peak-areas were made on electroprohesis diagrams enlarged 5 times, and then only after myosin and actomyosin had been completely resolved by prolonged electrophoresis.

To determine the concentration of tropomyosin extracted from myofibrils by the buffers, the protein was first released from its association with the F-actin moiety of actomyosin present in the extracts (Maruyama, 1964). This is an essential step, as tropomyosin would otherwise be precipitated with actomyosin during the dialysis to low salt concentration demanded by the method for determining the protein. Tropomyosin release from its association with actin was achieved by dialyzing the buffer extracts against 0.6*M* KI for 24 hr at 2°C.

The high iodide concentration has the effect of depolymerizing actin irreversibly into a globular form with concomitant dissociation of actomyosin (Szent-Györgyi, 1951). This was verified by similarly treating purified actomyosin. Moving-boundary electrophoresis showed that

myosin alone precipitates from KI-treated extracts on dialysis against 0.04*M* KCl, inactive G-actin remaining in solution. Further evidence that this method of KI treatment gives a true measure of the tropomyosin content was obtained from finding that purified tropomyosin added in 6 experiments to extracts before dialysis against KI was fully recovered (95%–100%).

The viscosity drop that occurs on adding salt to a tropomyosin solution was used to determine the concentration of this protein in the extracts (Perry *et al.*, 1958). The viscosities of the phosphate-KCl extracts and of the KI-treated extracts, exhaustively dialyzed against 0.078*M* borate buffer pH 7.1, were determined at 0°C with an Ostwald viscometer (water-outflow time, 46 sec). The drop in viscosity of the extracts was then measured after addition of 4.0*M* KCl (in the borate buffer) to a final concentration of 0.5*M* KCl.

The tropomyosin concentrations of the solutions were estimated from a standard curve obtained with highly-purified tropomyosin treated under identical conditions, over the range 0-1 mg protein/ml. This method was capable of determining tropomyosin concentrations of less than 0.015 mg/ml.

Preparation of purified proteins

Bovine myosin was prepared according to the method of Perry (1955). Actomyosin was obtained by extracting well-washed myofibrillar preparations from bovine LD muscle with Weber-Edsall buffer (0.6*M* KCl, 0.04*M* sodium bicarbonate, 0.01*M* sodium carbonate, pH 9.20) for 16 hr at 2°C (Perry *et al.*, 1958). Myrofibrillar debris was removed by centrifugation, and 10 vol of water was added. The pellet of protein obtained by centrifugation (2 hr, 30,000 × G) was redissolved in 0.5*M* KCl and adjusted with water to 0.3*M* KCl to reprecipitate actomyosin. After a further centrifugation the actomyocin was purified by 2 additional cycles of solution at 0.5*M* KCl and precipitation at 0.3*M* KCl.

Tropomyosin, prepared from bovine LD muscle as described for rabbit muscle by Bailey (1951), was found from electrophoresis to be approximately 70% pure. It was purified further by chromatography on a column of DEAE-cellulose (height, 25 cm; diameter 1.1 cm) using a linear gradient of KCl from 0.00*M* to 1.00*M* over 400 ml in tris buffer (0.02*M*, pH 8.2). Tropomyosin, free from less firmly held components, began emerging from the column at 0.55*M* KCl and was precipitated from the eluant by ethanol (Bailey, 1951).

The three purified myofibrillar proteins each gave a single symmetrical peak on prolonged electrophoresis. They were stored in 50% glycerol, 0.5*M* KCl at −20°C for at least 6 months without showing significant changes in their electrophoretic patterns.

Chromatography of extra protein

The heterogeneity of the extra protein extracted during the aging of isolated, washed myofibrils was examined by ion-exchange chromatography. The protein (50–100 mg) was dialyzed against 0.02*M* potassium phosphate buffer, pH 7.0 and applied to a column of DEAE-cellulose (height 25 cm; diameter 1.1 cm), equilibrated with the same buffer. The chromatogram was developed with a linear gradient of KCl from 0.00*M* to 1.00*M* over 420 ml in potassium phosphate buffer, 0.02*M* pH 7.0. Eluted protein was determined in 6 ml fractions by absorbancy measurements at 280 mμ.

Protein and pH determinations

The total protein concentrations of myofibrillar suspensions were determined by a micro-Kjeldahl method (AOAC, 1955). Protein extracted from the myofibrillar suspensions was determined by a biuret method (Gornall *et al.*, 1949). Estimations of pH were made, using a glass electrode, on homogenates (Marsh *et al.*, 1950) of meat samples (1–2 g) in 2m*M* sodium iodoacetate solution (10 ml, pH 7.0).

RESULTS

HS BUFFER HAS BEEN USED to extract myosin selectively from myofibrils prepared from rabbit muscle. The pyrophosphate and magnesium-ion constituents free myosin from its attachment to actin within the washed myofibrils, allowing solubilization at the high ionic strength (I = 0.74) of this buffer (Hasselbach *et al.*, 1951). An electrophoresis diagram, Fig. 1a (i), shows that HS buffer acts similarly in extracting myosin selectively from well-washed myofibrils of unaged bovine LD muscle. Essentially all the myosin is presumed to be released during the extraction period of 40 min, this quantity, (50–52% of the myofibrillar protein) being the recognized myosin content of the rabbit myofibril (Hanson *et al.*, 1957).

On the other hand the quantity of protein extracted from myofibrils prepared from aged muscle is much larger, rising throughout aging to a value greater than 70% of the myofibrillar protein (Davey *et al.*, 1968). A knowledge of the nature of the additional protein released from the myofibrils is essential to our understanding of the aging phenomenon. Table 1, typical of several such experi-

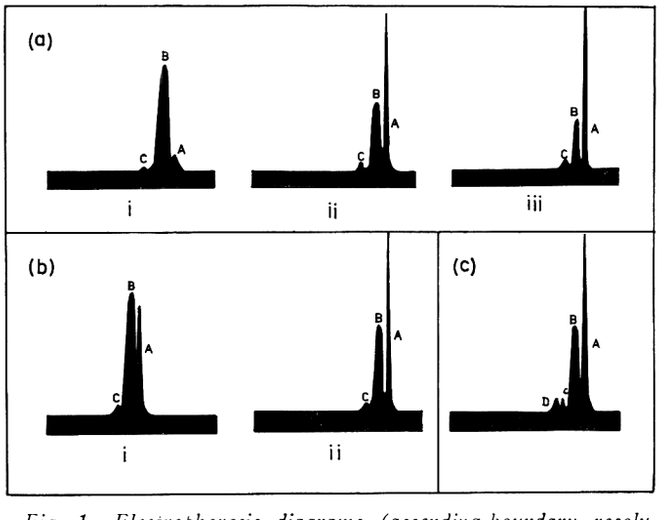

Fig. 1. Electrophoresis diagrams (ascending-boundary resolution) of protein precipitated from HS-buffer extracts of myofibrils by dialysis against 0.04M KCl: Direction of migration, left to right. (a) Prepared from bovine muscle aged for different times at 2°C, (i) no aging; (ii) aging 10 days; (iii) aging 24 days. (b) Prepared from bovine muscle and aged as well-washed myofibrils at 2°C, (i) no aging; (ii) aging 12 days. (c) Prepared from rabbit muscle aged for 10 days at 2°C.

Table 1. The fractionation at low ionic strength (I = 0.04) of proteins extracted by HS buffer at intervals during the aging of well-washed myofibrils from bovine LD muscles.

Days aging (2°C)	Percentage myofibrillar protein extracted	Fractionation of extracted protein at I = 0.04 (percentage of myofibrillard protein)		
		Precipitated component (and myosin)[1]	Precipitated component	Soluble component
0	52	51	1	1
3	61	55	5	6
5	65	57	7	8
8	69	59	9	10

[1] The myosin content is assumed to be 50% of the myofibrillar protein (see Fig. 2).

ments, shows that the additional material extracted is not a single protein, since it can be fractionated into a soluble component and (with myosin) a precipitated component by exhaustive dialysis of the HS-buffer extracts against 0.04*M* KCl. Table 1 also shows that both the soluble and the precipitated components increase in quantity during aging.

Moving-boundary electrophoresis (Fig. 1) shows that a number of proteins (peaks A-D in descending order of mobilities) are apparently precipitated at I = 0.04 from myofibrillar extracts of bovine and rabbit muscles. Actomyosin (peak A) is virtually absent from extracts of unaged material, but increases at the expense of myosin (peak B) to become the major component (with myosin) as the aging of either whole meat (Fig. 1a) or the myofibrils prepared therefrom (Fig. 1b) proceeds. This result shows clearly that actin is released from myofibrils into the HS-buffer extracts in increasing amounts during aging and that on dialysis against 0.04*M* KCl (removal of pyrophosphate and magnesium ions) it combines with 4 times its weight of myosin (also present) to be precipitated as the actomyosin of the electrophoresis diagrams.

The increase in the precipitated component (Table 1) is a measure of the progressive release of actin during the aging process of either whole meat or of well-washed myofibrils. A third component (peak C) always shows on the electrophoresis diagrams and, in extracts from the myofibrils of rabbit LD muscle (Fig. 1c), resolves on prolonged electrophoresis to give a fourth component (peak D). These latter proteins (peaks C and D) together contribute approximately 3% to the total protein precipitated and are possibly small amounts of soluble component occluded in the precipitated fraction. Similar results were obtained using whole meat samples of the LD muscles from 4 beef and 3 rabbit carcasses.

Tropomyosin is the third most plentiful myofibrillar component (after myosin and actin) and constitutes 10%–12% of the protein of the rabbit myofibril (Perry *et al.*, 1958). From electron-microscopic studies it is considered to be located both in the I bands of the myofibril in close association with actin (Hanson *et al.*, 1963), and in the Z bands (Huxley, 1963). Table 2 shows that tropomyosin remains resistant to release from myofibrils during aging.

It is not extracted by either phosphate-KCl buffer, which releases up to 10% of the myofibrillar protein, or by HS buffer, which extracts myosin and increasing amounts of actin during aging. On the other hand, at the higher ionic strength of P-P buffer, tropomyosin is wholly extracted from both unaged and aged myofibrils. Table 2 also shows that the tropomyosin content of bovine myofibrils (8.7–9.2%) is similar to the (10–12%) found for rabbit.

Table 2. Changes during aging of well-washed myofibrils from bovine LD muscles, in the tropomyosin content (expressed as a percentage of the total myofibrillar protein) of buffer extracts of the myofibrillar preparations.

Animal	Aging time at 2°C (days)	Buffer extract		
		Phosphate-KCl	HS	P-P
Jersey bull	0	0	0	8.7
(6 years old)	21	0	0	9.1
Aberdeen Angus steer	0	0	0	8.9
(3 years old)	22	0	0.8	9.2

Although actin is the major single myofibrillar component released in an extractable form during aging, Fig. 2, typical of numerous such experiments, shows that there is also a progressive and substantial increase in the quantity of extra protein extracted by buffers of low ionic strength. During the early period of aging, extra protein is extracted in greater quantity than is actin, although after 8 days of storage similar amounts of both (8–10% of the myofibrillar protein) are released. Also shown in Fig. 2 are the curves relating aging time with the extractabilities of myosin and actin (measured from electrophoresis diagrams) and of tropomyosin (measured viscometrically). Algebraic summation of the extractability curves shows that myosin, actin and extra protein make up most of the protein extracted by HS buffer, approximately 3% remaining unaccounted for.

The nature of the extra protein, which is assumed to be identical with the soluble component described in Table 1, remains undetermined, as myosin, actin and tropomyosin are the only well-characterized proteins of the

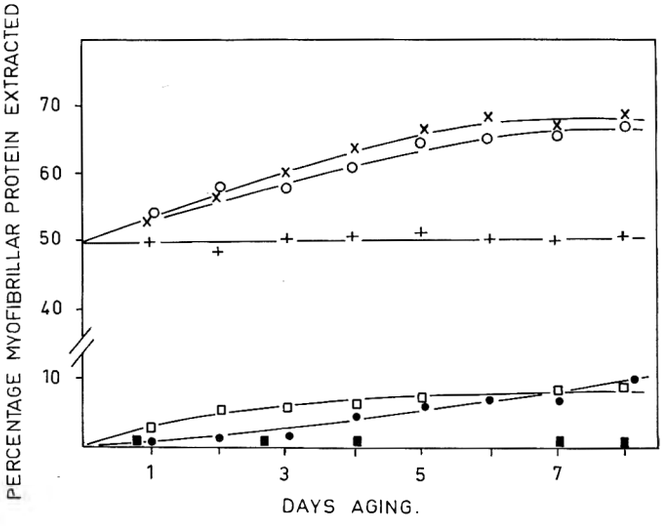

Fig. 2. Changes in the extractabilities of myofibrillar proteins during the aging of isolated myofibrils from bovine LD muscle. Protein extracted by HS-buffer, ×——×; actin, ●——●; extra protein, □——□; myosin, +——+; tropomyosin, ■. The sums of the percentage extractabilities of myosin, actin and extra protein at intervals of aging are given by the open circles.

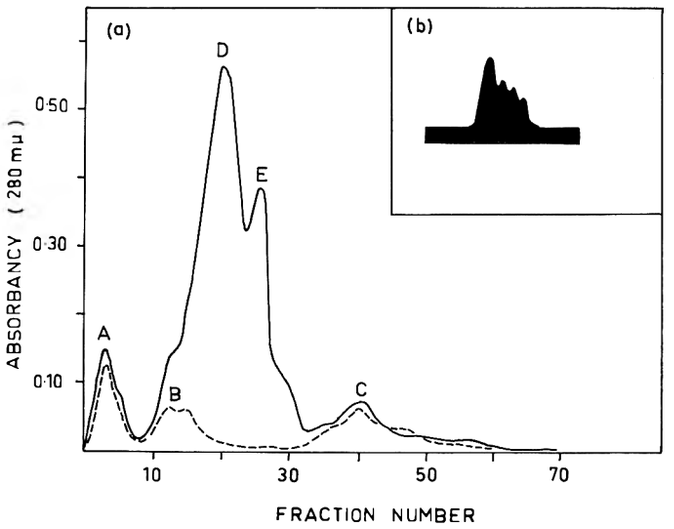

Fig. 3. The change in the ion-exchange chromatogram of extra protein during the aging of well-washed myofibrils (1 g) from bovine LD muscle. Unaged myofibrils, broken line; myofibrils aged 10 days at 2°C, continuous line.

myofibril. The extra protein is essentially colorless, and is considered to be derived from the myofibrillar components of the muscle cell and not from the sarcoplasm that would have been washed from the myofibrils during isolation, at least as judged by the complete removal of the red coloration of the sarcoplasmic protein, myoglobin. Fig. 3a, one of several such experiments, shows that the extra protein extracted from unaged myofibrils can be resolved by chromatography into at least three components (peaks A, B, C).

In contrast, the protein released from aged myofibrils is more complex and further resolves into two distinct components (peaks D and E), which constitute more than 90% of the extracted protein while accounting almost wholly for the increased extra protein from aged material. This extra protein extracted from the aged myofibrils (peaks D and E) could be further resolved by moving-boundary electrophoresis of a concentrated sample of the pooled fractions, 19–30 (Fig. 3b), and was shown to consist of at least 4 proteins.

DISCUSSION

IT IS CONSIDERED in this study that the mechanism of aging is a consequence of changes occurring within the myofibrillar component of meat and that such changes are related to the increase in myofibrillar protein extracted by HS buffer during storage.

The quantity of myosin extracted by HS buffer remains unchanged during aging at 50–51% of the myofibrillar protein. On the other hand the quantity of actin extracted increases and amounts to as much as 10% of the myofibrillar protein after a period of 21 days storage at 2°C. This is not sufficient to form actomyosin with all the extracted myosin, although muscle apparently contains enough actin to do so (Perry *et al.*, 1958). It is therefore possible that actin is also released in a form that is unable to combine with myosin. Under these circumstances such inactive actin would be expected to make up part of the extra protein extracted from aged material at low ionic strength.

Perry *et al.* (1958) have found that rabbit actin, on prolonged dialysis against 0.078*M* borate buffer pH 7.1, is converted into such an inactive form characterized by its insolubility in 0.75*M* potassium phosphate buffer pH 7.2—a fact confirmed by us for bovine actin. However this form of inactive actin (at least), is not present in the extra protein that remains soluble when dialyzed against the phosphate buffer.

In addition to the changes in actin extractability there is an increased extractability of extra protein during the aging of well-washed myofibrils. The location of this extra protein within the structure of the myofibril has not yet been ascertained. It is complex and possibly consists in part of the recently described α- and β-actinins (Ebashi *et al.*, 1965; Maruyama, 1965).

The present results have shown that tropomyosin remains resistant to extraction and is only released from both unaged and aged myofibrils by buffers of relatively high ionic strength. From electron-microscopic studies (Hanson *et al.*, 1963) and from evidence based on the finding that the tropomyosin content of extracted actin remains rather constant over a variety of extracting conditions (Corsi *et al.*, 1958; Maruyama, 1964), actin and tropomyosin have been considered to be in an associated form within the I bands of the sarcomeres. It is possible, therefore, that during meat aging the association of actin and tropomyosin not only within the I bands but also within the Z bands (Huxley, 1963) of the sarcomeres is weakened or destroyed leading to the more ready extraction of actin from myofibrils by HS buffer. Indeed histological evidence presented in a previous communication (Davey *et al.*, 1967), suggests that the aging changes giving rise to increased actin extractability are even more extensive, resulting in the weakening and final dissolution of the Z bands themselves.

Thus the aging of meat can be described from this evidence as a loss of the tensile strength of the myofibrillar component of the muscle cell, brought about by the disintegration of the Z bands. This dissolution would be expected to lead to a change in the extractability of proteins contained therein, while suggesting further that extra protein is located, at least in part, within the Z-band structures of the sarcomeres.

Although it has been shown (Davey *et al.*, 1967) that bacterial action enhances the extractability changes reported here, the present results were obtained in the virtual absence of microorganisms, which therefore cannot be implicated. On the other hand active proteolysis of bacterial origin undoubtedly can enhance the changes of aging. Such proteolysis might actually occur in the presence of quite low levels of live bacteria, being related to the time-integral of the bacterial population rather than to the numbers of viable organisms present at one time. Indeed diffusion of proteolytic enzymes from surface bacteria into sterile regions of meat is all that is required.

REFERENCES

Aberle, E. D. and Merkle, R. A. 1966. Solubility and electrophoretic behavior of some proteins of post-mortem aged bovine muscle. *J. Food Sci.* **31**, 151.

AOAC. 1955. Official Methods of Analysis. 8th ed. Assoc. Offic. Agr. Chemists, Washington, D. C.
Bailey, K. 1951. End-group assay in some proteins of the Keratin-myosin group. *Biochem. J.* **49**, 23.
Corsi, A. and Perry, S. V. 1958. Some observations on the localization of myosin, actin and tropomyosin in the rabbit myofibril. *Biochem. J.* **68**, 12.
Davey, C. L. and Gilbert, K. V. 1966. Studies in meat tenderness. II. Proteolysis and the aging of beef. *J. Food Sci.* **31**, 135.
Davey, C. L. and Gilbert, K. V. 1967. Structural changes in meat during aging. *J. Food Technol.* (British) **2**, 57.
Davey, C. L. and Gilbert, K. V. 1968. Studies in meat tenderness. 4. Changes in the extractability of myofibrillar proteins during meat aging. *J. Food Sci.* **33**, 2.
Ebashi, S. and Ebashi, F. 1965. α-Actinin, a new structural protein from striated muscle. I. Preparation and action of actomyosin-ATP interaction. *J. Biochem.* **58**, 7.
Fujimaki, M., Arakawa, N., Okitani, A. and Takagi, O. 1965. The changes of "myosin B" ("actomyosin") during storage of rabbit muscle. II. The dissociation of "myosin B" into myosin A and actin, and its interaction with ATP. *J. Food Sci.* **30**, 937.
Goll, D. E., Henderson, D. W. and Kline, E. A. 1964. Post-mortem changes in physical and chemical properties of bovine muscle. *J. Food Sci.* **29**, 590.
Gornall, A. G., Bardawill, C. J. and David, M. M. 1949. Determination of serum proteins by means of the biuret reaction. *J. Biol. Chem.* **177**, 751.
Hanson, J. and Huxley, H. E. 1957. Quantitative studies on the structure of cross-striated myofibrils. II. Investigations by biochemical techniques. *Biochim. Biophys. Acta* **23**, 250.
Hanson, J. and Lowy, J. 1963. The structure of F-actin and of actin filaments isolated from muscle. *J. Mol. Biol.* **6**, 46.
Hasselbach, W. and Schneider, G. 1951. Der L-myosin- und aktingehalt des Kaninchenmuskels. *Biochem. Z.* **321**, 462.
Huxley, H. E. and Hanson, J. 1957. Quantitative studies on the structure of cross-striated myofibrils. I. Investigations by interference microscopy. *Biochim. Biophys. Acta* **23**, 229.
Huxley, H. E. 1963. Electron microscope studies of natural and synthetic protein filaments from striated muscle. *J. Mol. Biol.* **7**, 281.
Khan, A. W. and van den Berg, L. 1964. Some protein changes during post-mortem tenderization in poultry meat. *J. Food Sci.* **29**, 597.
Longsworth, L. G. 1939. A modification of the Schlieren method for use in electrophoretic analysis. *J. Americ. Chem. Soc.* **61**, 529.
Marsh, B. B. and Snow, A. 1950. A simple tissue homogenizer. *J. Sci. Food Agr.* **1**, 190.
Maruyama, K. 1964. Interaction of tropomyosin with actin. A flow-birefringence study. *Arch. Biochem. and Biophys.* **105**, 142.
Maruyama, K. 1965. Some physico-chemical properties of β-actinin "actin factor" isolated from striated muscle. *Biochim. Biophys. Acta* **102**, 542.
Maruyama, K. and Gergely, J. 1962. Interaction of actomyosin with adenosine triphosphate at low ionic strength. I. Dissociation of actomyosin during the clearing phase. *J. Biol. Chem.* **237**, 1095.
Perry, S. V. 1955. "Methods in Enzymology," Vol. 2, p. 583. Academic Press, New York.
Perry, S. V. and Corsi, A. 1958. Extraction of proteins other than myosin from the isolated rabbit myofibril. *Biochem. J.* **68**, 5.
Scharpf, L. G. and Marion, W. W. 1964. Extraction of fibrillar and sarcoplasmic proteins of turkey muscle. *J. Food Sci.* **29**, 303.
Spicer, S. S. and Gergely, J. 1951. Studies on the combination of myosin with actin. *J. Biol. Chem.* **188**, 179.
Szent-Györgyi, A. G. 1951. A new method for the preparation of actin. *J. Biol. Chem.* **192**, 361.
Weinberg, B. and Rose, D. 1960. Changes in protein extractability during post-rigor tenderization of chicken breast muscle. *Food Technol.* **14**, 376.
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Frozen Geese Quality as Affected by Sex and Breed

SUMMARY—The eating quality of meat from Pilgrim, Hungarian and Chinese geese and their crosses was examined in 88 geese, equal numbers of males and females, in two experimental plans. Flavor and tenderness were ranked by an experienced taste panel. A modified Warner-Bratzler shearing device was used for tenderness determinations. Data were obtained for cooking rates, yield, color and moisture content. Variance components were estimated for tenderness measurements on the Warner-Bratzler Shear.

A difference among the genotypes was observed only for percent fat. Small, but statistically significant, differences between sexes were observed for cooking rate, percent meat, moisture of breast meat and color of breast and thigh meat.

INTRODUCTION

GEESE, MARKETED PROFITABLY for centuries in Europe for their meat and feathers, have not been popular with the American consumer because of excessive body fat and poor appearance. There is, however, no need to market excessively fattened geese. Young growing geese are not very fat (Deskins *et al.*, 1956).

Modern dressing and marketing practices have not generally been applied to geese and few plants have facilities for dressing waterfowl. Modern methods of production and marketing have shown a ready consumer acceptance for geese when attractively prepared and displayed (Snyder, 1959). Moderately fat, attractively marketed stock with distinctive flavor could find increasing use as a table delicacy. Geese are hardy and easy to raise. According to Snyder (1959) geese and ducks are among the most rapidly growing and efficient producers of food.

This study was undertaken to determine whether eating quality is related to breed, in order to assist breeders in developing better quality. Factors examined were: cooking rate, cooking loss, percent meat, percent fat, moisture, color, tenderness and flavor of breast and thigh meat.

Little information is available on the eating quality of goose meat. Bean *et al.* (1962), studying the tenderness of goose meat with shear apparatus and a taste panel, found a marked difference between the tenderness of commercially available geese and that of a group grown especially for their study. Deskins *et al.* (1956), studied dressing losses, evisceration losses, cooking losses and percent of edible meat in ready-to-cook carcasses of geese, 8 to 24 weeks old. Their work indicated that little is gained by growing geese to 24 weeks rather than 12 weeks. Snyder (1959) found that the highest percentage of edible meat was obtained at 12 weeks.

Considerable work has been done on the eating quality of other species of poultry. Gilpin *et al.* (1960), and Hanson *et al.* (1959), reported a series of studies in which "old style" slow growing chickens were compared with "modern style" fast growing ones. They found a noticeable difference in flavor between sexes but no difference in meat tenderness between the breeds. Morrison *et al.* (1954), found no significant difference in tenderness when comparing eight breeds and strains of chickens. Brant *et al.* (1962), studied eating quality differences which may be due to age, sex or genetic make-up of chickens and turkeys. The data did not provide a basis for one variety to claim superiority over another in eating quality.

Other workers (Kondra *et al.*, 1962, Harkin *et al.*, 1958, Goertz *et al.*, 1961, Peterson *et al.*, 1959) have examined the effects of age, sex and feeding practices on poultry quality but the major emphasis has been on the influence of post-mortem factors on poultry meat tenderness.

EXPERIMENTAL

MALE AND FEMALE PROGENY of three breeds, Pilgrim (P), Hungarian (H) and Chinese (C) mated reciprocally in all combinations were used for this study. The breeders were a random sample of the three purebred populations and consisted of 28 males and 80 females of each breed.

The progeny were from two hatches, one month apart, reared on pasture, fed a balanced diet, and full-fed for 14 days prior to slaughter at 18 weeks of age.

After slaughter, the geese were scalded at temperatures ranging from 144 to 150°F with an immersion time of 1½ to 2 min. Following plucking, the birds were eviscerated and maintained overnight (about 12 hr) in chill tanks, vacuum packed in Cryovac bags and placed in a brine freezing (−40°F) tank for 1½ hr. They were subsequently maintained in a freezer at −10°F for 1 to 4 months.

Progeny of each of the reciprocal crossbred matings were combined for sampling and considered as one genotype. There were therefore a total of six different genotypes: 3 breeds (HH, CC and PP) and 3 crosses (PH, HC and PC), considered for sampling and design purposes.

Because experimental material and facilities (4 ovens) were limited, two experimental plans, I and II were designed. In plan I, 48 birds, 4 of each sex of the 6 genotypes were accommodated in a triangular partially balanced incomplete block design with 12 blocks of 4 birds. The triangular design is possible whenever the number of treatments—6 genotypes—can be expressed in the form $L(L-1)/2$, where L is an integer; in our case $L = 4$. The design was constructed following the rules for formation of blocks stated by Bose *et al.* (p. 43, 1954).

In plan II, 40 birds, 4 of each sex of each of 5 genotypes (PP was excluded because of insufficient number)

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were set in a balanced incomplete block design with 10 blocks of 4 birds.

For both plans, the blocks refer to days of experimentation, and both sexes of two genotypes were tested on any one day. For each plan, each oven was used for all sexes and genotypes, thus ensuring orthogonality of ovens to sexes and genotypes.

The number of determinations, n, made on each bird varied with the characteristic being assessed. For cooking rate, cooking loss, fat and meat, $n = 1$; for moisture and color, $n = 2$; and for the Warner-Bratzler shear test for tenderness, $n = 4$.

The geese were thawed overnight in cold running water, then rinsed, weighed and cooked at 325°F according to the method described in Canada Agriculture Publication 1189 (1964). The ovens used were of the standard domestic type, calibrated to 325°F ± 15°. The geese were roasted until the internal breast temperature was 90°C, as measured by thermocouples. After cooking, the meat was cooled at room temperature for 30 min. and then removed from the breasts and thighs. Meat from the right breasts and thighs was used for objective evaluation and meat from the left for sensory evaluation.

The geese were weighed in the frozen, oven-ready and cooked state. In addition, weights of the following components were obtained: loose fat removed before cooking, accumulated fat (Deskins *et al.*, 1956), meat removed from the breasts and thighs. From these data, cooking rates, percent cooking losses, ratio of meat to oven-ready weight and ratio of fat (loose fat and melted fat) to oven-ready weight were calculated.

Sensory tenderness evaluations were made on samples cut parallel to the grain of the meat from the breast using a cork borer, 1 cm in diameter. A double scalpel with two blades, 1 cm apart, was used to obtain oblong samples from the thigh for texture evaluation. Thin slices were taken from the breast and thigh for flavor evaluation.

The samples from the breast and from the thigh were coded and presented to a panel of six selected tasters who ranked them for flavor and tenderness. Rank 4 was assigned to the most tender sample in the case of tenderness rating, or to the sample with better flavor in the case of flavor rating. Ties were not allowed. Flavor and tenderness were assessed on different samples.

Samples for objective measurement of tenderness of breast and thigh were cut in the same manner as for sensory evaluation. Shear force on four cores from each sample was measured by the modified Warner-Bratzler described by Voisey *et al.* (1967). Maximum force (g) during cutting was recorded.

The meat which remained after the samples had been taken for tenderness analysis was put through a meat grinder three times, keeping breast and thigh meat separate. The color of these ground samples was measured using the Hunter Color and Color Difference Meter with white standard. The amount of luminous reflectance (Rd) was recorded.

Moisture determinations were made on duplicate 10-g samples of ground meat using a combination of freeze-drying and vacuum oven drying. According to this method, developed by Emmons and Beckett (1965) for cottage cheese, weighed ground samples are frozen and dried in a freeze-drier for 16 hr, then dried in a vacuum oven at 100°C, 20 mm vacuum for 5 hr, cooled in a desiccator and weighed. This was found the most accurate method with the available equipment.

The data were subjected to intrablock analysis of variance (Cochran *et al.*, 1957).

In the case of sensory evaluations the ranks were first transformed using Table XX, p. 71, "Scores of Ordinal Data" (Fisher and Yates, 1953); thus 1, 2, 3, 4 were replaced by −1.03; −.30; .30; 1.03 respectively.

For the characteristics which were measured objectively, combined estimates of the sex means and of the five genotype means common to experiments I and II, were obtained by weighting the corrsponding estimates for I and II in inverse proportion to their variances. The error means squares—among birds—from I and II were pooled. In the case of sensory evaluations, where ranking was used, the sex and genotype means from experiments I and II were not combined because the PP genotype was not represented in experiment II. The "t" test was used for comparisons between sexes, and Tukey's studentized range test (Scheffé, 1961) for comparisons among genotypes.

RESULTS AND DISCUSSION

ESTIMATES OF SEX AND GENOTYPE means, for the objective measurements, are given in Table 1 for experiment I, along with combined estimates for experiments I and II. The estimates for the sensory evaluations are reported separately for experiments I and II. The standard errors reported in Table 1 are those associated with the sex means and the genotype means; they are respectively used in the "t" test for comparisons between sexes, and in Tukey's studentized range test for comparisons among genotypes. The results of the "t" tests are given in Table 1. For ease of presentation, the results of Tukey's test are given only for the combined estimates of genotype means corresponding to fat, which was the only case where a significant difference among genotypes was observed.

Female geese required about 4 min more cooking time per pound than males, which was to be expected since the oven-ready weights of females were lower. Females had a slightly higher (0.6%) percentage of meat (Table 1). The meat from the females was lighter than that from the males, and the cooked breast meat was moister (1.04%) (Table 1). Deskins *et al.* (1956) using pressure cooking reported an average cooking loss of 35.4%. The overall cooking loss of 39.8% in our study compares fairly well considering that the geese were roasted. The overall value for meat yield (23.5%) is considerably lower than those reported by Snyder (1959) and Deskins *et al.* (1956). However, both workers used the total available meat whereas in the present study only meat from the breasts, thighs and legs was used.

The only significant difference among genotypes was in percent fat, where the mean values ranged from 18.0% for the HH to 22.8% for the PC (Table 1).

The results of objective and subjective measures of tenderness showed a slight overall tendency for meat from females to be more tender than that from males (Table

Table 1. Means summarizing the characteristics used in assessing the eating quality of geese.

Characteristic	Experiment number	Sex			Genotypes						
		M	F	S.E.[1]	HH	PH	CC	HC	PC	PP	S.E.[1]
Oven-ready weight (lb)	I	6.9	5.9	.....	6.4	6.3	6.1	6.9	6.8	6.3	
	I & II	7.1	5.9		6.2	6.4	6.2	6.9	6.9		
Cooking rate (min/lb)	I				40	33	35	34	34	38	2.4
	I & II	31**	35	0.8	37	35	31	31	31		1.8
Cooking losses (%)	I				37.5	37.8	41.4	43.1	43.7	41.9	1.48
	I & II	39.7	40.0	0.55	37.5	39.6	39.6	40.9	41.3		1.22
Fat (%)	I				16.8	20.5	20.2	21.5	22.4	21.1	1.44
	I & II[2]	20.9	20.6	0.47	18.0	20.6	20.7	21.4	22.8		0.99
Meat (%)	I				23.0	23.4	23.5	21.9	23.1	22.6	0.59
	I & II	23.2*	23.8	0.19	23.9	23.6	24.1	22.9	23.4		0.43
Moisture breast (%)	I				62.20	62.25	61.63	60.67	62.03	62.13	0.639
	I & II	61.61**	62.65	0.207	62.24	62.39	62.03	61.58	62.27		0.458
Moisture thigh (%)	I				61.74	62.08	59.45	59.09	61.31	59.87	0.712
	I & II	60.46	61.06	0.230	61.49	61.06	60.25	60.43	60.90		0.510
Color breast (Rd. value)	I				17.1	16.9	17.2	17.0	16.9	16.7	0.48
	I & II	16.5**	17.3	0.16	17.0	17.3	17.0	16.7	16.2		0.34
Color thigh (Rd. value)	I				18.8	18.4	16.9	18.5	17.7	18.8	0.54
	I & II	17.7**	18.6	0.17	18.7	18.6	17.3	17.9	17.9		0.38
Tenderness (W-B)[3] breast	I				1596	1582	1466	1715	1593	1429	140.9
	I & II	1637	1605	45.6	1610	1483	1543	1875	1635		101.0
Thigh	I				1945	2056	2622	2689	2389	2242	213.0
	I & II	2406	2323	69.0	2162	2216	2646	2568	2269		152.8
Tenderness (panel)[4] Breast	I				M 0.65	0.02	−0.07	−0.01	−0.25	0.47	{0.385[5]
					F −0.12*	−0.38	−0.75*	0.05	0.32	0.06	{0.269[6]
	II	−0.08	0.08	0.120	−0.26	0.42	−0.17	−0.33	0.00		0.304
Thigh	I	−0.07	0.07	0.116	0.10	0.04	0.01	−0.42	−0.02	0.29	0.349
	II	−0.10	0.10	0.127	−0.26	0.24	−0.27	−0.03	0.31		0.225
Flavor (panel)[4] Breast	I	0.02	−0.02	0.078	0.07	0.04	0.01	−0.11	−0.10	0.9	0.224
	II	−0.03	0.03	0.086	−0.11	0.08	−0.12	0.00	0.15		0.217
Thigh	I	0.04	−0.04	0.079	−0.08	−0.16	−0.04	−0.06	0.19	0.15	0.237
	II	−0.12	0.12	0.087	−0.09	0.13	0.24	−0.16	−0.12		0.219

[1] Standard error based on 40 d.f.
[2] Variety means for percent fat underscored by the same line are not significantly different at the 5% level, according to Tukey's studentized range test.
[3] Maximum shear force (g) recorded by the Warner-Bratzler apparatus.
[4] Transformed ranks. The higher values denote greater tenderness and better flavor ratings.
[5] Standard error for comparison of varieties within a sex.
[6] Standard error for comparison of sexes within a variety.
*Differences between sexes $P < 0.05$. **$P < 0.01$.

1). However, in the two cases where differences were significant, the males were more tender than the females, i.e., panel ratings of breast texture of HH and CC genotypes in experiment I. Since in experiment I the interaction sex x genotypes was statistically significant, the results are displayed in Table 1 in the form of a two-way table. The shear values obtained in this study cannot be compared with those reported by Bean *et al.* (1962), since cores of a different diameter were used.

No significant differences in flavor were found among sexes or genotypes (Table 1).

For experimental design purposes, the within-bird variation for color and moisture measurements was negligible. However, the within-bird variation for tenderness measurements on the Warner-Bratzler shear was quite large. Since tenderness is considered the most important attribute of eating quality, the variance in tenderness measurements was examined. The components of error variance for tenderness measurements from the Warner-Bratzler shear test were estimated from the mean squares among birds and among determinations within birds, and are shown in Table 2. The variations within birds (S_w^2) was

Table 2. Estimates of variance components for shear test.[1]

	D.F.	Breast	Thigh
Among birds	40	59,464	157,180
Within birds	264	128,906	209,046

[1] Maximum shear force (g) recorded by the Warner-Bratzler apparatus.

greater for the thigh than for the breast meat ($P < 0.01$). This may be accounted for by the fact that the small amount of thigh meat and the many different muscles made it difficult to get samples cut parallel to the grain of the meat. For both breast and thigh, the variance component among birds (S_a^2) was significantly greater than zero ($P < 0.01$).

The contribution of the variation within-birds (S_w^2) and the variation among-birds (S_a^2) to the standard error of a mean (S_m) in n Warner-Bratzler shear test determinations in each of r birds, is given in a randomized complete block design, by $S_m = \sqrt{[S_a^2/r + S_w^2/(nr)]}$. Table 3 displays the values that the standard error of the mean (S_m) takes on for n, r = 1, 2, 4, 8. To reduce the standard error of the mean, it is apparent from Table 3 that, for a fixed number of observations (nr), the design strategy should be to have more birds. However, if the number of birds is fixed, replicate determinations within birds do help considerably to increase the precision of the mean estimates.

Although differences were observed between sexes for some attributes of eating quality, these differences were generally small. The difference among genotypes in percent fat might be of interest to breeders since excessive fat is rejected by the consumer. No effort was made to compare goose meat with other poultry, but it is felt that goose meat was well accepted by the panel of tasters.

Table 3. Standard error of the mean for shear test[1] n determinations in each of r birds using estimates of variance components in Table 2.

	n/r	1	2	4	8
Breast	1	434.0	306.9	217.0	153.4
Thigh		605.2	427.9	302.6	213.9
Breast	2	351.9	248.9	176.0	124.4
Thigh		511.6	361.5	255.7	180.8
Breast	4	302.8	214.1	151.4	107.0
Thigh		457.6	323.5	228.8	161.8
Breast	8	274.8	194.4	137.4	97.2
Thigh		428.1	302.7	214.0	151.4

[1] Maximum shear force (g) recorded by the Warner-Bratzler apparatus.

REFERENCES

Bean, M. F. and Hanson, H. L. 1962. Utilization of geese. I. Tenderness and yield. *Poultry Sci.* **41**, 236.

Bose, R. C., Clatworthy, W. H. and Shrikhande, S. C. 1954. Tables of partially balanced designs with two associate classes. North Carolina Agric. Expt. Sta. Tech. Bull. 107.

Brant, A. W. and Hanson, H. L. 1962. Ages, sex and genetic effects on poultry flavor. Proc. XII World's Poultry Congress.

Canada Agriculture (1964)—Publication 1189: Poultry, How to Buy/How to Cook.

Cochran, W. G. and Cox, C. M. 1957. "Experimental Designs." John Wiley and Sons, New York.

Deskins, Barbara, and Winter, A. 1956. Cooked edible meat in parts of poultry. II. Geese. *J. Dietetic Assoc.* **32**, 211.

Emmons, D. B. and Beckett, D. C. 1965. Unpublished data. Food Research Institute, Canada Department of Agriculture.

Fisher, R. A. and Yates, F. 1953. "Statistical Tables for Biological, Agricultural and Medical Research." Oliver and Boyd, London.

Gilpin, G. L., Harkin, A. M., Redstrom, R. A. and Dawson, E. H. 1960. Quality and yield of modern and old type chickens. *Poultry Sci.* **39**, 924.

Goertz, Grayce E., Weathers, B., Harrison, D. L. and Sanford, P. E. 1961. Tenderness scores and Warner-Bratzler shear values for broilers and Beltsville white turkeys fed different cereal grains. *Poultry Sci.* **40**, 488.

Hanson, H. L., Campbell, A. A., Kraft, A. A., Gilpin, G. L. and Harkin, A. M. 1959. The flavor of modern- and old-type chickens. *Poultry Sci.* **38**, 1071.

Harkin, A. M., Kitzmiller, C., Gilpin, G. L. and Marsden, S. J. 1958. Quality of cooked meat of turkeys fed animal or vegetable protein diets with vitamin and fat supplements. *Poultry Sci.* **37**, 1328.

Kondra, P. A., Richards, J. F. and Hodgson, G. C. 1962. The effect of sex, ration and strain on meat yield and its determination in chicken broilers. *Poultry Sci.* **41**, 922.

Morrison, M. A., Sauter, E. A., McLaren, B. A. and Stadelman, W. J. 1954. Some factors affecting yield and acceptability of chicken broilers. *Poultry Sci.* **33**, 1122.

Peterson, D. W., Simons, M., Lilyplade, A. L. and Martin, R. 1959. Some factors affecting intensity of flavor and toughness of chicken muscle. *Food Technol.* **13**, 53.

Scheffé, H. 1961. "The Analysis of Variance." John Wiley and Sons, New York.

Snyder, E. A. 1959. Duck and goose raising. Ontario Department of Agriculture Bulletin 532.

Voisey, P. W. and Hansen, H. 1967. A shear apparatus for meat tenderness evaluation. *Food Technol.*, **21**, 37A.

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Substrates and Intermediates in the Enzymatic Reduction of Metmyoglobin in Ground Beef

SUMMARY—Various substrates were tested for their ability to increase the reduction of metmyoglobin by donating electrons to NAD. Intermediates of the glycolytic pathway which proved to be effective were glyceraldehyde-3-phosphate and fructose-1,6-diphosphate. Other substrates, oxidized by NAD-linked dehydrogenases known to be present in meat are α-glycerophosphate, malate and glutamate. These also increased metmyoglobin reduction when added to meat.

The pathway of electron transport from NADH to metmyoglobin is believed to be mainly by way of DT diaphorase (menadione reductase) and quinones, since dicumarol, a specific inhibitor of this enzyme, partially blocked the reaction in meat. Higher levels of quinones did not accelerate the reaction.

INTRODUCTION

THE ACCEPTABILITY of meat is decreased when the brown pigment metmyoglobin is produced. This pigment can be reduced back to purplish myoglobin by enzymes present in meat (Stewart *et al.*, 1965). Watts *et al.* (1966) have shown that the reduction of both metmyoglobin and oxygen in meat is mediated through nicotinamide adenine dinucleotide (NAD) rather than succinic oxidase. Metmyoglobin is reduced only under anaerobic conditions. The nature of the substrates capable of reducing NAD and intermediates between NADH and metmyoglobin were unexplored; these areas will be considered here.

While there are a number of enzyme-substrate systems capable of reducing NAD in living muscle, their activity in post-rigor meat is unexplored. During the storage of intact beef muscle for 2 to 4 weeks, most of the glycolytic enzymes were found to be active (Andrews *et al.*, 1952). Similar studies have been reported in fish muscle (McLeod *et al.*, 1963).

Using histochemical techniques (Ogata *et al.*, 1964; Bodwell *et al.*, 1965), glycolytic, tricarboxylic acid cycle and electron transport chain enzymes have been detected in pork and beef muscles, although activities of some of these enzymes decreased with time (Bodwell *et al.*, 1965). Lack of substrates, rather than loss of enzymes, probably limits reductive capacity of meat after slaughter (Andrews *et al.*, 1952; Bodwell *et al.*, 1965; Watts *et al.*, 1966).

Enzymes may be involved in the transport of electrons from reduced pyridine nucleotide to metmyoglobin. An attempt to isolate a metmyoglobin reductase was not successful (Rossi-Fanelli *et al.*, 1957). However, a cytoplasmic flavoenzyme, DT diaphorase (also known as menadione reductase) was found in meat (Bodwell *et al.*, 1965). This enzyme catalyzes the reduction of various quinones to quinols, which in turn may reduce ferric heme compounds (Ernster *et al.*, 1962; Conover *et al.*, 1962).

The overall metmyoglobin-reducing ability in meat may be influenced by a number of factors such as concentration of NAD in meat, availability of various substrates and of intermediates of electron transport. Addition of NAD to meat was found to increase metmyoglobin reduction (Watts *et al.*, 1966). implying that this pyridine nucleotide could be a limiting factor. The NAD present at the time of slaughter is destroyed by the action of several enzymes which are brought into contact with the nucleotides upon maceration of the tissues (Severin *et al.*, 1963). Whether substrates and electron carriers are also limiting is not known.

A better understanding of the contributions of each of these factors could lead to an improvement in reducing ability by addition of the limiting factor. The purpose of this research is to study the effect of adding various substrates and intermediates on metmyoglobin reduction in meat.

MATERIALS AND METHODS

Chemicals and solutions

Sources of chemicals used in this study were: nicotinamide adenine dinucleotide (NAD), nicotinamide adenine dinucleotide phosphate (NADP), and fructose-1,6-diphosphate (FDP) from Calbiochem; DL-glyceraldehyde-3-phosphate (gly-3-p), DL-α-glycerophosphate, DL-isocitrate, malate, iodoacetate and dicumarol from Sigma Chemical Co.; fructose-6-phosphate, glucose-1-phosphate, glucose-6-phosphate, glutamate and menadione from Nutritional Biochemicals Co.; 1-2-naphthoquinone and p-benzoquinone from Eastman Organic Chemicals; 2-6-dimethylbenzoquinone from K and K Lab., Inc.

The stock solutions were prepared in distilled water except for quinones and dicumarol which were dissolved in propylene glycol or ethyl alcohol. Dilutions were made from the stock solution so that the appropriate amount of the test substance was contained in 1 to 2 ml volume. Controls (without test substance) contained the solvent alone.

DL-glyceraldehyde-3-phosphate solution was prepared from diethylacetal barium salt according to the method described by Sigma Technical Bulletin No. 10 (1961).

Preparation of meat samples

Beef semitendinosus (eye of round) from local packing plants was used. All meat was trimmed of external fat, ground twice and mixed just before each experiment. No attempt was made to adjust the pH of the meat, but solutions to be added were adjusted to the pH of the meat when necessary, so that all variations within a single experiment had the same pH. Fifty-gram portions were

placed in polyethylene bags and stored in the refrigerator at 1 to 5°C until the time of assay (2 to 6 hr).

Metmyoglobin reducing activity was measured as described by Watts *et al.* (1966).

RESULTS AND INTERPRETATION

Addition of substrates capable of supplying hydrogen to NAD

Intermediates of glycolytic pathway. Watts *et al.* (1966) demonstrated that NAD addition to meat increased the reduction of metmyoglobin, but its effectiveness varies considerably in samples of meat from different animals (Saleh, 1967). Varying amounts of suitable substrates may be the limiting factor. Since all of the glycolytic enzymes are still potentially active in post rigor meat, various intermediates of the glycolytic pathway were tested for their ability to increase metmyoglobin reduction.

Glyceraldehyde-3-phosphate. Three experiments on the addition of gly-3-p in the presence of NAD are summarized in Table 1. Metmyoglobin reduction was increased in the treated samples as compared to the controls.

The immediate response to gly-3-p is clearly shown in Fig. 1 when the reduction was measured every 2 min. Whereas 50% reduction of metmyoglobin was obtained in 15 min as a result of gly-3-p addition, only 8% of the pigment was reduced in the control within this time period. The magnitude of gly-3-p response tended to be more pronounced in those experiments in which the reducing ability of the control was low. It is probable that in the more active controls, greater amounts of endogenous substrates were present.

Iodoacetate in low concentration is known to inhibit glyceraldehyde-3-p-dehydrogenase (Cori *et al.*, 1948). The extent of inhibition in pure systems varies from 85 to 95% (Cori *et al.*, 1948; Dixon, 1937).

The effect of iodoacetate on metmyoglobin reduction was investigated in meat containing added gly-3-p. The results (Table 2) show a maximum inhibition of 73%. Other enzyme substrate systems, i.e., glutamate and its associated dehydrogenase (Singer *et al.*, 1954), and malic dehydrogenase (Dixon, 1937) which are not as sensitive to iodoacetate as gly-3-p, may also contribute electrons for the reduction of NAD.

Another possible reason for incomplete inhibition is that in the presence of excess gly-3-p, the enzyme is protected from inhibition by iodoacetate (Segal *et al.*, 1953). The extent of inhibition by iodoacetate was the same in the presence and in the absence of gly-3-p, indicating that this glycolytic intermediate may be an important endogenous substrate in meat.

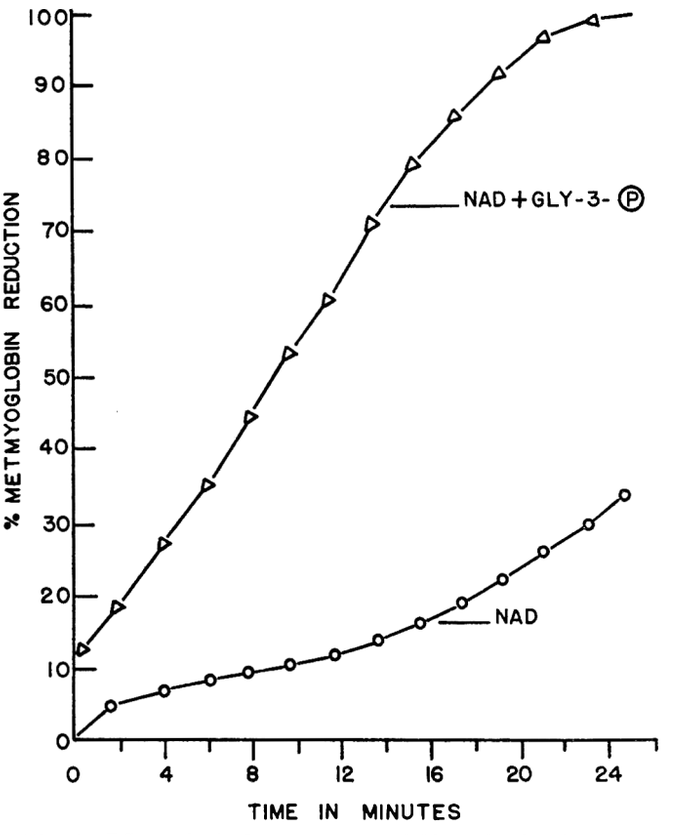


Fig. 1. Effect of gly-3-p on metmyoglobin reduction in ground beef. Both samples contained 40 mg % NAD. Concentration of gly-3-p in the test sample was 60 mg %.

Fructose-1,6-diphosphate (FDP). Gly-3-p can be derived by the hydrolytic cleavage of FDP by the action of aldolase shown to be present in meat (Andrews *et al.*, 1952). FDP was therefore tested for its effect on metmyoglobin reduction. A 2- to 3-fold increase in the rate of reduction of metmyoglobin was noticed in four experiments (Table 3). The results indirectly confirm the finding that aldolase is present in post-rigor meat (Andrews *et al.*, 1952).

When iodoacetate was added to meat along with FDP, a maximum inhibition of 60% in metmyoglobin reduction was observed.

DL-α-glycerophosphate. Another substrate indirectly related to the glycolytic pathway, whose oxidation is dependent on NAD, is α-glycerophosphate. The DL form

Table 1. Effect of glyceraldehyde-3-phosphate on metmyoglobin reduction.

Experiment No.	Gly-3-p added mg %	Metmyoglobin reduction in 15 min % of control	Time for 50% reduction (min)
1	0	100	30
	30	334	....
	60	1170	12
2	0	100	36
	60	650	15
3	0	100	15
	60	175	7
	120	178	5

All samples contained 40 mg % NAD.

Table 2. Effect of iodoacetate on metmyoglobin reduction in the presence of glyceraldehyde-3-phosphate.

Iodoacetate added mg %	Metmyoglobin reduction in 15 min % of control	Time for 50% reduction (min)
0	100	5
100	53	15
200	27	28
300	33	29

All samples contained 40 mg % NAD and 60 mg % Gly-3-p.

Table 3. Effect of fructose-1,6-diphosphate on metmyoglobin reduction.

Experiment No.	FDP added mg %	Metmyoglobin reduction in 15 min % of control	Time for 50% reduction (min)
1	0	100	17
	150	224	5
	300	237	5
2	0	100	28
	300	334	8
3	0	100	18
	300	248	5
4	0	100	20
	300	264	5

All samples contained 40 mg % NAD.

was tested for its effect on metmyoglobin reduction in meat. The enzyme, α-glycerophosphate dehydrogenase, located both in the soluble cytoplasm and in the mitochondria, may act as carrier of the NADH hydrogen across the mitochondrial membrane (Estabrook *et al.*, 1958). This dehydrogenase has been reported to be present in meat (Bodwell *et al.*, 1965). The results of the addition of various amounts of substrate along with NAD are reported in Table 4. Stimulation of metmyoglobin reduction was 1½- to 2-fold.

Glucose and some hexose monophosphates. The results of experiments on the addition to meat of glucose, glucose-6-phosphate, glucose-1-phosphate and fructose-6-phosphate were erratic; metmyoglobin reduction ranged from 25 to 170% of the control. No consistent increase in reduction was obtained with any of the compounds.

The observation that FDP addition resulted in stimulation of metmyoglobin reduction, whereas none of the monophosphate sugars gave such an effect, demonstrates the importance of the second phosphorylation. ATP is necessary for the conversion of monophosphorylated sugars to sugar diphosphates. Lack of ATP in post-rigor meat may limit this reaction.

Although lactate is present in large amounts in post-rigor meat and its oxidation is NAD dependent, the equilibrium of the lactic dehydrogenase reaction favors the oxidation rather than the reduction of NAD. Lactate was, therefore, not used as a test substance.

Some tricarboxylic acid cycle intermediates

Isocitrate. Early in this study it was observed that the addition of NADP to meat either had no effect or slightly

Table 4. Effect of DL-α-glycerophosphate on metmyoglobin reduction.

Experiment No.	DL-α-glycerophosphate added mg %	Metmyoglobin reduction in 10 min % of control	Time for 50% reduction (min)
1	0	100	15
	40	130	11
	200	197	6
	400	186	6
2	0	100	13
	200	156	8
	400	158	8

All samples contained 40 mg % NAD.

Table 5. Effect of DL-isocitrate on metmyoglobin reduction in presence of NADP.

DL-isocitrate added mg %	Metmyoglobin reduction in 15 min % of control	Time for 50% reduction (min)
0	100	51
20	136	41
40	236	26
60	264	23

All samples contained 20 mg % NADP.

inhibited metmyoglobin reduction. However, isocitrate addition, along with NADP, stimulated reduction as compared to NADP controls (Table 5). The presence of isocitrate dehydrogenase has been demonstrated in post-rigor meat (Bodwell *et al.*, 1965). From these results it appears that the ineffectiveness of NADP additions to meat may be ascribed to the lack of NADP-linked substrates.

Isocitrate addition, along with NAD to meat, inhibited metmyoglobin reduction. The reason for this inhibitory effect is not clear.

L-malate. Another substrate of the citric acid cycle, the oxidation of which proceeds via NAD, is malate. When L-malate was added to meat in the presence of NAD, it increased the reduction of metmyoglobin by a factor of 1½ to 2 (Table 6). This is presumptive evidence for the presence of malic dehydrogenase in post-rigor meat.

L-glutamate

Glutamate has been shown to be present as part of the free amino acid pool in meat (Gardner *et al.*, 1966). In addition, it is an inexpensive substrate, and is used widely in foods to enhance flavor. The enzyme catalyzing its oxidation, glutamic dehydrogenase, has also been reported in meat although its activity was somewhat low (Ogata *et al.*, 1964; Bodwell *et al.*, 1965).

The results of five experiments using different concentrations of L-glutamate are shown in Table 7. All five experiments were carried out under anaerobic conditions and additional NAD had been added to the meat samples. Results ranged from no effect in one experiment to marked stimulation.

Since L-glutamate gave promising results, it was tested further under both aerobic and anaerobic conditions and in the presence and absence of added NAD. The study under aerobic conditions was prompted by the fact that, in practice, meat is generally marketed in packaging material permeable to oxygen. The results are shown in Table 8. With all experimental variations, glutamate enhanced the reduction of metmyoglobin. The acceleration due to glutamate was greatest under aerobic conditions, without

Table 6. Effect of L-malate on metmyoglobin reduction.

L-malate added mg %	Metmyoglobin reduction in 15 min % of control	Time for 50% reduction (min)
0	100	24
140	164	16
280	193	14

All samples contained 40 mg % NAD.

Table 7. Effect of L-glutamate on metmyoglobin reduction.

Experiment No.	L-glutamate added mg %	Metmyoglobin reduction in 15 min % of control	Time for 50% reduction (min)
1	0	100	12
	20	191	8
	200	230	5
	400	200	8
2	0	100	13
	200	133	10
	400	197	7
3	0	100	12
	200	136	10
4	0	100	16
	200	113	14
5	0	100	14
	200	102	12

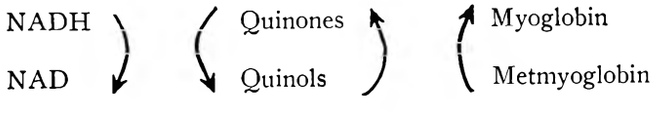
All samples contained 40 mg % NAD.

added NAD. Under these conditions the metmyoglobin reducing ability of the control itself was low.

In two additional experiments, meat treated with glutamate was stored for some days under both aerobic and anaerobic conditions and its reducing activity compared with untreated controls. The metmyoglobin-reducing activity of the glutamate-treated samples remained high over the storage period, whereas reduction in the controls decreased steadily. However, neither spectrophotometrically determined metmyoglobin nor subjective color ratings were significantly different in stored treated samples versus controls.

Role of quinones as possible intermediates

Quinones have been reported to mediate metmyoglobin reduction by NADH or NADPH in pure systems (Rossi-Fanelli *et al.*, 1957). DT diaphorase, a cytoplasmic enzyme, is present in meat (Bodwell *et al.*, 1965) and may be involved in metmyoglobin reduction. The purified enzyme has been shown to catalyze the oxidation of both NADH and NADPH with various quinones acting as electron acceptors (Ernster *et al.*, 1962). The role of quinones in the electron flow from NADH to metmyoglobin can be schematically presented as follows:



Various quinones were tested to determine whether insufficient quinone might be a limiting factor in metmyoglobin reduction in meat. Menadione, (2-methyl-1,4-naphthoquinone), 1,2-naphthoquinone, p-penzoquinone, and 2,6-dimethylbenzoquinone were tried. They either had no effect or somewhat inhibited reducing activity. During the mixing of some of the quinones (particularly naphthoquinone) with the ground meat, a rapid oxidation of meat pigments to metmyoglobin (brown color) was observed before the actual addition of the oxidizing agent (ferricyanide) to meat.

It is likely that there already are enough quinones present in meat to mediate the transport of electrons from

Table 8. Effect of L-glutamate on metmyogobin reduction under aerobic and anaerobic conditions.

Treatment	Time for 50% metmyoglobin reduction (min)	Metmyoglobin reduction % of control in 15 min	25 min
Anaerobic			
NAD-control	14	100	....
NAD + L-glutamate	11	130	....
Control—(no NAD)	50	100	....
Control + L-glutamate	28	342	....
Aerobic			
NAD-control	31	....	100
NAD-L-glutamate	17	....	220
Control—(no NAD)	50 [1]	....	100
Control + L-glutamate	29 [1]	....	600

[1] Time for 20% metmyoglobin reduction.
Concentrations used: NAD, 40 mg %; L-glutamate, 400 mg %.

NADH to metmyoglobin. Additional amounts might be expected to have an adverse effect on metmyoglobin reduction. Harley *et al.* (1962a,b) found the concentration of menadione to be critical in the enzymatic reduction of methemoglobin. Higher concentrations not only oxidize oxyhemoglobin to methemoglobin but also inactivate certain of the cellular enzymes concerned with the reduction of methemoglobin. Magos (1964) has shown that quinones not only oxidize ferrous hemes but also bring about the formation of globin hemochromogens.

The effect of dicumarol on metmyoglobin reduction.

DT diaphorase has been reported to be strongly inhibited by dicumarol (Ernster *et al.*, 1962). Moreover, dicumarol seems to be highly specific for this enzyme. Other diaphorase activities found in rat liver were not appreciably affected by even a 1,000-fold excess of the concentration needed for a complete inhibition of the DT diaphorase.

The results of two experiments on dicumarol addition to ground meat are shown in Table 9. The concentrations of dicumarol used ranged from 0.2 to 20 mg %. Inhibition varied from 0 to 63%. The dicumarol was difficult to dissolve; even at 0.2 mg/ml a true solution could not be obtained. The actual amount in solution at the higher concentration is not known.

The partial inhibition indicates that DT diaphorase plays a role as an intermediate in metmyoglobin reduction. The fact that inhibition was not complete may mean either that

Table 9. Effect of dicumarol on metmyoglobin reduction.

Experiment No.	Dicumarol added mg %	Metmyoglobin reduction in 15 min % of control	Time for 50% reduction (min)
1	0	100	17
	0.2	100	17
	0.4	66	22
	1.0	80	21
2	0	100	14
	0.2	100	14
	0.4	44	20
	1.0	62	19
	20	37	20

All samples contained 40 mg % NAD.

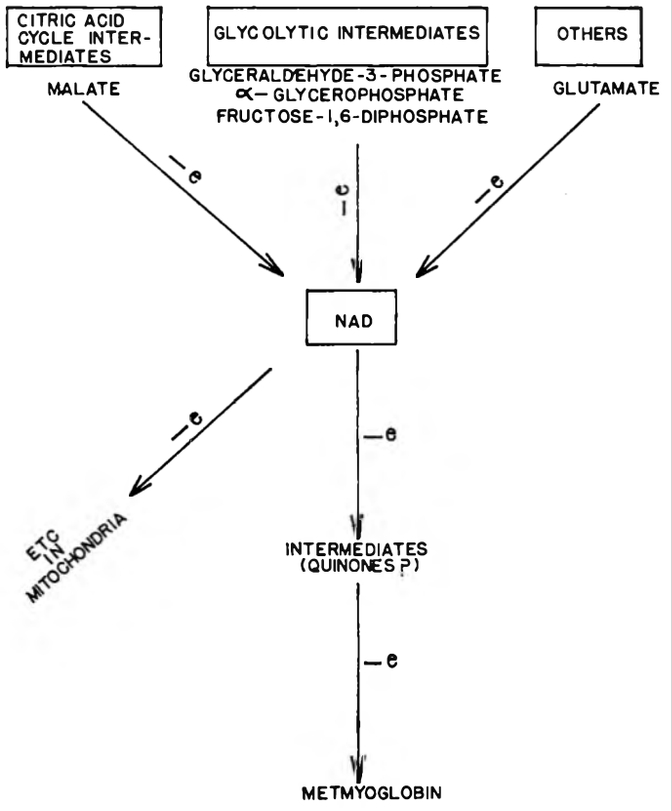


Fig. 2. Hypothetical scheme for the role of substrates and intermediates in metmyoglobin reduction in meat. ETC = electron transport chain.

DT diaphorase is not completely inhibited or that there is an additional pathway for the transport of electrons from NADH to metmyoglobin. Rossi-Fanelli *et al.* (1957) showed that the non-enzymatic reduction of metmyoglobin by NADH occurs at a measurable rate in the presence of quinones.

DISCUSSION

FROM THE RESULTS obtained in the present investigation, a number of substrates, any one of which may be present in trace amounts in meat, may be utilized to supply electrons to NAD and metmyoglobin according to the following scheme (Fig. 2). This work does not shed light on the actual endogenous substrates responsible for reducing activity in meat. However, it does demonstrate that, in meat, the rate of reduction of NAD controls the over-all rate of reduction of metmyoglobin. The step or steps between NADH and metmyoglobin apparently proceed rapidly, once NAD is reduced.

This work also demonstrates that the reducing activity of most samples of meat can be increased by the addition of appropriate substrates. From a practical standpoint, of the substrates used, only monosodium glutamate is cheap enough to be seriously considered.

Insufficient quantities of NAD also limit reducing activity of most samples of meat. Again NAD is far too expensive to be considered as an additive to meat except for experimental purposes. However, practical treatments which will protect the NAD normally present in meat have been investigated and will be described in the near future.

The larger question of the possible benefits to be derived from increasing the reducing activity of meats is still unclear. Hutchins *et al.* (1967) found a significant but not very high correlation between the reducing activity of meat from different animals and their tendency to resist browning (pigment oxidation) during refrigerated storage. However, in the present study, when the reducing activity of a single batch of meat was increased by the addition of glutamate, no significant improvement in appearance or decrease in metmyoglobin was noted in the treated samples after refrigerated storage.

The problem is complicated by the fact that autoxidation of myoglobin is much more rapid at low oxygen tensions than in air. Thus, the partial removal of oxygen from packaged meat by enzymatic activity could accelerate autoxidation. A systematic study of the relation of reducing activity to meat color under a variety of storage conditions is needed.

REFERENCES

Andrews, M. M., Guthneck, B. T., McBride, B. H. and Schweigert, B. S. 1952. Stability of certain respiratory and glycolytic enzyme systems in animal tissues. *J. Biol. Chem.* **194**, 715.

Bodwell, C. E., Pearson, A. M. and Fennell, R. A. 1965. Post mortem changes in muscle. III. Histochemical observations in beef and pork. *J. Food Sci.* **30**, 944.

Conover, T. E. and Ernster, L. 1962. DT diaphorase. II. Relation to respiratory chain of intact mitochondria. *Biochim. Biophys. Acta* **58**, 189.

Cori, G. T., Slein, M. W. and Cori, C. F. 1948. Crystalline D-glyceraldehyde-3-phosphate dehydrogenase from rabbit muscle. *J. Biol. Chem.* **173**, 605.

Dixon, M. 1937. Action of iodoacetate on dehydrogenases and alcoholic fermentation. *Nature* **140**, 806.

Ernster, L., Danielson, L. and Lyunggren, M. 1962. DT diaphorase. I. Purification from soluble fraction of rat liver cytoplasm and properties. *Biochim. Biophys. Acta* **58**, 171.

Estabrook, W. R. and Sackton, B. 1958. α-Glycerophosphate oxidase of flight muscle mitochondria. *J. Biol. Chem.* **233**, 1014.

Gardner, G. A. and Stewart, D. J. 1966. Changes in the free amino acid and other nitrogen compounds in stored beef muscle. *J. Sci. Fd. Agric.* **17**, 491.

Harley, J. D. and Robin, H. 1962a. The effect of menadione on the reduction of methemoglobin. *Austral. J. Exptl. Biol. Med. Sci.* **40**, 473.

Harley, J. D. and Robin, H. 1962b. Haemolytic activity of vitamin K_3: Evidence for a direct effect on cellular enzymes. *Nature* **193**, 478.

Hutchins, B. K., Liu, T. H. P. and Watts, B. M. 1967. Effect of additives and refrigeration on reducing activity, metmyoglobin and malonaldehyde of raw ground beef. *J. Food Sci.* **32**, 214.

McLeod, R. A., Jones, R. E. E. and Roberts, E. 1963. Glycolytic enzymes in the tissues of a salmonoid fish. *Canadian J. Biochem. Physiol.* **41**, 1971.

Magos, L. 1964. Globin haemochromogen formation caused by quinones. *Biochim. Biophys. Acta* **90**, 55.

Ogata, T. and Mori, M. 1964. Histochemical study of oxidative enzymes in muscle fibers of various animals. *J. Histochem. Cytochem.* **12**, 171.

Rossi-Fanelli, Antonini, A. E. and Madori, B. 1957. Enzymic reduction of ferrimyoglobin. *Arch. Biochem. Biophys.* **68**, 341.

Saleh, B. 1967. The role of pyridine nucleotides, substrates, and intermediates in the enzymatic reduction of metmyoglobin in ground beef. Ph.D. Thesis, Florida State University Library, Tallahassee.

Segal, H. L. and Boyer, P. D. 1953. The role of sulphydryl groups in the activity of D-glyceraldehyde-3-p dehydrogenase. *J. Biol. Chem.* **204**, 265.

Severin, S. E., Tseitlin, L. A. and Druzhinina, T. N. 1963. Enzymic breakdown of DPN in cardiac and skeletal muscle. *Biochem.* **28**, 112.

Sigma Technical Bulletin No. 10, September, 1961.
Singer, T. P. and Kearney, E. B. 1954. Oxidizing enzymes. In: "The Proteins, Chemistry, Biological Activity and Methods," eds. Neurath, H. and Bailey, K., Vol. 2, part A, p. 123. Academic Press, New York.
Stewart, M. R., Hutchins, B. H., Zipser, M. W. and Watts, B. M. 1965. Enzymic reduction of metmyoglobin by ground beef. *J. Food Sci.* **30**, 487.
Watts, B. M., Kendrick, J., Zipser, M., Hutchins, B. and Saleh, B. 1966. Enzymatic reducing pathways in meat. *J. Food Sci.* **31**, 855.
Ms. accepted 5/17/68.

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Polyphenolic Compounds in Canned Tomato Pastes

SUMMARY—Polyphenolic compounds in canned pastes made from VF-145 tomatoes (*Lycopersicum esculentum*, Mill) were extracted with methanol and ethyl acetate. The compounds were separated by two-dimensional paper chromatography with n-butanol-acetic acid-water (4:1:5 v/v) and 2% acetic acid as solvents. Twelve spots were found when the chromogenic reagent was $FeCl_3$-$K_3Fe(CN)_6$. The individual compounds were identified by their R_f values and color reactions with $FeCl_3$-$K_3Fe(CN)_6$, diazotized paranitroaniline, Hoepfner, sodium borohydride, and vanillin-HCl reagents, fluorescent behavior, and absorption spectra. Present in the extracts of tomato pastes were two chlorogenic acid isomers, two caffeic acid derivatives, rutin, naringenin, caffeic acid, and ferulic acid. Naringenin and *trans*-chlorogenic acid were present in larger amounts than the other polyphenolic compounds.

INTRODUCTION

POLYPHENOLIC COMPOUNDS play an important role in the biological functions, metabolism, and respiration of plant materials. In fruits they are related to astringent flavor and enzymic browning (Swain, 1962).

A defect of tomatoes (*Lycopersicum esculentum*, Mill) commonly known as "cloud" or "blotchy ripening" has been described (Walker, 1962). Kidson (1958) presented evidence that this defect was due to the action of the enzyme polyphenoloxidase. Fontaine *et al.* (1947) reported that a measurable amount of rutin was present in the leaves of the red currant tomato plant (*Lycopersicum pimpinelli folium*). This tomato variety, being almost immune to Fusarium wilt, has considerable importance in the development of wilt-resistant varieties.

Waggoner *et al.* (1956) reported that polyphenoloxidase preparations from tomato stems oxidized ortho-dihydroxy phenols but not meta- or para-dihydroxy phenols. Perkins *et al.* (1956) found chlorogenic and caffeic acids in the leaves of tomato plants. Flavonoid pigments in the skin of Ponderosa, Rutgers, and Sunny Ray tomatoes were identified as naringenin and quercitrin (Wu *et al.*, 1958). Leaf-with-petioles samples of Ponderosa and Sunny Ray contained quercitrin and rutin; Rutgers contained only quercitrin.

Walker (1962) separated the phenolic acids in the wall tissue of tomato fruit by paper chromatography. Caffeic, p-coumaric, ferulic, and chlorogenic acids were reported to be present in the alcohol extract of tomato wall tissue.

So-called cloud tissue showed polyphenoloxidase activity and reduced the levels of phenolic acids below those in equivalent healthy tissue. Van Bragt *et al.* (1965a) studied the effect of 2,4-dichlorophenoxyacetic acid (2,4-D) on the rutin content of stems and leaves of tomato plants. Four days after spraying with 2,4-D, rutin content was lower in sprayed plants than in the unsprayed control. Van Bragt *et al.* (1965b) identified neochlorogenic and 3-0-feruloyl quinic acids in the leaves, stems, and roots of tomato plants. Present in the same samples were chlorogenic acid and two unidentified phenolic compounds. Very little is known about polyphenols in tomato pastes.

In this work, polyphenolic compounds in pastes made from VF-145 tomatoes were separated by solvent extraction and purified by two-dimensional paper chromatography. Used to identify the compounds were R_f values in various solvent systems, color under ultraviolet radiation with or without exposure to ammonia vapor, color reactions with various chromogenic reagents, and ultraviolet absorption spectra.

MATERIALS AND METHODS

Tomato pastes

One thousand pounds of VF-145 tomatoes at canning ripeness were washed with tap water, crushed in a spike pump, and pumped immediately into a steam-jacketed swept-film heat exchanger operating at 230°F. The product was passed through a pulper with a 0.06-in. screen and a finisher with a 0.033-in. screen. The purée was concentrated in a vacuum pan at 100°F into paste of 26% total

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solids. The paste was heated in a swept-film heat exchanger at 204°F, held for 30 sec in a holding tube, and filled into 5-oz paste cans made of differential electrolytic tinplate and F-enamel ends. The sealed cans were inverted, held 3 min, cooled in running water to 110°F, and stored at 40°F.

Extraction of polyphenols

Canned tomato paste (180 g) was blended for 5 min with 250 ml of absolute methanol. The macerate was centrifuged and the supernatant was filtered in a Buchner funnel through Whatman No. 1 filter paper. The residue was extracted twice more with the same amount of solvent. The supernatants were combined, stored overnight at 32°F under a nitrogen atmosphere, filtered through Whatman No. 1 filter paper, and concentrated in a flash evaporator. The residue was suspended in distilled water and extracted several times with petroleum ether to remove carotenoids. The water extract was saturated with sodium chloride and then extracted three times with 250-ml portions of ethyl acetate. The acetate extracts were combined, dried with anhydrous sodium sulfate, filtered, and evaporated to a small volume in a flash evaporator. The residue was dissolved in 15 to 20 ml of methyl alcohol.

Two-dimensional paper chromatography

One hundred μl of the extract was spotted on the upper left corner of a Whatman No. 1 paper (46 × 57 cm). The chromatograms were developed in two dimensions at 20°C, with the upper layer of n-butanol-acetic acid-water (BAW, 4:1:5 v/v) as the first solvent for 18 hr, and 2% acetic acid as the second solvent for 3.5 hr. The air-dried chromatograms were first examined under ultraviolet radiation and examined again on exposure to ammonia vapor (Swain, 1953). One chromatogram was sprayed with freshly prepared $FeCl_3$-$K_3Fe(CN)_6$ reagent (Keppler, 1957). The chromatogram was rinsed with 2% HCl and then with distilled water. The blue spots of polyphenolic compounds were detected, and the R_f values were measured.

Four additional chromatograms were sprayed separately with diazotized p-nitroaniline (Swain, 1953), Hoepfner reagent (Walker, 1962), vanillin reagent (Swain *et al.*, 1959), and sodium borohydride (Horowitz, 1957). The color characteristics of the spots were noted.

Separation and purification of polyphenolic compounds

Bar technique. The methyl alcohol extract was applied with a 100-μl pipette along a line 30 cm long on the short direction of Whatman No. 3MM papers. The papers were developed in a descending direction with 2% acetic acid for 3.5 hr, and then dried in air. Four bands were detected under ultraviolet radiation in the presence of ammonia vapor. One additional band was detected when the $FeCl_3$-$K_3Fe(CN)_6$ reagent was used. Corresponding bands cut from eight unsprayed papers were eluted five times with 250-ml portions of 95% ethanol for 60 min each time. The eluates were concentrated to a small volume in a flash evaporator.

Two-dimensional paper chromatography of each eluted band was done to obtain pure individual polyphenolic compounds for spectral measurement. Each fraction was applied to 6 to 8 Whatman No. 1 papers. Two-dimensional descending paper chromatography was run in two solvent systems in the manner described above. Spots having identical R_f values were cut from the chromatograms, eluted with 95% ethanol, filtered through Whatman No. 1 filter paper, and concentrated in a flash evaporator.

R_f values and color reactions. The purified polyphenolic compounds were spotted on Whatman No. 1 papers. The papers were chromatographed at 20°C with BAW (4:1:5) in a descending direction for 18 hr and 2% acetic acid for 3.5 hr. The color reactions of each spot with various reagents were observed, and the R_f values were measured.

Absorption spectra. The spectral measurements were made with a Beckman DB recording spectrophotometer or a Beckman DK-2 recording spectrophotometer, using 1-cm silica cuvettes. The phenolic compounds were diluted with sufficient 95% ethanol to give proper absorbance in the ultraviolet region. For the purpose of measuring a bathochromic shift, three drops of 5% $AlCl_3$ in 95% ethanol was added to the cuvettes. The shifts in absorption spectrum were measured after standing for 1 min.

Chromogenic reagents

$FeCl_3$-$K_3Fe(CN)_6$ reagent. The reagent was prepared just before using by mixing equal volumes of aqueous 0.5% $FeCl_3$ and 0.5% $K_3Fe(CN)_6$ (w/v). It gives a blue color with cathechol and pyrogallol types of polyphenols (Keppler, 1957).

Diazotized p-nitroaniline (DPNA) reagent. It was prepared by mixing, in an ice bath, 0.5% p-nitroaniline in 2 *N* HCl, 5% sodium nitrite, and 20% sodium acetate (w/v) in a ratio of 1:10:30. The reagent gives characteristic colors with phenolic acids (Swain, 1953).

Hoepfner reagent. Equal volumes of 5% (w/v) acetic acid and 5% (w/v) sodium nitrite were mixed just before use. The reagent gives characteristic colors with phenolic acids (Walker, 1962).

Vanillin reagent. A 1% ethanolic solution of vanillin was mixed with an equal volume of concentrated hydrochloric acid immediately before use. It gives a pink to orange-red color with flavonoid compounds containing phloroglucinol nuclei (Swain *et al.*, 1959).

Sodium borohydride reagent ($NaBH_4$). A freshly prepared 2% solution of sodium borohydride in methanol was used. Flavanones are reduced under mild conditions by sodium borohydride in aqueous or alcoholic solution. The four hydroxyflavanes formed in the reduction give a brilliant purple or blue-red color upon treatment with strong acids (Horowitz, 1957).

Ethanolic aluminum chloride. A 5% ethanolic aluminum chloride solution was used to study the shift in absorption peak due to chelation of phenolic compounds containing ortho-dihydroxy configurations.

Relative amounts of polyphenolic compounds. The Folin-Denis colorimetric method described by Swain *et al.*, (1959) was modified to determine the relative amounts of polyphenolic compounds in the extract after two-dimensional paper chromatography (Luh *et al.*, 1967).

RESULTS

Two-dimensional paper chromatography

Fig. 1 represents a two-dimensional paper chromatogram of the polyphenolic compounds present in a methyl alcohol extract of pastes made from VF-145 tomatoes. Twelve blue-colored spots were detected when the chromatogram was sprayed with the $FeCl_3$-$K_3Fe(CN)_6$ reagent. The spots marked with full lines were present in larger amounts than spots marked with broken lines. The major compounds, and compounds Nos. 6 and 11, appeared on all two-dimensional chromatograms. Compound No. 5 did not show clearly on the two-dimensional chromatograms. It was isolated by the bar technique.

Separation and purification

The phenolic compounds in the methyl alcohol extract of canned tomato pastes were partially separated by one-dimensional paper chromatography with 2% acetic acid as solvent. The compounds were further purified by two-dimensional paper chromatography with the systems BAW (4:1:5) and 2% acetic acid.

Chromatographic separation of the extract with 2% acetic acid by the bar technique revealed 5 bands, designated *A, B, C, D* and *E,* from fastest-moving to slowest-moving. Spot 12 was detected on two-dimensional paper chromatography of band *A*. Band *B*, a stronger fluorescent band, consisted of spots 7, 8, 9, 10 and 11. Band *C*, which was invisible under ultraviolet light, but blue on exposure to ammonia vapor, gave spot 6. Band *D* contained spots 3, 4 and 5, with some contamination from spot 2. Band *E* yielded spots 1 and 2.

Chromatographic properties

Table 1 shows the R_f values of the polyphenolic compounds in canned tomato pastes in two solvent systems: BAW (4:1:5) and 2% acetic acid. Table 2 lists the color reactions of these compounds on a two-dimensional chromatogram when examined under ultraviolet radiation with or without ammonia vapor, or under visible light with ammonia vapor, or when sprayed with various chromogenic reagents.

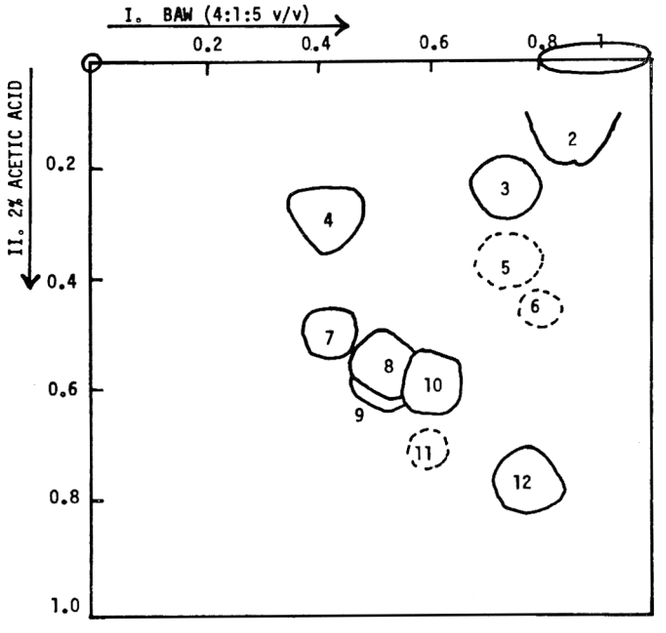

Fig. 1. Two-dimensional paper chromatogram of polyphenolic compounds in pastes made with VF-145 tomatoes.

Table 3 lists color reactions of some authentic phenolic samples. Spots 7, 9, 10 and 11 showed blue fluorescence under ultraviolet radiation, and the color changed to yellowish green when the chromatogram was exposed to ammonia vapor. On a separate chromatogram they appeared as four tan spots when sprayed with diazotized p-nitroaniline reagent. The spots showed a yellow color with Hoepfner reagent. The color characteristics of these compounds resembled those of derivatives of caffeic acid reported by Cartwright *et al.* (1955), Roberts (1962), and Walker (1962).

When BAW (4:1:5) and 2% acetic acid were used as solvents, spots 10 and 11 had R_f values respectively comparable to those of *trans-* and *cis*-chlorogenic acid (Williams, 1955). Williams stated that cinnamic acid derivatives give two spots on paper chromatograms when 2% acetic acid is the solvent. The two spots corresponded to the *trans* and *cis* isomers. Therefore, compounds 10 and 11 were respectively identified tentatively as *trans-* and *cis*-chlorogenic acid.

No known chlorogenic acid isomers have R_f values similar to those of spots 7 and 9 (Hanson *et al.*, 1963; Zane *et al.*, 1965; Van Bragt *et al.*, 1965b). Spots 7 and 9 were tentatively identified as caffeic acid derivatives.

Tsiang (1964) detected in tomato ketchup two unidentified compounds which showed fluorescence under ultraviolet radiation, and the same R_f values as compounds 7 and 9 when BAW (4:1:5) and 2% acetic acid were used as solvents.

Spot 2 displayed the same appearance as naringenin on the chromatogram. Its R_f values and color reaction with

Table 1. R_f values of polyphenolic compounds isolated from pastes made with VF-145 tomatoes.

Spot no.	Tentative identification	*Rf* value	
		BAW (4:1:5)	2% HOAC
1	Unknown	0.90	0.00
2	Naringenin	0.86	0.16
3	Caffeic acid	0.74	0.24
4	Rutin	0.42	0.30
5	Unknown	0.74	0.37
6	Ferulic acid	0.80	0.46
7	Caffeic acid derivative	0.43	0.50
8	Unknown	0.52	0.55
9	Caffeic acid derivative	0.53	0.59
10	*Trans*-chlorogenic acid	0.60	0.60
11	*Cis*-chlorogenic acid	0.60	0.71
12	Unknown	0.78	0.77
Authentic compounds			
	Naringenin	0.89	0.19
	Rutin	0.45	0.28
	Caffeic acid	0.75	0.26
	Ferulic acid	0.80 [1]	0.32–0.46 [1]
	Chlorogenic acid	0.60 [2]	0.57–0.73 [3]
	Neochlorogenic acid	0.55 [2]	0.65–0.79 [3]

[1] Values taken from Tsiang (1964).
[2] Values taken from Hanson *et al.* (1963).
[3] Values taken from Zane *et al.* (1965).

Table 2. Color reactions of polyphenolic compounds isolated from pastes made with VF-145 tomatoes.

Spot no.[1]	Tentative identification	UV light	UV + NH_3	Visible light + NH_3	$FeCl_3$-$K_3Fe(CN)_6$	DPNA	Hoepfner reagent	$NaBH_4$ HCl vapor
1	Unknown	Y	Y	Y	B	Y	Y	C
2	Naringenin	fP	Y	C	B	O	Y	M
3	Caffeic acid	B	B	C	B	T	R	C
4	Rutin	P	Y	Y	B	OY	Y	C
5	Unknown	B	B	C	B	.....	.....	.....
6	Ferulic acid	C	fB	C	B	.....	.....	C
7	Caffeic acid derivative	B	YG	Y	B	T	Y	C
8	Unknown	R	R	C	B	pkR	Y	C
9	Caffeic acid derivative	B	YG	Y	B	.....	Y	C
10	*Trans*-chlorog. acid	B	YG	Y	B	T	Y	C
11	*Cis*-chlorog. acid	B	YG	Y	B	T	Y	C
12	Unknown	C	C	C	B	Y	Y	C

[1] None of the compounds gave a color reaction with vanillin-HCl reagent. (Y, yellow; B, blue; P, purple; R, red; O, orange; G, green; M, magenta; T, tan; Br, brown; pk, pink; C, colorless; f, faint; l, light).

Table 3. Color reactions of authentic polyphenolic compounds.

Compound[1]	UV	UV + NH_3	Visible light + NH_3	$FeCl_3$-$K_3Fe(CN)_6$	DPNA	Hoepfner reagent	$NaBH_4$
Naringenin	fP	Y	C	B	O	.....	M
Rutin	P	Y	Y	B	YBr	Y	C
Caffeic acid	B	B	C	B	Br	R	C
Ferulic acid	B	B	C	B	R	YBr	C
Chlorogenic acid	B	YG	Y	B	T	Y	C

[1] None of the compounds gave a color reaction with vanillin-HCl reagent. (Y, yellow; B, blue; P, purple; R, red; O, orange; G, green; M, magenta; T, tan; Br, brown; pk, pink; C, colorless; f, faint; l, light).

$NaBH_4$ were the same as those of an authentic sample of naringenin. Spot 2 was therefore tentatively identified as naringenin.

Spot 3 showed a blue color under ultraviolet radiation, with or without ammonia. It gave a red color with the Hoepfner reagent and a tan color with diazotized p-nitroaniline. Its R_f values and color reactions were comparable to those of caffeic acid. Spot 3 was tentatively identified as caffeic acid.

Spot 4 showed a purple color under ultraviolet radiation. It changed to yellow when exposed to ammonia vapor. Its R_f values and color reactions with chromogenic reagents were similar to those of an authentic rutin sample. Spot 4 was tentatively identified as rutin.

Spot 6 was tentatively identified as ferulic acid. Its R_f values and color reactions matched those of an authentic sample.

Spectral characteristics

The polyphenolic compounds were further characterized by their absorption spectra in 95% ethanol in the ultraviolet region. The absorption maxima and minima of the compounds are presented in Table 4. The bathochromic shifts of compounds 4, 7 and 10 in the presence of $AlCl_3$ are shown in the same table.

Spot 1 has been reported by Tsiang (1964) as an isoflavone. The ultraviolet absorption spectrum of spot 1 isolated from tomato pastes resembles that of isoflavone (Jurd, 1962).

Spot 2 showed an absorption spectrum similar to that of an authentic naringenin sample, with a maximum peak at 290 mμ, an inflection at 330 mμ, and a minimum at 252 mμ. Wu *et al.* (1958) found naringenin and quercitrin in the fruit skins, but not in the flesh, of Ponderosa, Rutgers, and Sunny Ray tomatoes.

Since the tomato pastes used in this investigation were made from unpeeled whole tomatoes, naringenin in the skin could get into the paste during extraction and pulping. Quercitrin was not positively identified in our pastes. This difference might reflect the difference in varietal characteristics or the difference in methods of isolation of the polyphenols.

Wu *et al.* (1958) used a Magnesol column to separate the flavonoids present in a 500-g sample of dried tomato skin. This quantity was sufficient to identify quercitrin.

Table 4. Spectral characteristics of polyphenolic compounds isolated from pastes made with VF-145 tomatoes.

Compound	λmax (mμ)	λmin (mμ)	λmax (mμ) with $AlCl_3$	Tentative identification
1	273	256	.....	Unknown
2	290	252	.....	Naringenin
3	328,298	262	.....	Caffeic acid
4	258,360	283	273,400	Rutin
5	348,297	270	.....	Unknown
6	320	.....	.....	Ferulic acid
7	325,295	265	328,300	Caffeic acid derivative
8	282	249	290	Unknown
10	325,295	265	328,300	*Trans*-chlorogenic acid
12	256,260	246	.....	Unknown

Tsiang (1964) also found naringenin in tomato ketchup, but not quercitrin.

Spot 3 showed a maximum absorption peak similar to that of authentic caffeic acid. Spot 4 shows maximum absorption peaks at 258 and 360 mμ, and a minimum of 283 mμ. When $AlCl_3$ was added, the maximum respective peaks shifted to 273 and 400 mμ. The spectral characteristic was similar to that of authentic rutin. This is the first time that rutin has been demonstrated in tomato paste. There are reports of rutin in the stems and leaves of tomato plant (Fontaine *et al.*, 1947; Wu *et al.*, 1958; Van Bragt *et al.*, 1965a), and in tomato ketchup (Tsiang, 1964), but no positive identification of rutin in tomato fruit or products made exclusively with tomatoes.

Spot 7 had spectral characteristics similar to those of compound No. 10. Its maximum absorption peak was at 325 mμ, and the minimum at 265 mμ. The maximum peaks shifted to a longer wave length by 3 mμ when $AlCl_3$ was added, indicating an ortho-dihydroxy configuration. This further indicates that compound No. 7 was a caffeic acid derivative.

Spot 8 had a red fluorescence under ultraviolet radiation. It appeared to be a flavonoid compound.

Spot 10 had a maximum peak at 325 mμ and an absorption minimum at 265 mμ, with a shift of 3 mμ in the presence of $AlCl_3$. Its absorption spectrum is similar to that of authentic chlorogenic acid, and to those reported by Bradfield *et al.* (1952), Hanson *et al.* (1963), and Luh *et al.* (1967). This further substantiates the observation that compound No. 10 is *trans*-chlorogenic acid. This compound has been detected in tomato plant (Perkins *et al.*, 1956; Van Bragt *et al.*, 1965b), in wall tissue of tomato fruit (Walker, 1962), and in tomato ketchup (Tsiang, 1964).

Spot 12 had color characteristics and an ultraviolet absorption spectrum similar to those of simple phenols.

Relative amounts of polyphenolic compounds

Table 5 shows the relative amounts of polyphenolic compounds present in canned tomato pastes. Compounds 6 and 11 were not determined, being present only in trace amounts. Spot 12 was not determined, beause of difficulty in locating it on the paper chromatogram without the chromogenic reagent. It appears that naringenin and chlorogenic acid were the polyphenolic compounds present in larger amount in the paste sample. It should be noted that the Folin-Denis reagent determines only the aromatic hydroxyl groups in the polyphenols (Swain *et al.*, 1964; Singleton *et al.*, 1965). Since the phenolic compounds differ in the number of hydroxyl groups, the quantitative data presented here do no more than indicate the relative amount of polyphenols based on the color reaction.

Table 5. Relative amounts of polyphenolic compounds in pastes made from VF-145 tomatoes.

Compound	Tentative identification	Relative amount (%)
1	Unknown	8.4
2	Naringenin	28.2
3,5	Caffeic acid plus unknown 5	12.5
4	Rutin	12.2
7	Caffeic acid derivative	10.5
8,9	Unknown 8 plus caffeic acid derivative	11.8
10	*Trans*-chlorogenic acid	16.4

DISCUSSION

THE POLYPHENOLIC COMPOUNDS were extracted satisfactorily from the tomato paste with methanol. It was necessary to distill the methanol from the extract, remove the carotenoids with petroleum ether, and then extract the polyphenols with ethyl acetate. This procedure separated the polyphenols from sugars, amino acids, and other compounds which are more soluble in water than in ethyl acetate.

The ethyl acetate extract was concentrated almost to dryness in a flash evaporator and then redissolved in a small volume of ethyl acetate or methyl alcohol for two-dimensional chromatography. Methyl alcohol was more satisfactory than ethyl acetate as the solvent for redissolving the crude polyphenols.

Although the number of polyphenolic compounds found by two-dimensional chromatography of 100 μl was the same with either methyl alcohol or ethyl acetate extracts, the spots were more intense with the methyl alcohol extract. This can be explained by the lower solubility of the polyphenolic compounds (especially compound No. 4) in ethyl acetate than in methanol. Identification of compound No. 4 was successful only when methyl alcohol was used to redissolve the crude polyphenols from the flash evaporator.

Naringenin (4',5,7-trihydroxyflavanone) is the aglycone of the flavanone glycoside naringin. The latter is a 7-rhamnoglucoside of naringenin. Naringin is remarkably bitter. Its bitterness is easily detectable at concentrations as low as 10^{-4} or $10^{-5}M$. Horowitz *et al.* (1963) demonstrated that the sugar moiety in naringin is the disaccharide neohesperidose. Occasionally, some tomato juice samples have a slightly bitter taste. This might be related to the presence of bitter flavanone glycosides. It remains to be investigated whether naringin was present in some of the tomato products.

The chlorogenic acids are substrates for enzymatic oxidation by polyphenoloxidase. When tomatoes are harvested by mechanical means (removal of whole plants from the soil, followed by a mechanical shaking procedure), a certain percentage of the fruits are liable to be mechanically damaged or cracked. This may cause rapid enzymic oxidation of chlorogenic acids, caffeic acid, and other polyphenols in the damaged tomatoes by polyphenoloxidase to form quinones and their polymerization products. The present investigation reports the polyphenols present in tomato pastes made under controlled conditions. The results would be of interest to dietitians, nutritionists, and the medical profession.

The fact that fruits are rich storehouses of exotic phenolic compounds has led to many speculations concerning the metabolic significance of these substances. Some of them play an important role in respiration, disease resistance, enzymic oxidation, and metabolism. Rutin is shown to be present in the tomato paste. It appears to be an important ingredient in tomato products because it can reduce the fragility of blood vessels and regulate their permeability (Haley *et al.*, 1951). The medical profession

recommends patients suffering from blood circulation problems to eat more often those foods rich in rutin. Tomato products appear to be a good source of this bioflavonoid.

Caffeic acid has profound hormone-like effects on plant growth at extremely low concentrations (10^{-4} to 10^{-11} M). Phenolic acids affect the growth of plants in that they inhibit an enzyme that oxidatively destroys indoleacetic acid and thus its hormonal effect. Some of the polyphenolic compounds may act as chelating agents and play an important role in biological processes.

The present investigation identifies some of the polyphenols in tomato pastes. It is hoped that more information will be gathered in the future on the effect of climatic conditions, varietal characteristics, harvesting methods, and processing variables on the levels of polyphenols of physiological importance present in tomato products.

REFERENCES

Bradfield, A. E., Flood, A. E., Hulme, A. C. and Williams, A. H. 1952. Chlorogenic acid in fruit trees. *Nature* **170**, 168.

Cartwright, R. A., Roberts, E. A. H., Flood, A. E. and Williams, A. H. 1955. Suspected presence of p-coumaryl quinic acids in tea, apple, and pear. *Chem. & Ind.* (1955), 1062.

Fontaine, T. D., Ma, R., Poole, J. B., Porter, W. L. and Naghski, J. 1947. Isolation of rutin from tomatin concentrates. *Arch. Biochem. Biophys.* **15**, 89.

Haley, T. J. and Rhodes, B. M. 1951. Evaluation of "vitamin P-like" compounds for their ability to modify capillary permeability. *J. Am. Pharm. Assoc.* **48**, 179.

Hanson, K. R. and Zucker, M. 1963. The biosynthesis of chlorogenic acid and related conjugates of the hydroxycinnamic acids. *J. Biol. Chem.* **238**, 1105.

Horowitz, R. M. 1957. Detection of flavanones by reduction with sodium borohydride. *J. Org. Chem.* **22**, 1733.

Horowitz, R. M. and Gentili, B. 1963. Flavonoids of citrus. VI. The structure of neohesperidose. *Tetrahedron* **19**, 773.

Jurd, L. 1962. Spectral properties of flavonoid compounds. In "The Chemistry of Flavonoid Compounds," ed. Geissman, T. A., Chap. 5, p. 107. Macmillan Co., New York.

Keppler, H. H. 1957. The isolation and constitution of mollisacacidin, a new leucoanthocyanidin from the heartwood of *Acacia mollissima Wild. J. Chem. Soc.* (**1957**), 2721.

Kidson, E. B. 1958. Cloud or vascular browning in tomatoes. IV. Polyphenol oxidase activity in cloud-susceptible fruit. *New Zealand J. Agr. Research* **1**, 896.

Luh, B. S., Hsu, E. T. and Stachowicz, K. 1967. Polyphenolic compounds in canned cling peaches. *J. Food Science* **32**(3), 251.

Perkins, H. J. and Arnoff, S. 1956. Identification of the blue fluorescent compounds in boron-deficient plants. *Arch. Biochem. Biophys.* **64**, 506.

Roberts, E. A. H. 1962. Economic importance of flavonoid substances: tea fermentation. In "The Chemistry of Flavonoid Compounds," ed. Geissman, T. A., Chap. 15, p. 468. Macmillan Co., New York.

Singleton, V. L. and Rossi, J. A. Jr. 1965. Colorimetry of total phenolics with phospho-molybdic-phosphotungstic acid reagents. *Am. J. Enol. Vitic.* **16**(3), 144.

Swain, T. 1953. The identification of coumarins and related compounds by filter-paper chromatography. *Biochem. J.* **53**, 200.

Swain, T. 1962. Economic importance of flavonoid compounds: foodstuffs. In "The Chemistry of Flavonoid Compounds," ed. Geissman, T. A., Chap. 16, p. 513. Macmillan Co., New York.

Swain, T. and Goldstein, J. L. 1964. The quantitative analysis of phenolic compounds. In "Methods in Polyphenol Chemistry," ed. Pridham, J. B. p. 134. Macmillan Co., New York.

Swain, T. and Hillis, H. E. 1959. The phenolic constituents of *Prunus domestica*. I. The quantitative analysis of phenolic constituents. *J. Sci. Food Agr.* **10**, 63.

Tsiang, J. M. 1964. Chemical changes in tomato ketchup packed in aluminum and laminate pouches. M.S. thesis. University of California, Davis, Calif.

Van Bragt, J., Rohrbaugh, L. M. and Wender, S. H. 1965a. The effect of 2,4-dichlorophenoxyacetic acid on the rutin content of tomato plants. *Phytochemistry* **4**, 963.

Van Bragt, J., Rohrbaugh, L. M. and Wender, S. H. 1965b. Identification of neochlorogenic and 3-0-feruloyl quinic acids in tomato plants. *Phytochemistry* **4**, 977.

Waggoner, P. E. and Dimond, A. E. 1956. Polyphenol oxidases and substrates in potato and tomato stems. *Phytopath.* **46**, 495.

Walker, J. R. L. 1962. Phenolic acids in "cloud" and normal tomato fruit wall tissue. *J. Sci. Food Agr.* **13**, 363.

Williams, A. H. 1955. Paper chromatography of cinnamic acid derivatives. *Chem. & Ind.* (1955), 120.

Wu, M. and Burrell, R. C. 1958. Flavonoid pigments of the tomato. (*Lycopersicum esculentum* Mill). *Arch. Biochem. Biophys.* **74**, 114.

Zane, A., Steck, W. and Wender, S. 1965. Identification of 4-0-caffeoyl quinic acid and related depsides in tobacco from cigarettes. *Tobacco* **160**(24), 31.

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Formation of Nitric Oxide Myoglobin: Mechanisms of the Reaction with Various Reductants

SUMMARY—The concentration, temperature and pH dependences of the formation of nitric oxide myoglobin (NOMb) from metmyoglobin nitrite (MetMb · NO_2) were determined for nitrite and the reductants, ascorbic acid, cysteine, hydroquinone, nicotinamide adenine dinucleotide (NADH) and glyceraldehyde. The reaction for all reductants except glyceraldehyde involves the production of a nitroso-reductant intermediate which breaks down to release nitric oxide. The latter forms a nitric oxide metheme complex (Fe^{+++}) which is then reduced to the ferrous state (Fe^{++}). With cysteine and NADH there is a second pathway which probably involves the direct reduction of MetMb · NO_2. Ascorbate and hydroquinone form nitroso intermediates that are stabilized in alkali. The effects of oxygen, ethylenediaminetetraacetic acid and cytochrome *c* on the reaction were determined. Oxygen slows or inhibits the reaction, while the latter two have no effect on the reaction as studied.

INTRODUCTION

TO DATE, IMPROVEMENTS in cured meat color technology have been made on a largely empirical basis since the mechanisms of chromophore production in meat or meat products are unknown. It is known that the process is one of reduction of nitrous acid (from added nitrite) to nitric oxide and of metmyoglobin and methemoglobin from the ferric (met) to the ferrous state. But there are a number of reducing systems and/or reductants in meat which, in addition to or conjunction with added reductants, are capable of effecting the reduction.

Although isolated studies of the effects of various reductants and/or reducing systems have been made (Kelley *et al.*, 1957; Siedler *et al.*, 1959; Fox *et al.*, 1963; Walters *et al.*, 1965; Borys, 1965; Watts *et al.*, 1966), the accumulated data do not cover all conditions of pH, temperature and reactant concentrations encountered in cured meat production. The development of comprehensive data on the foregoing conditions for endogenous or added reductants can have a two-fold value, (1) by helping establish which reductants or systems are principally involved in meat curing, and (2) indicating the mechanism(s) of the reactions involved. Once these mechanisms are known it will be possible to determine the optimal conditions for obtaining maximal rates of color production conversions and stability.

We therefore undertook an *in vitro* study of the kinetics and thermodynamics of the formation of NOMb with the aim of producing coherent data on the pH, temperature and concentration dependences of the rate of nitric oxide heme pigment formation using reductants either normally added or endogenous to meat, or else potentially useful in the process. The data were used to develop equations that express the rate dependence of the reaction in terms of these various factors and reductants, and to determine the mechanisms of the reactions where possible.

EXPERIMENTAL

THE TECHNIQUES USED for the preparation of myoglobin have been described previously (Fox *et al.*, 1963). NADH was obtained from Nutritional Biochemicals Corporation; the rest of the chemicals were reagent grade. The nitric oxide came from the Matheson Company and the nitrogen was water-pumped prepurified grade.

The course of the reactions was followed by the change in absorption at 547.5nm as this was the wavelength of maximal absorption (β peak) in the visible portion of the spectrum as determined with a Cary Model 14 recording spectrophotometer calibrated to within $\pm$ 0.02nm. The Cary Model 14 or a Gilford attachment for a Beckman DU spectrophotometer was used to record the change in absorption; the Cary was used to record spectra. Temperature control was attained by means of a YSI thermoregulator with the thermistor probe either immersed in the cuvette or used as a surface probe fastened to the cuvette holder. The YSI unit controlled a Waco Refrigerated Visibility Bath. With this arrangement it was possible to control the cuvette to within plus or minus 0.05°C.

Reactions were run under three different conditions: (1) under nitrogen; (2) in open cuvettes without prior removal of oxygen; and (3) with air bubbling through the cuvette during the reaction. One centimeter cuvettes were fitted at the top with short sections of 6 mm tubing so that the cells might be sealed with size 000 serum bottle caps. Gases and solutions were introduced into the cuvettes through hypodermic needles inserted in the caps. Nitrogen gassing was conducted for a period of 10 min. Reagent solutions were made with nitrogen-gassed water.

NOMetMb was produced by bubbling nitric oxide through MetMb solutions after oxygen removal. A criterion of the conversion to NOMetMb was that the 575nm absorption maximum be higher than the 535nm absorption; lesser conversions gave poor results.

The reductants used in this study were ascorbate, cysteine, NADH, hydroquinone, and glyceraldehyde, chosen as being endogenous to meat or as containing functional groups corresponding to endogenous reductants. Table 1 summarizes the order of the dependences of the reaction rate on the concentration of the various reactants excepting glyceraldehyde. Table 2 lists a number of empirical equations for the various dependences on pH and temperature. With the exception of the reaction with NADH at pH 5.0

Table 1. Reaction rate on concentration.

Reductant	From metmyoglobin nitrite order of dependence, n				From nitric oxide myoglobin order of dependence, n			
			mM Nitrite					
	[R]	pH	<0.7	>0.7	[MMb]	[R]	pH	[MMb]
Ascorbate	0.50	−1.43[1]	1.0	0.41	0	1.0	0	1.0
Cysteine	0.48	−0.60	1.0	0.22	0	1.0	0	1.0
Hydroquinone	1.00	−1.2[1]	1.0	0.62	0	1.0	0	1.0
NADH	0.58	−1.19	~1.0		1.0[2]	1.0	0.1	1.0
					0[3]			

[1] 20°C.
[2] pH 4.5.
[3] pH 5.5–6.5.

and below, the reactions were all zero order with respect to the concentration of pigment.

To determine the order of dependence of the reaction on the concentrations of reductant and nitrite, these concentrations were varied and the effect on the zero order rate constant (k_0) observed. The dependence was fractional order with respect to the concentration of cysteine and NADH and it was found necessary to make use of the logarithmic form (2) of the basic equation (1):

$$(1)\quad y = a\,x^n$$
$$(2)\quad \log y = \log a + n \log x$$

The plots are shown in Fig. 1. The value for n of 0.481 for cysteine is not significantly different from 0.500, and indicates that the reaction rate is dependent upon the square root of the cysteine concentration. The figure of n = 0.578 for the NADH dependence does differ significantly from 0.500, however. Fig. 2 shows the reaction sequences involved, and is as follows:

Nitrous acid, in the form of the anhydride reacts with the reluctant to form a semi-stable intermediate, as proposed by Dahn *et al.* (1958, 1960), which may then react either to release free NO (reaction 4) or with another molecule of intermediate to reform the initial reactants (backward reaction, 3). Reaction 6, being the formation of the nitric oxide met-heme complex, is fast compared to the other reactions. Reaction 7 is a reduction step, and either it or reaction 4 is the rate-limiting step. Reaction 3 is the step which introduces the square root dependence on the reductant concentration into the rate expression, and if $k_3 > k_4$, the dependence will be exactly 0.5, i.e., cysteine and ascorbate. If $k_4 > k_3$ the reaction will be dependent directly upon the concentration of the reductant (n = 1.0), i.e., hydroquinone. It therefore follows that where $k_4 \cong k_3$ the dependence will be somewhere between 0.5 and 1.0, which is the case with NADH.

If $k_4 < k_7$ the overall rate expression will be zero order with respect to the pigment, but if $k_4 > k_7$ or if the reaction mechanism proceeds by some other pathway, for example, reaction 5, or reactions 8 and 9, the overall rate expression will be dependent upon the first power of the pigment concentration. The reaction sequence does not include the reduced form (ferrous) of the heme pigment, an omission based on the observation that at these pH values nitrite oxidizes the reduced heme pigments faster

Table 2. Coefficients of the equations describing the dependence of the rate of formation of nitric oxide myoglobin on pH and temperature.

Reductant	pH	t°C	Equation for log $10^2 k_0$[1,2]	ΔHa kcal/mole
Ascorbate	4.5	....	9.625 − 1804/T°K	8.26
	5.0	....	9.746 − 2050/T°K	9.38
	5.5	....	9.965 − 2323/T°K	10.63
	6.0	....	10.169 − 2593/T°K	11.87
	....	20°	9.907 − 1.43 pH	.....
	....	40°	9.805 − 1.32 pH	.....
Cysteine	4.5	....	16.212 − 4225/T°K	19.3
	....	20°	4.423 − .599 pH	.....
Hydroquinone	4.5	....	9.616 − 1902/T°K	8.71
	5.5	....	10.343 − 2549/T°K	11.67
	6.5	....	18.091 − 5092/T°K	23.30
NADH	....	20°	7.931 − 1.19 pH[3]	.....
			log $10^3 k_{1st}$[4]	
NADH	4.5	....	12.467 − 2900/T°K	13.28

[1] $k_0 = \mu M/min$.
[2] The standard deviation ranged from 2 to 10%; the average and modal S. D. was 5%.
[3] pH 5.0–6.5.
[4] $k_{1st} = min^{-1}$.

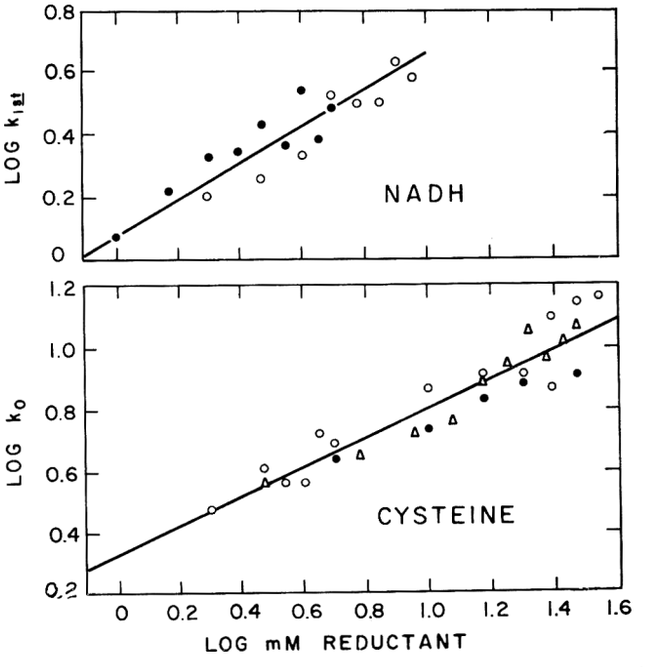


Fig. 1. Dependence of the rate constant for the formation of NOMb on the concentration of the reductants NADH and cysteine.

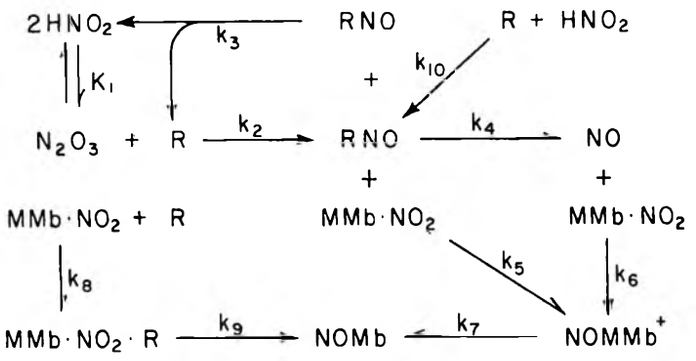


Fig. 2. Reaction sequences for the formation of NOMb from MetMb·NO_2.

than the nitric oxide heme pigments are formed, i.e., until the nitric oxide pigments are formed, the reduced heme pigments do not exist in solution in the presence of nitrite.

On the other hand nitric oxide combined with the oxidized pigment is probably stabilized against oxidation by oxygen and nitrite. The presence of the intermediate (NOMetMb) formed is observed spectrally during the course of the reaction from the position of the β peak (530–550 nm region), as follows. Since the maximum of NOMetMb appears at 532 nm, while the corresponding maximum for its reduction product NOMb lies at 547.5 nm, during the reaction a peak in this region appears first around 538–540 nm (indicating the presence of NOMetMb) and shifts towards a longer wavelength during the reaction until at the end of the reaction the final value of 547.5 nm is reached.

The variation with pH was fractional order with all four reductants as shown in Table 1. This fractional order may be the result of one or more of three causes: (1) the backward reaction 3 in Fig. 2 when the formation of the intermediate depends on pH; (2) two or more independent pathways to the same product with different pH dependences; and/or (3) two different forms of a semistable reaction intermediate, each form having a different heat of activation for breakdown. In the first case, the derived rate expression would be a precise mathematical derivation of the mechanism, but in the last two cases the expressions for the rate dependence are empirical. At the extremes of either pH or temperature the overall kinetics will be characteristic of one reaction mechanism or intermediate; at intermediate values the kinetics will reflect the behavior of all mechanisms and/or intermediates.

The reduction by ascorbate

The mechanism of this reaction has been discussed previously (Fox *et al.*, 1963) and was found to involve reactions 1, 2, 3, 4, 6 and 7. Reaction 4 was rate limiting, and, from the pH dependence, it was determined that the reaction was mainly dependent upon the unionized (acid) forms of the two acids, nitrous and ascorbic.

In the present study, the pH dependence was variable and somewhat less than 1.5 (1.43 at 20°C), in this case probably reflecting a variation in the stability of the nitroso-ascorbic acid intermediate. Since the heat of activation is the energy required to cleave the intermediate complex into an ascorbate radical and nitric oxide, its variation with pH is probably due to the ionization of the ascorbate carboxyl group, the ionized (alkaline) form being the more stable. It is important to note that the equations in Table 2 hold true only for the pH range 4.5 to 6.0. At 40° and pH 4.5 some cleavage of the protein-heme bond occurred producing some nitric oxide hemochrome; above pH 6.0 the reaction rate begins to increase with pH.

Reduction by cysteine

The basic assumption in solving the reaction sequence from the observed dependences is that the intermediates occur at constant concentration, "steady state," (Fox *et al.*, 1963). The desired rate expression which describes the principal reaction at pH 4.5 is the same for cysteine as for ascorbate, with different rate constants. The solution is obtained by assuming the reaction sequence includes reaction steps 1, 2, 3, 4, 6 and 7, with steady state concentrations of N_2O_3, AHNO, NO and NOMetMb. Reaction 4 is assumed to be rate limiting. The pH dependence $k_0 \sim [H]^{0.6}$, is very much different from what would be expected from a reaction involving only the unionized forms of the reactants.

Since the sulfhydryl group does not ionize appreciably in this pH range, it would be expected that the pH dependence would be due to the nitrous acid ionization and that k_0 would be proportional to $[H]^{1.0}$. From the low value of the order of dependence and the lack of variation with pH of the heat of activation, it is apparent that there is a second reaction which is an important pathway, particularly at the higher pH values. Since there were at least two mechanisms operative, we attempted to further elucidate the reaction mechanism by varying the concentration of the reactants at pH 6.5 where the effect of the reaction of the unionized forms would be minimal.

The rate of conversion to NOMb tended to be intermediate between zero and first order with respect to the pigment at higher pH values when the concentrations of cysteine and nitrite were 5m*M*. At nitrite concentrations above 5m*M* at pH 6.5, the reactions were all exactly first order with respect to pigment, the nitrite dependence disappeared completely, and the dependence of the first order rate constant on the cysteine concentration was very low ($n = 0.15$ in equation [2]).

The results indicate saturation of the system with both reductant and nitrite at the concentrations studied, and are consistent with a reaction sequence represented by reactions 8 and 9 in Fig. 2, showing the direct reduction by cysteine of MetMb·NO_2. The heat of activation did not vary with pH, indicating that the scission of the S-N bond was not affected by ionizations in this pH range, regardless of the mechanism of the reaction. The value obtained is normal for the reduction reactions of cysteine, ca. 20 kcal/mole (Tarbell, 1961).

Reduction by hydroquinone

Hydroquinone was chosen as representative of the quinoid-type reductants that occur in animal tissue such as the tocopherols (E-Vitamins), K-vitamins, coenzyme Q and ubiquinone. Using the usual method of steady state analysis, it was determined that the reduction involved, in sequence, pathways 10, 4, 6 and 7, with reaction 4 again

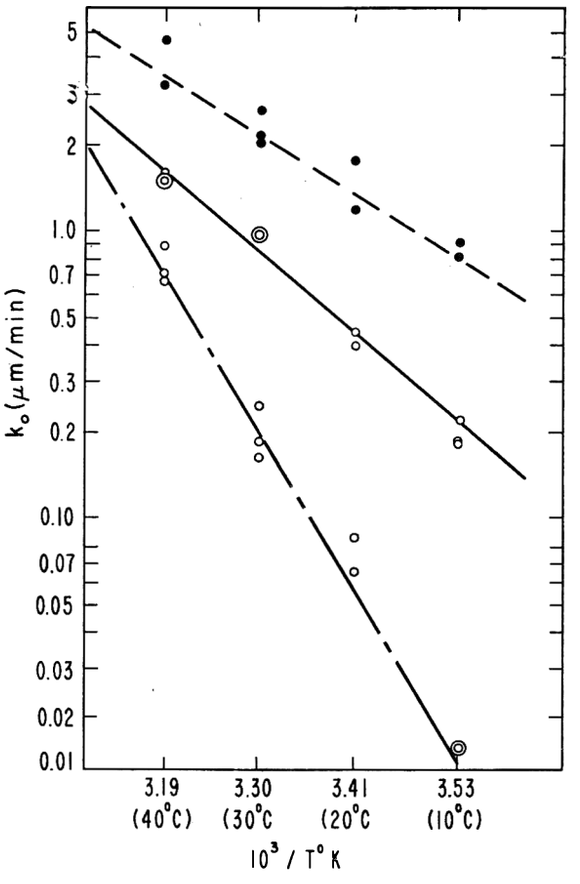
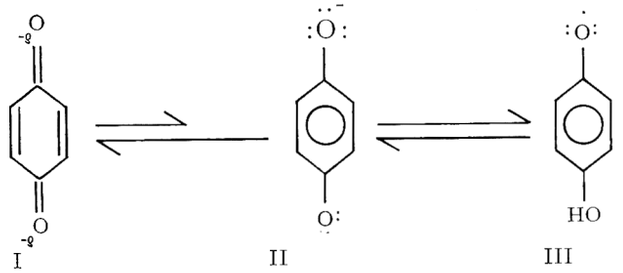


Fig. 3. Arrhenius plots for the formation of NOMb with hydroquinone as reductant. pH 4.5, - - -; pH 5.5, ——; pH 6.5, ——.

the limiting step. The release of NO and the formation of NOMetMb was attested to by the appearance of the β peak at 540 nm. The variation with temperature of the rate of the reaction increased sharply with decreasing hydrogen ion concentration, as shown in Fig. 3.

The heats of activation calculated from the Arrhenius plots are, in kcal/mole: pH 4.5, 8.7; pH 5.5, 11.7, and pH 6.5, 23.3. This increase is the result of the increased stability of the quinone intermediate at higher pH values as was also noted for the ascorbate intermediate. This increased stability in alkali is typical of quinoid structures containing oxygen and is the result of the delocalization of the charge of the ionized species in the π-bond structure of the semi-quinone (Gould, 1959).

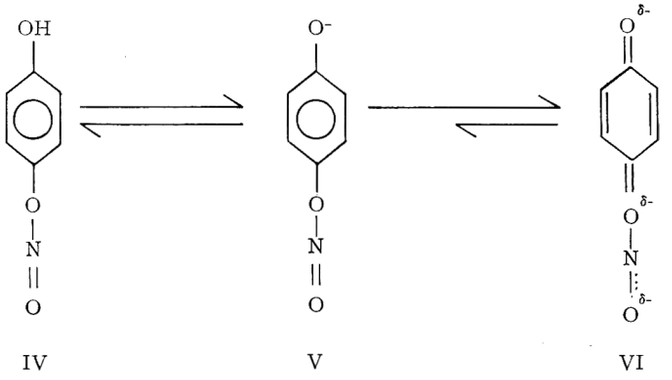
The theory of the resonance stability of the alkaline semi-quinones is represented in equation [3]:



I II III

In I the undissociated proton interferes with the participation of the p-orbital electrons of the oxygen in the resonant structure. In III, the structure is stabilized by the further inclusion of the two oxygen atoms in the π-bond system and the delocalization of the charge.

We therefore postulate the nitroso-semiquinone intermediate to be a structure containing both hydro-quinone and nitric oxide which may be formulated as follows:



IV V VI

Structure VI is the classical resonance representation of the stabilized structure and indicates the extensive delocalization of the π-bond electrons over the entire moelcule. It was not possible to assign specific heats of activation to either form of the intermediate because in the narrow range of pH 4.5 to 6 the reaction involved mixtures of both forms in unknown quantities.

The cleavage which releases nitric oxide is a one electron transfer presumably resulting in the formation of a semiquinone. We postulate that it is the ease of formation and stability of the semi-quinone as compared with the relative difficulty of formation of the radical products of the other reductants which accounts for the linear dependence of the rate constants on the concentration of hydroquinone and the fractional order dependences of ascorbate, cysteine, and NADH.

In a bimolecular reaction between two intermediate reductant-nitric oxide complexes, the bimolecular intermediate may either react to produce nitric oxide and the radical form of the reductant, or revert to the original electronic states of the reduced reductant and nitrous acid. The first reaction is in the forward direction. When the steady state analysis is made, the order of this reaction disappears and does not affect the order of the total reaction. The second reaction is in the backward direction and, if favored, introduces a fractional order dependence on the reductant. The quinone bimolecular intermediate will readily form the relatively stable radical product (first reaction) with the release of nitric oxide. In the case of the acid quinone, the formation of the meriquinone dimer may further stabilize the semi-quinoid product resulting from the release of nitric oxide, and contribute toward the ease of electron transfer.

In contrast, the other reductants, whose radical forms are not as fully stabilized as is the semiquinone structure, would tend to revert to their original state of reduction from the dimer rather than form the radical product, hence introducing a fractional order dependence.

Reduction by NADH

The total reaction sequence, as determined from the dependences of Table 1, involves reaction steps 1, 2, 3, 4,

6 and 7. At pH 4.5 the reaction is first order with respect to the pigment, but at pH 5.5 and higher values the conversion of pigment is zero order. The rate of the reaction at pH 4.5 must therefore be governed by a slow reaction after the involvement of the pigment. In the sequence of Fig. 2 it would be reaction 7, the reduction of NOMetMb. If the reduction of the last named pigment is the rate limiting step, then its rate should be equal to the rate of the reduction of MetMb·NO_2.

Table 3 summarizes the rate constants for the two reactions, and, as can be seen, at pH 4.5 the rates of the two reactions are the same. Comparing the turnover rates at higher pH values for the two reactions (0 order rate versus $k_{1st} \times 50\ \mu M$ pigment), it is seen that the rate of NOMetMb reduction is several magnitudes greater than the rate of MetMb·NO_2 reduction, hence the reduction of the latter is zero order. Such a situation did not prevail with the other reductants; at all pH values studied the rate of NOMetMb reduction was greater than the rate of nitric oxide production.

As in the case of cysteine the fractional order dependence of the NADH reduction is probably due to two or more different reaction mechanisms since the heat of activation did not vary with pH. However, we could not obtain data to support any further conclusions as to possible sequences, in part due to the formation of a reaction product between nitrite, NADH and heme pigment, which was not NOMb. The first reaction observed was the formation of NOMb followed by a reaction resulting in the formation of a stable heme pigment with a distinctive absorption spectrum, Fig. 4. The product is water-soluble and therefore presumably contains native protein, but we have not yet been able to convert it to any other recognizable heme pigment form.

Reduced nicotinamide adenine dinucleotide phosphate (NADPH, TPNH) was tried as a reductant. Although a reddening of the solutions was observed, the NADPH denatured the protein with concomitant increase in turbidity.

Reduction by glyceraldehyde

The solutions of pigment, glyceraldehyde and nitrite did not show any spectral changes for periods up to 3 days at any pH between 4.5 to 6.5 and 20°C. It was necessary to raise the temperature to 40°C before any appreciable reactions were observed. At pH 4.5, the observed reaction was the production of nitrimetmyoglobin, the green heme pigment observed by Fox *et al.* (1964), while at pH 6.0 conversion to NOMb occurred overnight. The reaction rate increased with increasing pH values above pH 6.0 and is probably a reaction similar to that observed for the other reductants above pH 6.5. At intermediate pH values, no reactions were observed up to 40°C.

Table 3. Rates of reduction of metmyoglobin nitrite and nitric acid myoglobin by NADH.

pH	Pigment	Order	Rate constant	Turnover number [MMb]=50 μM
4.5	MMb·NO_2	1	0.187 min^{-1}	9.35
	NOMMb	1	0.211 min^{-1}	10.55
5.5	MMb·NO_2	0	0.71 μM/min	0.71
	NOMMb	1	0.224 min^{-1}	11.2
6.5	MMb·NO_2	0	0.083 μM/min	0.083
	NOMMb	1	0.182 min^{-1}	9.10

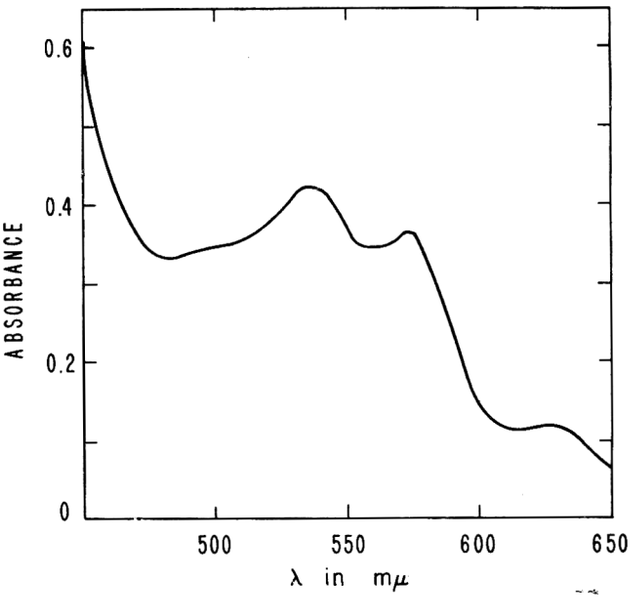


Fig. 4. Spectra of final product of reaction between NADH, nitrite and MetMb.

Effect of nitrite

The concentration of nitrite was varied over a range of 0.05 to 50.0 m*M* at pH 4.5, 20°C in nitrogen, with each of the four reductants. The results were plotted on a log-log scale and the calculated values of n (Equation 2) are shown in Table 1. Two effects are noted: (1) the fractional dependence was observed only above 0.7 m*M* nitrite and (2) the fractional order varied with the reductant. The 0.7 m*M* nitrite corresponds to ca. 0.05 m*M* undissociated nitrous acid at pH 4.5, the same concentration as the pigment, and suggests an activation process involving the pigment. The fractional order dependences suggest multiple reactions between the reductant and the nitrite, with nitric oxide being but one of a number of products. In fact Evans *et al.* (1956) have found this to be true with the reaction between nitrite and NADH or ascorbate producing N_2O and N_2 as well as NO.

Reduction of NOMetMb

The reduction of NOMetMb was first order with respect to the pigment and with respect to the various reductants tested (ascorbate, cysteine, hydroquinone and NADH). The calculated rate constants were consistent with the observed rates of NOMb formation from MetMb·NO_2 and, except with NADH, did not vary with pH. The reduction of NOMetMb by NADH showed a low order dependence on pH (Table 1) which may be an effect of steric hindrance. NADH is a large molecule, and may not fit as well or as easily into the cleft in which the heme lies (Kendrew *et al.*, 1960; Perutz *et al.*, 1960). The pH dependence thus reflects changes in the protein conformation making the heme more accessible as the pH decreases.

Table 4. Effect of air on the reaction, $MMb \cdot NO_2 \rightarrow NOMb$.

Reductant	Gas	pH	Temp	k_0 μM/min	k_{1st} min^{-1}	Initial turnover rate μM/min
Asc	Air	4.5	20	....	0.315	13.3
	N_2	4.5	20	22.7 ± 2.5	....	22.7
	Air	4.5	30	....	0.649	23.1
	N_2	4.5	30	40.3 ± 3.6	....	40.3
	Air	4.5	40	....	1.23	33.0
	N_2	4.5	40	61.4 ± 0.3	....	61.4

Effect of oxygen

The reaction studied by Fox *et al.* (1963) took place in open cuvettes with no precautions to remove dissolved oxygen. All reactions discussed in this paper were run in closed absorption cells that had prepurified nitrogen bubbled through the solutions to remove oxygen. The rates of the latter reactions showed less variation and the rate constants were higher than in the previous study. In studying the effect of oxygen on the reaction, we bubbled air through the reacting solutions to maintain a constant level of oxygen in the solutions. The results are summarized in Table 4.

Two principal effects were noted; the reactions in air were first order with respect to pigment and the reaction rates were reduced, as indicated by turnover numbers in Table 4. This reduction in rate is probably a result of the relatively rapid oxidation of nitric oxide by oxygen. When the proposed nitroso-ascorbic acid intermediate breaks down to yield nitric oxide, in the absence of oxygen the only reactant available is the heme pigment.

With oxygen present, a competition between heme pigment and oxygen exists, and the relative amounts of nitric oxide consumed by the two pathways are dependent on the relative concentrations of pigment and oxygen, and the respective rate constants of the two reactions. With nitric oxide produced at a constant rate and the oxygen concentration constant, the observed fate of NOMb formation will depend on the concentration of MetMb available for complexing nitric oxide. Since this latter concentration decreases as the pigment is converted, the reaction becomes first order.

Effect of cytochrome c

Walters *et al.* (1965), studying the reduction of nitrite by mitochondria, found that ferrocytochrome *c* was oxidized by the system with the formation of nitric oxide. Since experience had shown us that our myoglobin preparations were sometimes contaminated with small amounts of cytochromes, we considered it advisable to determine what effect such contamination might have on the reaction. Our studies of the chromatography of myoglobin on carboxymethyl-cellulose showed that cytochrome *c* adhered firmly to the top of the column.

We placed myoglobin preparations on carboxymethyl cellulose columns under conditions that cause cytochrome *c* to adhere (0.05 *M* phosphate, pH 7.0), but found none, probably because it was completely dialyzed out of the solution in the two dialysis steps applied in preparing myoglobin. We therefore tested the effect of reduced cytochrome *c* on the reaction rate at 0.05 m*M*, 0.0005 m*M*, and zero concentration levels, but found no differences in the reaction rates. We therefore conclude that cytochrome *c* would have had no effect on the formation of NOMb in our reaction mixtures.

Effect of high pH

All the reductants exhibited a decreasing rate of $MetMb \cdot NO_2$ reduction with increasing pH up to pH 6.0 to 6.5. Above this pH the rate versus pH curve rose sharply. Even the reduction by glyceraldehyde showed an appreciable reaction rate above pH 7.0 at 20.0°C. From preliminary studies it appeared that the reaction mechanisms above pH 6.5 are different from those below pH 6.5 and that further study will be required to characterize them.

Effect of ethylenediamine tetraacetic acid (EDTA)

Siedler *et al.* (1959) and Weiss *et al.* (1953) reported that the addition of iron increased the formation of nitric oxide hemochrome; Pascal *et al.* (1957) reported that the oxidation of cysteine by peroxide was probably metal-catalyzed. We wished to know whether or not contamination was affecting our results, so we examined the effect of the addition of EDTA on our systems. The results were negative; the addition of EDTA neither changed the rate of the reaction, nor improved the precision of the measurements. This result is consistent with the observed negative pH dependence of the reactions, since metal catalyses are generally associated with positive rate-pH dependences (Pascal *et al.*, 1957).

DISCUSSION

Our results emphasize some of the important aspects of the role performed by reductants in cured meat color production. Some of these aspects are (1) the effectiveness of any given reductant over the range of pH encountered in meat products; (2) its effectiveness in the elimination of oxygen and/or forming the nitric oxide pigment in the presence of oxygen; (3) the temperatures required to produce the nitric oxide heme pigments within reasonable time periods, and (4) the number of side reactions which take place that either use up the available reductants or produce nitrogen oxides no longer available for the production of cured meat pigments.

With regard to (1) and (3) it has been observed (Fox *et al.*, 1967) that cysteine is fully as effective as ascorbate in color production in frankfurter emulsions, after the oxygen has been eliminated and the emulsions are cooked. This is a happenstance of the pH and temperature dependences of cysteine and ascorbate. Thus, from the data of Table 2, it may be calculated that at pH 6.0 and 100°F (40°C) cysteine reduces $MetMb \cdot NO_2$ as fast as does ascorbate.

Sufficient oxidizable sulfhydryl groups do exist in meat to account for the observed rates; Hamm *et al.* (1965, 1966) found 17.5 m*M* (1965) and 25 m*M*-SH (1966) by amperometric titration. Assuming a 1:1 dilution in frankfurter emulsions, the sulfhydryl content of the added meat alone could account for the observed rates of nitric oxide hemochrome formation in frankfurter emulsions (Fox *et al.*, 1967). Sulfhydryl groups alone are only part of the available reductants in muscle tissue; Thomson *et al.*

(1962) and Regier *et al.* (1956) reported total reducible substance concentrations ranging from 50 to 100 mM.

Chromophore production itself is only one of a number of requirements. As Watts *et al.* (1966) and Fox *et al.* (1967) have shown, removal of oxygen, when present, is a vital part of cured meat color production. Ascorbate is more effective than cysteine in this respect, since the former reacts faster with oxygen and is capable of driving the reaction of $MetMb \cdot NO_2 \rightarrow NOMb$ to completion in the presence of oxygen. The latter reaction was accomplished only at the highest temperature studied (40°C) by cysteine.

Side reactions have been given little if any consideration to date, but may be of great importance. ten Cate (1962) obtained evidence of N_2O in the gases above curing pickles, showing that such side reactions do occur in practice. Our studies show that these reactions are particularly important at nitrite concentrations common to cured meat products (30-200 ppm, 0.7–5.0 m*M*) and that the relative rates vary with the reductant.

Since these side reactions produce nitrogen in oxidation states no longer available for the formation of nitric oxide, part of the overall effectiveness of any given reductant may depend upon the extent to which the reductant produces these compounds. As a general conclusion then, these studies suggest that no one reductant may be optimally effective in all cured meat products, but that consideration should be given to using different reductants or combinations thereof, depending on what kind of products are made and how they are made.

REFERENCES

Borys, A. 1965. The influence of the curing process on the conversion of meat pigments to nitroso-compounds. Proceedings of the XIth Conference of European Meat Research Workers, Belgrade.

Dahn, H. and Loewe, L. 1958. Uber die Reaktion von Ascorbinsäure mit salpetrige Säure. *Chimia (Aarau)* **12**, 362–363.

Dahn, H. and Loewe, L. 1960. Uber die oxydation von Ascorbinsäure durch salpetrige Säure. Teil IV: Die Reaktion nullter Ordnung. *Helv. Chim. Acta* **43**, 310–317.

Evans, H. J. and McAuliffe, C. 1965. Identification of nitric oxide, nitrous oxide, and nitrogen as products of the non-enzymic reduction of nitrite by ascorbate or reduced diphosphopyridine nucleotide. In "Symposium on Inorganic Nitrogen Metabolism," ed. McElroy, W. D. and Glass, H. B. p. 189. Johns Hopkins Press, Baltimore.

Fox, J. B. Jr. and Thomson, J. S. 1963. Formation of bovine nitrosylmyoglobin. I. pH 4.5–6.5, *Biochem.* **2**, 465–470.

Fox, J. B. Jr. and Thomson, J. S. 1964. The formation of green heme pigments from metmyoglobin and methemoglobin by the action of nitrite. *Biochem.* **3**, 1323–1328.

Fox, J. B. Jr., Townsend, W. E., Ackerman, S. A. and Swift, C. E. 1967. Cured color development during frankfurter processing. *Food Technol.* **21**, 386–392.

Gould, E. S. 1959. "Mechanism and Structure in Organic Chemistry." Holt, Rinehart and Winston, New York.

Hamm, R. and Hofmann, K. 1965. Changes in the sulphydryl and disulphide groups in beef muscle proteins during heating. *Nature* **207**, 1269–1271.

Hamm, R. and Hoffman, K. 1966. Schwefelhaltige Verbindungen des Fleisches. III. Mitteilung Bestimmung von Sulfhydryl- und Disulfid-Gruppen in Myofibrillen und Muskelgewebe mit Hilfe der amperometrischen Titration. *Z. Lebensm. Untersuch. und Forsch.* **130**, 133–145.

Kelley, G. G. and Watts, B. M. 1957. Effect of reducing agents on cured meat color. *Food Technol.* **11**, 114–116.

Kendrew, J. C., Dickerson, R. E., Strandberg, B. E., Hart, R. G., Davies, D. R., Phillips, D. C. and Shore, V. C. 1960. Structure of myoglobin. A three-dimensional Fourier synthesis at 2 Å resolution. *Nature* **185**, 422–427.

Pascal, I. and Tarbell, D. S. 1957. The kinetics of the oxidation of a mercaptan to the corresponding disulfide by aqueous hydrogen peroxide. *J. Am. Chem. Soc.* **79**, 6015–6020.

Perutz, M. F., Rossmann, M. G., Cullis, A. F., Muirhead, H., Will, G. and North, A. C. T. 1960. Structure of haemoglobin. A three-dimensional Fourier synthesis at 5.5-Å resolution, obtained by X-ray analysis. *Nature* **185**, 416–422.

Regier, L. W. and Tappel, A. L. 1956. Freeze-dried meat. III. Non-oxidative deterioration of freeze-dried beef. *Food Res.* **21**, 630–639.

Siedler, A. J. and Schweigert, B. S. 1959. Biochemistry of myoglobin. Effects of heat, nitrite level, iron salts, and reducing agents on formation of denatured nitrosomyoglobin. *J. Agr. Food Chem.* **7**, 271–274.

Tarbell, D. S. 1961. The mechanism of oxidation of thiols to disulfides. In "Organic Sulfur Compounds," Vol. I, ed. Kharasch, N., p. 97. Pergamon Press, New York.

ten Cate, L. 1962. Elementare Pökelversuche V. Teil. Einfluss von Temperatur und Keimzahl auf die Reifung der Pökellaken. *Fleischwirtschaft* **14**, 964–969.

Thomson, J. S., Fox, J. B. Jr. and Landmann, W. A. 1962. The effect of water and temperature on the deterioration of freeze-dried beef during storage. *Food Technol.* **16**(9), 131–136.

Walters, C. L. and Taylor, A. McM. 1965. The reduction of nitrite by skeletal-muscle mitochondria. *Biochem. Biophys. Acta* **96**, 522–524.

Watts, B. M., Kendrick, J., Zipser, M. W., Hutchins, B. and Saleh, B. 1966. Enzymatic reducing pathways in meat. *J. Food Sci.* **31**, 855–862.

Weiss, T. J., Green, R. and Watts, B. M. 1953. The effect of metal ions on the formation of nitric oxide hemoglobin. *Food Res.* **18**, 11–16.

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Gel Strength of Kappa-Carrageenan as Affected by Cations

SUMMARY—Kappa carrageenan was ion-exchanged with Al^{+3}, NH_4^{+1}, Ca^{+2}, Fe^{+2}, Fe^{+3}, Mg^{+2}, Na^{+1} and Sn^{+2}. The gelling temperature and gel strength were determined for 1% exchanged kappa carrageenan gels prepared with 0.0375, 0.075 and 0.15 *N* solutions of each of the exchanging cations; 1% exchanged kappa carrageenans with 0.0375, 0.075 and 0.15 *N* KCl, and non-exchanged kappa carrageenan with the same concentrations of $AlCl_3$, NH_4Cl, $CaCl_2$, $FeCl_3$, $FeCl_3$, $MgCl_2$, KCl, NaCl and $SnCl_2$.

Ion-exchange with Al, Fe^{+2}, Fe^{+3} and Sn prevented gel formation. The other ion-exchanged carrageenans had significantly lower gel strength than the non-exchanged carrageenan in a corresponding cation solution. Furthermore, the Mg- and Ca-exchanged carrageenans had greater gel strengths than the NH_4- and Na-carrageenans. Use of KCl with the NH_4-, Ca-, Mg- and Na-exchanged carrageenan resulted in increased gel strength, but the Al, Fe^{+2}, Fe^{+3} and Sn-exchanged carrageenans still did not form a gel. The rank order of the gel strength of the NH_4-, Ca-, Mg- and Na-exchanged carrageenan dispersed in KCl was similar for the exchanged carrageenan in solutions of the exchanging cation, but the gelling temperatures and gel strength of the former approached that of the non-exchanged carrageenan in similar concentrations of KCl.

For the non-exchanged carrageenan at the 0.075 *N* level of salt concentration, the effect of cation was K > Ca, Mg > Fe^{+2}, NH_4 > Na, Sn > Fe^{+3}, Al ($P < 0.001$). Use of increasing concentrations of NH_4Cl, NaCl, KCl and $SnCl_2$ resulted in increased gel strength, whereas increasing concentrations of $CaCl_2$, $FeCl_3$ and $MgCl_2$ decreased gel strength. These differences were reflected in a slightly different rank order for the 0.15 *N* level of salt.

INTRODUCTION

MANY OF THE COMMERCIAL USES of carrageenan are based on the gelation properties of the macromolecule. Glicksman (1962) recently reviewed most of the important commercial applications.

The gelling fraction of carrageenan, designated as kappa, is selectively precipitated from the non-gelling lambda carrageenan with potassium salts (Smith *et al.*, 1953). Kappa carrageenan is composed of 3, 6-anhydro-D-galactopyranose units linked through C_4 to D-galactopyranose-4-sulfate units with a glycosidic linkage at C_3 (O'Neill, 1955; Anderson *et al.*, 1965). Smith *et al.* (1955) state that the galactose 4-sulfate and 3, 6-anhydro-D-galactose occurred in kappa carrageenan in nearly equal amounts; from X-ray diffraction studies, however, Bayley (1955) concluded galactose and anhydrogalactose units occur in a 2:1 ratio. Rees (1963) pointed out the structure of the gelling kappa component differs from the non-gelling lambda in two ways: (1) it contains 3,6-anhydro-D-galactose in place of the 1,4 linked D-galactose-2,6 disulfate units occurring in lambda, and (2) it contains a higher proportion of 4-sulfated-D-galactose units.

Gel formation with kappa carrageenan is a precipitation phenomenon involving ionic bonding between certain metallic cations and the negative charge of the ester sulfate group. Calcium, potassium, ammonium, rubidium, and cesium salts have been reported to gel kappa carrageenan sols (Smith *et al.*, 1953; Stoloff, 1959). They stated that bivalent calcium cation probably forms a cross link between carrageenan molecules, whereas the mechanism of the monovalent cations is regarded as a zipper arrangement between aligned sections of linear polymer sulfates with the ion forming the lock between alternating surface radicals from each section. The four monovalent ions reported to be effective gelling agents have similar ionic diameters; however, sodium and lithium, which do not have the ability to gel kappa carrageenan sols, are thought to have ionic diameters too large to fit into the crystal lattice of the carrageenan molecule.

Gel strength increases with increasing concentrations of potassium ions until the concentration of potassium affects the solubility of the carrageenan (Stoloff, 1959). Potassium chloride is the most commonly used gelling agent for dessert gels (Baker, 1949, 1954; Campbell, 1962; Standard Brands, 1960). An early study by Rice (1946) indicated KCl to be more effective than $CaCl_2$, which was, in turn, more effective than NaCl in precipitating and setting carrageenan sols. Marshall *et al.* (1954) reported K to be a more effective gelling cation than Mg, Na, NH_4, Ca or Li, while Stoloff (1954) observed the rank order for effectiveness of cations for developing gel strength to be K, NH_4, Ca, Mg, Al, and Na. None of these investigators estimated the amount of kappa carrageenan in the sample.

The purpose of the study reported here was to quantify the effect of increasing concentrations of various cations on both ion-exchanged and non-exchanged kappa carrageenan and to determine whether the use of increasing concentrations of potassium chloride in the dispersing media of the ion-exchanged kappa carrageenan will be effective in returning the gel strength to that of the non-exchanged kappa carrageenan.

EXPERIMENTAL

KAPPA CARRAGEENAN, the chemical analysis of which was published previously (Zabik *et al.*, 1965), was submitted to ion-exchange with the cations Al^{+3}, NH_4^{+1}, Ca^{+2}, Fe^{+2}, Fe^{+3}, Mg^{+2}, Na^{+1} and Sn^{+2}, using the procedure outlined by Zabik *et al.* (1967). Moisture analyses of the original sample and the ion-exchanged samples were determined by drying to a constant weight in a vacuum oven at 70–75°C with a vacuum of 29 in Hg. The Plant Analysis Laboratory, Department of Horticulture, per-

formed potash determinations and spectrographic analysis on all samples.

For the first series, 1.0% exchanged kappa-carrageenans (weights corrected for differences in moisture to that of the original sample) were dispersed in 0.0375, 0.075 and 0.15 *N* chloride solutions of each of the exchanging cations. For the second series, 1.0% kappa-carrageenans (original sample) were dispersed in 0.0375, 0.075 and 0.15 *N* solutions of $AlCl_3$, NH_4Cl, $CaCl_2$, $FeCl_2$, $FeCl_3$, $MgCl_2$, KCl, NaCl and $SnCl_2$. For the third series 1.0% exchanged kappa-carrageenans (corrected for moisture differences) were dispersed in 0.0375, 0.075 and 0.15 *N* KCl.

Three hundred g of carrageenan dispersions were prepared by mixing the carrageenan with each 80°C salt solution for 3 min in an Osterizer blender, set on high speed, and connected to a powerstat set at 70. The dispersion was allowed to stand 1 min to allow entrapped air bubbles to surface and this foam was skimmed off. Part of the dispersion was immediately poured into an 8.5 × 9.2 × 6.4 cm stainless steel mold to a depth of 3.5 cm. Subsequently, 10 ml of the dispersion were poured into a 6-in. pyrex test tube. The stainless steel mold was securely covered with Saran and refrigerated at 4–5°C for 20 to 22 hr before gel strength measurements were performed. The pH of the liquid formed by syneresis was determined after the gel strength had been measured.

The gelling temperature was determined for each sample by immersing the test tube containing the dispersion in an ice water bath. The sample was considered gelled when it did not flow on tipping the test tube to a 45° angle. The temperature, to the nearest 0.2°C, was immediately taken.

Gel strength was determined using the upper assembly of the fixed blade standard shear-compression cell of the Allo-Kramer shear press, model SP12, equipped with an electronic recorder, model E-2EZ. A 100-lb proving ring, 25-lb pressure, and 30-sec downstroke were used in this operation. Due to the differences in gel strength obtained, ranges from 2- to 20-lbs were used to obtain appropriate readings on the recorder.

The carrageenan gels were removed from the refrigerator, uncovered, and positioned directly on a supporting plate under the test cell after which the fixed blade assembly was immediately lowered into the gel to a depth of 3.0 cm. The cell was rinsed with cold water before each evaluation. The gel strength was calculated as the maximum graph reading X range. An area-under-the-curve index was calculated using a modification of the method described by Brown *et al.* (1967). To compensate for the use of different ranges, the actual area of the curve was multiplied by the range to determine the area-under-the-curve index.

The data were analyzed for variance. Duncan's Studentized range test was used to establish significant differences among cations (Duncan, 1957). The z statistic, described by Dixon *et al.* (1957), was used to compare the effect of cations between series. Simple correlation coefficients were calculated where appropriate.

RESULTS AND DISCUSSION

TABLE 1 GIVES THE POTASH and spectrographic analyses of the original sample and the ion-exchanged carrageenans. Even though many cations were present, the ion-exchanged samples were appreciably higher in their respective cations. Since the quantities of NH_4 and Sn were not included in the spectrographic analyses, it can only be assumed that the ion-exchange procedures also produced samples with significantly increased amounts of the respective cations.

The pH of the kappa carrageenans either exchanged with or dispersed in NH_4, Ca, K, Mg or Na were approximately neutral (Table 2). Use of Al, Fe^{+2}, Fe^{+3} or Sn to either ion-exchange or disperse the kappa carrageenan resulted in low pH's of 2–3. These latter pH values are sufficiently acidic enough to cause hydrolysis of the carrageenan molecule (Stoloff, 1950).

Gelling temperature

Al, Fe^{+2}, Fe^{+3}- and Sn-exchanged kappa carrageenans dispersed in increasing concentrations of $AlCl_3$, $FeCl_2$, $FeCl_3$, and $SnCl_2$, respectively, remained viscous even at temperatures below 2°C. Gels were not formed for these exchanged carrageenans dispersed in increasing concentrations of KCl. In contrast in the latter series, the Mg-exchanged carrageenan dispersed in 0.15 *N* KCl gelled almost instantly making it impossible to obtain, during preparation, a valid gelling temperature. The non-exchanged kappa carrageenan dispersed in 0.15 *N* KCl also gelled during preparation.

Analysis of variance showed very highly significant differences among gelling temperatures in each of the series. The Studentized range test was used to establish differences among cations for each concentration of dispersing media in each series.

Table 1. Potash and spectrographic analyses of the original sample and ion-exchanged carrageenan.

Cation used in exchange	%				ppm							
	K	P	Ca	Mg	Na	Mn	Fe	Cu	B	Zn	Mo	Al
None	7.40	0.023	0.410	0.260	5120+	28	170	79.4	106.8	222	2.20	117
Al^{+3}	3.50	0.023	0.270	0.070	3648	12	108	35.0	42.8	54	1.58	1659+
NH_4^{+1}	3.50	0.023	0.300	0.160	4144	20	143	65.8	104.6	164	1.80	96
Ca^{+2}	3.50	0.023	1.540	0.140	3168	22	156	60.0	105.5	134	6.40	86
Fe^{+2}	3.50	0.112	0.300	0.110	3840	30	726+	10.2	14.6	69	1.60	84
Fe^{+3}	3.92	0.102	0.315	0.130	4032	42	726+	17.6	11.5	30	1.60	54
Mg^{+2}	3.60	0.023	0.300	1.170	3840	38	132	60.0	97.4	182	1.90	148
Na^{+1}	3.00	0.023	0.270	0.120	5120+	20	137	62.2	102.0	195	1.85	92
Sn^{+2}	3.92	0.010	0.380	0.180	4784	19	37	17.6	11.5	47	2.10	58

Values marked + denote those exceeding range of spectrographic instrument.

Table 2. pH values of kappa carrageenan gels.

Series	*N* of salt solution	Cation used for ion-exchange and/or dispersing media								
		NH_4^{+1}	Ca^{+2}	Mg^{+2}	K^{+1}	Na^{+1}	Al^{+3}	Fe^{+2}	Fe^{+3}	Sn^{+2}
Exchanged kappa	0.0375	6.2	7.0	7.0		7.8	2.0	2.1	2.0	1.8
in exchanging	0.075	6.0	7.1	6.9		7.9	1.9	2.0	1.8	1.7
cation solutions	0.15	5.8	7.3	7.0		7.9	1.9	2.1	1.8	1.5
Non-exchanged	0.0375	6.8	6.9	7.3	7.4	8.0	3.2	4.3	2.2	2.1
kappa in	0.075	6.6	6.9	7.4	7.3	8.0	3.0	3.5	2.1	2.1
cation solutions	0.15	6.4	6.9	7.4	7.3	7.9	3.0	3.0	1.9	2.0
Exchanged kappa	0.0375	6.6	7.5	7.8		7.8	2.1	2.4	2.2	2.2
in KCl	0.075	6.7	7.5	7.7		8.1	2.1	2.4	2.2	2.1
	0.15	7.3	7.9	7.7		8.3	2.0	2.3	2.2	2.1

As shown in Table 3, the Na-exchanged kappa carrageenans dispersed in NaCl had significantly lower gelling temperatures ($P \leqslant 0.01$ for 0.0375 *N* and 0.05 *N*; $P \leqslant 0.001$ for 0.075 *N*) than the NH_4-, Ca- or Mg-exchanged carrageenans gelled with NH_4Cl, $CaCl_2$, or $MgCl_2$, respectively. The Mg-exchanged carrageenan gelled with 0.075 *N* MgCl had a higher gelling temperature than the NH_4-exchanged carrageenan gelled at the highest temperature (significant at 0.1% level), showing a reversal of the previous trend.

Non-exchanged kappa carrageenan gelled with KCl exhibited significantly higher gelling temperatures (0.1% for 0.0375 *N* and 1.0% for 0.075 *N*) than all other kappa carrageenans. Non-exchanged carrageenan in NaCl or $SnCl_2$ gelled at the lowest temperatures (Table 3).

In contrast, few significant differences occurred among the gelling temperatures of exchanged kappa carrageenans in KCl. However, in all but one instance the gelling temperature of the exchanged kappa carrageenan in KCl was very significantly higher at 1.0% level than the respective gelling temperature of the exchanged carrageenan gelled with the exchanging cation (Table 3). Moreover, all the gelling temperatures in this series were similar to those of the non-exchanged kappa carrageenan gelled with corresponding concentrations of KCl. Stoloff (1959) also reported carrageenan gels with both K and Ca present gelled at temperatures similar to gels with only K present.

Increasing the concentration of gelling cation resulted in few significant differences in gelling temperatures (Table 4). The following combinations resulted in significantly higher gelling temperatures: higher concentrations of NH_4Cl with either exchanged or non-exchanged carrageenan; $SnCl_2$ with non-exchanged carrageenan; and $CaCl_2$ with Ca-exchanged carrageenan. In addition, kappa carrageenan gelled with 0.075 *N* KCl formed a gel at a highly significantly higher temperature than with 0.0375 *N* KCl. These latter results agree with those of Stoloff (1959) who reported increasing gelling temperatures with increasing potassium concentration. He postulated that the controlling factor was the ionic strength. Results of the present study also agree with his findings that sodium ions had no effect on gelling temperature and that NH_4 ions behave similarly to K in their effect on gelling temperature.

In the present investigation calcium ion concentration was found to affect the gelling temperature, at least for the exchanged carrageenan. However, the lack of significant effect of calcium ion concentration on the gelling temperature of the non-exchanged kappa carrageenan would support Stoloff's statement that calcium ions do not affect gelling temperatures.

Gel strength

Means and significant differences among the exchanged and non-exchanged kappa carrageenan series for gel strength data based on maximum force and area-under-the-curve index are presented in Table 5. Both measures of gel strength showed similar results. Only carrageenans exchanged with NH_4, Ca, Mg and Na were capable of forming gels; this was true with either the exchanging cation or KCl used in the gelling medium. The Ca- and Mg-exchanged carrageenans exhibited the greatest gelling ability and the Na-exchanged carrageenans were generally the weakest. Calcium and magnesium ions form gels with kappa carrageenan by cross linking the macromolecules. These data show them to be equally effective as gelling cations at 0.0375 and 0.075 *N* levels of concentration; however Mg was more effective than Ca at the 0.15 *N* level.

Both the Ca- and Mg-exchanged carrageenan exhibited significantly lower gel strengths at the 0.15 *N* level of concentration than at the 0.0375 *N* level (Table 4). Thus, it appears that although a certain concentration of Ca or Mg is necessary for gel formation, increasing the concentration of the cation over this amount decreases the gel strength. The probable explanation is that as the concentration of bivalent cation increases, more carrageenan-bivalent cation-chloride bonding occurs and less carrageenan-bivalent cation-carrageenan cross links result, thus weakening the structure.

Table 5 also shows very highly significant differences between the non-exchanged carrageenan in $CaCl_2$ and the corresponding values for the Ca-exchanged carrageenan and significant differences between the non-exchanged carrageenan in $MgCl_2$ and the corresponding values for Mg-exchanged carrageenan (1.0, 5.0, and 1.0% level of probability for 0.0375, 0.075 and 0.15 *N*, respectively). Again, increasing the concentration of cation through ion-exchange reduced the gel strength. The percentage of Ca in the original sample was slightly higher than Mg and the percentage of Ca in the Ca-exchanged carrageenan was higher than the percentage of Mg in the Mg-exchanged carrageenan (Table 1). These sample differences may

Table 3. Means[1] and significant differences among gelling temperatures (°C) for exchanged and non-exchanged kappa carrageenan series.

Series	Salt solution N	Cation used for ion-exchange and/or dispersing media									Significant differences		
		NH_4^{+1}	Ca^{+2}	Mg^{+2}	K^{+1}	Na^{+1}	Al^{+3}	Fe^{+2}	Fe^{+3}	Sn^{+2}	At 0.1%	Additional at 1.0%	Ad at
anged kappa	0.0375	19.2	23.0	22.8		9.3	[3]	[3]	[3]	[3]	Ca Mg NH_4 Na	$NH_4 > Na$	
d with	0.075	23.0	28.7	31.5		11.7	[3]	[3]	[3]	[3]	Mg Ca $NH_4 > Na$	Mg Ca NH_4	Ca >
anging tion	0.15	40.2	32.0	27.0		13.3	[3]	[3]	[3]	[3]	NH_4 Ca Mg Na	$NH_4 > Ca$ Mg $> Na$	
exchanged	0.0375	26.7	25.3	27.0	36.2	12.8	27.3	22.3	21.0	14.0	$K > AlMgNH_4$ Ca $Fe^{+2}Fe^{+3} > SnNa$	None	
a gelled with	0.075	33.3	31.3	28.3	42.3	15.0	27.7	29.5	25.3	20.3	KNH_4Ca $Fe^{+2}MgAlFe^{+3}$ SnNa	$K > NH_4Ca$ Ca $> SnFe^{+3} > Na$	NH_4 >
ted cations	0.15	40.3	32.0	30.8		19.3	30.0	30.0	25.3	24.3	NH_4Ca $MgAlFe^{+2}Fe^{+3}SnNa$	$NH_4 > Ca$ $MgAlFe^{+2} > Na$	$Fe^{+3}S$
		***	***	***		***							
anged kappa	0.0375	31.7	34.7	35.0		29.3	[3]	[3]	[3]	[3]	None	Mg Ca $> Na$	
		***	***	**		***							
d with	0.075	43.5	42.2	41.5		39.7	[3]	[3]	[3]	[3]	None	None	NH_4
		***	***			***							
	0.15	51.0	50.7			51.3	[3]	[3]	[3]	[3]	None	None	

ased on 3 replications.
ems underscored by one consecutive line are not significantly different (Duncan, 1957).
Values for this series differ significantly ($P \leq 0.001$) from values of corresponding ion-exchanged carrageenan gelled with ex-
g cation; ** significant at $P \leq 0.01$.
els did not form.

Table 4. Significant effects of concentrations [1] of gelling cations.

Factor	Cation	Non-exchange kappa carrageenan gelled with various cations			Exchanged kappa carrageenan gelled with exchanging cation			Exchanged kappa carrageenan gelled with KCl		
		0.1%	Additional at 1.0%	Additional at 5.0%	at 0.1%	Additional at 1.0%	Additional at 5.0%	at 0.1%	Additional at 1.0%	Additional at 5.0%
Gelling temperature	NH_4	H M L	None	None	H > M = L	None	None	H > M > L	None	None
	Ca	None	None	None	None	H M L	M > L	H > M > L	None	None
	Mg	None	None	None	None	M H L	None	M > L	None	None
	K	None	M > L	None						
	Na	None	None	None	None	None	None	H > M > L	None	None
	Al	None	None	None						
	Fe^{+2}	None	None	H = M > L						
	Fe^{+3}	None	None	None						
	Sn	None	H M L	None						
Maximum force	NH_4	H > M > L	None	None	H > M = L	None	None	None	None	None
	Ca	L M H	L > M > H	None	L = M > H	L > M	None	M = L > H	None	None
	Mg	None	L M H	None	L M H	None	M > H	None	M > L	None
	K	M > L	None	None						
	Na	H M L	H > M	None	H M L	None	None	M = L > H	None	None
	Al	None	None	None						
	Fe^{+2}	M H L	None	None						
	Fe^{+3}	None	L > M = H	None						
	Sn	H > M = L	None	None						
Area-under-the-curve-index	NH_4	H > M = L	M > L	None	H > M = L	None	None	None	None	None
	Ca	None	L M H	M > H	L M H	None	L > M > H	M = L > H	None	None
	Mg	None	None	None	None	L M H	None	None	M > L	None
	K	M > L	None	None						
	Na	None	H M L	H > M	H M L	None	M > L	M = L > H	None	None
	Al	None	None	None						
	Fe^{+2}	None	None	None						
	Fe^{+3}	None	None	L > M = H						
	Sn	H > M = L	None	None						

[1] H = .15 *N*; M = 0.075 *N*; L = 0.0375 *N*.

account for the slight difference in effect of Ca and Mg found in this study.

The NH_4-exchanged carrageenan was expected, on the basis of work by Smith *et al.* (1953), to have greater gel strength than the Na-exchanged carrageenan. These investigators reported the ammonium ion to have an ionic diameter capable of forming a zipper arrangement between aligned sections of linear polymer sulfates, whereas the sodium ion has an ionic diameter too large to fit into the crystal lattice of the carrageenan molecule. Stoloff (1958) advocated use of a potassium salt of a sequestering agent, preferably potassium metaphosphate, to bind extraneous ions of Na^{+1}, Ca^{+2} and Mg^{+2} present in tap water in order to improve the texture of the carrageenan water gel.

A more complete rank order of cation effect can be obtained from the series of non-exchanged carrageenan in increasing concentrations of various cations (Table 5). Potassium chloride produced the strongest gels. The next most efficient salts were $CaCl_2$ and $MgCl_2$. $FeCl_2$ and NH_4Cl produced gels of medium strength; and NaCl and $SnCl_2$ produced somewhat weaker gels. Very little gelation occurred when $FeCl_3$ or $AlCl_3$ were used. The pH of the gels with $AlCl_3$, $FeCl_3$, $SnCl_2$ and possibly $FeCl_2$ were sufficiently low to have resulted in acid hydrolysis of the carrageenan macromolecule (Table 2).

The order of gel strength for the 0.15 *N* level of concentration differed slightly from that of the lower two levels reflecting differences that increasing concentration of cation had on gel strength. Use of increasing concentrations of NH_4Cl, NaCl, KCl and $SnCl_2$ resulted in increased gel strength, whereas increasing concentrations of $CaCl_2$, $FeCl_3$ and $MgCl_2$ resulted in decreased gel strength. The data obtained in this investigation agreed with the effect of cations reported by Rice (1946) and Marshall *et al.* (1954). The rank order reported by Stoloff (1954) is similar to that obtained at the 0.15 *N* level of salt, except that in the present study NaCl was more effective than $AlCl_3$. At the two lower levels of concentration the $MgCl_2$ and $CaCl_2$ were more effective than NH_4Cl, which is the reverse of the rank order reported by Stoloff.

All non-exchanged carrageenan gels exhibited significantly higher gel strengths than the corresponding exchanged carrageenan gels (Table 5). Since increasing concentrations of Na and NH_4 had previously increased gel strength, the increase in concentration of cation alone does not seem to explain these reduced gel strengths. Thus

Table 5. Means[1] and significant differences among gel strength for exchanged and non-exchanged kappa carrageenan series.

Series	Salt solution N	Cation used for ion-exchange and/or dispersing media									At 0.1%	Additional at 1.0%	Additio at 5.0
		NH_4^{+1}	Ca^{+2}	Mg^{+2}	K^{+1}	Na^{+1}	Al^{+3}	Fe^{+2}	Fe^{+3}	Sn^{-2}			
Exchanged kappa	0.0375	1.40	2.69	2.69		0.88					CaMg > NH_4 > Na	None	No
gelled with	0.075	1.58	2.26	2.51		1.22					MgCa > NH_4Na	NH_4 > Na	Mg > Ca
exchanging cation	0.15	2.13	1.73	2.14		1.51					MgNH$_4$ > CaNa	None	No
Non-exchanged		***	***	**		***							
kappa	0.0375	1.71	3.53	3.07	5.53	1.31	0.53	1.79	0.64	1.15	K > Ca > Mg > $Fe^{+2}NH_4$ > NaSn > Fe^{+3}Al	None	No
		***	***	*		**							
gelled with	0.075	2.19	3.10	2.88	6.67	1.52	0.30	2.19	0.26	1.28	K > CaMg > $Fe^{+2}NH_4$ > NaSn > Fe^{+3}Al	None	No
		**	***	**		*							
elected cations	0.15	2.79	2.68	2.65		1.92	0.38	1.99	0.17	2.42	NH_4CaMgSnFe^{+2}Na > AlFe^{+3}	Sn > Fe^{+3}Na	No
		***	***	***		***							
Exchanged kappa	0.0375	3.72	5.28	4.88		4.21					CaMg NaNH$_4$	Ca > Na	Mg > Na
		***	***	***		***							
gelled with	0.075	4.21	5.65	5.72		4.87					MgCa NaNH$_4$	MgCa > Na	Na ≥ NH_4
			***										
KCl	0.15	2.87	3.25			1.67					CaNH$_4$ > Na	None	No
Exchanged kappa	0.0375	24.9	55.9	56.5		15.5					MgCa > NH_4Na	None	NH_4 > Na
gelled with	0.075	28.5	47.3	52.5		23.5					MgCa > NH_4Na	None	No
exchanging cation	0.15	42.0	37.6	46.2		29.7					MgNH$_4$ Ca Na	NH_4 > Na	Ca > Na
Non-exchanged		*	*	***		***							
kappa	0.0375	31.2	71.4	66.2	126.5	23.4	10.4	37.3	12.3	18.8	K > CaMg > $Fe^{+2}NH_4$NaSn Fe^{+3}Al	NH^4 > Sn Na > Fe^{+3}Al	NH_4 > Na
		***	***	***		*							
gelled with	0.075	42.3	67.5	58.9	153.2	30.0	4.5	45.1	2.8	25.6	K > CaMg > $Fe^{+2}NH_4$ > NaSn > Fe^{+3}Al	None	No
		***	***	***		*							
elected cations	0.15	57.2	59.3	60.3		38.7	7.7	42.4	2.1	41.6	MgCaNH_4 > $Fe^{+2}Sn^{+2}$Na > AlFe^{+3}	None	Mg > NH_4
			***	***		***							
Exchanged kappa	0.0375	80.9	112.0	108.7		94.7					Ca Mg Na NH$_4$	CaMg > NH_4	Ca > Na
		***	***	***		***							
gelled with	0.075	90.5	125.2	134.1		112.2					MgCa Na NH$_4$	Mg > Na > NH_4	No
		*	***			*							
KCl	0.15	66.9	74.2			38.4					None	None	CaNH$_4$ > N

ed on 3 replications.
ns underscored by one consecutive line are not significantly different (Duncan, 1957).
Values for this series differ significantly ($P \leq 0.001$) from corresponding values of ion-exchanged carrageenan gelled with exchanging cation; ** significant at $P \leq 0.01$; *
$P \leq 0.05$.

Table 6. Correlation coefficients of gelling temperature with gel strength.

Series	Exchanged kappa gelled with exchanging cation			Non-exchanged kappa gelled with selected cations			Exchanged kappa gelled with KCl		
	Gel. temp.	Max. force	Area	Gel. temp.	Max. force	Area	Gel. temp.	Max. force	Area
Gel. temp.		.134	.211		.593**	.617**		—.545	—.502
Max. force	.134		.629*	.593**		.796***	—.545		.940***
Area	.211	.629*		.617**	.796***		—.502	.940***	

* Significant at the 5% level of probability.
** Significant at the 1% level of probability.
*** Significant at the 0.1% level of probability.

it is possible that the ion-exchange procedure may have caused slight degradation of the carrageenan macromolecule and contributed to the reduced gel strength values.

Use of 0.0375 *N* and 0.075 *N* KCl to gel the exchanged kappa carrageenans resulted in very highly significant increased gel strengths when compared to corresponding values for the exchanged carrageenan gelled with the exchanging cation (Table 5). These values, in fact, approached the gel strengths of the non-exchanged kappa carrageenan in 0.0375 and 0.075 *N* level of KCl, respectively. These data disagree with the statement of Stoloff (1959) that use of Ca and K salts together tend to reduce the gel strength toward that predicted by use of the calcium ions alone. The rank order for effect of cation for this series was similar to that previously mentioned for the exchanged carrageenan gelled with the exchanging cations so, although use of potassium chloride greatly increased the resulting gel strength, the cations used in the ion-exchange also affected the final strength of the gel.

In this series of exchanged carrageenans gelled with KCl, those gels with 0.15 *N* concentration all exhibited significantly lower gel strength than corresponding carrageenans with lower levels of KCl (Table 4). Because all of these gelled at 51°C (Table 3), the imperfections in the gel produced by partial gelation during pouring may account for the reduced gel strength. Viscousness of the dispersions and difficulty in the removal of entrapped air may have contributed to reduced gel strength.

Significant correlation coefficients were obtained for all series between maximum force and area-under-the-curve indexes (Table 6). However, as shown in Table 6, the relationship between gelling temperature and gel strength varied among the series. Thus, gelling temperature alone was not always an indication of gel strength.

REFERENCES

Anderson, N. S., Dolan, T. C. S. and Rees, D. A. 1965. Evidence for a common structural pattern in the polysaccharide sulfates of the Rhodophyceae. *Nature* **205**, 1060–1062.

Baker, G. L. 1949. Edible gelling composition containing Irish moss extract, locust bean gum, and an edible salt. U. S. Patent 2,466,146.

Baker, G. L. 1954. Gelling compositions. U. S. Patent 2,669,519.

Bayley, S. 1955. X-ray and infrared studies on carrageenan. *Biochim. et Biophys. Acta* **17**, 194–205.

Brown, S. L. and Zabik, M. E. 1967. Effect of heat treatments on the physical and functional properties of liquid and spray-dried egg albumen. *Food Technol.* **21**, 87–92.

Campbell, A. D. 1962. Irish moss food product. U. S. Patent 3,031,308.

Dixon, W. J. and Massey, F. J. Jr. 1957. "Introduction to Statistical Analyses" 2nd ed., pp. 119–124. McGraw-Hill Book Company, Inc., New York.

Duncan, D. 1957. Multiple range test for correlations and heteroscedastic means. *Biometrics* **13**, 164–176.

Glicksman, M. 1962. Natural polysaccharide gums in the food industry. *Advances in Food Research* **11**, 124–138.

Marshall, S. M. and Orr, A. P. 1954. Effect on different ions on gel strength of red seaweed extractives. *Adv. in Chem.* **11**, 101–103.

O'Neill, A. N. 1955. Derivatives of 4-0-β-D-galactopyranosyl-3,6-anhydro-D-galactose from K-carrageenan. *Jour. Am. Chem. Soc.* **77**, 6234–6236.

Rees, D. A. 1963. The carrageenan system of polysaccharides. Part I. The relation between the K- and λ-components. *J. of Chem. Soc.* 1963, 1821–1829.

Rice, F. A. H. 1946. The effect of solvent and temperature on the viscosity of the polysaccharide of Irish moss and the effect of solvent on its initial gelation. *Can. J. Res., Sect. B* **24**, 12–19.

Smith, D. B. and Cook, W. H. 1953. Fractionation of carrageenan. *Arch. Biochem. Biophys.* **45**, 232–233.

Smith, D. B., O'Neill, A. N. and Perlin, A. S. 1955. Studies on heterogeneity of carrageenan. *Can. J. Chem.* **33**, 1352–1360.

Standard Brands, Inc. 1960. Irish moss food product. British Patent 841,973.

Stoloff, L. 1950. Solution of Irish moss extractives by acidulation. *Food Technol.* **4**, 138–141.

Stoloff, L. 1954. Irish moss extractives. *Adv. in Chem.* **11**, 92–100.

Stoloff, L. 1958. Method and compositions for the preparation of carrageenan water gels. U. S. Patent 2,864,706.

Stoloff, L. 1959. Carrageenan. In "Industrial Gums," ed. Whistler, R. L., pp. 83–116. Academic Press, New York.

Zabik, M. E. and Aldrich, P. J. 1965. The effect of selected anions of potassium salts on the gel strength of carrageenan high in the kappa fraction. *J. Food Sci.* **30**, 795–800.

Zabik, M. E. and Aldrich, P. J. 1967. The effect of cations on the viscosity of lambda-carrageenan. *J. Food Sci.* **32**, 91–97.

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The Isolation and Characterization of an α 1,4 (4,5 Dehydrogalacturonosyl) Galacturonate Hydrolase

SUMMARY—An unsaturated oligogalacturonate hydrolase was isolated from the cell extracts of a *Bacillus* sp. This enzyme attacked only the α 1,4 glycosidic bond adjacent to the terminal 4,5-dehydrogalacturonate of unsaturated oligogalacturonides and preferentially attacked short chain unsaturated uronides. The rate of activity was maximal with unsaturated dimer followed by trimer (65% that of dimer), tetramer (47%) and pentamer (35%). The pH optimum was 6.3 to 6.6 and the enzyme did not require calcium ions for its activity. The enzyme was relatively stable below 30°C but lost 90% of its activity after 10 min at 40°C.

INTRODUCTION

IN RECENT YEARS, exocellular pectolytic enzymes of bacterial origin have been investigated intensively by several groups. It has become increasingly evident that transeliminases (lyases) appear to be the major components. However, information is limited on the characteristics of intracellular pectolytic enzymes.

It has been reported that, in addition to transeliminases, cell extracts of several bacteria possess hydrolases (Nagel *et al.*, 1961; Okamoto *et al.*, 1963; Preiss *et al.*, 1963). In a more recent study, we have partially purified the hydrolase from the cell extract of *Bacillus*, isolate B (Hasegawa *et al.*, 1967). This partially purified preparation, which was free of transeliminase activity, appeared to be quite different from known pectic enzymes in that the enzyme preferentially attacked short chain uronides. The preparation attacked both saturated and unsaturated oligouronides liberating monomers from the non-reducing end of the substrates. Although the preparation was active on both types of substrates, activities on the two groups of substrates were significantly different. Therefore, it was quite likely that there were at least two different types of hydrolases present in the preparation. One is an oligogalacturonate hydrolase and the other is an unsaturated oligogalacturonate hydrolase which attacks only the α 1–4 glycosidic bond adjacent to the terminal 4,5-unsaturated galacturonate.

The purification and characterization of the latter type of hydrolase is reported in this paper. The enzyme has been isolated from the cell extract of *Bacillus*, isolate 5, and found to be free of transeliminase and oligogalacturonate hydrolase activities.

MATERIALS AND METHODS

Preparation of substrates

Saturated and unsaturated oligogalacturonides were prepared and characterized by the methods described previously (Hasegawa *et al.*, 1966; Nagel *et al.* 1965).

Growth of cells

The strain of *Bacillus* sp., identified as isolate 5, used in this study was isolated from soil on a pectic acid medium similar to the one used by Priess *et al.* (1963). The culture was grown under the same conditions used for mass cultivation of *Bacillus*, isolate B, (Hasegawa *et al.*, 1966). In this case, however, cells were harvested after 48 hr of incubation at 23 ± 1°C.

Assay methods

Protein concentration was measured by the procedure of Lowry *et al.* (1951). Oligogalacturonides were determined by the carbazole method (McComb *et al.*, 1952). Unsaturated galacturonic acid, which gives no color formation when it is reacted with the carbazole reagent, was detected by the thiobarbituric acid (TBA) method of Weissbach *et al.* (1959). Transeliminase activity was assayed by measuring absorbancy changes at 232 mμ under the conditions described in a previous publication (Hasegawa *et al.*, 1966).

Hydrolase activity was assayed by determining the decrease in absorbancy at 232 mμ with unsaturated oligogalacturonides as the substrates. Activity was measured in 0.6 ml of a reaction mixture consisting of 0.067*M* potassium phosphate buffer, pH 6.5, $5 \times 10^{-3}M$ unsaturated uronide and proper concentrations of the enzyme preparation. Absorption changes at 232 mμ were measured automatically in a Beckman DU spectrophotometer equipped with a Gilford Multiple Sample Absorbance Recording System containing a constant temperature jacket for the cuvette compartment. The reaction was carried out in a standard silica cuvette with a spacer to give a 1 mm light path. The reaction temperature was 25°C. Initial rate determinations were started within 20 to 30 sec after addition of enzyme to the reaction mixture, and the rate was determined from the linear portion of the curve obtained. One unit of hydrolase is that amount of activity that causes the hydrolysis of 0.1 μmole of unsaturated digalacturonic acid per minute under the above conditions. A molar extinction coefficient of 4,600 (Nagel *et al.*, 1965) was used for conversion of the absorbancy values to molar concentrations.

Preparation of enzyme

All operations were carried out in a cold room maintained at 5 ± 1°C. The frozen bacterial cells were suspended in 4 volumes of 0.1*M* potassium phosphate buffer, pH 7.5, containing 0.1% cysteine-HCl and disrupted by sonic vibration for 2 min in a 20kc oscillator. After centrifugation at 12,800 × g for 10 min, the supernatant solu-

tion was used as the starting material for isolation of the enzyme.

The supernatant solution was brought to 60% saturation with ammonium sulfate by the addition of the solid salt with continuous stirring. The resulting precipitate, containing the hydrolase activity, was collected by centrifugation at 12,800 × g for 10 min, dissolved in a minimum portion of water and dialyzed against distilled water for 2 hr. Then, the enzyme solution was applied to a 1.5 × 14.0 cm column of DEAE cellulose, which had been equilibrated with 0.01*M* Tris buffer, pH 7.5. The protein was eluted from the column with 0.1*M* Tris buffer, pH 7.5, containing $5 \times 10^{-4}M$ dithiothreitol and increasing concentrations of sodium chloride by a stepwise method as follows: 75 ml of the buffer followed by 50 ml of the buffer containing 0.1*M* NaCl, 25 ml containing 0.15*M* NaCl, 50 ml containing 0.2*M* NaCl, 50 ml containing 0.4*M* NaCl and 50 ml containing 0.7*M* NaCl. Fractions containing 2.8 ml were collected. The flow rate was approximately 25 ml/hr..

Paper and column chromatography

The action of the hydrolase on various substrates was followed with the use of paper and column chromatography. A 2.4-ml reaction mixture was used containing 12 μmoles of substrate, 0.067*M* potassium phosphate buffer, pH 6.5 and 0.2 unit of hydrolase. After 2, 4, 6 and 10 hr of incubation at 30°C, 0.5 ml of the reaction mixture was withdrawn, treated with Dowex 50 (H^+ form) to inactivate the enzyme, evaporated to dryness and rehydrated in 0.2 ml of water. A 40 μl sample was spotted on Whatman No. 4 paper and chromatographed with an ethyl acetate-pyridine-water-acetic acid (5:5:3:1) solvent. Spots were detected by treatment with periodate followed by benzidine (Gordon *et al.*, 1956).

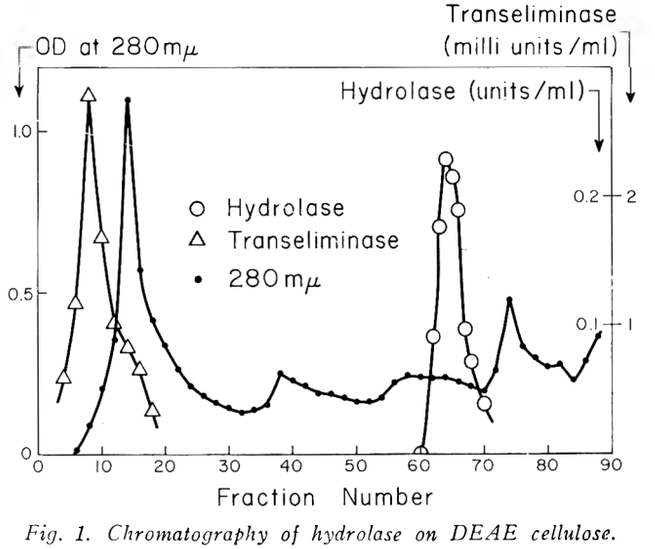

Fig. 1. Chromatography of hydrolase on DEAE cellulose.

Since the paper chromatographic method could not detect unsaturated galacturonic acid, the reaction products were examined also by column chromatography on Dowex-1 × 8 (Nagel *et al.*, 1967a). Identification of the peaks and hydrolytic split of the glycosidic bond by the hydrolase have been determined by using suitable standards (Hasegawa *et al.*, 1966; Nagel *et al.*, 1967a).

Relative rates of attack of unsaturated oligouronides

Reaction mixtures consisted of 0.067*M* potassium phosphate buffer, pH 6.5, $5 \times 10^{-3}M$ or $1 \times 10^{-2}M$ substrate and 0.045 unit of the enzyme in 0.6 ml. The reaction was followed by the standardized procedure.

RESULTS

Purification of enzyme

A typical elution pattern obtained from fractionation of the hydrolase by DEAE-cellulose column chromatography is shown in Fig. 1. The hydrolase was collected in fractions 60–70, whereas pectic acid transeliminase activity was poorly retained on the column and was eluted in fractions 5–20. Fractions 62–68 were combined and used as the purified enzyme. A summary of the purification is presented in Table 1.

Purity of the preparation

The purity of this preparation with respect to freedom from contamination with other pectic enzymes such as pectic acid transeliminase and oligogalacturonate hydrolase was further examined. The cell extract of the bacterium contained approximately 1.5×10^{-2} unit of transeliminase activity per milliliter. On the basis of breakdown of the glycosidic bonds, the extract contained about 170 times more transeliminase activity than that of unsaturated oligogalacturonate hydrolase. Ammonium sulfate fractionation removed 57% of the transeliminase activity and the remainder was completely eliminated by the column separation. In order to confirm the above, the purified preparation was incubated with ASPA and oligogalacturonides and the reaction was followed by measuring changes in absorbancy at 232 mμ. No increase in optical density was observed. The reaction was carried out at pH 9.5 where the unsaturated hydrolase is inactive.

The cell-free extract also contained oligogalacturonate hydrolase activity. When 45 μmoles of di- or tri-galacturonic acid were incubated in a 9 ml mixture containing 33.3% (v/v) of the cell extract and 0.067*M* potassium

Table 1. Purification of the hydrolase.

Purification step	Total volume (ml)	Activity		Protein (mg/ml)	Specific activity (units/mg protein)	Purification	Recovery %
		units/ml	Total				
Cell extract	66.7	0.0846	5.46	0.91	0.093	1	100
0.6 $(NH_4)_2SO_4$ after dialysis	5.0	0.880	4.40	3.35	0.263	2.8	78
Fractions 62–68	19.6	0.176	3.45	0.22	0.800	8.6	61

Table 2. Reaction products obtained from different substrates.

Substrate	Products
Dimer	None
Trimer	None
Tetramer	None
Unsat. dimer	Unsat. monomer, monomer
Unsat. trimer	Unsat. monomer, dimer
Unsat. tetramer	Unsat. monomer, trimer

phosphate buffer, pH 6.5, it was found that approximately 5.85 μmoles of the dimer and 6.75 μmoles of the trimer were hydrolyzed during 20 hr of incubation at 30°C. This activity was approximately one-fifth that of the unsaturated oligogalacturonate hydrolase. All the activity, however, was lost during the preparation procedures. In order to confirm whether the preparation was free from oligogalacturonate hydrolase activity, the preparation was incubated with digalacturonic acid for 20 hr at 30°C and the resulting mixture was assayed for the presence of galacturonic acid by the NADH linked enzymic method (Nagel *et al.*, 1967b). The results showed that no galacturonic acid was produced.

The above evidence coupled with the results of paper and column chromatographic analyses of reaction products obtained from various oligogalacturonides (Table 2 and Fig. 2) confirmed that the purified preparation was free of transeliminase and oligogalacturonate hydrolase activities.

Effects of pH and temperature

Activities of the enzyme at various hydrogen ion concentrations on unsaturated digalacturonic acid showed that the optimum was in the range of pH 6.3–6.6 (Fig. 3).

The heat stability of the enzyme is shown in Fig. 4. The enzyme was stable at temperatures below 30°C, but was almost completely inactivated by heating at 40°C for 10 min. Preliminary studies indicated that this unsaturated oligogalacturonate hydrolase was most stable at pH 7.5.

Characterization of reaction products

The results of paper and column chromatographic analyses of the reaction products are summarized in Table 2. No products were detected from the reaction mixtures of saturated oligogalacturonides. As previously mentioned, this confirms the absence of both oligogalacturonate hydrolase and transeliminase activities in the purified preparation. Unsaturated digalacturonic acid was hydrolyzed to galacturonic acid and unsaturated galacturonic acid. After 10 hr of incubation the reaction was complete. As shown in Table 2 and Fig. 2, unsaturated galacturonic acid and digalacturonic acid were the products obtained from unsaturated trigalacturonic acid. Unsaturated tetragalacturonic acid was hydrolyzed to unsaturated galacturonic acid and trigalacturonic acid. None of the saturated oligouronides produced from unsaturated uronides were further hydrolyzed. The two peaks of unsaturated monomer shown in Fig. 2 have also been observed by Priess *et al.* (1963). If the reaction mixture is held for a period of time prior to chromatography, peak B is not observed.

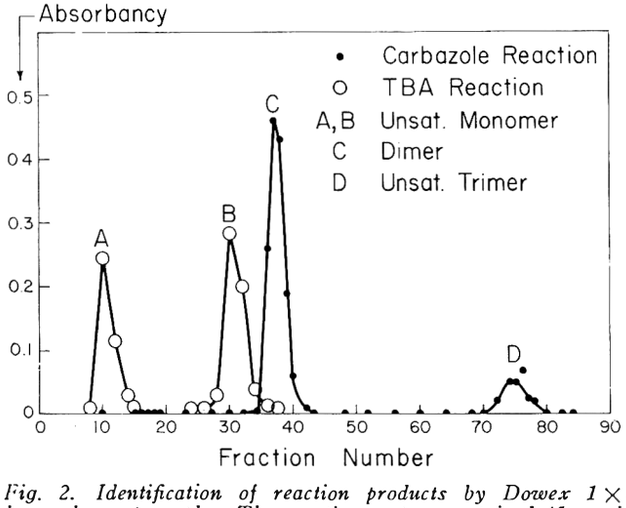

Fig. 2. Identification of reaction products by Dowex 1 × 8 column chromatography. The reaction system contained 45 μmoles of unsaturated trigalacturonic acid, 0.067M potassium phosphate buffer, pH 6.5 and 0.53 unit of the enzyme in a volume of 9.0 ml. After 12 hr of incubation at 30°C, the resulting mixture was chromatographed by procedures described by Nagel et al. *(1967a).*

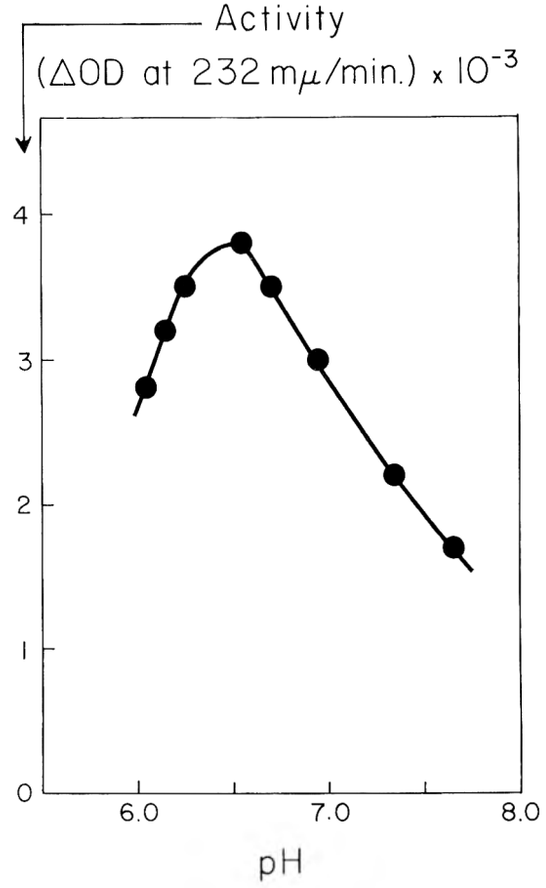

Fig. 3. Effect of pH on hydrolase activity. The reaction mixture consisted of 5 × 10^{-3}M unsaturated digalacturonic acid, 0.067M potassium phosphate buffer and 0.045 unit of the hydrolase per 0.6 ml.

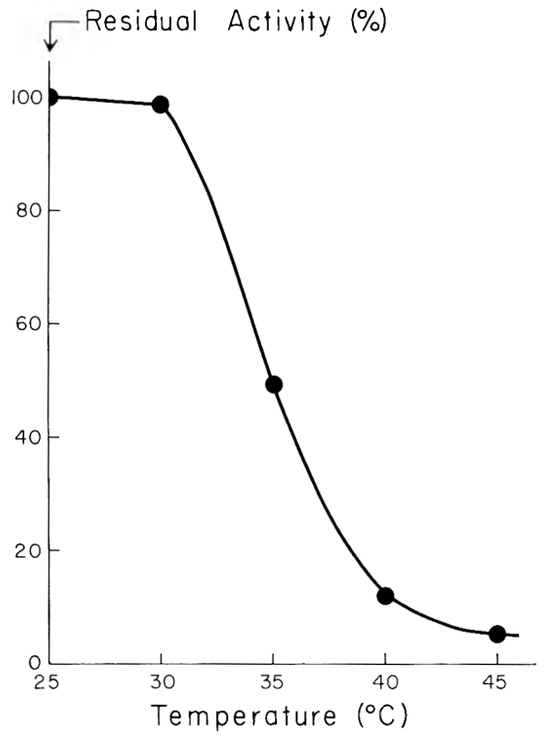

Fig. 4. Heat stability of hydrolase. Enzyme solution in 0.5M potassium phosphate buffer, pH 7.5, was treated at various temperatures for 10 min. The residual activity was assayed with unsaturated digalacturonic acid by the standard method.

Relative rates of attack of unsaturated oligouronides

Results of paper chromatographic studies suggested that unsaturated digalacturonic acid was attacked at the fastest rate followed by trimer and tetramer, in that order. The relative rates of attack were further studied by measuring absorbancy changes at 232 mμ. As summarized in Table 3, the relative rates of hydrolysis of unsaturated oligouronides were inversely proportional to the chain length. The activity was maximal with unsaturated dimer, followed by unsaturated trimer (65% that of dimer), unsaturated tetramer (47%) and unsaturated pentamer (35%).

Effect of EDTA

Reaction was carried out in 0.6 ml of the mixture containing $5 \times 10^{-3}M$ unsaturated digalacturonic acid, 0.067*M* potassium phosphate, pH 6.5 and 0.045 unit of the enzyme in the presence of $1 \times 10^{-4}M$ or $1 \times 10^{-3}M$ EDTA. The results show that EDTA had no effect on enzyme activity (Table 4). It is apparent that, unlike transeliminases, the enzyme requires no divalent cations for activity.

Table 3. Relative rates of attack on the unsaturated substrates.

Substrates	Activity ΔOD 232 mμ/min	Relative rate %
Unsaturated dimer	3.4×10^{-3}	100
Unsaturated trimer	2.2×10^{-3}	65
Unsaturated tetramer	1.6×10^{-3}	47
Unsaturated pentramer	1.2×10^{-3}	35

Table 4. Effect of EDTA on hydrolase activity.

Treatments	Activity ΔOD 232 mμ/min
Control	3.3×10^{-3}
10^{-4}M EDTA	3.4×10^{-3}
10^{-3}M EDTA	3.3×10^{-3}

DISCUSSION

THE HYDROLASE PURIFIED from the cell extract of *Bacillus,* isolate 5, was proved to be free of transeliminase and oligogalacturonate hydrolase activities. This hydrolase attacked specifically the α 1,4 glycosidic linkage adjacent to the terminal unsaturated galacturonate of unsaturated uronides but was inactive toward saturated uronides. So far as we know, this is the first time that the unsaturated oligogalacturonate hydrolase has been isolated.

The results show also that the enzyme preferentially attacked short chain uronides. This behavior is in contrast to other polygalacturonases (Demain *et al.,* 1954; Luh *et al.,* 1956; McCready *et al.,* 1954) and transeliminases (lyases) (Nagel *et al.,* 1965; Hasegawa *et al.,* 1966), which degrade oligogalacturonic acids at rapidly declining rates as the chain length of the substrate decreases. In the case of an exo-transeliminase, the enzyme attacks uronides of different chain length at the same rate (Macmillan *et al.,* 1964b). The same type of hydrolase activity, however, has been shown with horse serum maltases, which hydrolyze maltose most rapidly and higher oligoglucosides, starch and glycogen at slower rates (Lieberman *et al.,* 1957; Rutter *et al.,* 1961).

The range of optimum pH, 6.3 to 6.6, differs also from other pectolytic enzymes. It is slightly higher than polygalacturonases of fungi (Luh *et al.,* 1953; Saito, 1955) and plants (McColloch *et al.,* 1948) but much lower than bacterial transeliminases (Hasegawa *et al.,* 1966; Macmillan *et al.,* 1964a; Nagel *et al.,* 1965).

From the limited survey of pectic enzymes of bacterial origin, it is now apparent that there are at least three basically different types of bacterial pectic enzymes, that is, transeliminases including both exo- and endo-types, oligogalacturonate hydrolases and unsaturated oligogalacturonate hydrolases. Isolation and characterization of the saturated oligogalacturonate hydrolase of *Bacillus, i*solate B, is described elsewhere (Hasegawa *et al.,* 1968).

REFERENCES

Demain, A. L. and Phaff, H. J. 1954. Hydrolysis of the oligogalacturonides and pectic acid by yeast polygalacturonase. *J. Biol. Chem.* **210**, 381.

Gordon, H. T., Thornburg, W. and Werum, L. N. 1956. Rapid paper chromatography of carbohydrates and related compounds. *Anal. Chem.* **28**, 849.

Hasegawa, S. and Nagel, C. W. 1966. A new pectic acid transeliminase produced exocellularly by a *Bacillus. J. Food Sci.* **31**, 838.

Hasegawa, S. and Nagel, C. W. 1967. Separation of a hydrolase from a pectic acid transeliminase in cell extracts of a *Bacillus. Nature* **213**, 207.

Hasegawa, S. and Nagel, C. W. 1968. Isolation of an oligogalacturonate hydrolase from a *Bacillus* species. *Arch. Biochem. Biophys.* **124**, 513.

Lieberman, I. and Eto, W. H. 1957. Purification and properties of equine serum maltase. *J. Biol. Chem.* **225**, 899.

Lowry, C. H., Rosebrough, N. J., Farr, A. L. and Randal, R. J. 1951. Protein measurement with the folin phenol reagent. *J. Biol. Chem.* **193**, 265.

Luh, B. S., Leonard, S. J. and Phaff, H. J. 1956. Hydrolysis of pectic materials and oligouronides by tomato polygalacturonase. *Food Res.* **21**, 448.

Luh, B. S. and Phaff, H. J. 1953. Properties of yeast polygalacturonase. *Arch. Biochem. Biophys.* **48**, 23.

Macmillan, J. D. and Vaughn, R. H. 1964a. Purification and properties of a polygalacturonic acid-trans-eliminase produced by *Clostridium multifermentans*. *Biochem.* **3**, 564.

Macmillan, J. D., Phaff, H. J. and Vaughn, R. H. 1964b. The pattern of action of an exopolygalacturonic acid-trans-eliminase from *Clostridium multifermentans*. *Biochem.* **3**, 572.

McColloch, R. J. and Kertesz, Z. I. 1948. An unusually heat-resistant pectolytic factor from tomato. *Arch. Biochem.* **17**, 197.

McComb, E. A. and McCready, R. M. 1952. Colorimetric determination of pectic substances. *Anal. Chem.* **24**, 1630.

McCready, R. M. and Seegmiller, C. G. 1954. Action of pectic enzymes on oligogalacturonic acids and some of their derivatives. *Arch. Biochem. Biophys.* **50**, 440.

Nagel, C. W. and Vaughn, R. H. 1961. The degradation of oligogalacturonides by the polygalacturonase of *Bacillus polymyxa*. *Arch. Biochem. Biophys.* **94**, 328.

Nagel, C. W. and Anderson, M. M. 1965. Action of a bacterial transeliminase on normal and unsaturated oligogalacturonic acids. *Arch. Biochem. Biophys.* **112**, 322.

Nagel, C. W. and Hasegawa, S. 1967a. Kinetics and site of attack of oligogalacturonic acids by an endo-pectic acid transeliminase. *Arch. Biochem. Biophys.* **118**, 590.

Nagel, C. W. and Hasegawa, S. 1967b. The enzymic determination of galacturonic acid. *Anal. Biochem.* **21**, 411.

Okamoto, K., Hatanaka, C. and Ozawa, J. 1963. On the saccharifying pectolytic enzymes of *Erwina aroideae*. *Agr. Biol. Chem.* **27**, 596.

Priess, J. and Ashwell, G. 1963. Polygalacturonic acid metabolism in bacteria. I. Enzymic formation of 4-deoxy-L-5-hexoseulose uronic acid. *J. Biol. Chem.* **238**, 1571.

Rutter, W. J. and Brosemer, R. W. 1961. Glucose production by isolated rat liver cells. An amylose-oligoglucosidase patnway for glycogen breakdown. *J. Biol. Chem.* **236**, 1247.

Saito, H. 1955. Pectic glycosidases of *Aspergillus niger*. *J. Gen. Appl. Microbiol.* **1**, 38.

Weissbach, A. and Hurwitz, J. 1959. The formation of 2-keto-3-deoxyheptonic acid in extracts of *Escherichia coli* B. *J. Biol. Chem.* **234**, 709.

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Sarcoplasmic and Myofibrillar Protein in Skeletal Muscle of Two Breeds of Pig

SUMMARY—The sarcoplasmic and myofibrillar proteins were extracted from two muscles of Landrace and Large White pigs using solutions of low and high ionic strengths. The breed of pig did not affect the extractability of the proteins. It was concluded that the genetic background did not influence the inherent extractability of the muscle proteins before differences in rate of post-mortem glycolysis (considered to exist between the types of pig studied) could induce changes in protein solubility.

INTRODUCTION

WHEN PIG SKELETAL MUSCLE goes into rigor mortis under acidic conditions soon after death, i.e., when pH values at or below 6.0 are reached before the temperature of the muscle has fallen below 35°C, a marked reduction occurs in the solubility (1) of the sarcoplasmic proteins at low ionic strength, and (2) of the myofibrillar proteins at high ionic strength (McLoughlin, 1963; Sayre *et al.*, 1963; Briskey *et al.*, 1964).

Such changes in the solubility of the proteins have been associated with pale, soft, exudative post-rigor muscle (Hart, 1962; Sayre *et al.*, 1963; Sayre *et al.*, 1964; Borchert *et al.*, 1964 and 1965; Sayre *et al.*, 1966).

While information is available on changes which occur in the solubility of muscle proteins during rigor, there is relatively little information about the absolute amounts of sarcoplasmic and myofibrillar protein in pre-rigor pig muscle. Similarly, there is very little evidence to indicate whether there are genetically-determined differences in the protein extractability of pre-rigor muscle between different breeds of pig. Sayre *et al.* (1963), Borchert *et al.* (1965) and Sayre *et al.* (1966) published figures for the amounts of sarcoplasmic and myofibrillar protein in pig longissimus dorsi muscle excised at death; Sayre *et al.* (1966) compared the amounts of these proteins in the longissimus dorsi muscle of adult Poland China and Chester White pigs.

The object of the work reported here was to determine whether there are inherent, i.e., genetically determined, differences in the amounts of sarcoplasmic and myofibrillar protein which can be extracted from pre-rigor muscle of Large White and Landrace pigs. There is evidence (Lawrie, 1960; Bendall *et al.*, 1963) which suggests that there are differences in the rate of glycolysis post-mortem between these breeds of pig.

EXPERIMENTAL

Animals

Twenty-two pure-bred pedigree Landrace and Large White pigs from two herds kept together on the same farm under the same conditions were slaughtered in the live-weight range 160 to 180 lb. The pigs were transported individually to the laboratory the day before slaughter.

Slaughter

The pigs were stunned by shooting through the forebrain with a captive-bolt pistol and were then exsanguinated.

Extraction of proteins

Specimens of the longissimus dorsi muscle at the level of the last rib, and the gastrocnemius muscle, were removed immediately after exsanguination. All subsequent operations were carried out at 2°C. Superficial fat and connective tissue were quickly removed, the muscle was chopped into pieces and duplicate samples (15 g) were placed in cold extracting solutions and homogenized. The extraction procedure was based on that described by Helander (1957).

Sarcoplasmic protein was extracted (3 extractions, each 1 hr) by stirring with 10 volumes of cold potassium phosphate buffer (0.03 *M*; pH 7.4); myofibrillar protein was extracted (4 extractions, each 2 hr) from the residue with 10 volumes of cold potassium phosphate buffer (0.01 *M*; pH 7.4) containing potassium iodide (1.1 *M*). Soluble nonprotein nitrogen was obtained following precipitation of the proteins in the sarcoplasmic extract using an equal volume of ice-cold trichloroacetic acid (20% w/v).

Chemical analysis

The total nitrogen contents of the muscle tissue and of the protein extracts were determined by macroKjeldahl analysis. Intramuscular fat was determined by soxhlet extraction of the dried tissue with petroleum ether for 6 hr.

RESULTS AND DISCUSSION

THE RESULTS FOR THE longissimus dorsi muscle are shown in Table 1. There were no significant differences between Landrace and Large White pigs either in the amounts of extractable sarcoplasmic and myofibrillar protein or in the soluble non-protein nitrogen and total nitrogen contents of the muscle. The small standard errors about the mean values in Table 1 indicate that muscle composition was markedly constant between individual animals.

Combining the figures for all pigs, the sarcoplasmic proteins constituted 30.4 ± 0.3%, the myofibrillar proteins 51.5 ± 0.5% of the total nitrogen of the muscle on a fat-free basis. The percentage of the total protein extracted was the same for Landrace and Large White pigs (92.9% and 93.2% respectively).

The relatively small standard errors in Table 1 suggest that the salt extraction technique used can give quite reproducible results. The extractions were carried out on duplicate samples of muscle; the precision of a single-sample extraction was calculated from an analysis of variance. The error for single-sample extraction of the

Table 1. Composition of porcine longissimus dorsi muscle.

Breed of pig	Mean values[1] for: Sarcoplasm	Myofibrils	Soluble non-protein nitrogen	Total nitrogen
Landrace (11)[2]	11.2 ± 0.1	18.7 ± 0.2	4.3 ± 0.1	36.4 ± 0.3
Large White (11)[2]	11.0 ± 0.1	18.9 ± 0.2	4.6 ± 0.1	36.7 ± 0.2

[1] Expressed as mg nitrogen/1 g wet tissue on a fat-free basis.
[2] Number of animals.

Table 2. Composition of porcine gastrocnemius muscle.

Breed of pig	Mean values[1] for: Sarcoplasm	Myofibrils	Soluble non-protein nitrogen	Total nitrogen
Landrace (5)[2]	10.5 ± 0.2	18.5 ± 0.3	4.0 ± 0.1	34.8 ± 0.3
Large White (5)[2]	10.5 ± 0.1	18.5 ± 0.2	4.0 ± 0.2	35.6 ± 0.5

[1] Expressed as mg nitrogen/1 g of wet tissue on a fat-free basis.
[2] Number of animals.

sarcoplasmic proteins was ± 0.3 mg/1 g tissue for both types of pig; the errors for the myofibrillar proteins were ± 0.6 mg/1 g and 0.5 mg/1 g for Landrace and Large White pigs respectively.

The results for the gastrocnemius muscle are shown in Table 2. Breed of pig did not influence the quantities of sarcoplasmic and myofibrillar protein extracted from this muscle.

Sayre *et al.* (1963) gave figures for the longissimus dorsi muscle of Poland China pigs. The muscle samples were taken immediately after death. The amounts of sarcoplasmic protein varied between approximately 30% and 34%, the myofibrillar protein between about 42% and 48% of the protein nitrogen (these figures are derived from the histograms which the authors employed to present their results).

In this study, the average values (22 pigs) for sarcoplasmic and myofibrillar protein were, respectively, 34.6% and 58.6% of the protein nitrogen (as distinct from percent of the total nitrogen, the form in which these figures have already been expressed above). The value for extractable myofibrillar protein is appreciably higher than that reported by Sayre *et al.* (1963). Borchert *et al.* (1965) obtained low values for the extractable myofibrillar protein (33% of the protein nitrogen) of longissimus dorsi muscle excised at 15 min post-mortem: the value for the sarcoplasmic protein (also 33% of the protein nitrogen) was comparable to that found for the Landrace and Large White pigs.

This observation emphasizes that considerable variation can occur in the extractability of the muscle proteins. The breed of pig used by Borchert *et al.* was not specified but, since it was described as a genetic strain previously shown to have a high incidence of pale, soft, exudative muscle, it was presumably Poland China.

Sayre *et al.* (1966) found no difference in the amounts of sarcoplasmic and myofibrillar protein between Poland China and Chester White pigs of similar live-weight (about 125 kg, i.e., heavier than the pigs used in this study). This comparison was apparently made because earlier results (Sayre *et al.*, 1963) had suggested that the former type of pig was much more likely to develop pale, soft, exudative muscle than the latter. The amount of sarcoplasmic protein in the Poland China and Chester White pigs (about 30% of the total nitrogen) was similar to that found here for the Landrace and Large White (30.4% of the total nitrogen); the amount of extractable myofibrillar protein was appreciably lower.

The average value for myofibrillar protein reported by Sayre *et al.* (1966) was about 40% ± 8% of the total nitrogen (figure deduced from a histogram), while the corresponding average for Landrace and Large White was 51.5% ± 0.5%. The Landrace and Large White pigs used here were lighter than the pigs used by Sayre *et al.* (1966), but this is hardly likely to account for the difference in the quantities of myofibrillar protein because these authors showed that both the sarcoplasmic and myofibrillar protein content of Poland China longissimus dorsi muscle increased with increasing live-weight. The values for myofibrillar protein in the longissimus dorsi and in the gastrocnemius muscles of the Irish pigs were, however, close to that reported by Dickerson *et al.* (1960) for thigh muscles of adult English pigs (19.9 mg N/1 g tissue).

The myofibrillar content of longissimus dorsi muscle of the Landrace and Large White pigs was remarkably constant, as indicated by the relatively small standard error (± 0.5%) about the average value. The larger standard errors shown in histogram form by Sayre *et al.* (1963), Borchert *et al.* (1965) and Sayre *et al.* (1966) suggest that the amount of myofibrillar protein extracted varied considerably between individual Poland China and Chester White pigs.

The quantity of sarcoplasmic protein extracted from pre-rigor longissimus dorsi muscle of Landrace and Large White pigs was similar to that extracted from Poland China and Chester White muscle by Sayre *et al.* (1963), Borchert *et al.* (1965) and Sayre *et al.* (1966). More myofibrillar protein, however, was extracted from Landrace and Large White muscle than from Poland China or Chester White.

The extraction techniques employed in these studies were similar in principle but the preparation of muscle tissue for extraction differed. In this work, the fresh tissue was placed in cold extracting solution immediately after excision, whereas the authors quoted above froze their materials before extraction. The freezing in liquid nitrogen and the subsequent storage and treatment of the frozen tissues described by these authors seem unlikely to alter the

properties of the myofibrillar proteins. On the other hand, if thaw-rigor occurred, with consequent strong contraction of the myofilamental proteins, it is possible that the solubility of the myofibrils in solutions of high ionic strength might be reduced.

Aberle *et al.* (1966) observed that the solubility of beef myofibrillar protein in a potassium iodide (1.1 *M*) and phosphate buffer (0.1 *M*; pH 7.4) mixture decreased initially after death, i.e., as the actin and myosin filaments slid together to give the weak, sustained contraction which is rigor.

Earlier workers (Deuticke, 1932; Weber *et al.*, 1933) also observed a decrease in protein solubility as muscle went into rigor. Whole carcasses or large sections of meat can be frozen and thawed without thaw contracture occurring because the rigid structure of the material prevents shortening of the muscle (Marsh *et al.*, 1958). This is probably why Borchert *et al.* (1965) did not observe (as they themselves pointed out) any change in protein extractability in the exposed ends of longissimus dorsi muscle which underwent thaw-rigor compared to central parts which only experienced accelerated cooling, when the lumbar sections of pig carcasses were immersed for short periods in liquid nitrogen. Small sections of isolated, frozen pre-rigor muscle would readily contract and shorten if any thawing occurred. However, it must be pointed out that Borchert *et al.* (1965) and Sayre *et al.* (1966) employed procedures specifically designed to prevent this happening.

In conclusion, the amounts of sarcoplasmic and myofibrillar protein which were extracted from pre-rigor skeletal muscle of Landrace and Large White pigs does not appear to be related to the genetic background of the animals. The amount of sarcoplasmic protein which was extracted from the longissimus dorsi muscle of Landrace and Large White pigs was similar to that obtained by other workers from Poland China and Chester White muscle; the amount of myofibrillar protein extracted from the former breeds of pig was appreciably higher than from the latter.

REFERENCES

Aberle, E. D. and Merkel, R. A. 1966. Solubility and electrophoretic behavior of some proteins of post-mortem aged bovine muscle. *J. Food Sci.* **31**, 151.

Bendall, J. R., Hallund, O. and Wismer-Pedersen, J. 1963. Post-mortem changes in muscles of Landrace pigs. *J. Food Sci.* **28**, 1.

Borchert, L. L. and Briskey, E. J. 1964. Prevention of pale, soft, exudative porcine muscle through partial freezing with liquid nitrogen post-mortem. *J. Food Sci.* **29**, 203.

Borchert, L. L. and Briskey, E. J. 1965. Protein solubility and associated properties of porcine muscle as influenced by partial freezing with liquid nitrogen. *J. Food Sci.* **30**, 138.

Briskey, E. J. and Sayre, R. N. 1964. Muscle protein extractability as influenced by conditions of post-mortem glycolysis. *Proc. Soc. Exptl. Biol. Med.* **115**, 823.

Deuticke, H. J. 1932. Kolloidzustandsänderungen der Muskelproteine bei der muskeltätigkeit. *Z. Physiol. Chem.* **210**, 97.

Dickerson, J. W. T. and Widdowson, E. M. 1960. Chemical changes in skeletal muscle during development. *Biochem. J.* **74**, 247.

Hart, P. C. 1962. Physico-chemical characteristics of degenerated meat in pigs. II. *Tijdschr. Diergeneesk.* **87**, 3.

Helander, E. 1957. On quantitative muscle protein determination. *Acta Physiol. Scand.* **41**, Suppl. 141.

Lawrie, R. A. 1960. Post-mortem glycolysis in normal and exudative longissimus dorsi muscles of the pig in relation to so-called white muscle disease. *J. Comp. Path. Therap.* **70**, 273.

Marsh, B. B. and Thompson, T. F. 1958. Thaw rigor and the delta state of muscle. *Biochem. Biophys. Acta* **24**, 427.

McLoughlin, J. V. 1963. Studies on pig muscle II. The effect of rapid post-mortem pH fall on the extraction of the sarcoplasmic and myofibrillar proteins of post-rigor muscle. *Irish J. Agr. Res.* **2**, 115.

Sayre, R. N. and Briskey, E. J. 1963. Protein solubility as influenced by physiological conditions in the muscle. *J. Food Sci.* **28**, 675.

Sayre, R. N., Kiernat, B. and Briskey, E. J. 1964. Processing characteristics of porcine muscle related to pH and temperature during rigor mortis development and to gross morphology 24 hr post-mortem. *J. Food Sci.* **29**, 175.

Sayre, R. N., Para, J. and Briskey, E. J. 1966. Protein alterations and associated changes in porcine muscle as influenced by maturity, genetic background, and post-mortem muscle temperature. *J. Food Sci.* **31**, 819.

Weber, H. H. and Meyer, K. 1963. Das kolloidale Verhalten der muskeleiweisskörper. *Biochem. Z.* **266**, 137.

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Major Volatile Components of the Juice of American Cranberry

SUMMARY—Volatiles from the juice of the American cranberry (*Vaccinium macrocarpon* Ait.) were investigated using gas chromatography, mass spectrometry and infrared spectrophotometry. Forty-two compounds comprising over 95% of the aroma complex were identified. These consist of 14 aromatic compounds, 7 terpenes, 9 aliphatic alcohols, 6 aliphatic aldehydes and 6 other compounds including the 2 acids, benzoic and 2-methylbutyric. The aromatic compounds (benzaldehyde, benzyl and benzoate esters) and the terpenes appear to be the major contributors to the aroma of cranberry juice. The remaining 5% of the aroma complex contains over 200 components. Although these compounds occur in very small concentrations, they appear to be important in the overall aroma.

INTRODUCTION

AT THE TIME THE STUDY WAS INITIATED, a review of the literature revealed no previous investigations of the volatiles of the cranberry. The cranberry acids, benzoic, quinic, citric and malic have been characterized (Fellers *et al.*, 1955). Markley *et al.* (1934), in an analysis of cranberry wax, found glycerides of linolenic, linoleic and oleic acid in addition to the hydrocarbons nonocosane and hentriacontane. Virtanen *et al.* (1955) found a number of keto and hydroxyketo acids in the cranberry. Wu *et al.* (1956) isolated ursolic acid from this fruit, and Vahatalo *et al.* (1957) found homoserine in cranberries. The above acids could be precursors of aroma components in the cranberry.

Most recently, Anjou *et al.* (1967a) have analyzed the volatiles of the lingonberry (*Vaccinium vitis-idaea* L.) and the American cranberry (*Vaccinium macrocarpon* Ait.). In the former study, 74 compounds comprising 85% of the concentrate of volatiles were identified; in the latter study (*V. macrocarpon* Ait.), 88 compounds comprising 82% of the concentrate were identified. The concentrates in both studies were obtained from the berry residue (press cake) remaining after the juice had been expressed.

The purpose of the present study was to identify the volatiles in the juice of the American cranberry.

EXPERIMENTAL

Reagents

Untreated cranberry juice was acquired from the Ocean Spray Co. at Hanson, Mass. This juice was obtained by thawing frozen berries (Howes and Early Blacks) then cold-pressing with a Willmuth Press at 84 p.s.i.

Reference compounds were obtained from commercial sources and purified by gas chromatography where necessary.

Ethyl ether (analytical grade) was further purified by treating with 5% aqueous ferrous sulfate, followed by phase separation and redistillation.

Procedures

Concentration of volatiles. To minimize solvent impurity contribution, a continuous liquid-liquid extractor, similar to that described by Heinz *et al.* (1966) was utilized.

Approximately 38 l of salt-saturated cranberry juice was passed through the extractor and extracted with 1.5 l of ethyl ether. The ether phase was periodically collected from the extractor and further concentrated in a large fractional distillation apparatus consisting of a 75 cm × 3 cm ID column packed with glass helices and fitted with a vacuum-jacketed automatic vapor-splitting head, maintained at a 24:1 reflux ratio. Further ether stripping was carried out in a semi-micro fractional distillation apparatus consisting of a 30 cm × 1 cm ID column packed with glass helices and operated at an 80:1 reflux ratio.

When the volume was reduced to 10 ml, the acidic compounds were removed by repeated washing with sodium carbonate solution. The ether extract was dried over sodium sulfate, and further concentrated to a volume of 0.3 ml in a cooled centrifuge tube under nitrogen. The concentrate was then refrigerated at −15°C until used. This 0.3 ml concentrate represented 8 ppm of the original juice. Total volatiles (with acids) comprised 11 ppm of the juice.

To determine acidic compounds, the sodium carbonate extract was neutralized with dilute sulfuric acid and extracted with ethyl ether. Concentration was carried out in a manner similar to that employed for the acid-free extract. This concentrate was then refrigerated until used.

Gas chromatographic analysis. A Varian-Aerograph Model 1200 gas chromatograph with flame ionization detector was utilized for separation and retention time analysis. The following columns and parameters were employed:

(a) Column: 0.02 in. OD × 200 ft stainless steel capillary, coated with diethylene glycol succinate polyester (designated DEGS).

Injection block temperature: 235°C.

Helium flow rate: 20 cc/min.

Temperature program: 10 min at 40°C, then 4°C/min to 170°C.

(b) Column: 0.2 in. OD × 200 ft stainless steel capillary, coated with Carbowax 20M (designated CW).

Injection block temperature: 235°C.

Helium flow rate: 20 cc/min.

Temperature program: 6 min at 40°C, then 8°C/min to 200°C.

(c) Column: 0.01 in. OD × 150 ft stainless-steel capillary, coated with polypropylene glycol (designated PPG).

Injection block temperature: 235°C.

Helium flow rate: 5 cc/min.

Temperature program: 3 min at 40°C, then 10°C/min to 100°C, then 6°C/min to 150°C.

Used in trapping fractions for infrared analysis was a Perkin-Elmer 800 gas chromatograph with flame ionization detector. The parameters employed with this system were as follows:

Column: ⅛-in. OD × 6-ft stainless steel. 8% diethylene glycol succinate polyester with 2% phosphoric acid on 80- to 100-mesh Chromasorb W.

Injection block temperature: 240°C.

Helium flow rate: 40 cc/min.

Temperature program: 10 min at 40°C, then 4°C/min to 170°C.

Detector-exit port split ration: 1:4.

Infrared analysis. Infrared spectra were recorded on a Perkin-Elmer 337 infrared spectrometer. Subfractions were collected from the Perkin-Elmer 800 by the method of Edwards *et al.*, (1965) and analyzed in chloroform using a "D" type sodium chloride cavity cell with 0.1-mm path (Connecticut Instrument Corp., Wilton, Conn.), or sodium chloride disk. Confirmation of unknowns was then made by comparison of spectra to those of authentic standards.

Mass spectral analysis. The effluent from the Varian Aerograph gas chromatograph was connected in tandem to a Hitachi Perkin-Elmer RMU-6A mass spectrometer. Fifty percent of the column effluent was directed to the gas chromatograph flame ionization detector, and 50% was led via a 0.01 in. OD × 3 ft. stainless-steel heated line to a Biemann-type separator (Watson *et al.*, 1964), thence to the mass spectrometer. This system employed both the flame ionization detector and total ion current monitor. The following parameters were employed with the mass spectrometer:

Ion source pressure: 5×10^{-6} mm Hg
Connecting line temperature: 180°C
Biemann-separator temperature: 180°C
Ion source temperature: 225°C
Electron energy: 70 eV
Ionization current: 60 ua
Scan: M/e 12–M/e 400 in 5 sec

Unknown compounds were identified by comparison of their spectra to published standards.

RESULTS AND DISCUSSION

A SEPARATION OF CRANBERRY JUICE VOLATILES on the capillary PPG column is shown on a chromatogram in Fig. 1. The peak marked S is due mostly to solvent. Other more volatile compounds were, undoubtedly, masked by this peak, but no attempts were made to further examine these compounds.

Identication was carried out by mass spectrometry for all compounds successfully identified. When the concentration of the component permitted, infrared spectra were recorded. The data obtained were checked for retention-time agreement on the various columns. The compounds identified are presented in Table 1, in which the peak numbers refer to those in Fig. 1. Those compounds deriving, at least in part, from the solvent are annotated (s). A (+) symbol in the retention time column indicates retention time agreement with an authentic sample on the designated column. A (+) symbol in the mass spectra column indicates mass spectral agreement with published spectra. A (+) symbol in the infrared spectra column indicates confirmation by comparison of spectrum to that of an authentic sample. Approximate percentages of compounds present are also indicated. Acidic compounds identified in the sodium carbonate extract are included at the end of the table.

Forty-two compounds comprising 95% of the volatile concentrate have been identified.

Table 1 shows that aromatic (benzenoid) compounds dominate the volatile aroma complex. Although such aro-

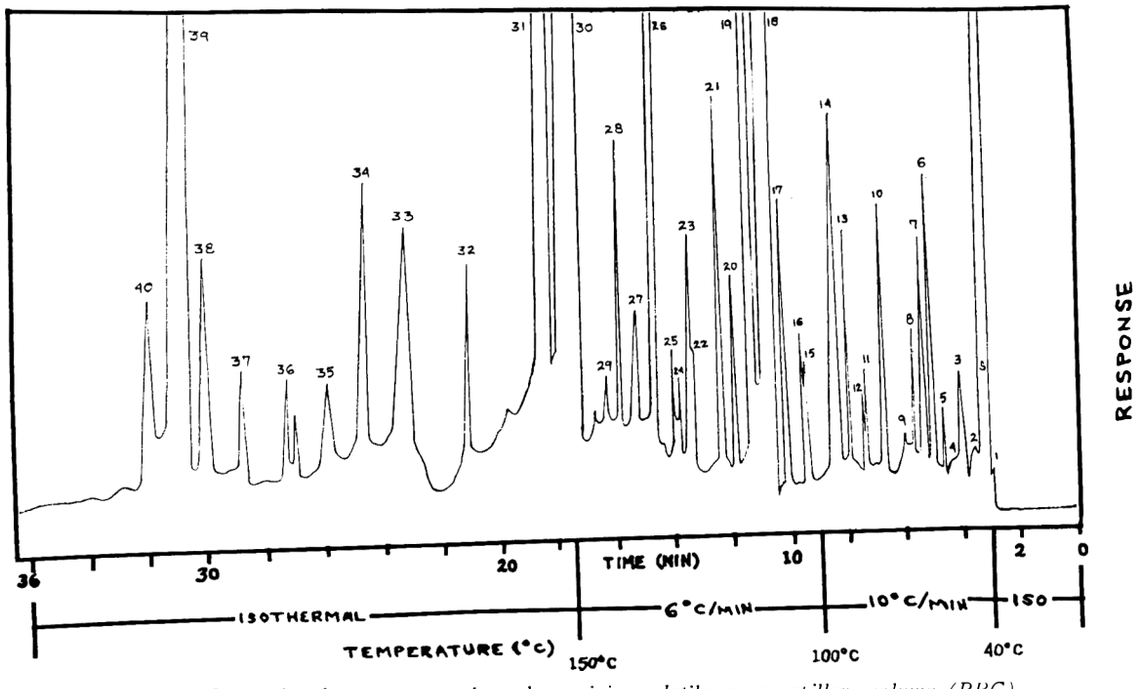

Fig. 1. Composite chromatogram of cranberry juice volatiles on a capillary column (PPG).

matic compounds have occasionally been found in fruit—peaches (Sevenants *et al.*, 1966; Jennings *et al.*, 1964), black currants (Andersson *et al.*, 1964, 1966), apples (Flath *et al.*, 1967), and strawberries (McFadden *et al.*, 1965)—such a large number of volatile aromatic compounds as found in the cranberry is unusual. As benzoic acid and benzyl alcohol are significant components of the volatile complex, it is not surprising to find many esters of these compounds present.

Despite the abundance of benzyl alcohol and benzoic acid, they do not appear to be important contributors to the overall aroma. Benzyl alcohol is reported to have a

Table 1. Volatile compounds identified in cranberry juice.

		Gas chromatography					
Compound	Peak no.	DEGS	CW	PPG	M.S.	I.R.	Percentage of concentrate
Aromatic							
Benzene(s)	4	+	+		+		.1
Benzaldehyde	18	+	+	+	+	+	9.6
Benzyl ethyl ether	19	+	+		+		1.0
Acetophenone	22	+	+	+	+		.8
Methyl benzoate	23	+	+		+		1.0
Benzyl formate	24	+		+	+		.7
Ethyl benzoate	28	+			+	+	1.0
Benzyl acetate	29	+	+		+		.7
Benzyl alcohol	31	+		+	+	+	6.0
2-Phenyl ethanol	33		+	+	+		2.2
4-Methoxy benzaldehyde	36	+	+		+		.8
2-Hydroxy diphenyl	38	+		+	+		1.2
Benzyl benzoate	39	+		+	+	+	11.9
Dibutyl phthalate(s)	40	+	+	+	+		1.1
Terpenes							
Alpha-pinene	9		+	+	+		.1
Beta-pinene	11		+	+	+		.2
Myrcene	12	+	+		+		.2
Limonene	14	+	+		+	+	1.1
Linalool	25		+	+	+		.6
Alpha-terpineol	30			+	+	+	13.0
Nerol	34			+	+		1.1
Aliphatic alcohols							
2-Methyl-3-buten-2-ol	6		+	+	+		.9
2-Pentanol	7			+	+		.8
Pentanol	10	+	+		+		.9
Hexanol	15	+	+		+		.7
1-Octen-3-ol	20			+	+		.8
Octanol	26		+	+	+		2.3
Nonanol	32		+	+	+		.8
Decanol	35		+	+	+		.7
Octadecanol	37	+		+	+		.8
Aliphatic aldehydes							
Acetaldehyde(s)	1	+	+		+		.1
Pentanal	5		+	+	+		.2
Hexanal	8		+	+	+		.8
Octanol	17		+	+	+		.9
Nonanal	21		+	+	+		1.0
Decanal	27		+	+	+		.8
Other compounds							
Diacetyl	2			+	+		.3
Ethyl acetate	3		+	+	+		.7
2-Furaldehyde	13		+		+		.8
Methyl hepanoate	16	+	+		+		.6
Acids							
Benzoic acid	....	+	+		+	+	26.6
2-Methylbutyric acid	....	+	+		+		.3
						Total	95.2

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low specific odor intensity. Benzoic acid, although unimportant in the aroma of cranberries, probably contributes to the tart flavor. Many of the other aromatic compounds present, such as benzaldehyde, methoxy benzaldehyde, the benzoate and benzyl esters, have characteristic odors and higher odor intensities, and probably contribute significantly to the total aroma. Benzene and dibutylphthalate could be found in blank runs through the system with purified ether.

Terpenes and their derivatives occur very widely in fruits. Because of their characteristic odors, these compounds, particularly the alcohols, may be important in the flavor of cranberries. Alpha-terpineol, one of the four major components of the volatile extract, was found to dehydrate to limonene at high injection block temperatures (above 250°C). Combination of alpha-terpineol dehydration and isomerization of the hydrocarbon formed can give rise to additional amounts of limonene, myrcene, alpha-pinene and beta-pinene, and for this reason gas chromatograph injection block temperatures below 250°C were employed. The thermal decomposition of terpene hydrocarbons and alcohols has also been noted by von Sydow *et al.* (1963) and von Sydow (1963).

The aliphatic alcohols are also common constituents of fruit. The saturated alcohols are probably less important in cranberry aroma than the unsaturated compounds (2-methyl-3-buten-2-ol and 1-octen-3-ol) which have higher specific odor intensities.

The six aliphatic aldehydes identified probably also contribute to the aroma of cranberry juice. The possible formation of these compounds from fatty acids in the cranberry has been discussed by Anjou *et al.* (1967a). Acetaldehyde was found in the solvent, yet its location here does not necessarily exclude its natural occurrence, as it very commonly occurs in fruits and berries, and the amount found in the extract appears more than could be accounted for from solvent contribution.

The remaining compounds, diacetyl, ethyl acetate, methyl heptanoate and 2-furaldehyde, have all been reported in one or more of the many fruits previously investigated. Some of these, particularly 2-furaldehyde, contribute to some extent to the aroma.

In the acid concentrate, benzoic acid and 2-methyl butyric acid were found; the concentration of benzoic being over 100 times greater than that of 2-methylbutyric. 2-methylbutyric acid has been implicated as a primary component in the aroma of lingonberries (Anjou *et al.*, 1967a). In contrast to the lingonberry, the aroma concentrate of the American cranberry contained little 2-methylbutyric acid, which is in agreement with Anjou *et al.* (1967b). There was no apparent aroma difference between a total extract and an acid-free extract, discounting 2-methylbutyric acid as a primary aroma component of the American cranberry.

Anjou *et al.* (1967b) in their work on the "press cake" of the American cranberry (*V. macrocarpon* Ait.) Early Black variety, have identified most of the above mentioned compounds in addition to many others. Identified in the juice of the American cranberry, but apparently not present in the "press cake" are beta-pinene, methyl heptanoate, benzyl ethyl ether, benzyl acetate, octadecanol, and 4-methoxybenzaldehyde. The relative abundances of compounds differed also in the two studies. These quantitative and qualitative differences can best be explained in terms of the portion of the berry extracted (juice vs. presscake), the different extraction and concentration techniques employed, the varietal difference in berries studied and the possibility of seasonal or locational variation.

An attempt was made to duplicate the aroma complex with proportional amounts of commercial reagents. Although this synthetic mixture was somewhat reminiscent of cranberry juice, it lacked the delicate aroma of the natural extract and was easily distinguishable from it. This might indicate the importance to the aroma of the many compounds occurring in smaller quantity that remained unidentified. Over 250 components were visible by gas chromatography, of which only the largest 42 peaks, comprising 95% of the extract, were identified.

REFERENCES

Andersson, J. and von Sydow, E. 1964. The aroma of black currants. I. *Acta Chem. Scand.* **18**, 1105–1114.

Andersson, J. and von Sydow, E. 1966. The aroma of black currants. II. *Acta Chem. Scand.* **20**, 522–529.

Anjou, K. and von Sydow, E. 1967a. The aroma of cranberries. I. *Vaccinium vitis idaea*. L. *Acta Chem. Scand.* **21**, 945–952.

Anjou, K. and von Sydow, E. 1967b. The aroma of cranberries. II. *Vaccinium macrocarpon Ait. Acta Chem. Scand.* In press.

Edwards, R. A. and Fagerson, I. S. 1965. Collection of gas chromatographic fractions for infrared analysis. *Anal. Chem.* **37**, 1630–1631.

Fellers, C. R. and Esselen, W. B. 1955. Cranberries and cranberry products. Agr. Exp. Sta. Univ. of Massachusetts, Bull. 481, 13–20.

Flath, R. A., Black, D. R., Guadagni, D. G., McFadden, W. H. and Schultz, T. H. 1967. Identification and organoleptic evaluation of compounds in Delicious apple essence. *J. Agr. Food Chem.* **15**, 29–36.

Heinz, D. E., Sevenants, M. R. and Jennings, W. G. 1966. Preparation of fruit essences for gas chromatography. *J. Food Sci.* **31**, 63–69.

Jennings, W. G. and Sevenants, M. R. 1964. Volatile components of peach. I. *J. Food Sci.* **29**, 796–801.

Markley, K. S. and Sando, E. C. 1934. Petroleum ether and ether soluble constituents of cranberry pomace. *J. Biol. Chem.* **105**, 643–653.

McFadden, W. H., Teranishi, R., Corse, J., Black, E. R. and Mon, T. R. 1965. Volatiles from strawberries. II. Combined gas chromatography and mass spectrometry on complex mixtures. *J. Chrom.* **18**, 10–19.

Sevenants, M. R. and Jennings, W. G. 1966. Volatile components of peach. II. *J. Food Sci.* **31**, 81–85.

Vahatalo, M. L. and Virtanen, A. I. 1957. Homoserine in fruits of cranberry and cranberry. *Acta Chem. Scand.* **11**, 747–748.

Virtanen, A. I. and Alftan, M. 1955. New alpha-keto arids in green plants. *Acta Chem. Scand.* **9**, 188–190.

von Sydow, E. and Ryhage, R. 1963. Mass spectrometry of terpenes. I. Monoterpene hydrocarbons. *Acta Chem. Scand.* **17**, 2025–2035.

von Sydow, E. 1963. Mass spectrometry of terpenes. II. Monoterpene alcohols. *Acta Chem. Scand.* **17**, 2504–2512.

Watson, J. T. and Biemann, K. 1964. High-resolution mass spectra of compounds emerging from a gas chromatograph. *Anal. Chem.* **36**, 1135–1137.

Wu, B. Y. and Parks, L. M. 1956. Ursolic acid from cranberries. *J. Am. Pharm. Assoc.* **42**, 602–606.

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Quantitative Determination of Carrageenan in Milk and Milk Products Using Papain and Cetyl Pyridinium Chloride

SUMMARY—Proteins in milk, chocolate milk, evaporated milk, and ice cream containing added carrageenan were digested with papain at 70°C in the presence of 1.0 *M* NaCl. The digest was adjusted to pH 8.0 to 8.5 with NaOH. Celite was added and the mixture filtered over glass wool. Carrageenan in the filtrate was precipitated with cetyl pyridinium chloride (C.P.C.) in the presence of 0.5 to 1.0 *M* KCl and Celite. The carrageenan-C.P. precipitate was washed with 0.1% C.P.C.-0.05 *M* KCl until the filtrate was negative to the Benedict's test. Then, it was dissolved in 30% H_2SO_4 and the carbohydrate content determined by the phenol-H_2SO_4 method.

At concentrations of 0.01 to 0.2% carrageenan, average recoveries of 92 to 102% were obtained from milk. For chocolate milk, evaporated milk and ice cream, and at a level of 0.1% carrageenan, recoveries of 90, 94 and 96%, respectively, were obtained. Optimum conditions for the isolation of the carrageenan-cetyl pyridinium complex were established.

INTRODUCTION

SEVERAL METHODS ARE AVAILABLE for the isolation, identification and quantitative determination of carrageenan (Ewart *et al.*, 1952; Hansen *et al.*, 1960; Graham *et al.*, 1962; Stoloff *et al.*, 1964; Graham, 1966). However, because of the strong interaction of the hydrocolloid with proteins, the determination of this sulfated polyanionic phytocolloid in foodstuffs containing a high percentage of protein has been tedious.

Proteolytic enzymes such as papain, trypsin, pepsin or pancreatin have been used in the preliminary digestion of proteins in mixtures from which sulfated mucopolysaccharides structurally related to carrageenan were subsequently isolated (Scott, 1960; Korn, 1959a, 1959b; Schiller *et al.*, 1961). In the isolation step, quaternary ammonium detergents have been employed because they can selectively precipitate the sulfated polymers in the presence of salt solutions of fairly high molarity. The insolubility of the carrageenan cetyl pyridinium complex has been mentioned (Scott, 1956, 1960; Slack, 1958).

This paper reports efforts to determine carrageenan in some high protein food products using papain to digest the protein and subsequently determining the carrageenan in the digest after its precipitation with cetyl pyridinium chloride in the presence of relatively high salt concentrations. Various factors which influence the proposed method were investigated and data are given on determination of carrageenan in the presence of other food gums.

EXPERIMENTAL

Materials

A 1% stock dispersion of carrageenan was dialyzed as outlined previously (Graham, 1960).

Buffer: 1.0 *M* sodium acetate-acetic acid, pH 6.5.

Sulfuric acid: (Fisher Scientific Co.) 95 to 98%, sp. gr. 1.84.

Celite 535: (Johns-Manville Co., New York, N. Y.) This was washed with distilled water, 1% sodium acetate, sodium chloride (¼ saturated) and finally deionized water until the washings gave a negative phenol-H_2SO_4 test.

Filtration column: The column, 18 mm in diameter and 40 cm high, was made from borosilicate glass and was fitted with a stopcock. It was packed tightly with glass wool to a height of 10 cm. Filtration was done at the rate of 10 to 15 ml per min. The glass wool was washed consecutively with concentrated H_2SO_4, and deionized water until the washings gave a negative phenol-H_2SO_4 test. After this, 25 ml of 0.1% C.P.C.–0.05 *M* KCl was poured over it, with the stopcock closed. The column was soaked in this mixture for at least 30 min and was kept wet with this solution until ready for use.

Papain: This was obtained from the Mann research laboratories as a powdered concentrate assaying 72 milk-clotting units per gram by the method of Balls *et al.* (1937). Any other highly purified concentrate may be used.

Papain-EDTA-cysteine hydrochloride mixture. Papain (20.0 g), cysteine hydrochloride (1.0 g), and EDTA (1.0 g), were dispersed in 250 ml of warm (70°C) acetate buffer (pH 6.5).

Equipment

Beckman DU-2 spectrophotometer or Bausch and Lomb Spectronic 20 spectrophotometer; pH meter, Beckman Zeromatic.

All glassware was washed thoroughly in water, soaked in dichromate-H_2SO_4 cleaning solution, washed and finally rinsed with distilled water.

Procedure

Digestion of the protein with papain. Five g of milk or milk product was placed in a 250-ml centrifuge bottle and 1.25 to 10.0 mg of carrageenan, dispersed in distilled water, was added. Enough solid sodium chloride to provide a 1.0 *M* solution was added and the total volume made up to 100 ml with distilled water. The mixture was shaken and heated for 10 min in a water bath (98 ± 1°C), cooled to 70°C and 5.0 ml of 1.0 *M* sodium acetate-acetic acid buffer, pH 6.5 was added. The mixture was incubated

at 70°C for 30 min and then 25 ml of the papain-EDTA-cysteine hydrochloride mixture was added. The centrifuge bottle was stoppered with a hard No. 6 rubber stopper, shaken well and the mixture incubated at 70°C for 16 hr. At the end of this period, 2.0 ml of 1.0 *N* NaOH (or enough to adjust the pH of the mixture to 8.5), and 1.0 g of Celite 535 were added. The mixture was shaken well and filtered over glass wool. The residue was washed 3 times with 10-ml portions of distilled water, the washings collected and pooled with the filtrate in a 250-ml centrifuge bottle. The residue was discarded.

Precipitation of the carrageenan. To the filtrate containing the carrageenan, enough solid KCl was added to give a final concentration of 1.0 *M*, then 10 ml of a 1% solution of C.P.C. in water was added drop-wise, with constant shaking. The mixture was shaken well and incubated for 30 min at 45°C. One g of Celite 535 was added, the mixture was shaken and incubated for another 10 min. At the end of this period, the mixture was centrifuged for 10 min at 2,500 r.p.m. and the supernatant filtered over glass wool. The residue containing the carrageenan-C.P. precipitate was retained.

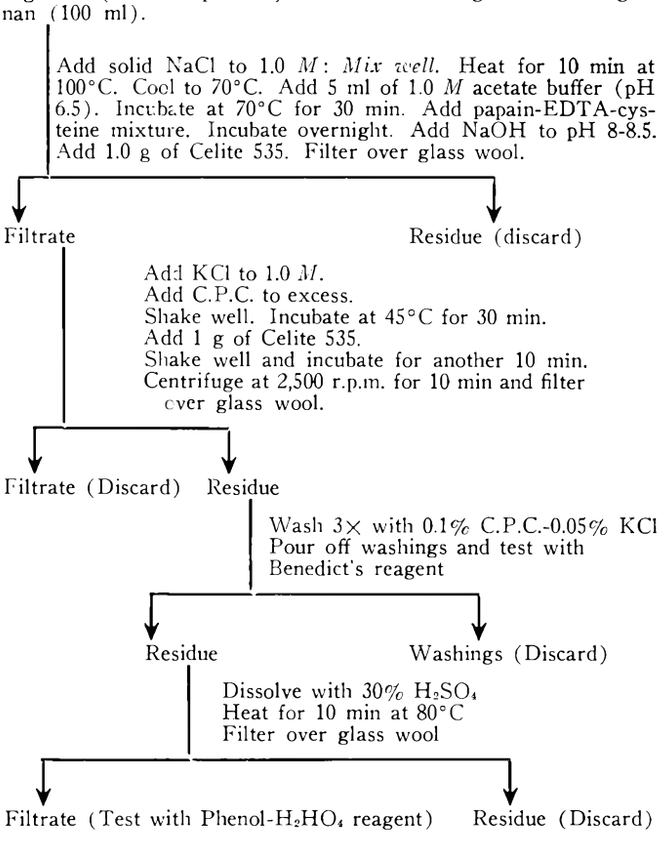
Removal of sugars from the carrageenan-C.P. precipitate. The residue described above contains the carrageenan-C.P. complex adsorbed onto the Celite. Admixed with this are sugars and probably other water-soluble carbohydrates contained in the product. In order to free the precipitate of these interferences, the residue was washed three times with 15-ml portions of 0.1% C.P.C.—0.05% KCl and centrifuged at 2,500 r.p.m. The supernatants were poured over the column of glass wool. The filtrate from the column was tested with the Benedict's reagent and, if necessary, the washing was continued until a negative test was obtained. The washings were discarded.

Solution of the carrageenan-C.P. precipitate and determination of the carbohydrate content. To the sugar-free residue, 20 ml of 30% H_2SO_4 was added, the precipitate stirred with a glass rod and the mixture held for 10 min in a water bath at 80°C. The mixture was centrifuged for 10 min at 2,500 r.p.m., the supernatant poured over the column of glass wool and the filtrate collected in a 50-ml glass-stoppered volumetric flask. The residue was washed twice more with 12-ml portions of hot (80°C) 30% H_2SO_4, centrifuged, filtered, and the washings also collected. The filtrate and washings was made up to 50 ml with 30% H_2SO_4, mixed well, and a 2-ml portion used for determination of the carbohydrate content by the phenol-H_2SO_4 method. The entire process is summarized in Table 1.

Phenol-H_2SO_4 test. The procedure used, outlined by Dubois *et al.* (1956), is as follows:

Two ml of the carrageenan suspension dispersed in 30% H_2SO_4 and containing 50 to 400 μg of the phytocolloid was placed in a series of 30-ml borosilicate test tubes. One ml of a solution of 5% phenol in water was added and the tubes were shaken to mix the contents well. Five ml of concentrated H_2SO_4 were added rapidly from a burette, allowing the acid to fall directly into the center of the tube in order to obtain good mixing. The tubes were shaken and left at room temperature (28 to 30°C) for 10 min. At the end of this period, the tubes were held in a water bath for 10 min at 28 ± 2°C. The color developed was measured at 490 μ against a reagent blank. This constituted the standard curve.

Table 1. Procedure for separation and determination of carrageenan. 5 g milk (or other product) in 250-ml centrifuge bottle carrageenan (100 ml).



For the determination of carrageenan recovered from milk or other products, 2.0 ml of the filtrate (appropriately diluted, if necessary) were used and the procedure for color development was the same. The blank was treated in the same way as the samples.

Variables affecting the method

Several factors may influence the isolation of carrageenan from any one of the products. These include the time of incubation with the papain, the amount of papain (of known activity) used, the pH of the system, the temperature of incubation, the time of incubation during digestion of the proteins, the completeness of washing of the residue, the pH of the buffered system during digestion of the proteins, the protein content of the sample, the presence of salt in the digestion mixture, the presence of salt during precipitation and the heating of the sample prior to the addition of the papain.

In order to assess the influence of these variables, a constant amount of milk (5.0 g) containing a constant amount of carrageenan (5.0 mg) was subjected to the general procedure while varying the above-mentioned factors one at a time (Table 2).

The optimum conditions for the variables were established by determining the amount of carrageenan recovered. For the first seven variables, the completeness of digestion

Table 2. Influence of variables on the determination of carrageenan in milk

Variable investigated	Range investigated	Condition for reproducibility		Value or condition selected
		min.	max.	
Final concentration of NaCl added (M)	0.0 to 1.5	0.5	1.0	1.0
Time of pre-heating at 99 ± 1°C (min)	0.0 to 30	5.0	10.0	10.0
Final concentration of buffer during incubation (*M*)	0.0 to 0.5	0.1	0.2	0.2
pH of buffered system during digestion	4.0 to 7.0	6.0	6.5	6.5
Temperature of incubation (°C)	40 to 70	65	70	70.0
Time of incubation at 70°C (hr)	1.0 to 36	4.0	16	16.0
Amount of papain added (g)	0.0 to 5.0	1.5	2.0	2.0
Final adjusted pH of digest	6.5 to 10.0	8.0	8.5	8.5
Amount of Celite added before filtering off carrageenan (g)	0.0 to 2.0	0.5	1.0	1.0
Final concentration of KCl added (*M*)	0.0 to 2.0	0.5	1.0	1.0
Amount of C.P.C. added (mg)	1.0 to 100	25	100	100
Amount of Celite added to adsorb precipitate (g)	0.0 to 2.5	0.75	1.0	1.0
Temperature of incubation (°C)	28 to 60	37	45	37.0
Number of washings of precipitate	0.0 to 6.0	Till negative to Benedict's test		Till negative to Benedict's test

was also determined according to the method outlined by Moore *et al.* (1954) and employed by Hill *et al.* (1952).

Recovery of carrageenan

After optimum conditions were established, the recovery of carrageenan from milk, various milk products and from milk to which other gums were added was attempted. The protein content of the various products was determined by the micro-Kjeldahl method as modified by Rao *et al.* (1960). The conversion factor of 6.38 was used to convert nitrogen to protein. Recovery from milk (3.2% protein) was tested by adding varying levels of carrageenan (0 to 10.0 mg) to 5.0 g of milk (Table 3).

Table 3. Recovery of various amounts of carrageenan from milk.

Milk added	Carrageenan added	Carrageenan recovered		Recovery (Average)
		Range	Average [1]	
g	mg	mg	mg	%
5.0	0.00	0.0	0.0	0.0
5.0	10.00	9.6 –10.5	10.2	102.0
5.0	5.00	4.6 – 5.3	4.9	98.0
5.0	1.50	2.2 – 2.6	2.4	96.0
5.0	1.25	1.10– 1.24	1.15	92.0

[1] Average of five different trials.

Recovery from various milk products was tested by adding 5.0 mg of carrageenan to 5.0 g of each product (Table 4).

Recovery of carrageenan from milk in the presence of other hydrocolloids was tested by adding 5.0 mg of carrageenan to 5.0 g of milk (protein content, 3.38%) in the presence of 5.0 mg of each of the hydrocolloids listed in Table 5.

RESULTS AND DISCUSSION

PRELIMINARY HEATING OF THE MIXTURE of milk and carrageenan denatures the milk proteins, thus rendering them more susceptible to digestion by the papain (Scott, 1960). There might be considerable interaction between hydrocolloids like carragenan and papain, but, in the presence of relatively high concentrations of sodium chloride this does not occur (Scott, 1960).

Experiments in which the amount of added sodium chloride was varied within the limits indicated in Table 2, demonstrated that much better digestion was attained in the presence of at least 0.5 *M* of the salt. The minimum time necessary for digestion of the milk proteins, under the conditions used, was 4 hr. However, under laboratory practices, it is quite convenient to incubate the mixture

Table 4. Recovery of carrageenan from various products.

Product	Amount of product added	Protein content	Carrageenan recovered		Recovery (Average)
			Range	Average	
	g	%	mg	mg	%
Milk (commercial, homogenized, and pasteurized)	5.0	3.38	4.6–5.3	4.9	98.0
Chocolate milk (3% chocolate added to commercial milk)	5.0	3.44	4.2–4.6	4.5	90.0
Evaporated milk (commercial brand)	5.0	7.33	4.5–5.1	4.7	94.0
Ice cream (commercial, vanilla flavor)	5.0	3.88	4.5–5.2	4.8	96.0

[1] Average of five different trials.

Table 5. Recovery of carrageenan from milk in the presence of other gums.

Milk added	Other gum added	Carrageenan recovered		Recovery (Average)
		Range	Average[1]	
g	mg	mg	mg	%
5.0	Quince seed (5)[2]	4.4–4.9	4.7	94
5.0	Sodium carboxymethyl cellulose (5)	4.3–4.8	4.6	92
5.0	Starch (soluble) (5)	3.5–4.2	3.9	78
5.0	Pectin (5)	4.4–4.9	4.6	92
5.0	Locust bean gum (5)	4.5–4.8	4.6	92
5.0	Sodium alginate (5)	4.4–4.9	4.6	92

[1] Average of three different trials. 5 mg of carrageenan added in each case.
[2] Numbers in parentheses indicate amount in milligrams of other gum added.

overnight (12 to 16 hr). Therefore, an incubation time of 16 hr was used throughout the investigation.

The addition of sodium hydroxide after the incubation aids in the dissociation of any carrageenan-protein complexes present in the mixture. Although the digestion of proteins by papain can occur over a wide pH range, a level of 6.5 was chosen since under this condition and at 70°C the probability of the hydrolysis of carrageenan was greatly minimized. Protein hydrolysis was more rapid at 70°C than at 40°C. It is known that with most substrates, papain exhibits its optimum activity at 60 to 70°C. Buffer strengths between the levels of 0.1 and 0.2 *M* had no measurable influence on the degree of digestion of the milk proteins and, under the experimental conditions and for the papain sample used, less than 1.5 g led to reduced protein hydrolysis.

Celite added at this stage serves to adsorb proteins, protein degradation products and pigments and thus remove them from the bulk of the liquid containing the water-dispersible carrageenan.

Addition of 0.5 to 1.0 g of Celite prior to filtering off the carrageenan in suspension produced a clear filtrate. Use of less than 0.5 g resulted in a slightly colored filtrate due, probably, to the presence of protein degradation products and other materials in the digest. This was particularly true with chocolate milk. However, addition of more than 1.0 g of Celite led to reduced recoveries of carrageenan, due undoubtedly to the adsorption of some of the hydrocolloid by the Celite. This possibility has been pointed out by Scott (1960).

Addition of KCl enhances the flocculation of carrageenan. Sodium chloride or other salts would suffice but KCl was chosen because it is known that the kappa fraction of the hydrocolloid is flocculated by this salt, hence the overall precipitation by the C.P.C.-KCl mixture was potentiated. Though concentrations of less than 0.5 *M* will suffice, this minimum is recommended because under these conditions other hydrocolloids such as sodium alginate, pectin and sodium carboxymethylcellulose will not be precipitated by C.P.C. At the maximum concentration of carrageenan used, 100 mg of C.P.C. was enough to provide an excess of the precipitant in the system and 0.75 to 1.0 g of Celite allowed for rapid and complete sedimentation of the precipitate.

Maximum precipitation, as indicated by recoveries using carrageenan in aqueous suspensions, occurred at 37 to 45°C in concurrence with previous observations (Graham *et al.*, 1962). The number of washings of the precipitate necessary in order to remove extraneous carbohydrates differs from product to product. A negative Benedict's test served as a useful end point. The phenol-H_2SO_4 test was used also. However, when large amounts of water-soluble carbohydrates are present, the precipitate must be washed several times. Since this precipitate is not absolutely insoluble in water (Scott, 1960), the phenol-H_2SO_4 test, being extremely sensitive, may at times erroneously indicate incomplete washing. The use of a 0.1% C.P.C.-0.05 *M* KCl solution for washing of the precipitate minimizes this possibility.

Good recoveries of carrageenan from milk were obtained (Tables 3 and 4). Recovery appeared to decrease as the concentration of added carrageenan decreased. Evaporated milk and chocolate milk gave lower recoveries (Table 4). In the latter product, the chocolate on being adsorbed to the Celite added before filtration of the carrageenan in suspension, probably occluded some of the hydrocolloid, thus leading to a lowered recovery.

In the presence of other food gums, the recovery of carrageenan from milk was consistently lower than when the hydrocolloid alone was present. Lowest recovery was obtained in the presence of starch. In all cases, increased viscosity and probably some interaction between the other gums and carrageenan could have subscribed to the results obtained.

Experiments on the recovery of carrageenan from soups gave extremely low results. This might have been due to the large amount of starch in such products. If so, this is in accord with the low recoveries obtained in the presence of starch (Table 5). This difficulty could probably be overcome by pre-digesting the mixture with amylase to degrade the starch. Cursory trials with beer gave average recoveries of 92 to 96% at levels of added carrageenan of 0.02 to 0.2% of the beer.

High results were obtained with the resorcinol, anthrone and Molisch methods due to interference from the C.P.C. and probably traces of proteins (enzymes) in the filtrate. The phenol-H_2SO_4 method was suitable because neither proteins nor C.P.C. interfered. Attempts to further purify the filtrate with charcoal led to low results.

Good recovery of carrageenan from the products used depends greatly on the digestion of the proteins and the removal of degradation products and other colored materials such as those which are present in chocolate milk. After an incubation period of from 4 to 32 hr at 70°C, the mixture of milk and carrageenan gave no increased absorbance at 570 mμ (Moore *et al.*, 1954, Hill *et al.*, 1962), indicating complete hydrolysis of the milk proteins.

As compared to milk, protein digestion in the other products was calculated to be: chocolate milk 91%, evaporated milk 95%, ice cream 96%. In the presence of starch, the digestion of milk proteins was 84%, indicating some retardation of proteolysis which probably was partially contributory to the lowered recovery in the presence of starch.

Trypsin, pepsin and pancreatin, or combinations thereof,

gave much lower recoveries of carrageenan due to poor hydrolysis of the milk proteins.

The proposed method is highly specific because in the presence of 1.0 *M* KCl, other common non-sulfated food gums will not be precipitated by C.P.C. However, its application to individual products should be preceded by a thorough investigation of all variables concerned since the recovery of carrageenan can be influenced by the quantity of one or more ingredients. Possible interference from other sulfated polysaccharides such as furcellaran should not be overlooked.

REFERENCES

Balls, A. K. and Hoover, S. R. 1937. The milk clotting time of papain. *J. Biol. Chem.* **121**, 737–745.

Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A. and Smith, F. 1956. Colorimetric method for determination of sugars and related substances. *Anal. Chem.* **28**, 350–357.

Ewart, M. H. and Chapman, R. A. 1952. Identification of stabilizing agents. *Anal. Chem.* **24**, 1460–1464.

Graham, H. D. 1960. Quantitative aspects of the interaction of carrageenan with cationic substances. 1. Interaction with methylene blue. *Food Research* **25**, 720–730.

Graham, H. D. 1966. Spectrophotometric determination of carrageenan ester sulfate in milk and milk products with barium chloranilate. *J. Dairy Sci.* **49**, 1102–1108.

Graham, H. D. and Thomas, L. B. 1962. Quantitative aspects of the interaction of carrageenan with cationic substances. II. Precipitation with long-chain quaternary ammonium detergents. *J. Food Sci.* **27**, 98–105.

Hansen, P. M. T. and Whitney, R. M. 1960. A quantitative test for carrageenin ester sulfate in milk products. *J. Dairy Sci.* **43**, 175–186.

Hill, R. L. and Schmidt, W. R. 1962. The complete enzymic hydrolysis of proteins. *J. Biol. Chem.* **237**, 389–397.

Korn, E. D. 1959a. The synthesis of heparin by slices of mouse mast cell tumor. *J. Biol. Chem.* **234**, 1321–1324.

Korn, E. D. 1959b. The isolation of heparin from mouse cell tumor. *J. Biol. Chem.* **234**, 1325–1329.

Moore, S. and Stein, W. 1954. A modified ninhydrin reagent for the photometric determination of amino acids and related compounds. *J. Biol. Chem.* **211**, 907–914.

Rao, M. B. and Whitney, R. M. 1960. An improvement in the direct nesslerization method for the determination of total nitrogen in stored sterile milk. *J. Dairy Sci.* **43**, 563–564.

Schiller, S., Slover, G. A. and Dorfman, A. 1961. A method for the separation of acid mucopolysaccharides: Its application to the isolation of heparin from the skin of rats. *J. Biol. Chem.* **236**, 983–987.

Scott, J. E. 1956. The preparation and fractionation of acidic polysaccharides using long-chain quaternary ammonium compounds. *Biochem. J.* **62**, 31P.

Scott, J. E. 1960. Aliphatic ammonium salts in the assay of acidic polysaccharides from tissues. In "Methods of Biochemical Analysis," ed. Glick, D., pp. 145–197. Interscience Publ., New York.

Slack, G. B. 1958. Connective tissue growth stimulated by carrageenin. 3. The nature of and amount of polysaccharides produced in normal and scorbutic guinea pigs and the metabolism of a chondroitin sulphuric acid fraction. *Biochem. J.* **69**, 125–134.

Stoloff, L. and Blethen, J. 1964. Use of a quaternary ammonium compound for quantitative estimation of active sites in water-soluble polyanions. *Anal. Chem.* **36**, 2192–2194.

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Whiskey Composition: Identification of Components by Single-Pass Gas Chromatography-Mass Spectrometry

SUMMARY—A single-pass gas-liquid chromatography method is described for identifying compounds not reported previously in distilled alcoholic liquors. Water-free concentrates of ether-pentane extracts of the distillates were injected into a gas chromatographic column train and the column effluent transported to a mass spectrometer. Several hydrocarbons, and compounds apparently resulting from the reaction of acrolein with ethyl alcohol, were identified in the samples.

INTRODUCTION

GAS-LIQUID CHROMATOGRAPHY (GLC) has been employed by numerous workers for the analysis of alcoholic beverages. Much work has been done on the GLC analysis of wines and beer. Recent work includes that of Webb *et al.* (1961); Sihto *et al.* (1963), and Powell *et al.* (1966). These products were shown to contain many volatile compounds in addition to those found in whiskey. A few compounds have been reported in distilled liquors which are not found in beer and wine.

Some studies have been made on the determination of low-boiling components (Austin *et al.*, 1960), esters of aliphatic acids (Martin *et al.*, 1964; Nykanen *et al.*, 1963) and aromatic high-boiling aldehydes (Baldwin *et al.*, 1967) in whiskies. Most of the work has been directed toward the detection and determination of the components of fusel oil (Kamibayashi *et al.*, 1963; Brunelle, 1967; Kayahara *et al.*, 1964; Kabot *et al.*, 1962; Scott *et al.*, 1966; Singer, 1966). Recently, de Becze *et al.* (1967) detected 12 volatile compounds commonly present in whiskies. Four separate columns were required to resolve these compounds in four passes. Other workers (*loc. cit.*) have determined fusel oil components, separated from each other to various degrees, on single or combination columns in a single pass.

We determine on a routine basis acetaldehyde, acetal and ethyl acetate, all of the common fusel oil components, and ethyl lactate in a single pass. The liquid phase of this column consists of glycerol and hexanetriol. Details of this method will be described in a future publication.

Although ethyl formate had been identified by several of the cited investigators, there is some question as to whether this compound was present in the samples being examined, or was formed by the interaction of ethyl alcohol with the Carbowax columns employed. Thus, Weurman *et al.* (1959) reported that formates are produced on polyethylene glycol (Carbowax) columns when alcohols are injected into them. To avoid this difficulty, Kieser *et al.* (1960) suggested that Carbowax be pretreated to remove formic acid.

The purpose of the present work was to devise a method for separating and detecting a wide variety of compounds in distilled alcoholic beverages. A technique is described by which many components never before reported in whiskies have been detected and identified. By connecting two different columns in series, both polar and non-polar compounds were separated in a single pass. Samples may be analyzed "as is" if the compounds are of sufficient concentration to be sensed by the flame ionization detector. For compounds present in insufficient quantities, a concentration step was employed which also removes all of the water and most of the ethyl alcohol from the samples.

MATERIALS AND METHODS

Equipment

A Varian Aerograph Model 1520 gas chromatograph, equipped with a hydrogen flame ionization detector (F.I.D.) was used in exploratory work for selection of columns and conditions. The thermal conductivity detector (T.C.) of the same instrument was used for determining feasibility and parameters for analyzing the separated peaks by mass spectrometry.

Many columns packed with various liquid phases were tried singly and in series to attempt to separate the many compounds present in the alcoholic distillates. Columns packed with the widely used liquid phases deposited on Chromosorb did not give the desired separation.

Tests were carried out on two less common liquid phases, Tergitol NPX and Surfonic N-300, with the same results. A chance trial of two columns in series, the first one packed with 10% w/w Tergitol and the other with 10% w/w Surfonic (Applied Science Laboratories) yielded the desired separation. The liquid phases were deposited on 100/120 mesh acid-washed Chromosorb W and two 10-ft ⅛-in. OD copper columns in series were used.

Operating conditions were: carrier gas, helium at 100 lbs inlet pressure and flow of 38 ml per min. Column oven temperature was programmed from 60° to 135°C at 3° per min. Injector and detector temperatures were 165°. The T.C. detector with WX filaments was operated at 200 ma current. A 1 mv 1 second Westronics recorder was operated at a speed of 24 in. per hr. Two 10-ft ⅛-in. columns in series packed with SE-30 on Chromosorb W were used for the reference side of the filaments.

Identification of compounds by mass spectrometry involved the use of the same columns and conditions with an F and M Model 700 lab chromatograph connected to an Atlas CH-4 mass spectrometer equipped with an EC-1 inlet valve for monitoring the chromatographic effluent. Fast-scan spectra were obtained using a Honeywell oscillographic recorder.

Samples

This work was initiated by the analysis of a vent condensate (see Sample A) believed to contain a higher concentration of the more volatile components of whiskey and other compounds which are not condensed in whiskey manufacture. When some of these same compounds were also found in a typical unaged Bourbon (see Sample B), it was decided to investigate what compounds are present in some Bourbon and Canadian whiskies.

Sample A was an ether-pentane extract of a condensate obtained by chilling the vapors from the vent of a conventional beer still. The condensation was achieved by passing the vent vapors through a stainless steel coil immersed in an ice and salt bath. This condensate contained a higher percentage of the more volatile components than does the regular beer still product. The alcohol concentration, as determined by a hydrometer, was 184° proof or 92% by volume (Fig. 1).

Sample B was an ether-pentane extract of a typical unaged Bourbon whiskey, and serves as a control for Sample A. Unaged whiskey is prepared by redistilling the beer still product without rectification in a simple still called a doubler (Fig. 2).

Sample C was an ether-pentane extract of a typical Bourbon whiskey. It was aged four years in the conventional manner in new charred white-oak barrels (Fig. 3).

Sample D was an ether-pentane extract of a 3-year-old Bourbon whiskey which initially had an acrolein content of approximately 10 g per 100 l at 100° proof. (Fig. 4).

Sample E was an ether-pentane extract of a low-boiling fraction, or heads cut, distilled from a Canadian beer still product which contained acrolein (Fig. 5).

Sample F was the same product as Sample E analyzed "as is" (Fig. 6).

Sample G was an ether-pentane extract of Canadian unaged whiskey which contained acrolein (Fig. 7).

Sample preparation and analysis

Samples were extracted by a modification of the method of Mecke *et al.* (1959). Since the alcohol content of the samples did not permit formation of two layers upon addition of ether, 250 ml water was added to 350 ml of each sample to reduce the concentration of alcohol. One hundred ml of ether-pentane (2:1) was added for the first extraction and to allow for ether saturation of the alcohol-water layer. The ether-pentane extraction was repeated twice again with 50-ml portions.

The combined ether-pentane solutions were shaken three times with 15-ml portions each of water to remove ethyl alcohol. After drying over anhydrous sodium sulfate, most of the ether-pentane solution was evaporated under reduced

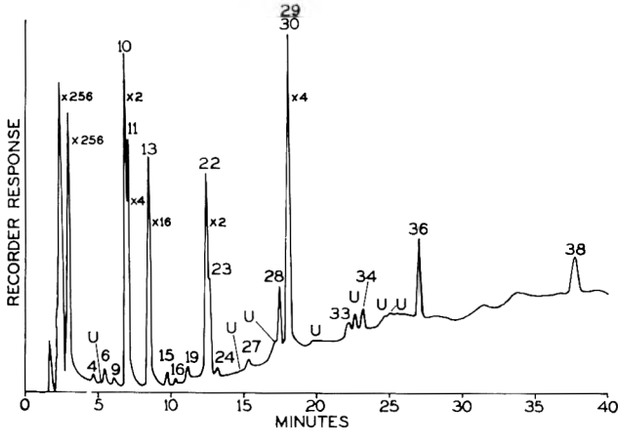

Fig. 1. Chromatogram of ether-pentane extract of a vent condensate (Sample A).

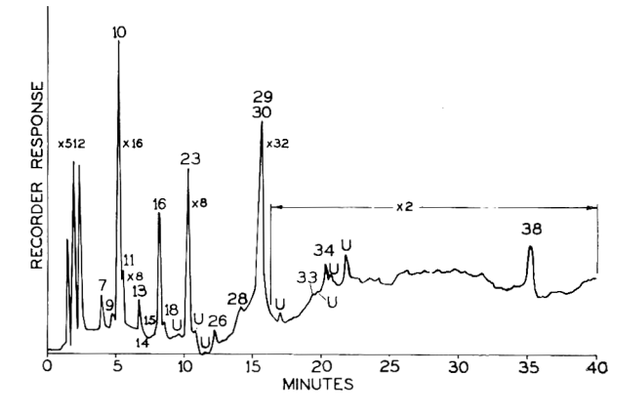

Fig. 2. Chromatogram of ether-pentane extract of a typical unaged Bourbon whiskey (Sample B).

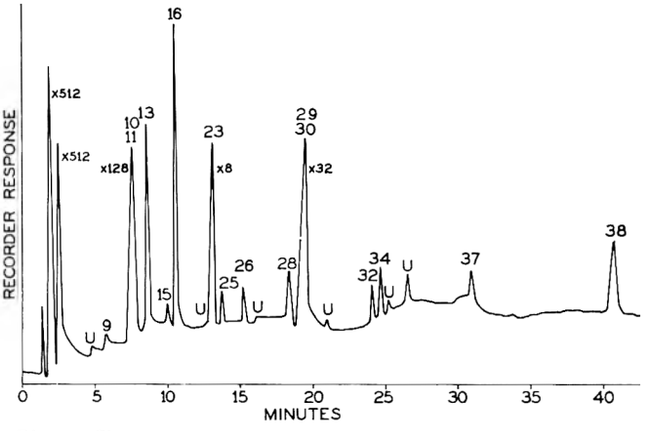

Fig. 3. Chromatogram of ether-pentane extract of a typical 4-yr-old Bourbon (Sample C).

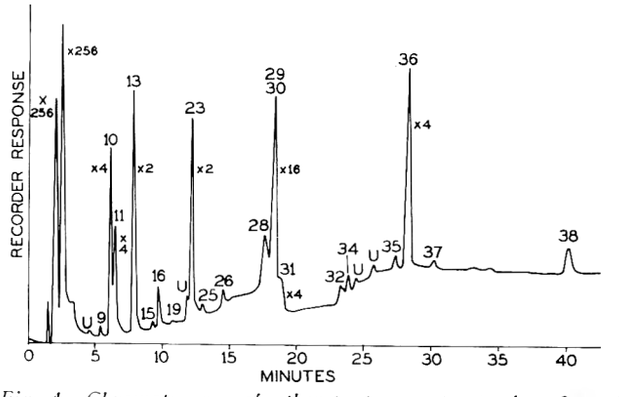

Fig. 4. Chromatogram of ether-pentane extract of a 3-yr-old Bourbon which initially contained acrolein (Sample D).

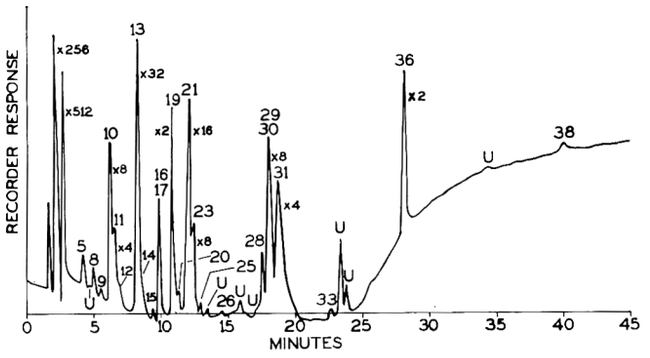

Fig. 5. Chromatogram of ether-pentane extract of heads cut from unaged Canadian whiskey (Sample E).

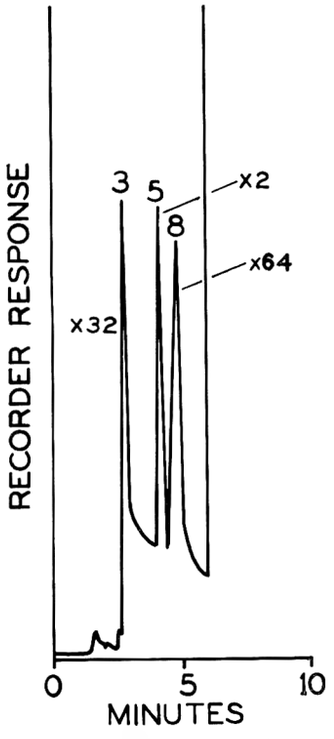

Fig. 6. Chromatogram of heads cut from unaged Canadian whiskey analyzed "as is" (Sample F).

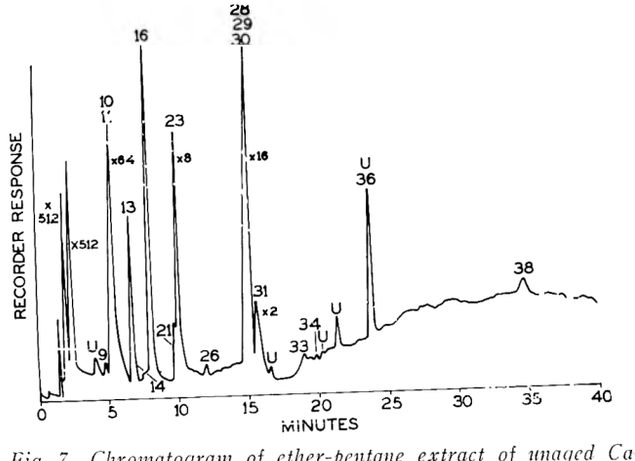

Fig. 7. Chromatogram of ether-pentane extract of unaged Canadian whiskey which contained acrolein (Sample G).

pressure without heating. The concentrates were stored under nitrogen in vials with tinfoil lined caps. Sample sizes used were 3 μl for the FID and 6 μl for the TC detector.

To insure that no contaminants such as hydrocarbons were present in the solvents, and thus erroneously detected in the samples, a large volume of an ether-pentane mixture was evaporated and concentrated as described above and the concentrate run on the same combination of columns. No contaminants of any kind were found.

Extracts were prepared of all samples except "F" which was analyzed "as is" in order to determine what low-boiling compounds, normally masked by the ether-pentane solvent peaks, are present. The analysis of Sample F was done in the usual manner except that the column was disconnected from the T. C. detector just as the ethyl alcohol peak appeared. Fig. 6 shows the partial chromatogram of Sample F using the flame ionization detector.

Compounds such as 1,1-diethoxypropane and 1,1-diethoxy-2-methyl-propane, which are not commercially available, were synthesized in the laboratory. The mass spectra of these laboratory preparations were compared with those of the suspected compounds in the samples in order to confirm their identification.

RESULTS AND DISCUSSION

THE TECHNIQUE, AS DESCRIBED, allows the detection of approximately 50 compounds in alcoholic distillates. Of these compounds, the identities of 35 have been confirmed by the comparison of their mass spectra and GLC retention times with those of authentic samples. Two additional compounds have been identified tentatively. Sixteen of the positively identified compounds have not been reported previously in whiskey (Table 1).

Unavoidably, some GLC peaks consisted of mixtures of compounds, as determined by their mass spectra, and some of these peaks could not be identified. It is anticipated that by varying the parameters of the chromatographic separation, additional compounds can be separated from peaks which represent mixtures.

In the concentration step involving the evaporation of the solvents, some low-boiling compounds may be lost along with the ether and pentane. Small quantities of ethyl methyl sulfide, acetal, isobutyl alcohol, C_5 alcohols and larger quantities of ethyl alcohol and ethyl acetate were found in a dry ice trap used for Sample C.

Table 1 shows the compounds which have been confirmed as well as those which have been identified tentatively. It must be cautioned that even though any number of these compounds appear in the vent condensate or any other sample tested, it can not necessarily be assumed that each and every compound tabulated will be present in any one or all unaged or aged whiskies.

Although the presence of hydrocarbons such as benzene, heptane, toluene, 2-pinene and styrene was unexpected, these compounds have been identified in extracts of various fruits. (Nursten *et al.*, 1967.) No contaminants were found in the solvents. Since all samples were treated alike, contamination in procedure or equipment would have

Table 1. Identification of compounds in samples and key to peaks in figures.

Peak		Samples** A	B	C	D	E	F	G
						Extract "as is"		
1	Carbon bisulfide (Tent.)		x					x
2	Unknown		x					x
3	Acetaldehyde						x	
4	*Heptane	x						
5	Propionaldehyde					x	x	
6	Isobutyraldehyde	x						
7	Ethyl formate		x					
8	Acrolein					x	x	
9	*Ethyl methyl sulfide	x	x	x	x	x		x
10	Ethyl alcohol	x	x	x	x	x		x
11	Ethyl acetate	x	x	x	x	x		x
12	*2-Butanone					x		
13	Acetal	x	x	x	x	x		x
14	*Benzene		x			x		x
15	Ethyl propionate	x	x	x	x	x		
16	n-Propyl alcohol	x	x	x	x	x		x
17	sec.-Butyl alcohol					x		x
18	*2-Pentanone		x					
19	*1,1-Diethoxypropane	x			x	x		
20	*1-Ethoxy-1-propoxyethane					x		
21	*1,1-Diethoxy-2-propene					x		x
22	*1,1-Diethoxy-2-methylpropane	x						
23	Isobutyl alcohol	x	x	x	x	x		x
24	*Toluene	x						
25	Ethyl butyrate			x	x	x		
26	1-Butanol		x	x	x	x		x
27	*2-Pinene	x						
28	Isopentyl acetate	x	x	x	x	x		x
29	Isopentyl alcohol	x	x	x	x	x		x
30	2-Methyl butanol	x	x	x	x	x		x
31	*3-Ethoxypropionaldehyde				x	x		x
32	*Triethyl orthoformate (Tent.)			x	x			
33	*Styrene	x	x			x		x
34	Ethyl hexanoate	x	x	x	x			x
35	*Ethyl 3-ethoxypropionate				x			
36	*1,1,3-Triethoxypropane	x			x	x		x
37	2-Furaldehyde			x	x			
38	Ethyl octanoate	x	x	x	x	x		x
U	Unidentified compound							

* Compound not previously reported in whiskey.
** See text for description of samples.

resulted in the detection of some of the hydrocarbons in all of the samples. This was not the case. Finding these hydrocarbons in trace quantities in distilled liquors remains unexplained even though positive identifications were made. Unidentified hydrocarbons were reported in grain spirits by Austin *et al.* (1960). The number of acetals and esters found was not surprising as aldehydes, alcohols, and acids are always present in whiskey.

A most interesting and novel result of this work is the finding of compounds related to acrolein. This aldehyde which has a pungent odor and lachrymatory property, is responsible for the "peppery" smell rarely found in distilled liquors (Mills *et al.*, 1954 and Serjak *et al.*, 1954). Determinations in this laboratory by the method of Circle *et al.* (1945) have shown that the acrolein content of peppery whiskey decreases over a 2 to 3-year aging period. The fate of the acrolein was not known.

Acrolein can react with ethyl alcohol to form several products as illustrated below:

(a) 1,1-diethoxy-2-propene by acetal formation with acrolein:

$$CH_2 = CH - CHO + 2\ C_2H_5OH \rightarrow CH_2 = CHCH(OC_2H_5)_2 + H_2O$$

(b) 3-ethoxypropionaldehyde by addition of ethyl alcohol to the vinyl double bond of acrolein:

$$CH_2 = CH - CHO + C_2H_5OH \rightarrow C_2H_5OCH_2CH_2 - CHO$$

(c) 1,1,3-triethoxypropane by acetal formation with the intermediate 3-ethoxypropionaldehyde:

$$C_2H_5OCH_2CH_2CHO + 2C_2H_5OH \rightarrow C_2H_5OCH_2CH_2CH(OC_2H_5)_2 + H_2O$$

The above products have been prepared and studied by Smith (1962) and the Shell Chemical Corporation (1959).

All three of the reaction products of acrolein with ethyl alcohol were found in one or more of the samples analyzed. The final reaction product of acrolein and ethyl alcohol, 1,1,3-triethoxypropane, does not have the unpleasant odor nor the lachrymatory effect of acrolein. Ethyl 3-ethoxypropionate was found in Sample D. This compound may be derived from 3-ethoxypropionaldehyde, by oxidation and esterification (Smith, 1962). To our knowledge, none of these compounds have been identified or reported in unaged or aged whiskey, or in any other naturally occurring product.

The occurrence of 1,1,3-triethoxypropane in the vent condensate from a typical acrolein-free beer still product was not expected. Acrolein is produced by the action of bacteria on the glycerol formed as a by-product of yeast metabolism. Acrolein-producing bacteria occur naturally in most corn, rye and barley malt grains and are found in small numbers in many alcoholic fermentations of malt-converted distillery mashes. Since acrolein-producing bacteria require rather exacting conditions for the production of acrolein, detectable amounts of the compound are seldom found under normal conditions in distillates from grain mashes (Mills *et al.*, 1954). The occurrence of 1,1,3-triethoxypropane and the absence of detectable acrolein suggests that the former is an immediate reaction product of the latter with ethyl alcohol during the fermentation or in subsequent distillation.

No conclusive evidence of the actual reaction mechanisms of acrolein with ethyl alcohol in fermentation products has been found. It is postulated, however, that the reactions, as described by Smith (1962), do proceed in a similar manner in distilled liquors because the same reaction products were found and proper pH conditions were present.

Ethyl lactate, acetone and other compounds thought to be present in most of the samples analyzed would not be detected by our technique. Their peaks would either be masked by other components or would be too polar to be extracted from the ethanolic-aqueous layer by ether-pentane.

Carbon bisulfide was identified tentatively by its mass spectrum. It was eluted just after pentane near unidentified compound 2 using the F and M chromatograph. However, the same columns in the Varian chromatograph resolved no peaks in the area just following pentane. Since the figures were derived from the latter instrument they do not show peaks 1 and 2.

The compound triethyl orthoformate was positively identified in several samples by its mass spectrum; however, the retention time of the authentic material's peak is somewhat shorter than that of the peak identified by GLC-MS as triethyl orthoformate. Possibly the presence of large quantities of pentyl alcohols interferes with proper matching of retention times. It is feasible that peak "U" just before peak "9" in Fig. 7 is, in fact, acrolein. Even though this peak could not be identified by the mass spectrometer, its retention time coincides with that of acrolein.

It is difficult to draw a correlation between aroma and the detected compounds. It is not known, for example, what effect trace quantities of ethyl methyl sulfide would have on the sample. It is possible that compounds represented by some of the many still unidentified peaks may have a significant influence on the aroma and flavor of the samples.

REFERENCES

Austin, F. L. and Boruff, C. S. 1960. Concentration of congeners of grain spirits and their analysis by gas chromatography. *J. Assoc. Offic. Agric. Chemists* **43**, 675.

Baldwin, S., Black, R. A., Andreasen, A. A. and Adams, S. L. 1967. Aromatic congener formation in maturation of alcoholic distillates. *J. Ag. Food Chem.* **15**(3), 381.

Brunelle, R. L. 1967. Collaborative study of the quantitative determination of ethyl acetate and fusel oil by GLC. Presented at the 81st Annual Meeting, Association of Official Analytical Chemists, Washington, D. C.

Circle, S. J., Stone, L. and Boruff, C. S. 1945. Acrolein determination by means of tryptophane. A colorimetric micro method. *Ind. Eng. Chem. Anal. Ed.* **17**, 259.

de Becze, G. I. Smith, H. F. and Vaughn, T. E. 1967. Use of gas chromatography in distillery research and control. *J. Assoc. Offic. Anal. Chemists* **50**, 311.

Kabot, F. J. and Ettre, L. S. 1962. Gas chromatography gives direct analysis of wide variety of alcoholic beverages. Perkin-Elmer Instrument News **13**(4).

Kamibayashi, A., Miki, M., Yamaguchi, M. and Ono, H. 1963. Gas chromatographic studies on fermentation products. 8. Analysis of small quantities of lower alcohols in water. *Report of the Fermentation Research Institute (Japan)* **24**, 173.

Kayahara, K., Mori, S., Taguchi, T. and Miyachi, N. 1964. The flavor components of whiskey. II. Analysis by gas chromatography. 2. The liquid phase and resolution of the column. *Hakko Kogaku Zasshi* **42**(3) 149 (C.A. **64**, 20586).

Kayahara, K., Mori, S., Taguchi, T. and Miyachi, N. 1964. Flavor components of whiskey. III. Analytical methods by gas chromatography. 3. Concentration of the high-boiling components by extraction with an ether-pentane solvent system. *Hakko Kogaku Zasshi* **42**(6), 375 (C.A. **64**, 20587).

Kayahara, K., Mori, S., Taguchi, T. and Miyachi, N. 1964. Flavor components of whiskey. IV. Detection of the flavor components by gas chromatography. *Hakko Kogaku Zasshi* **42**(8), 512 (C.A. 66, 74905).

Kieser, M. E. and Sissons, D. J. 1960. Formation of volatile compounds on gas-liquid chromatography columns. *Nature.* **185** (4712), 529.

Martin, G. E., Schoeneman, R. L. and Schlesinger, H. L. 1964. Determination of esters in whiskey by gas-liquid chromatography. *J. Assoc. Offic. Agric. Chemists* **47**, 712.

Mecke, R. and DeVries, M. 1959. Gaschromatographische Untersuchung von alkoholischen Getraenken. *Z. Anal. Chem.* **170**, 326.

Mills, D. E., Baugh, W. D. and Conner, H. A. 1954. Studies on the formation of acrolein in distillery mashes. *Appl. Microbiol.* **2**(1), 9.

Nursten, H. E. and Williams, A. A. 1967. Fruit aromas: A survey of components identified. *Chemistry and Industry*, March 25, 486.

Nykanen, L. and Suomalainen, H. 1963. The aroma compounds of alcoholic beverages. *Teknillisen Kemian Aikakauslehti* **20**(19–20) 789 (C.A. **61**, 2437).

Powell, A. D. G. and Brown, I. H. 1966. The determination of beer components by gas-liquid chromatography. *J. Inst. Brew.* **72**, 261–5.

Scott, C., Hadden, N. and Bonelli, E. J. 1966. Fusel oil analysis in brandy. Varian Aerograph Previews and Reviews (8) 6.

Serjak, W. C., Day, W. H., Van Lanen, J. M. and Boruff, C. S. 1954. Acrolein production by bacteria found in distillery grain mashes. *Appl. Microbiol.* **2**(1), 14.

Shell Chemical Corporation, New York. 1959. "Acrolein, Its Chemistry and Its Applications."

Sihto, E. and Arkima, V. 1963. Proportions of some fuel oil components in beer and their effect on aroma. *J. Inst. Brew.* **69**, 20.

Singer, D. D. 1966. The analysis and composition of potable spirits: determination of C_3, C_4 and C_5 alcohols in whiskey and brandy by direct gas chromatography. *Analyst,* **91** (1079). 127.

Smith, C. W. 1962. "Acrolein," pp. 110–115. John Wiley and Sons, Inc., New York, N. Y.

Webb, A. D. and Kepner, R. E. 1961. Fusel oil analysis by means of gas-liquid partition chromatography. *Am. J. Enol. Vitic.* **12**(2), 51.

Weurman, C. and Dhont, J. 1959. Unexpected formation of volatile compounds on gas-liquid chromatography columns. *Nature* **184** (4697), 1480.

Ms. accepted 4/3/68.

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Peanut Alcohol Dehydrogenase. 2. Physico-Chemical and Kinetic Properties

SUMMARY—Some of the physical, chemical, and kinetic properties of peanut alcohol dehydrogenase have been investigated. A molecular weight of 112,000 and a sedimentation coefficient of 5.46 S were obtained in aqueous salt solutions. The enzyme preparation contained 1.5 g-atoms of Zn per mole of enzyme and was inactivated by 1,10-phenanthroline, EDTA, 8-hydroxyquinoline-5-sulfonic acid, and iodoacetate. Comparison of the peanut enzyme kinetic properties with those of yeast and liver alcohol dehydrogenases indicated generally a greater similarity to the yeast enzyme.

INTRODUCTION

In Pattee *et al.* (1968) the isolation and purification of alcohol dehydrogenase (alcohol:NAD^+ oxidoreductase, EC 1.1.1.1) from peanut kernels (*Arachis hypogea* var. N.C. 2) was reported.

The high degree of purification attained provides the first opportunity to compare the properties of the alcohol dehydrogenases as obtained from single celled organisms, mammals, and higher plants. The present communication reports some of the physiochemical and kinetic properties of the peanut alcohol dehydrogenase. The peanut enzyme, although distinctly different from both the yeast and mammalian enzymes, has some properties in common with each of these.

EXPERIMENTAL

Chemicals

The enzyme was prepared by the procedure of Pattee *et al.* (1968). The purified protein was precipitated with ammonium sulfate, and the experiments were performed on dialysed solutions within several days of the initial isolation. The ammonium sulfate was a special grade, low in metal ion impurities, obtained from Mann Research Laboratories. Solutions containing NAD^+, NADP, and NADH, obtained from Calbiochem, were prepared just before use. Dialysis tubing (Visking Company) was cleaned (Hughes *et al.*, 1956), and thoroughly rinsed with deionized distilled water before use. The water was distilled over glass and passed through a mixed-bed resin (Rexyn I-300). All other reagents and buffer chemicals were analytical grade and used without further purification.

Physical methods

Analytical sedimentation experiments were performed in a Beckman Spinco Model E Analytical Ultracentrifuge which was equipped with a phase plate for schlieren optics and a rotor temperature-control unit. The schlieren diagrams were photographed on metallographic plates (Kodak) and distances were measured with a Bausch and Lomb microcomparator.

A Gelman Rapid Electrophoresis Apparatus was used to observe the zone electrophoretic properties. Cellulose acetate strips (Sepraphore III) were used for the solid support media. The total protein was stained with Amido Black 10B or Ponceau S.

Measurements of pH were obtained with a Radiometer PHM 25, equipped with a scale expander, using a G202B glass electrode or a GK2641 C combined electrode. The isoionic pH of the protein was determined by using the combination electrode to monitor the pH of the effluent from a column of Bio-Rad AG-501-X8 mixed-bed resin to which 20 mg of the enzyme was applied. The reliability of the method was first confirmed by studies on crystalline ovalbumin (Sigma Chemical Company).

The initial rate kinetics were obtained by spectrophotometrically observing the appearance or disappearance of NADH in 3 ml of reaction mixture at 23°C (Racker, 1950). The reaction was initiated by the addition of 0.1 ml of enzyme solution to the substrate solution containing 0.015*M* pyrophosphate buffer, pH 8.5. Enzyme concentrations were chosen to be within the range of linear rate dependence. Absorbance readings at 340 mμ were made at 15-sec intervals for several minutes. Generally the 15-

[a] Department of Food Science.
[b] U. S. Department of Agriculture, ARS, MQRD.

sec and 45-sec readings were used together with the molar extinction coefficient for NADH (Theorell *et al.* 1951) to calculate the change in NADH concentration per unit time. Absorbances were determined using a Beckman DU spectrophotometer equipped with a Gilford Adaptor for digital read-out.

The specific activity of the preparations was assayed at room temperature by the above procedure using a reaction medium containing 0.1*M* ethanol, 0.5m*M* NAD^+, and 0.01*M* pyrophosphate buffer (pH 8.5).

Protein concentration was determined by the method of Lowry *et al.* (1951), using crystalline bovine plasma albumin as a standard. The experimentally determined extinction coefficient was used to measure the protein concentration of solutions of purified preparations.

A Cary Model 15 recording spectrophotometer was utilized to study the ultraviolet absorption characteristics of the purified enzyme.

RESULTS AND DISCUSSION

Physical properties

The protein from the central zone of enzymatic activity resulting from the second elution from Sephadex G-150 was used for these studies. These preparations were shown to be homogeneous with regard to molecular weight. Velocity sedimentation demonstrated a single boundary, which sedimented at a rate given by the relation

$$1/s_{20,w} = 0.183\ (1 + 0.041C) \times 10^{13}$$

where C is the concentration in mg/ml. These data, obtained in phosphate buffer (pH 6.60), are shown in Fig. 1. The value calculated for the sedimentation coefficient at zero protein concentration, $s^\circ_{20,w}$, was 5.46S.

Weight homogeneity was demonstrated further by the linearity of equilibrium sedimentation data plotted according to the method of Van Holde *et al.* (1958) (Fig. 2). Also, as shown in Fig. 3, the schlieren curve at equilibrium

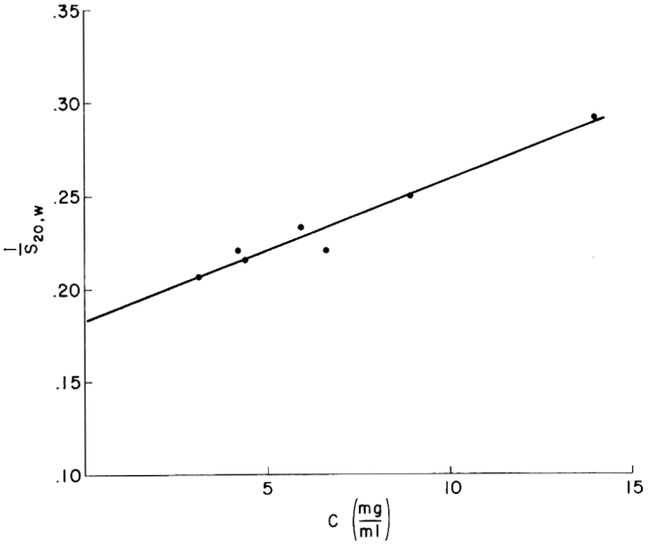

*Fig. 1. Concentration dependence of the sedimentation coefficient for peanut alcohol dehydrogenase. The data were obtained for the enzyme in 0.1*M *phosphate buffer (pH 6.60) containing 0.4*M *NaCl, 0.01*M *cysteine and 0.001*M *2-mercaptoethanol. Sedimentation was observed at 19°C at a rotor speed of 59,780 rpm.*

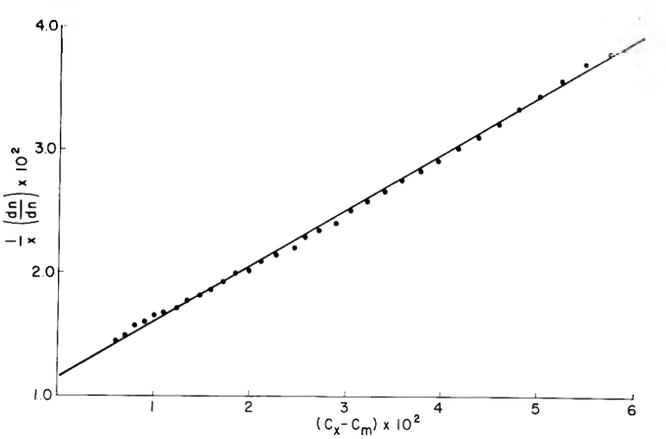

*Fig. 2. Van Holde-Baldwin plot for the schlieren data of a peanut alcohol dehydrogenase solution after attainment of equilibrium at 7.0°C using a rotor speed of 9,341 rpm. The protein was dissolved to a concentration of 10 mg/ml in 0.1*M *phosphate buffer (pH 6.98) containing 0.4*M *NaCl, 0.01*M *cysteine, and 0.001*M *2-mercaptoethanol.*

does not reveal any abrupt curvature near the bottom of the solution column as should be the case for significant weight heterogeneity. The apparent weight average molecular weight calculated from this equilibrium experiment at a protein concentration of about 10 mg/ml in phosphate buffer, pH 6.98, was 112,000.

The absorption spectrum of the enzyme showed a peak at 278 mμ. No absorption was observed above 300 mμ

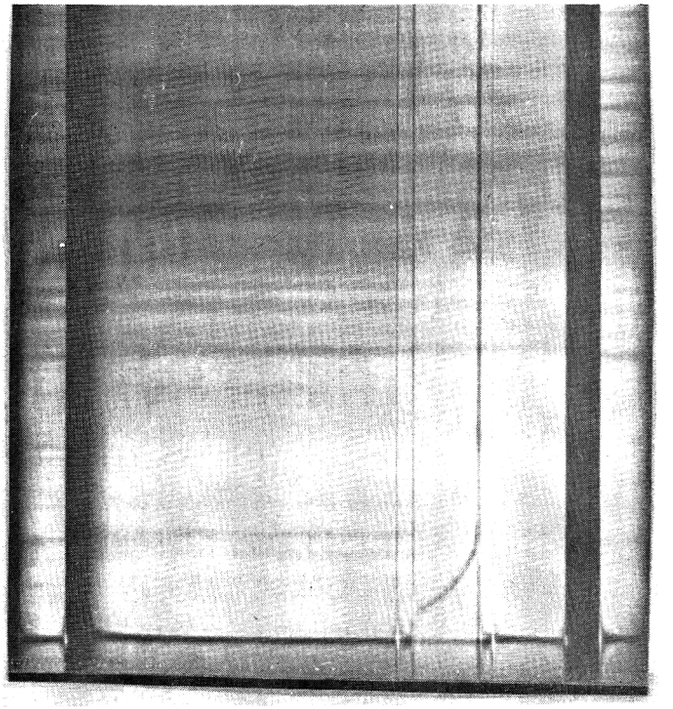

*Fig. 3. Photograph of the equilibrium schlieren diagram for peanut alcohol dehydrogenase. The experiment was performed at 7°C using a protein concentration of 10 mg/ml in 0.1*M *phosphate buffer (pH 6.98), containing 0.4*M *NaCl, 0.01*M *cysteine, and 0.001*M *2-mercaptoethanol. The photograph was taken after 24 hr at a rotor speed of 9341 rpm using a phase plate angle of 70 degrees. Fluorocarbon oil, FC 43, was used to form a false bottom for the solution and solvent in a Kel F double sector centerpiece.*

indicating the absence of reduced coenzyme. Also the A_{280}/A_{260} ratio, 1.7, suggested the absence of nucleotides in these preparations. The extinction coefficient, $E^{1mg/ml}_{1cm}$ at 278 mμ was calculated to be 0.64. Protein concentration was determined by drying aliquots of both the solution and the solvent to constant weight *in vacuo* over P_2O_5 at 105°.

Electrophoretic analyses, both zonal and free boundary, have indicated that the preparations are essentially electrophoretically homogeneous. Cellulose acetate electrophoretograms of the protein in 0.05 ionic strength phosphate buffer, pH 5.46, 6.04, 6.46, 6.99, and 7.52 and in 0.03 ionic strength barbital-acetate buffer, pH 7.07, 7.99 and 9.03, have generally shown one anodal band. In some instances heterogeneity was suggested, especially if the preparation included a larger fraction of the peak from Sephadex G-150 (Pattee *et al.*, 1968). One electrophoretogram obtained in a Tris-barbital buffer, pH 8.8, displayed 3 or 4 bands. However this may have been caused by metallic ion impurities in the Tris (Watts *et al.*, 1962). The present observation of a single anodal band for peanut alcohol dehydrogenase even at pH 5.46 indicates the binding of buffer ions since the observed isoionic point was 6.3. In fact, as the pH approached 9 there appeared to be an increase in the cathodal movement that might be attributed to a change in ion binding.

A number of bands have been observed on the zonal electrophoretograms of both liver and yeast alcohol dehydrogenase. For example, McKinley-McKee *et al.* (1965) observed four cathodal bands upon electrophoresis of the horse liver enzyme in phosphate buffer, pH 7.1. They attributed the cathodal movement to electroendosmosis and the multiple banding to differences in coenzyme binding. However, Pietruszko *et al.* (1966) have found five cathodal bands by cellulose acetate electrophoresis in Tris buffer, pH 8.5, which they interpreted to represent different horse liver enzymes. The cathodal movement at this high pH is rather surprising in view of the reported isoelectric point. The number of anodal bands observed in starch-gel electrophoresis of the yeast enzyme decreased from eighteen to essentially one when the buffer system (phosphate, pH 7.5 and Tris-citrate buffer, pH 8.9) was treated to remove metal ion impurities (Watts *et al.*, 1962).

The electrophoretic mobility of peanut alcohol dehydrogenase in 0.1*M* phosphate buffer, pH 7.31 was determined from the descending boundary to be -0.68×10^{-5} cm^2V^{-1} (Pattee *et al.*, 1968). This value is consistent with the isoionic pH of about 6.3 obtained by passing the protein over a column of mixed-bed resin in the H, OH form. Von Wartburg *et al.* (1964) have reported a mobility of -0.80×10^{-5} cm^2 V^{-1} sec^{-1} for the human liver enzyme in phosphate buffer, pH 7.4. An iso-electric point of 6.8 has been reported by Dalziel (1958) for horse liver alcohol dehydrogenase at 0.1 ionic strength. However, the enzyme isolated from yeast was reported to have an isoelectric point of 5.4 (Hayes *et al.*, 1954).

Some of the physical properties of the peanut enzyme are compared with those of the liver and yeast enzymes in Table 1. The molecular size, as indicated by equilibrium molecular weight studies and the values of the sedimentation coefficients, is intermediate between those of the other enzymes. Based on a molecular weight of 112,000, the zinc content of purified peanut alcohol dehydrogenase was at least 1.5 g-atoms/mole (Pattee *et al.*, 1968). This value is only slightly lower than that reported for the human liver enzyme by Von Wartburg *et al.* (1964) and that found for crystalline horse liver alcohol dehydrogenase (1.7–2.2 g-atoms/mole) by Vallee *et al.* (1957). Recently, however, Akeson (1964) has reported four zinc atoms per 84,000 g of horse liver alcohol dehydrogenase. The yeast enzyme has been found to have four zinc atoms per mole, one for each mole of coenzyme found (Vallee *et al.*, 1955).

Table 1. Some physical properties of alcohol dehydrogenases.

Property	Peanut	Liver	Yeast
M_w	112,000	83,300[1]; 87,000[2]	150,000[3]
$S^\circ_{20,w}$	5.46S	5.11S[1]; 4.80S[2]	6.72S[3]
$E^{1\ mg/ml}_{1\ cm,\ 278\ m\mu}$	0.64	0.455[4]	1.26[3]
$Zn^{g\text{-}atoms}$/mole	1.5	2.2[2]; 4.2[5]	(3.3–4.7) 4.1[6]

[1] Ehrenberg *et al.* (1958).
[2] Value for human LADH, Von Wartburg *et al.* (1964).
[3] Hayes *et al.* (1956).
[4] Theorell *et al.* (1951).
[5] Akeson (1964).
[6] Vallee *et al.* (1955).

Kinetic properties

The peanut enzyme was observed to have a specific activity of about 2 units/mg. (A unit of activity is defined as one μmole NADH formed per minute.) This value is within the range of those exhibited by the liver enzymes (Table 2). The yeast enzyme is about 100 times as active, having specific activities ranging from about 100 to 300 units/mg.

The initial rates of reaction were determined at various concentrations of NAD^+, ethanol, and NADH. The results, when plotted according to Lineweaver *et al.* (1934), gave a linear relationship in each case as shown in Figs. 4, 5 and 6. As illustrated in Fig. 4, the narrow range of intersection of the curves for a series of NAD^+ concentrations with the reciprocal substrate concentration axis indicates that the Michaelis constant for ethanol is not greatly dependent upon the coenzyme concentration. The Michaelis constants, obtained by fitting the data to a straight line by the method of least squares, are compared to the constants for liver and yeast alcohol dehydrogenase in Table 3.

Results for the peanut enzyme show values of Km for both coenzymes several orders of magnitude larger than

Table 2. Specific activities of various alcohol dehydrogenases.

Enzyme	Specific Activity μmole NADH/min/mg	Reference
PADH	2.3	This work
Human LADH	0.5*	Blair *et al.* (1966)
	0.5*	Von Wartburg *et al.* (1964)
Horse LADH	5.8*	Theorell *et al.* (1951)
	6.1*	Li *et al.* (1965)
YADH	179*	Hayes *et al.* (1954)
	76*	Racker (1950)
	100*	Watts *et al.* (1962)
	260*	Kagi *et al.* (1960)

* Calculated from the data originally reported in other units, usually the turnover number in moles NADH/min/mole enzyme.

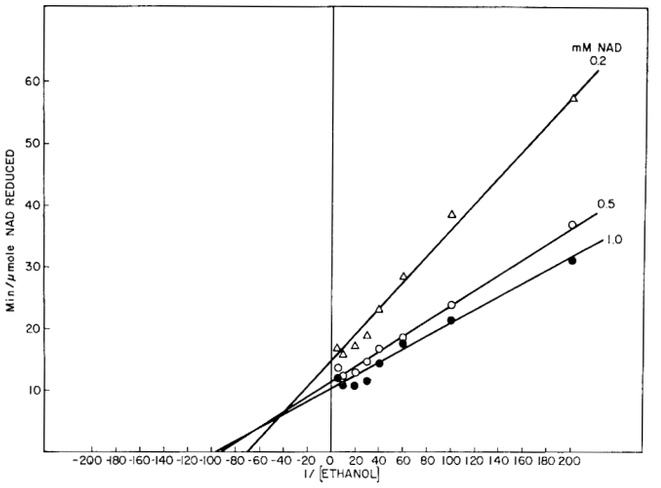

Fig. 4. Lineweaver-Burk plot for the determination of the Michaelis constant for ethanol. The reaction mixture contained 0.015M pyrophosphate buffer (pH 8.5), and 63 μg/ml of enzyme in addition to the ethanol and NAD. The symbols represent: ●, 1.0 mM NAD⁺; ○, 0.5 mM NAD⁺; and △, 0.2 mM NAD⁺.

the values for the other enzymes. Although these are not true dissociation constants, the magnitude suggests that coenzyme is less strongly bound to the peanut enzyme than to the other alcohol dehydrogenases. The Km values obtained with ethanol are essentially the same for peanut and yeast alcohol dehydrogenases.

Peanut alcohol dehydrogenase shows considerable specificity for ethanol. The relative rates of oxidation presented in Table 4 indicate similar specificities for the peanut and yeast enzymes. Both of the mammalian enzymes show increased activity on n-butanol, and human liver alcohol dehydrogenase shows increased activity on methanol.

The peanut enzyme was not active when NADP⁺ was substituted for NAD⁺. A similar observation was made for both the mammalian and the yeast enzymes (Pullman *et al.*, 1952).

Initial reaction rates obtained in Tris-EDTA buffer as a function of pH indicate a maximum rate of ethanol oxidation around pH 8.6, as shown in Fig. 7. The pH optimum is identical with that reported for yeast alcohol dehydrogenase (Wallenfels *et al.*, 1958) whereas the optimum for ethanol oxidation by the mammalian enzymes was reported to be pH 11 at high ethanol concentrations (Von Wartburg *et al.*, 1964; Theorell *et al.*, 1951).

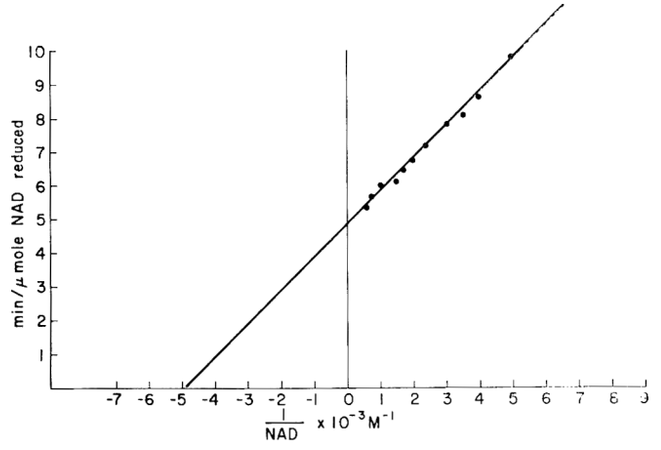

Fig. 5. Lineweaver-Burk plot for the determination of the Michaelis constant for NAD⁺. The reaction mixture contained 0.1M ethanol, 0.015M pyrophosphate buffer (pH 8.5) and 45 μg/ml of enzyme in addition to the NAD⁺.

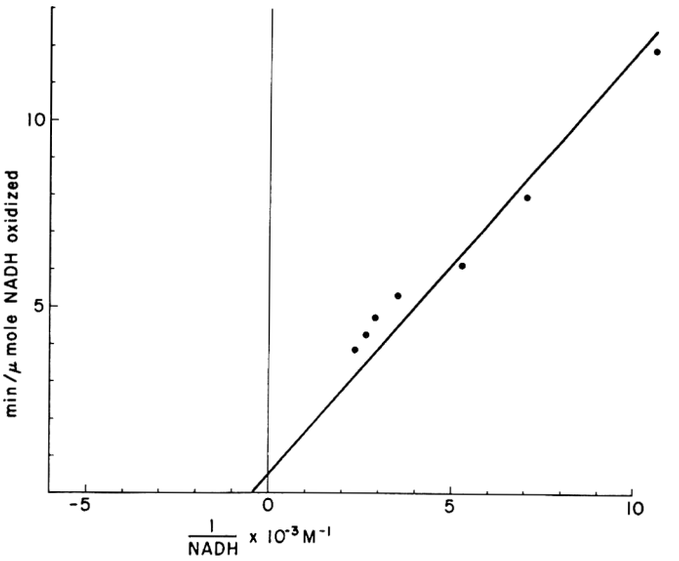

Fig. 6. Lineweaver-Burk plot for the determination of the Michaelis constant for NADH. The reaction mixture contained 0.133M acetaldehyde, 0.015M pyrophosphate buffer (pH 8.5) and 50 μg/ml of enzyme in addition to the NADH.

The importance of metal ions to enzymatic activity was ascertained by observing the effect of several chelating agents on NAD⁺ reduction. The data, presented in Fig. 8, demonstrate an initial rapid inactivation of about 12% followed by a time-dependent inactivation of the enzyme by 1,10-phenanthroline. A similar effect has been observed in the case of the yeast enzyme (Williams *et al.*, 1958); horse liver alcohol dehydrogenase, however, was rapidly inactivated to 54% of its initial activity with no further time-dependent inactivation (Vallee *et al.*, 1957).

The inactivation of peanut alcohol dehydrogenase was prevented by the incorporation of NAD⁺ in the reaction mixture prior to the addition of 1,10-phenanthroline. Similar results for the yeast enzyme (Hoch *et al.*, 1956) and for the enzyme from horse liver (Wallenfels *et al.*, 1957) have been interpreted to suggest the binding of coenzyme to the zinc. With regard to inactivation by incubation with EDTA, the peanut enzyme gave results similar to those for horse liver alcohol dehydrogenase (Vallee *et al.*, 1957), which showed a first order initial rate of inactivation. By contrast, yeast alcohol dehydrogenase is not significantly

Table 3. Michaelis constants for various alcohol dehydrogenases.

Km M	Human Liver[1] pH 8.2, 23°	Horse Liver[2] pH 7, 26°	Yeast pH 7.9, 26°	Peanut pH 8.5, 23°
K_{EtOH}	1.2×10^{-3}	5.4×10^{-4} (pH 8.2)	1.8×10^{-2}	1.3×10^{-2}
K_{NAD}	1.1×10^{-4}	1.2×10^{-5}	1.7×10^{-4}	2.0×10^{-2}
K_{NADH}	2.2×10^{-5}	1.3×10^{-5}	2.3×10^{-5}	2.8×10^{-3}

[1] Von Wartburg *et al.* (1964).
[2] Theorell *et al.* (1951).

Table 4. Substrate specificity of alcohol dehydrogenases.

	Peanut	Human[1] Liver	Horse[2] Liver	Yeast[3]
Assay conditions	0.01*M* pyrophosphate pH 8.5, 23°C 0.5 mM NAD alcohol, 700 mM	0.016*M* pyrophosphate pH 8.8, 23° 1.66 mM NAD 1.16 mM alcohol	0.1*M* Glycine-NADH pH 9.5, 23.5° 0.12 mM NAD 1 mM alcohol	0.02*M* pyrophosphate pH 8.9, 25° 0.2 mM NAD 200 mM alcohol
Substrate	Relative rates of oxidation			
Ethanol	1.0	1.0	1.0	1.0
Methanol	0	1.2(0.5*M* MeOH)	0	0
n-Butanol	0.33	1.7	1.6	0.175
Isopropanol	0	0.4	0	0.055
n-Pentanol	0.07	.....	1.18	0.125

[1] Von Wartburg *et al.* (1964).
[2] Data of A. D. Winer as given by Sund *et al.* (1963).
[3] Data of E. S. G. Barron and S. Levine as given by Sund *et al.* (1963).

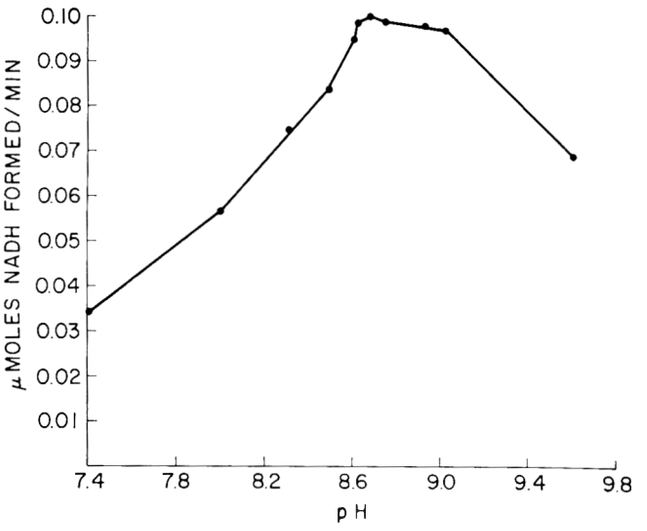

*Fig. 7. Influence of pH on the rate of ethanol oxidation by peanut alcohol dehydrogenase. The initial rates were determined at room temperature in 0.1*M *Tris-HCl containing 1 mM EDTA.*

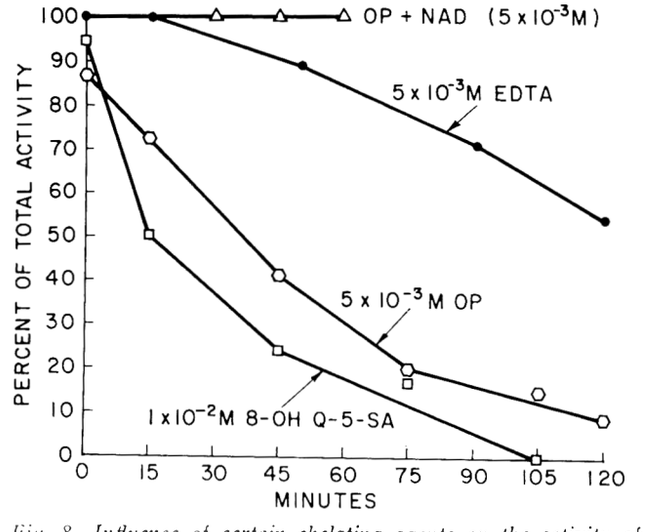

*Fig. 8. Influence of certain chelating agents on the activity of peanut alcohol dehydrogenase. The enzyme was pre-incubated with the chelating agent at room temperature in 0.1*M *phosphate buffer (pH 7.5) for the designated periods prior to the addition of 0.1 ml of this mixture to the standard assay system.*

affected by EDTA (Wallenfels *et al.*, 1957). Peanut alcohol dehydrogenase was also readily inactivated by 8-hydroxyquinoline-5-sulfonic acid as are other alcohol dehydrogenases (Sund *et al.*, 1963).

The sulfhydryl groups of yeast (Whitehead *et al.*, 1964) and horse liver (Li *et al.*, 1965) alcohol dehydrogenase have been shown to be necessary for enzymatic activity. Preliminary investigation of the effect of iodoacetate on the activity of peanut alcohol dehydrogenase has shown that the enzyme is very sensitive to inhibition by this reagent.

Enzymatic activity was found to be highly temperature-sensitive (0.01*M* pyrophosphate buffer, pH 8.5). When the temperature was increased to 40°C, using a 15-min incubation, the activity was completely lost. In this regard, the enzyme is very similar to yeast alcohol dehydrogenase, whereas the enzyme from horse liver is quite temperature stable (Brand *et al.*, 1962).

CONCLUSIONS

The physical characteristics of peanut alcohol dehydrogenase differ somewhat from those of yeast and mammals. Enzymatically the peanut enzyme has a specific activity similar to that from liver; however, the specificity, pH optimum and Km (ethanol) correspond to the yeast enzyme. Inactivation of the enzyme by heat and 1,10-phenanthroline is characteristic of the effects of these agents on the yeast enzyme, but the loss of activity in the presence of EDTA resembles the behavior of the mammalian enzyme.

REFERENCES

Akeson, A. 1964. On the zinc content of horse liver alcohol dehydrogenase. *Biochem. Biophys. Res. Commun.* **17**, 211–214.

Blair, A. H. and Vallee, B. L. 1966. Some catalytic properties of human liver alcohol dehydrogenase. *Biochemistry* **5**, 2026–2034.

Brand, L., Everse, J. and Kaplan, N. O. 1962. Structural characteristics of dehydrogenases. *Biochemistry* **1**, 423–434.

Dalziel, K. 1958. On the purification of liver alcohol dehydrogenase. *Acta Chem. Scand.* **12**, 459–464.

Ehrenberg, A. and Dalziel, K. 1958. The molecular weight of horse liver alcohol dehydrogenase. *Acta Chem. Scand.* **14**, 465–469.

Hayes, J. E. and Velick, S. F. 1954. Yeast alcohol dehydrogenase: molecular weight, coenzyme binding, and reaction equilibria. *J. Biol. Chem.* **207**, 225–244.

Hoch, F. L. and Vallee, B. L. 1956. Kinetic studies on the role of zinc and diphosphopyridine nucleotide in the activity of yeast alcohol dehydrogenase. *J. Biol. Chem.* **22**, 491–499.

Hughes, T. R. and Klotz, I. M. 1956. Analysis of metal-protein complexes. *Methods Biochem. Analy.* **3**, 265–299.
Kagi, J. H. R. and Vallee, B. L. 1960. The role of zinc in alcohol dehydrogenase. V. The effect of metal-binding agents on the structure of the yeast alcohol dehydrogenase molecule. *J. Biol. Chem.* **235**, 3188–3192.
Li, T. K. and Vallee, B. L. 1965. Reactivity and function of sulfhydryl groups in horse liver alcohol dehydrogenase. *Biochemistry* **4**, 1195–1202.
Lineweaver, H. and Burk, D. 1934. The determination of enzyme dissociation constants. *J. Am. Chem. Soc.* **56**, 658–666.
Lowry, D. H., Rosebough, N. J., Farr, A. L. and Randell, R. J. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**, 265–275.
McKinley-McKee, J. S. and Moss, D. W. 1965. Heterogeneity of liver alcohol dehydrogenase on starch-gel electrophoresis. *Biochem. J.* **96**, 583–587.
Pattee, H. E. and Swaisgood, H. E. 1968. Peanut alcohol dehydrogenase. 1. Isolation and purification. *J. Food Sci.* In press.
Pietruszko, R., Clark, A., Graves, J. M. H. and Ringold, H. J. 1966. The steroid activity and multiplicity of crystalline horse liver alcohol dehydrogenase. *Biochem. Biophys. Res. Commun.* **23**, 526–534.
Pullman, M. E., Colowick, S. P. and Kaplan, N. O. 1952. Comparison of diphosphopyridine nucleotide with its deaminated derivative in various enzyme systems. *J. Biol. Chem.* **194**, 593–602.
Racker, E. 1950. Crystalline alcohol dehydrogenase from baker's yeast. *J. Biol. Chem.* **184**, 313–319.
Sund, H. and Theorell, H. 1963. Alcohol dehydrogenases. In "The Enzymes," ed. Boyer, P. D., Lardy, H. and Myrback, K., Vol. 7, Second Edition, pp. 25–83. Academic Press, New York.
Theorell, H. and Bonnichsen, R. 1951. Studies on liver alcohol dehydrogenase. I. Equilibria and initial reaction velocities. *Acta Chem. Scand.* **5**, 1105–1126.
Vallee, B. L. and Hoch, F. L. 1955. Zinc, a component of yeast alcohol dehydrogenase. *Proc. Nat. Acad. Sci. U. S.* **41**, 327–338.
Vallee, B. L. and Hoch, F. L. 1957. Zinc in horse liver alcohol dehydrogenase. *J. Biol. Chem.* **225**, 185–195.
Van Holde, K. E. and Baldwin, R. L. 1958. Rapid attainment of sedimentation equilibrium. *J. Phys. Chem.* **62**, 734–743.
Von Wartburg, J. P., Bethune, J. L. and Vallee, B. L. 1964. Human liver alcohol dehydrogenase. Kinetic and physicochemical properties. *Biochem.* **3**, 1775-1782.
Wallenfels, K. and Sund, H. 1957. Über den Mechanismus der Wasserstoffübertragung mit Pyridinnucleotiden. I. Freie SH-Gruppen und Akitivität bei Alkoholdehydrogenase aus Hefe. *Biochem. Z.* **329**, 17–30.
Wallenfels, K., Sund, H. and Diekmann, H. 1957. Über den Mechanismus der Wasserstoffübertragung mit Pyridinnucleotiden. IV. Hemmstoffe für DPN-abhängige Zinkenzyme. *Biochem. Z.* **329** 48–58.
Watts, D. C. and Donninger, C. 1962. Starch gel electrophoresis as a criterion for the purity of crystalline yeast alcohol dehydrogenase. *Anal. Biochem.* **3**, 489–497.
Whitehead, E. P. and Rabin, B. R. 1964. The thiol groups of yeast alcohol dehydrogenase. *Biochem. J.* **90**, 532–539.
Williams, R. J. P., Hoch, F. L. and Vallee, B. L. 1958. The role of zinc in alcohol dehydrogenase. III. The kinetics of a time dependent inhibition of yeast alcohol dehydrogenase by the 1,10-phenanthroline. *J. Biol. Chem.* **232**, 465–474.

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Growth of Salmonellae on Irradiated and Non-Irradiated Seafoods

SUMMARY—*Salmonella give* grew competitively in crabmeat at 22°C but not at 11°, 8°, or 5°C. At 22°C the cells grew rapidly reaching high numbers in all samples; but decreased in numbers at all lower temperatures. On English sole tissue, *S. heidelberg, S. typhimurium* and *S. derby* all grew rapidly at temperatures as low as 8°C, from inocula as low as 10^1 cells/g and even in the presence of 10 to 100-fold higher numbers of competing saprophytes. Ionizing radiation at relatively low levels, by reducing the numbers of competitive saprophytes, enhances the growth of salmonellae on fish fillets. No growth was obtained under any condition when the temperature was held below 6°C.

INTRODUCTION

SALMONELLAE DO NOT OCCUR normally on marine fish caught in the open sea; however, from the small amount of data available, it is clear that fish are potential vehicles for all the more important types of bacterial food poisoning (Shewan *et al.*, 1955; Shewan, 1962). Adventitious contamination of fish during handling and processing by bacteria of public health significance can occur (Guelin, 1962). Little is known concerning the actual potential for growth of salmonellae on seafoods.

Radiation pasteurization appears to be a useful and feasible process for seafoods since it significantly extends the shelf life (Masurovsky *et al.*, 1963; Spinelli *et al.*, 1965). The beneficial effects are due to the elimination of the radiation-sensitive bacteria normally causing spoilage and an apparent extension of the lag phase for surviving bacteria.

A number of studies of the bacterial changes involved in this process have been published (Miyauchi *et al.*, 1963). However, little information is available concerning the fate of pathogenic or food poisoning bacteria which may occur as adventitious contaminants on seafoods.

Enterotoxigenic staphylococci have been shown to grow well on sterilized crabmeat held at 12° and 22°C but fail to grow and, indeed, actually decline on non-sterile (untreated) crabmeat held at the same temperature (Slabyj *et al.*, 1965). The same staphylococci were found to grow well in non-sterile crabmeat in which the number of viable spoilage bacteria had been reduced by pasteurizing radiation. This indicated that one effect of such processing might be to relieve the competitive pressure from other bacteria, which normally limits staphylococcal growth.

Salmonellosis is a problem of increasing magnitude in the USA (Salmonella Surveillance Report, Anonymous, 1966) and the origins of many outbreaks are obscure. There is reason to suspect food as the transmission route in many cases.

Factors such as these indicated a need for more information on the growth of salmonellae on seafoods and in particular on the effects of radiation processing on salmonellae on seafoods. This paper describes the results of an investigation into these factors.

MATERIALS AND METHODS

Test organisms

Salmonella serotypes—*heidelberg*, ATCC 8326; *typhimurium*, ATCC 6994; and *derby*, ATCC 6966 were studied because they were among the five serotypes most frequently isolated from human sources (Morbidity and Mortality Reports, Anonymous, 1965). *Salmonella give* was also used in many of the experiments because it was found to be more radiation-resistant than other strains in our laboratory collection.

Inoculum

The inoculum was prepared by adding 18-hr brain heart infusion broth (Difco) culture of the organisms to 0.1% peptone water to obtain 55% transmittency at 660 mμ in a Bausch and Lomb Spectrophotometer. Appropriate serial decimal dilutions for inoculation were prepared in 0.1% peptone water.

Sample preparation

English sole *(Parophrys vetulus)* were caught in Puget Sound by the College of Fisheries research vessel. The fish were iced while still alive and transported to the laboratory within 2 to 4 hr. Fish were either filleted and used immediately or held in iced storage for up to 16 days before filleting and use.

Fresh picked Dungeness crab body meat *(Cancer magister)* was obtained from a local crab processor on the morning of use. Both English sole fillets and crabmeat were distributed in 45 g quantities in 6 mil polymylar pouches, inoculated, heat sealed and stored at the appropriate temperature. In many of the experiments the inoculum was adjusted so that approximately equal numbers of viable cells remained in both irradiated and non-irradiated samples. For microbial enumeration, the entire contents of the pouch were added to 180 ml of 0.1% peptone water and homogenized for 1½ min at high speed in a Waring Blendor; the sample homogenate was then appropriately subcultured.

Tissue slurry

Crabmeat and English sole tissue slurries were prepared by blending 1 part tissue and 4 parts distilled water in a Waring Blendor at high speed for 2 min. The slurry was added to screw cap tubes and autoclaved.

Storage temperatures

Crabmeat and English sole tissue inoculated with salmonellae were stored at 5°, 8°, 11° and 22°C and 6°, 8°, 12° and 22°C respectively.

Enumeration

Salmonellae counts were obtained by the drop plate method on brilliant green agar (Difco) using 0.1 or 0.01 ml volumes of the appropriate homogenate dilution and by the MPN technique using a selenite broth (Raj and Liston, 1965). Both media were incubated at 37°C. Total viable counts of the mixed flora were obtained by the pour plate method using trypticase phytone yeast extract agar (Miyauchi *et al.*, 1963) and an incubation temperature of 22°C. The composition of the TPY agar is: trypticase, 15 g; phytone, 5 g; yeast extract, 5 g; NaCl, 5 g; agar, 15 g; water, 1 l; pH, 7.2.

Radiation

The packaged English sole fillets and crabmeat were irradiated in the College of Fisheries with 50 to 100 Krads of gamma radiation in the Cobalt 60 Mark II Food Irradiator at a dose rate of 300 Krads/hr and a temperature of 24°C.

RESULTS

Growth in crabmeat

The growth of *S. give* was tested in untreated crabmeat and crabmeat irradiated at 50 and 100 Krad. The organism grew well in all samples held at 22°C, reaching maxima between 10^6 and 10^8 cells/g of tissue in 6 days (Fig. 1). The increase in *Salmonella* count occurred concomitantly with an increase in total microbial count. *Salmonella* reached higher numbers in samples irradiated with 100 Krads than in either non-irradiated samples or samples irradiated with 50 Krad. This appears to be due to a greater reduction in numbers of competing spoilage organisms in the samples irradiated with the higher dose. However, the samples irradiated with 50 Krad also had a lower initial *Salmonella* inoculum, further weakening their competitive position.

Since *S. give* was able to grow and compete with the saprophytic population on fresh crabmeat at 22°C, its ability to grow at lower temperatures on this seafood product was also tested. After irradiation at 0, 50 and 100 Krads and storage at 5, 8 and 11°C, there was no increase in viable count of *S. give* inoculated into crabmeat as shown in Fig. 2 and Table 1. Indeed, under these conditions an actual decline in numbers of *Salmonella* was observed during storage but concurrently the total saprophytic count increased in both the irradiated and non-irradiated samples.

Table 1. Growth of *Salmonella give* on crabmeat.

	0	50	100
		Log no./g sample Dose, Krad	
Storage (days) 5°C			
0	.....	6.1	6.7
7	5.9	5.0	6.0
14	5.2	4.3	5.1
21	5.1	3.6	5.4
28	5.5	4.4	5.5
Storage (days) 11°C			
0	5.5	5.1	5.1
7	4.6	3.0	2.1
14	4.6	2.7	2.1
21	4.6	2.7	2.0
28	4.4	0	0

The same general pattern was obtained with *S. heidelberg, S. typhimurium* and *S. derby* when inoculated into crabmeat, irradiated at 50 Krad and stored at 8°C (Table 2). At this temperature salmonellae counts showed a continued decline while the total counts increased throughout storage.

The salmonellae tested were thus unable to grow at low temperatures in the presence of the competing spoilage flora on non-sterile crabmeat. However, when these serotypes were inoculated into sterile crabmeat slurry and incubated at 8°C, the organisms survived and grew reaching counts as high as 10^7/g in 14 days (Table 3).

Growth in English sole

The growth pattern on non-sterile English sole was quite different from that obtained in non-sterile crabmeat.

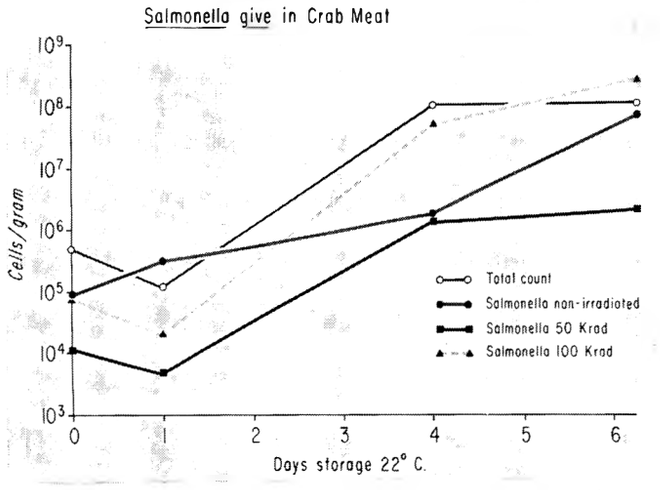

Fig. 1. Growth of Salmonella give *on crabmeat at 22°C.*

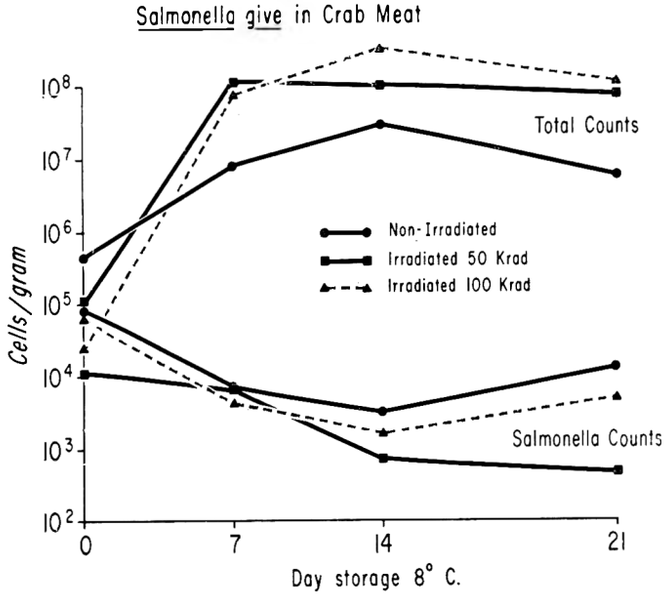

Fig. 2. Changes in Salmonella give *and total counts on crabmeat during storage at 8°C.*

Table 2. Growth of salmonellae and saprophytes on crabmeat incubated at 8°C.

Test organism	Storage (days)	Total counts (saprophytes)		Salmonellae	
		Non-irradiated	Irradiated 50 Krad	Non-irradiated	Irradiated 50 Krad
		Log no./g sample			
Salmonella heidelberg	0	4.98	4.67	4.59	4.08
	3	6.25	6.98	4.32	3.53
	10	6.65	8.70	4.32	1.95
Salmonella typhimurium	0	4.81	3.93	3.93	2.20
	3	6.28	6.95	<3.0	<2.0
	10	6.28	8.41	<2.0	<1.0
Salmonella derby	0	4.90	3.93	3.11	2.25
	3	6.28	7.15	2.95	<2.0
	10	6.32	8.50	<2.0	<1.0

Table 3. Growth of salmonellae in sterile crabmeat slurry at 8°C.

Test organism	Days storage			
	0	3	6	14
	Log no./g slurry			
S. typhimurium	4.96	4.51	5.31	6.20
S. derby	4.90	5.55	5.20	6.40
S. heidelberg	5.06	4.92	5.91	7.81
S. give	6.1	6.1	6.0	7.2

Table 4. Growth of salmonellae in sterile English sole slurry at 8°C.

Test organism	Days storage			
	0	3	6	14
	Log no./g slurry			
S. typhimurium	4.98	5.31	5.80	6.28
S. derby	5.32	4.88	4.83	6.19
S. heidelberg	5.46	5.36	5.92	8.30

Salmonellae and spoilage bacteria grew more rapidly, and salmonellae grew more competitivly at lower temperatures on English sole than in crabmeat. *S. heidelberg* grew on English sole at 22°, 12° and 8°C but not at 6°C (Table 5). At 22°C salmonellae populations increased by over 1,000-fold in about 1 day on both irradiated and non-irradiated samples. The spoilage flora increased by over 10,000-fold in about one day in all samples.

Salmonellae inoculated at levels of 10^4 cells/g made up the majority of the bacterial population on the fillets which contained only 10^2 to 10^3 spoilage organisms/g and this may account for their rapid growth. In samples stored at 12° and 8°C, *S. heidelberg* was still able to compete effectively with the saprophytes and increased approximately 1,000-fold in 18 to 21 days in both irradiated and non-irradiated samples. At 6°C, *S. heidelberg* declined in both irradiated and non-irradiated samples, while spoilage saprophytes continued to grow.

The growth of salmonellae serotypes in sterile fish slurry was very similar to that obtained with the same serotypes in sterile crabmeat slurry (Table 4). The organisms

Table 5. Growth of *Salmonella heidelberg* and saprophytes on English sole fillets.

Incubation temperature	Storage (days)	Total counts (saprophytes)		Salmonellae	
		Non-irradiated	Irradiated 50 Krad	Non-irradiated	Irradiated 50 Krad
		Log no./g sample			
6°C	0	3.78	3.53	5.84	5.81
	7	7.26	7.78	5.34	5.40
	14	7.95	8.41	4.30	<3.0
8°C	0	2.93	2.11	5.81	5.93
	8	8.04	7.74	8.54	7.78
	13	8.57	8.08	8.51	8.48
	22	8.28	8.43	7.90	7.81
	28	8.30	8.53	7.81	7.70
12°C	0	3.11	2.36	4.71	5.53
	7	7.3	6.18	.....	.....
	14	8.56	8.83	9.08	8.40
	21	9.71	8.36	9.04	8.56
22°C	0	2.93	2.11	5.81	5.93
	1	7.45	6.58	9.20	9.11
	2	8.93	8.70	8.98	8.98
	5	8.78	8.93	8.40	9.00

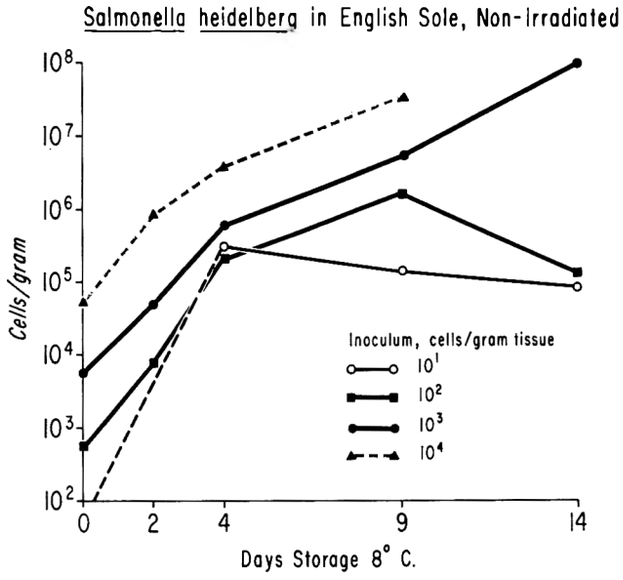

Fig. 3. Growth of low levels of Salmonella heidelberg *on English sole tissue at 8°C non-irradiated.*

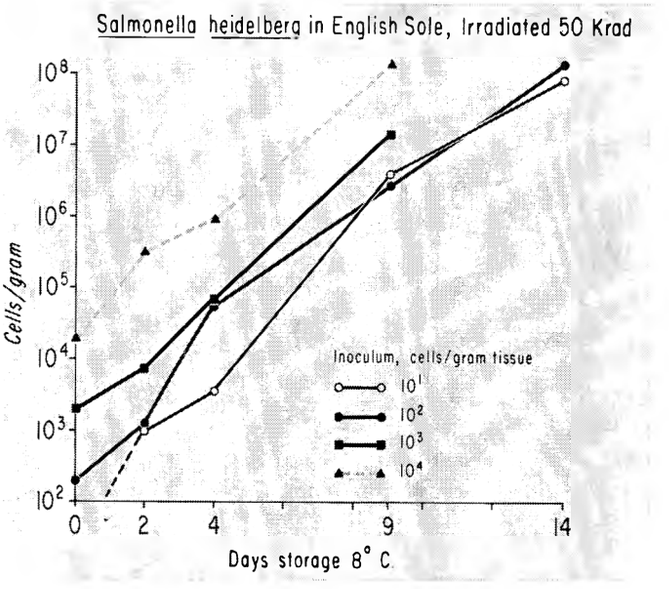

Fig. 4. Growth of low levels of Salmonella heidelberg *on English sole tissue at 8°C irradiated with 50 Krad.*

reached log counts between 6 and 8 in 14 days at 8°C in the heat sterilized slurry. *S. heidelberg* grew at a faster rate than the other serotypes tested in both slurries.

The high levels of salmonellae inocula (10^4 to 10^5 cells/g) used in the earlier experiments facilitated enumeration and provided advantageous conditions for competitive growth. However, it was recognized that they are quite unrepresentative of levels of contamination likely to occur on seafoods. Some experiments were, therefore, performed in which lower levels of inocula were used.

Growth from lower levels of initial inocula (10^1, 10^2, 10^3, 10^4 cells/g) of *S. heidelberg* on English sole fillets at 8°C is shown in Fig. 3. Storage of samples inoculated with 10^4/g was terminated after 9 days while the other samples were held an additional 5 days. In the sample with the lowest inoculum (10^1 cells/g), the *Salmonella* population increased and reached a maximum after 4 days, but declined thereafter, probably due to competitive growth of the spoilage microflora. When inoculated with 10^2 cells/g the *Salmonella* population continued to increase for 9 days before it started to decline. With higher levels of inoculum, the pathogen continued to increase throughout the storage period (14 days).

The effects of irradiation on the growth of low levels of *Salmonella* can be seen in Fig. 4. In these experiments, irradiation reduced the numbers of saprophytes sufficiently to allow low numbers of *Salmonella* to grow uninterruptedly even when the lowest level of inoculum was used. The numbers increased steadily throughout the storage period reaching high levels within 9 to 14 days.

Two other serotypes were inoculated at levels between 10^1 and 10^5 cells/g of fish tissue. *Salmonella derby* grew rapidly, reaching high numbers after 9 days storage at 8°C (Table 6) The growth of *S. typhimurium* was neither as rapid as the growth of *S. derby* nor the increase in numbers of cells as large after 9 days incubation. The suppressing effects of saphophytes on the growth of *S. heidelberg* and *S. typhimurium* were more pronounced than their effect on *S. derby*. In these cases, all levels of salmonellae inocula grew throughout the 9 days of storage at 8°C in both irradiated and non-irradiated samples.

Table 6. Growth of low levels of salmonellae and saprophytes on English sole fillets at 8°C.

		Total counts (saprophytes)		salmonellae					
Test organism	Storage (days)	Non-irradiated	Irradiated 50 Krad	Non-irradiated			Irradiated 50 Krad		
		Log no./g sample							
Salmonella typhimurium	0	1.7	.....	3.18	5.26		2.0	4.56	
	2	3.11	1.65	3.0	5.63		.....	3.70	
	5	7.23	5.26	4.18	5.18		2.65	4.78	
	7	7.04	8.18	5.28	6.32		3.04	5.46	
	9	7.81	7.78	6.04	5.87		2.93	6.60	
Salmonella derby	0	2.81	1.48	0.88	1.88	2.88	1.08	2.08	3.08
	3	6.0	5.08	3.90	4.48	5.08	3.88	4.3	4.0
	6	7.78	7.63	6.88	6.26	7.49	6.15	5.78	7.08
	9	8.48	8.49	7.70	7.74	8.18	7.43	7.40	7.74

DISCUSSION

Salmonellae will apparently grow at low temperatures on seafoods if the correct conditions are present. In the case of crabmeat, the required condition appears to be the absence of other organisms, but English sole, which might be considered reasonably representative of low fat fish, seems to provide the required condition *per se*. It is doubtful that competition provides the sole explanation for the failure of salmonellae to grow on crabmeat though the higher temperature experiments with *S. give* indicate that reduction of the competing saprophytic population by radiation will affect the extent of salmonellae growth.

Berry (1952) reported that *S. aertrycke* and *S. morgani* failed to grow on autoclaved crabmeat at 5°C but grew at 25° and 37°C. In our experiments, slurries provided a somewhat different environment from picked crabmeat in which, due to the brine immersion treatment during processing, the salt content varies between 0.35 and 0.72 molal NaCl and water content 75.8 to 79.5% (Nelson and Thurston, 1964). It is possible that this treatment renders crabmeat unsuitable for salmonellae growth at low temperatures, though experiments with salt-containing media indicate that the calculated a_w in crabmeat is not low enough to inhibit growth. Tests in which nutrient broth was added to crabmeat inoculated with the pathogen established that nutrient limitation was not the primary growth restricting factor.

It is interesting that reduction in the size of the saprophytic population on English sole by irradiation affected the growth level of salmonellae. Very low inocula of 10^1 cells/g showed essentially unlimited growth at 8°C on fillets given 50 Krad treatment, while similar inocula on untreated fillets grew only to a limited maximum of nearly 10^6 cells/g and then declined on untreated fillets.

This observation and the similar results for *S. give* in crabmeat at 22°C conform to the pattern reported by Slabyj *et al.* (1965) for staphylococci and suggest that a competitive ratio which may be qualitative as well as quantitative is significant in the natural control of outgrowth of food poisoning organisms on seafoods.

Fish spoilage organisms have a much shorter generation time at low temperatures than salmonellae and can compete more effectively for the available nutrients. Pasteurizing radiation eliminates many of the saprophytic bacteria and apparently impairs the competitive effectiveness of the survivors. This may account for the depression of the serotypes tested; however, it does not expain why the growth of *S. heidelberg* is depressed to a greater extent than either *S. typhimurium* or *S. derby*.

In the experiments, salmonellae were added to the tissue before irradiating. The effects of adding non-irradiated salmonellae to irradiated tissue and adding irradiated salmonellae to non-irradiated tissue were not tested. These variations may produce different *Salmonella* growth responses and should be tested.

In all of the experiments, temperatures a few degrees below 8°C (e.g., 5° or 6°C) were found to inhibit completely the growth of salmonellae and indeed to bring about a progressive reduction in their numbers. Since most seafood products are held at temperatures below 8°C this provides an explanation (in part) of the very low reported incidence of salmonellosis attributed to consumption of seafoods. However, in view of the reduction of the competing saprophytes by radiation, it is important that radiation processed foods be rendered free of, as well as protected from, subsequent infection by salmonellae and stored at temperatures below 6°C.

REFERENCES

Anonymous. 1965. Communicable Disease Center morbidity and mortality weekly report 14:210, U. S. Dept. of Health, Education and Welfare, Washington, D. C.

Anonymous. 1966. Communicable Disease Center *Salmonella* Surveillance Report, Annual Summary, 1965, U. S. Dept. of Health, Education and Welfare, Washington, D. C.

Berry, C. 1942. The multiplication of bacteria in sterile crabmeat. *J. Bacteriol.* **44**, 145.

Guèlin, A. 1962. Polluted waters and the contamination of fish. In "Fish as Food," Vol. II, edited by George Borgstrom, pp. 481–502.

Masurovsky, E. B., Goldblith, S. A. and Nickerson, J. T. B. 1963. Effects of substerilization doses of Co^{60} gamma radiation on the cold storage life extension of shucked soft-shelled clams and haddock fillets. *Appl. Microbiol.* **11**, 220–228.

Miyauchi, D., Eklund, M., Spinelli, J. and Stoll, N. 1963. Application of radiation-pasteurization processes to Pacific crab and flounder. Report No. TID 19585, Isotopes-Industrial Technology TID 4500 (19th ed.), Office of Technical Services, Dept. of Commerce, Washington, D. C., U. S. A.

Nelson, R. W. and Thurston, C. E. 1964. Proximate composition, sodium, and potassium of Dungeness crab. *J. Am. Diet. Assn.* **45**, 41–43.

Raj, H. and Liston, J. 1965. A highly sensitive enrichment medium for *Salmonella* from foods. *Bacteriol. Pro. p.* 3.

Shewan, J. M. 1962. Food poisoning caused by fish and fishery products. In "Fish as Food," Vol. II, edited by George Borgstrom, pp. 443–466.

Shewan, J. M. and Liston, J. 1955. A review of food poisoning caused by fish and fishery products. *Appl. Bacteriol.* **18**, 522–534.

Slabyj, B. M., Dollar, A. M. and Liston, J. 1965. Post-irradiation survival of *Staphylococcus aureus* in seafoods. *J. Food Sci.* **30**, 344–350.

Spinneli, J., Eklund, M., Stoll, N. and Miyauchi, D. 1965. Irradiation preservation of Pacific Coast fish and shellfish. III. Storage life of Petrale sole fillets at 33° and 42°F. *Food Technol.* **19**, 1016–1020.

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Thermal Resistance of Spores of Five Strains of Clostridium botulinum Type E in Ground Whitefish Chubs

SUMMARY—Thermal death-time determinations in the temperature range of 165°F (73.9°C) to 185°F (85°C) were made of spores of five strains of *Clostridium botulinum* type E in tubed fish paste prepared from whole Great Lakes whitefish chubs. Estimated F_{170} (80°C) values for the destruction of approximately 1×10^{11} spores per gram of fish paste were as follows: for strain Alaska, 34.2 min; Beluga, 17.0 min; 8E, 14.0 min; Iwanai, 12.5 min; and Tenno, 13.2 min. Estimated 95% confidence limits about these F_{170} values were for strain Alaska, 23.4—49.5 min; Beluga, 14.2—20.2 min; 8E, 8.2—17.7 min; Iwanai, 10.2—15.4 min; and Tenno, 9.3—18.5 min. D_{176} values calculated from our F_{170} values by Schmidt's (1957) equation were for strain Alaska, 4.3 min; Beluga, 2.1 min; 8E, 1.8 min; Iwanai, 1.6 min; and Tenno, 1.6 min. Z_F values obtained from thermal death-time determinations were for strain Alaska, 13.2; Beluga, 13.3; 8E, 10.3; Iwanai, 13.6; and Tenno, 13.1°F.

INTRODUCTION

THE RESISTANCE of spores of *C. botulinum* type E to heat has been of major interest since a high incidence of type E botulism (Dolman *et al.*, 1963) culminated in the 1963 outbreak in the United States, in which 17 cases and 7 deaths (U. S. Dept. of Health, 1963) were attributed to the consumption of smoked Great Lakes whitefish chubs.

Heat is the most inexpensive and widely used effective agent in the destruction of food spoilage microorganisms; hence, it is a natural first choice as a suitable control method for ensuring a safe food product. In lightly smoked fish, however, smoking is not carried out to preserve the product, and the thermal exposures in the smoking process traditionally were not directed toward producing a safe product.

The present study was carried out to contribute data on the thermal resistance of five recognized strains of *C. botulinum* type E in a fish menstrum, as a step toward better understanding of the degree of thermal processing necessary to reduce the hazards from type E botulism in this type of product, apart from brining and other measures that conceivably may prove useful in enhancing its safety.

MATERIALS AND METHODS

Cultures

Strains Beluga, Alaska, Iwanai and 8E were obtained as spore suspensions through the courtesy of Dr. C. F. Schmidt, Continental Can Company, Chicago, Ill. Strain Tenno in lyophilized form had been supplied the senior author by Dr. G. Sakaguchi in 1956 and was retained in the original form until subcultured in 1964 for use in the present studies.

Cultures were maintained in the frozen state. They were subcultured in tubes of a laboratory-prepared cooked-meat medium prepared from lean beef (neck meat, beef heart, or veal heart) according to a previous edition of the Manual of Methods for the Pure Culture Study of Bacteria (Society of Amer. Bacteriologists, 1936). After 36 to 48 hr at 35°C, cultures were transferred to bottles containing 150 ml of the same medium and were again incubated for the same period. They were then shaken, allowed to settle, and the fluid portion together with small meat particles was dispensed in 2.0-ml quantities into 5.0-ml pharmaceutical vials. These were flame sealed, quick frozen at −60°C, and stored in the deep freeze.

Production of spore suspensions

Spore suspensions were prepared according to the method of Schmidt *et al.* (1962), except that stock cultures for inoculation of the trypticase-peptone-glucose sporulation medium were made by seeding the frozen-thawed meat cultures into tubes of the sporulation broth to which was added 0.5 g per liter of Bacto-agar (incubated at 29.5 to 30°C for 24 hr). This was followed by preparing a second transfer into tubes of sporulation broth without added agar (incubated at 29.5 to 30°C for about 40 hr). These second subcultures were then heat-treated by immersion in a water bath at 60° for 15 min, and inoculated into bottles of the trypticase-peptone-glucose medium. The remaining steps of the procedure were carried out essentially as described by Schmidt *et al.* (1962).

Preparation of fish paste

Whole frozen Great Lakes whitefish chubs were ground in a commercial Hobart Model A120 food grinder (Hobart Mfg. Co., Troy, Ohio), and 200 g were dispensed into 12-oz wide-mouth refrigerator jars. The filled jars were autoclaved at 121°C for 30 min, allowed to cool to room temperature, and stored in the refrigerator until needed. They were tested for sterility by inoculating representative samples into thioglycollate sterility test medium.

Viable counts

Viable counts of stock spore suspensions were made by the method of Schmidt (1964) in a medium composed of Bacto-peptone, 5.0%; Bacto-agar, 1.2%; and with a pH of 7.0. The medium was put up in 10-ml quantities in clear 16-mm × 150-mm screw-capped test tubes. Sterile tubes

of media were steamed and allowed to equilibrate to 50°C, following which 10% sterile sodium thioglycollate and 10% sterile (Seitz-filtered) sodium bicarbonate were aseptically added to final concentrations of 0.2% and 0.14%, respectively.

Initial 1:4 dilutions of stock spore suspensions were made in sterile distilled water with 1.0 ml of stock suspension. Two ml of this initial suspension in a 16-mm × 150-mm screw-capped tube was heat-shocked by immersing the tube in a water bath at 60°C for 15 min, then rapidly cooling it in a container of cold water. The initial suspensions, both heated and unheated, were then diluted, and 0.1, 0.2 and 0.3 ml of the 10^{-5}, 10^{-6} and 10^{-7} dilutions were seeded into the complete medium, equilibrated at 50°C, and mixed.

The seeded tubes were immediately placed in a rack immersed in tap water to which ice cubes were added to bring the water to about 10 to 15°C. The seeded tubes were overlayed with 3.0 ml of sterile 1.5% Bacto-agar to which sterile 0.2% sodium thioglycollate solution was added during equilibration at 50°C. Tubes were incubated at 30°C, and the resulting deep agar colonies were counted at 48 hr, and at intervals up to 14 days. Counts were most reproducible in the range of 10 to 30 colonies per tube. This range was subsequently employed as the best standard of validity.

Seeding of the sterile fish paste was based on that volume of concentrated stock spore suspension which, when added to 200.0 g of fish paste, yielded 10^6 heat-resistant spores per gram (based on surviving spores in 2 ml of a 1:4 suspension capable of surviving an immersion time of 15 min at 60°C in a 16 × 150-mm screw-cap Pyrex test tube immersed to the lower rim of the cap).

Stock suspensions were enumerated at periodic intervals to ensure against changes in viability or heat resistance that might alter their characteristics. Refrigerated, stored tubes of spore suspensions in distilled water remained quite stable for as long as 21 months.

Preparation and treatment of thermal death-time tubes

The method of Angelotti *et al.* (1961) was used to prepare, fill, and seal the thermal death-time (TDT) tubes with 1.0 ± 0.1 g of seeded fish paste containing about 10^6 spores. In the present study, however, the grease gun filling apparatus was modified by eliminating the metal ball valve in the nozzle to prevent jamming by fish-bone fragments, and substituting a Fisher Electro Hosecock, A. C. Model (Fisher Scientific Co., Pittsburgh, Pa.) activated by a togle switch clamped to the grease gun handle. The hosecock was adjusted to open and close on a short length of rubber tubing placed between the outlet of the grease gun and a glass nozzle through which fish paste was delivered into TDT tubes. After repeated trials a further modification was made by adding machined spacer bushings on the pressure piston to increase the accuracy of delivery that was lost in the removal of the ball valve.

The grease gun was chilled during filling operations by means of two medium-sized pillows of Sno-Gel Non-Melting Reusable Ice (Sno-Gel Co., Lodi, Calif.) molded in the frozen state to enclose the barrel of the grease gun.

Heat resistance tests were run on all strains at 73.9°C (165°F), 79.4°C (175°F), and 85°C (185°F). Tests at 76.7°C (170°F) also were run on strains Alaska and Tenno.

Following treatment, the seeded fish paste was recovered from the sealed tubes by scoring the tubes with a motor-driven glass tubing cutter, snapping them open, and with an inoculating needle shaped into a helix, removing approximately 0.25 to 0.5 g of the contents for subculturing. Ten tubes at each interval were subcultured into tubes of a Noyes veal-broth medium (Angelotti *et al.*, 1962), modified by the addition of 0.2% soluble starch and 0.5% glucose (incorporated before autoclaving).

The recovery medium was put up in 16 × 150-mm tubes each containing 8.0 ml of broth and 1.0 g of veal. Meat in the medium provided a spongy mass ideal for trapping and holding small bubbles of gas, thus facilitating detection of growth especially during long periods of delayed incubation.

The recovery cultures were incubated at 30 ± 0.5°C and examined for gas production and growth after 24, 48, 72, 120 and 168 hr. The cultures were then removed from the incubator and further incubated at room temperature (approximately 25°C) to allow for delayed germination. During this period they were observed at 14 days, 21 days, 1 month, and subsequently at convenient intervals for a total of 6 months.

Presence of toxin and absence of contaminants were confirmed as follows: in recovery cultures from each experiment, 5 cultures from the last heating interval showing growth in all 10 tubes and all tubes showing growth in recoveries from subsequently longer heating intervals were tested for specific type E toxicity by the mouse test. Control animals were protected with type E anti-toxin (purchased from the Communicable Disease Center, Atlanta, Georgia). Recovery cultures also were streaked on plates of trypticase soy agar to detect aerobic contamination. All cultures that showed specific type E toxicity were included in the data.

Measurement of the rate of heat penetration

Measurements of the rate of heat penetration were made using copper-constantan thermocouples essentially as described by Angelotti *et al.* (1961). Corrections for thermal lag and lethality during lag were computed as previously described (Anellis *et al.*, 1954; Angelotti *et al.*, 1961) except that they were applied for the range from 125°F to the various test temperatures.

The calculated correction factors representing additional minutes of lethal exposure occurring during come-up and cool-down for each of the five spore strains ranged from 1.0 to 1.3 at 165°F, 0.9 to 1.2 at 175°F, and from 1.0 to 1.1 at 185°F. Values at 170°F were obtained only for Tenno and Alaska strains and were both 1.1.

RESULTS AND DISCUSSION

TABLE 1 SHOWS the raw data in terms of the last time interval at which some cubes were positive and the first interval at which all tubes were negative. The original experimental design was based on replicates run at three temperatures. The extra values at 165°F for Alaska were determined for comparison with results for the other strains because the results for this strain were found to be

Table 1. Thermal death-time data based on observation of viability after 6-month incubation of recovery cultures of *Clostridium botulinum* type E in veal broth medium seeded with heat-treated whitefish chub paste containing 1×10^6 spores per g.

	Time (min) of the longest heating interval showing at least one viable out of 10 recovery cultures, and the shortest time interval showing no viable recovery cultures at temperatures of:							
	165°F		170°C		175°F		185°F	
Strain	Viability	Kill	Viability	Kill	Viability	Kill	Viability	Kill
Alaska	155	165	90	100	71	71	8	9
			105	111	43	56	6	7
Beluga	110	115	.....	.....	24	26	4	5
	105	110			22	24	5	6
8E	140	147	.....	.....	21	24	4	5
	146	153			21	24	2	3
Iwanai	68	80	.....	.....	10	12	4	5
	76	84			16	18	4	5
	86	93						
	101	108						
Tenno	130	145	32	34	12	15	3	4
	113	120	25	26	21	24	6	7
	108	114	24	26				

higher than the others at 170, 175 and 185°F. No attempt was made to present a balanced statistical design because of the large amount of time and labor involved.

The data include results of all experiments in which valid end points were attained. Initially it was difficult to reach end points when the few published Z_F values for type E *C. botulinum* were applied to the first data at 170 and 175°F for predicting heating intervals at other temperatures. Many unsuccessful experiments resulted from missed end points due to this factor. Also, some experiments in which an end point was reached after incubation of several days or weeks were later nullified by delayed germination after prolonged incubation and had to be repeated in order to locate the true end point.

Each pair of values, one for viability and one for lethality, was averaged to yield a single thermal death-time value. When these thermal death-time values were corrected for come-up and cool-down of samples as specified by Anellis *et al.* (1954), they yielded the average corrected F values shown in Table 2. The estimated thermal death-time curves were computed from statistical analysis of the data in Table 2.

Townsend *et al.* (1938) suggested a procedure for treating thermal death-time data based on rules used to fit a best visual line. The pair of points representing the longest heating interval yielding viable cultures and the shortest heating interval yielding complete kills are plotted on the $\log_{10}$ axis versus temperature, and a line is drawn between the points over the temperature range. More recent studies (Pflug *et al.*, 1953; Read *et al.*, 1966) utilize the least squares technique to fit the regression of $\log_{10}D$ and $\log_{10}F$ respectively versus temperature.

The statistical method employed in this study was least squares linear fit, which was further extended to include certain probability statements. First, limits were calculated for the line so that the true theoretical line would fall between the bounds 95% of the time. Z_F and an appropriate set of limits were then calculated for each strain where Z_F is the temperature change (°F) required to effect a one-log drop in F.

The general method for fitting a linear regression, and calculating confidence intervals is given by Ostle (1963). Assumptions for this analysis are given below.

a). Temperature (X) was measured essentially without error.

b). For any temperature, the possible $\log_{10}F$ (Y) values were assumed to be independently and normally distributed with mean $= \alpha + \beta X$ and $\sigma^2_{y/x}$. Alpha (α) is the true population intercept and β the true population slope where X represents temperature (°F).

c). The variance of Y_i's are the same for all values of temperature (X).

Table 2. The average value between longest heating interval showing at least one viable culture (min) and shortest heating interval showing no viable recovery cultures (min) corrected for come-up and cool-down time.

	Temperature (F°)			
Strain	165	170	175	185
Alaska	158.5	93.6	73.5	7.0
		106.6	45.5	5.0
Beluga	111.0		23.7	2.9
	106.0		21.7	3.9
8E	141.8		21.2	2.9
	147.8		21.2	0.9
Iwanai	72.6		9.8	3.0
	78.6		15.8	3.0
	88.1			
	103.1			
Tenno	136.1	31.5	12.2	2.0
	115.1	24.0	21.2	5.0
	109.6	23.5		

Although the assumptions may not exactly represent the actual situation, the test is enough for most practical purposes. The regression lines were calculated from the data in Table 2. Z_F values were calculated by inverting the linear equation slope b_1 (i.e. $Z_F = 1/b_1$). The estimated equations are,

$$Y = b_o + b_1 X$$

where b_o = the estimate of the true intercept α,
b_1 = the estimate of the slope β,
X = temperature (°F)
$Y = \log_{10} F$.

Approximate 95% confidence limits around Z_F were obtained by inverting the limits calculated for b_i as described by Ostle (1963). These limits are not symmetrical. They do, however, give an indication of the variability associated with measuring Z_F.

Data from Table 2 were used, as shown by Ostle (1963), to calculate the linear regression lines and confidence intervals. Data points from these lines are presented, for convenience, in tabular form in Table 3 together with the confidence limits applicable to each point. From the data presented, the estimated F_{176} values for destruction of approximately 1×10^6 type E spores per gram of fish were for strain Alaska, 34.2 min; Beluga, 17.0 min; 8E, 14.0 min; Iwanai, 12.5 min; and Tenno, 13.2 min.

In Table 4 are recorded the Z_F, the intercept, slope of line, and the approximate confidence intervals around Z_F, of the thermal death-time curves from which the data in Table 3 were taken. The 95% confidence intervals are smallest at the point $(\overline{X}, \overline{Y})$ X mean, Y mean, because a term used to calculate the limits, $(X_o - \overline{X})^2$, becomes zero at $X_o = \overline{X}$. The limits are larger as X_o becomes greater or smaller than $\overline{X}$. Values of X_o used to calculate the estimated line are the same as those observed in the experiments at 165, 170, 175 and 185°F.

The coefficient of determination (r^2), proportion of the sum of squares due to linear regression, gives an indication of the linearity of the data. For the five sets of data r^2's ranged from 0.92 (Tenno) to 0.99 (Beluga). Most replicate determinations are in close agreement.

Comparisons of these data with previously published results are difficult because of the paucity of the earlier data and, as well, differences in the substrates and methods employed. Most of the earlier information is presented in the form of D values calculated from survivor curves rather than in the form of the thermal death-time data presented here. For comparison, we used the Schmidt (1957) interconversion equation for calculating D from F values, $D = \frac{F}{\log A + 2}$ where A is the number of spores in the initial inoculum. At 80°C (176°F), D values in our experiments for the strains tested were: Alaska, 4.3; Beluga, 2.1; 8E, 1.8; Iwanai, 1.6; and Tenno, 1.6 min. Our values were not greatly divergent from the decimal reduction time at 80°C reported by Ohye *et al.* (1957) of 3.3 min for their 103 strain (the Nanaimo strain isolated by Prof. Dolman), but differed markedly from their D value of 0.4 min for their 108 strain (a Russian isolate obtained from Dr. K. F. Meyer). Ohye *et al.* (1957) heated their suspensions in M/15 phosphate buffer at pH 7.0 with recovery in pork infusion thioglycollate starch agar incubated for 2 weeks at 25°C. Schmidt (1964) reported a D_{176} value for the Minneapolis strain heated and subcultured in trypticase-peptone-glucose broth of 1.8 min and for suspensions treated in M/15 phosphate buffer, pH 7.0, a value of 2.3 min. These values are similar

Table 3. Heat resistance of five strains of *C. botulinum* type E spore suspensions in sealed tubes containing 1.0 ± 0.1 g of sterile ground whole whitefish chub seeded with approximately 1×10^0 spores.[1]

	Calculated F[2] values and approximate 95% confidence limits around each value									
Strain	F_{165}	Confidence limits	F_{170}	Confidence limits	F_{175}	Confidence limits	F_{180}	Confidence limits	F_{185}	Confidence limits
Alaska	233.6	126.5–431.6	97.5	63.4–149.9	40.7	28.7–57.7	17.0	10.5–27.4	7.1	3.8–13.1
Beluga	114.8	88.3–149.1	48.3	39.0– 59.3	20.3	17.2–23.9	8.6	6.9–10.5	3.6	2.8– 4.7
8E	160.9	72.5–356.9	54.0	27.5–100.0	17.2	10.4–28.4	5.7	2.9–10.7	1.8	0.8– 4.1
Iwanai	81.1	63.4–130.9	34.5	28.0– 50.0	14.8	12.3–18.0	6.4	4.9– 8.2	2.7	2.0– 3.8
Tenno	91.1	59.1–140.5	37.9	27.6– 52.0	15.8	11.5–21.6	6.5	4.1–10.3	2.7	1.5– 5.0

[1] Data based on linear regression and calculation of confidence limits according to Ostle (1963).
[2] Minutes of exposure necessary to reduce the inoculum in 10 tubes to a level not detectable by our methods after 6 months incubation.

Table 4. Z_F, intercept, slope of line, and approximate confidence intervals around Z_F of the thermal death-time curve calculated for heat resistance of five strains of spores of *Clostridium botulinum* type E. Comparison with previously published Z values.

					Previously published data			
Strain	b_0 intercept	b_1 slope	Z_F (°F)	Approx. confidence intervals about Z_F	r^2	Reference	Suspending medium	Z value
Alaska	14.8948	−0.0759	13.2	10.2–18.5	0.94	Roberts *et al.* (1965)	Water	10.5
Beluga	14.4808	−0.0753	13.3	11.9–15.0	0.99	Roberts *et al.* (1965)	Water	9.5
8E	18.2373	−0.0972	10.3	8.1–14.2	0.96	Roberts *et al.* (1965)	Water	7.4
Iwanai	14.0777	−0.0738	13.6	12.0–15.6	0.98	Roberts *et al.* (1965)	Water	10.0
Tenno	14.5303	−0.0762	13.1	10.6–17.2	0.92	Sakaguchi *et al.* (1954)	M/15 phosphate buffer pH 7.0	9.3

to ours, except for the greater resistance of the Alaska strain.

On the other hand, Roberts *et al.* (1965) report estimated D_{176} values for four of the strains studied by us as follows: strain Alaska, 0.6 min; Beluga, 0.75 min; Iwanai, 1.0 min; and 8E, 0.8 min. Their suspensions were heated in water and recovered in Reinforced Clostridial Agar (Oxoid) incubated for 4–5 days. Sakaguchi *et al.* (1954) have reported a D_{176} value of 0.8 for the Tenno strain heated in M|15 phosphate buffer, pH 7.0. Somewhat lower than our results, the values of Roberts *et al.* (1965) and Sakaguchi *et al.* (1954) nevertheless show a general pattern of heat sensitivity as compared with the widely accepted heat resistance of types A and B.

One of the authors (Peeler) considered a mathematical model suggested to explain the F-D interconversion. The intrinsic value of the equation appears to be adequately bolstered by the interconversion suggested by Schmidt (1957) whose equation was obtained from experimental data. Further, this equation may explain the presence of skips and the necessity for their inclusion in thermal-death data.

Several assumptions are necessary to develop the required mathematical relation between D and F values. First, we assume that the death rate is logarithmic over the range of our experiment. Second, we assume that organisms within a tube are Poisson distributed in the region of the endpoint. Although the true average concentration (λ), organisms per gram, may be 0.5 (i.e., 5 viable organisms distributed over 10 tubes), one tube might contain four organisms while others may have three, two, one, or none. This can be expressed as follows:

$$\Pr(x) = \frac{\lambda^x e^{-\lambda}}{x!} \qquad \lambda > 0 \qquad x = 0, 1, 2, \ldots \quad [1]$$

λ = expected number of organisms per tube.
x = number of organisms in the tube.

Third, we assume that reaction of organisms to heat is independent, tube to tube. Thus, the probability that k out of n tubes show no growth is given to the binomial distribution.

$$\Pr(k) = \binom{n}{k} \left[\Pr(x = 0) \right]^k \left[1\text{-}\Pr(x = 0) \right]^{n-k} \qquad k = 0, 1, \ldots n \quad [2]$$

k = number of tubes showing no growth
n = number of tubes
$\Pr(x = 0)$ = Probability that no organisms survive within a tube.

The required probability of $x = 0$ for a single tube is

$$\Pr(x = 0) = e^{-\lambda} \quad [3]$$

Consequently, $1 - e^{-\lambda}$ will be the probability that a tube has one or more organisms.

Now, letting $k = 10$ and $n = 10$ we can obtain (4) from (2), which relates the probability that all 10 tubes are negative to the concentration of λ organisms per gram.

$$\Pr(k = 10) = \left[e^{-\lambda} \right]^{10} \quad [4]$$

Table 5 indicates the probability that all 10 tubes show no growth related to 7 concentrations.

One can see that below $\lambda = 0.1$, heating intervals resulting in a completely negative series of recovery tubes are likely to occur before the last heating interval showing viable cultures. These intervals are often referred to as "skips." Thus an end point based on that heating interval resulting in positive tubes after several such skips may be quite valid down to $\lambda = 0.001$ or even lower. At $\lambda = 0.001$, however, there is only one chance in a hundred that any positive tubes will occur in a series of 10 tubes.

Table 5. Probability that all 10 tubes will show no growth when λ organisms per gram are present in the sample.

Heating interval (min)[1]	Average concentration (organisms per gram) λ	Probability that all 10 tubes are negative
t	1.000	4.54×10^{-5}
$t + c_1$	0.500	6.74×10^{-3}
$t + c_2$	0.100	0.368
$t + c_3$	0.050	0.607
$t + c_4$	0.010	0.905
$t + c_5$	0.005	0.951
$t + c_6$	0.001	0.990

[1] t = time in min up to the point where $\lambda = 1.000$.
c_1 = the additional increment of time in min.
$t + c_6$ = defined as end point, $F = D \log_{10} A + 3$.

We can thus define this as our end point and the time to reach $\lambda = 0.001$ as our F value. If n (number of tubes) were equal to one hundred or one thousand, λ could be expected to be lower and F somewhat larger before all tubes show negative results. This means that a drop of 2 or 3 log cycles below 1 could occur before the chance of detecting growth becomes small. Therefore, we can suggest the following relation between F and D.

$$D = \frac{F}{(\log_{10} A + 3)} \quad [5]$$

This relation between F and D is essentially the same as that determined experimentally by Schmidt (1957).

Our Z_F values generally fall in the range of 10.1 to 13.6 which is somewhat higher than that of Roberts *et al.* (1965). The exception is our Alaska strain suspensions in which our lower confidence limit for Z_F of 10.2 overlaps their value of $Z = 10.5$. $Z_F = Z_D$ using a conversion factor of Schmidt (1957) if we assume $\log_{10} A$ is constant for all experiments. Our values, on the other hand, are lower than the Z_D values of 17 and 14 calculated from the data of Ohye *et al.* (1957) for their strains 103 and 108, respectively, and lower than the 14 and 15 reported by Schmidt (1964) for the Minneapolis strain heated in trypticase-peptone-glucose medium and M|15 phosphate buffer, respectively. These differences may reflect somewhat the differences between their methods and our method of heating in fish paste and recovery after long incubation in a complex meat medium.

In the present study extended incubation over a 6-month period revealed a considerable number of recovery cultures showing germination and outgrowth and subsequent toxin production by viable spores, many of which had the effect of altering the viability-kill end points. These are presented in Table 6. Extended incubation was evidently beneficial in ascertaining the values reported here, and it is possible that further changes may have taken place with even longer incubation periods had the time and facilities for

Table 6. Increases in the viability-kill end points after 7 days incubation at 30°C observed during extended incubation of recovery cultures at 25°C over a 6 month period.

Test temperature (°F)	Type E strain	Incubation time (days)	Heating interval (min)	Number of heating intervals increase in end point
165	Beluga	150–180	5	2
	Iwanai	7–30	7	1
		120–150	8	1
	Tenno	7–30, 90–120	6	1, 4 (skip)
170	Alaska	7–30	10	1
		90–120	6	1
	Tenno	60–90	2	1
		90–120	2	2
		90–120, 120–150	1	1, 1
175	Alaska	60–90, 150–180	7	1, 1
	Beluga	30–60, 60–90	6	1, 1
	Iwanai	120–150	2	1
	Tenno	60–90	3	1
		150–180	3	2
	8E	7–30	3	5
		90–120	3	1
185	Alaska	7–30	1	3
		60–90	1	1

carrying them out been available. Conceivably a recovery system such as ours in which a sizable portion of inoculated natural food is placed in a recovery medium composed largely of meat may well contain germination inhibitors that make extended incubation necessary. The end points observed are likely to reflect the sum total of nutrients, inhibitors, etc. present in a recovery system.

The Z_D values reported by Schmidt (1957) for spores are approximately 18 ± 2 (for the temperature range 220 to 270°F) and for vegetative cells 10 ± 2 (for the temperature range 130 to 150°F). We obtained Z_D values between 10 and 18 for the temperature range 160 to 180°F which is interesting because they fall between the values reported for the temperatures at which vegetative cells and spores were tested. Thus our Z_D values tend to reinforce somewhat the common impression that the heat resistance of type E spores is greater than vegetative bacterial cells and less than the spores of *C. botulinum* types A and B and PA3679.

Our data for the most resistant strain, Alaska, indicate that at 180°F 1×10^6 spores of any of the five strains of *C. botulinum* we studied would be killed in approximately 17 min, provided that the spores are produced and subjected to conditions of water activity, low salt content, etc., approaching those present in the current experiments.

REFERENCES

Anellis, A., Lubas, J. and Morton, M. R. 1954. Heat resistance in liquid egg of some strains of the genus Salmonella. *Food Res.* **19**, 377.

Angelotti, R., Foter, M. J. and Lewis, K. H. 1961. Time-temperature effects on Salmonellae and Staphylococci in foods. III. Thermal death-time studies. *Appl. Microbiol.* **9**, 308.

Angelotti, R., Hall, H. E., Foter, M. J. and Lewis, K. H. 1962. Quantitation of *Clostridium perfringens* in foods. *Appl. Microbiol.* **10**, 193.

Dolman, C. E., and Iida, H. 1963. Type E botulism; its epidemiology, prevention, and specific treatment. *Can. J. Publ. Health* **54**, 293.

Ohye, D. F. and Scott, M. J. 1957. Studies in the physiology of *Clostridium botulinum* type E. *Austral. J. Biol. Sci.* **10**, 85.

Ostle, B. 1963. "Statistics in Research." Iowa State University Press, Ames, Iowa.

Pflug, I. J. and Esselen, W. B. 1953. Development and application of an apparatus for study of thermal resistance of bacterial spores and thiamine at temperatures above 250°F. *Food Technol.* **7**, 237.

Read, R. B. and Bradshaw, J. G. 1966. Thermal inactivation of staphylococcal enterotoxin B in veronal buffer. *Appl. Microbiol.* **14**, 130.

Roberts, T. A., Ingram, M. and Skulberg, A. 1965. The resistance of spores of Clostridium botulinum type E to heat and radiation and the resistance of Clostridium botulinum type E toxin to radiation. *J. Applied Bacteriol.* 28, 125.

Sakaguchi, G., Tokyama, Y., Saito, S., Fugjisawa, S. and Wada, S. 1954. An outbreak of type E botulism in Akita prefecture due to giltheadizushi. *Japan J. Med. Sci. Biol.* **7**, 539.

Schmidt, C. F. 1957. Thermal resistance of microorganisms. In "Antiseptics, Disinfectants, Fungicides and Chemical and Physical Sterilization," ed. Reddish, G. F., pp. 831–884. 2nd edition. Lea and Febiger, Philadelphia, Pa.

Schmidt, C. F., Nank, W. K. and Lechowich, R. V. 1962. Radiation sterilization of food. II. Some aspects of the growth, sporulation, and radiation resistance of spores of *Clostridium botulinum*, type E. *J. Food Sci.* **27**, 77.

Schmidt, C. F. 1964. Personal communication.

Society of American Bacteriologists. 1936. "Manual of Methods for the Pure Culture Study of Bacteria, Leaflet II (Preparation of Media)," 6th ed. p. 12. Biotech Pub., Geneva, N. Y.

Townsend, C. T., Esty, J. R. and Baselt, F. C. 1938. Heat resistance studies on spores of putrefactive anaerobes in relation to determination of safe processes for canned foods. *Food Res.* **3**, 323.

U. S. Dept. Health, Educ. and Welfare. 1963. Morbidity and Mortality. **12**(41), 337.

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Mention of commercial products does not imply endorsement by the Public Health Service.

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Salt, Moisture and Aging Time Effects on the Viability of Trichinella spiralis in Pork Hams and Shoulders

SUMMARY—Twenty-four weanling pigs were infested with *Trichinella spiralis*, by dosing with trichina larvae. The hogs were raised to market weight and slaughtered. Lean samples from each ham and shoulder were digested in a pepsin-HCl solution and microscopically examined. The samples contained respectively an average of 192 and 175 larvae per gram.

The hams and shoulders were dry-cured for 2 days per pound, using 8 pounds of cure per 100 pounds of meat. The mixture contained salt, sugar, potassium nitrate, and sodium nitrite. After being cured, the meat was hung for 30 days for salt equalization, rinsed, allowed to dry, smoked for approximately 24 hr at 90 to 100°F, and aged at 75°F.

Cores were taken from randomly selected hams and shoulders at weekly intervals and analyzed for salt and moisture while part was digested and examined for live trichinae. Live trichinae persisted through the curing and salt equalization periods but began to be reduced in number after one week of aging. Samples taken from hams and shoulders aged for one month were found to be void of any live trichina larvae.

Meat from similar samples was force-fed to rats for 5 days. After 8 weeks the rats were sacrificed and the carcasses digested and examined. No trichina larvae were found.

INTRODUCTION

CONSIDERABLE INTEREST has been developed in the Southern United States in the production of quick-aged country-style hams. According to Kemp *et al.* (1961), Christian (1962), Cecil and Woodroof (1954) and Hunt *et al.* (1939) hams aged for 3 to 6 months under environmentally controlled temperatures and humidity were comparable to regularly aged hams which had been held for one year.

With the increased use of this accelerated method of aging dry-cured hams a new look must be taken at the federal regulations governing the processing of dry-cured hams to destroy trichinae. Present regulations designed to kill trichinae in dry-cured hams are based on work reported by Ransom *et al.* (1920) and are not conducive to the production of a uniform high quality product.

This project was designed to test the effect of salt concentration and aging time on the viability of trichina larvae in dry-cured hams and shoulders and to evaluate a method of curing and aging which will produce a high quality product, free from disease-producing trichinae.

MATERIALS AND METHODS

TWENTY-FOUR WEANLING PIGS were infected with trichinae by orally dosing each with approximately 20,000 excysted larvae obtained by artificial digestion of infested rats. The hogs were raised to an average market weight of 204 lb and slaughtered. The hams and shoulders were removed from each carcass, individually identified, and a fresh lean sample obtained from each for total larvae count determination. They were then dry-cured for 2 days per pound at 36°F, using 8 pounds of cure per 100 pounds of meat. The curing mixture contained 73.6% salt, 24.5% sugar, 1.2% potassium nitrate and 0.6% sodium nitrite.

After curing, the meat was hung for 30 days at 36°F to allow for salt equalization. After that the hams and shoulders were soaked for 30 min in lukewarm water and scrubbed free of any mold on the surface, allowed to dry, and then were smoked at 90 to 100°F for approximately 24 hr. They were then placed in an aging room and aged at 75°F with a relative humidity of 60 to 65% until the trial was terminated.

Randomly selected hams and shoulders were cored as illustrated in Figs. 1 and 2 at 10 different intervals during the curing process. These intervals consisted of removal from cure, one week salt equalization, 2 weeks salt equalization, 3 weeks salt equalization, before smoking, after smoking, one week aging, 2 weeks aging, 3 weeks aging,

The core holes were filled with melted lamb fat to prevent excessive moisture loss and to prevent mold growth. A one-inch section was removed from the exterior of each core and discarded.

The remainder was defatted and analyzed for salt and moisture and a portion artificially digested to determine the number of live trichina larvae. The larvae were excysted for counting by placing 25 g of defatted finely chopped meat in a quart mason-type jar and adding 50 ml granulated pepsin, 8 ml concentrated HCl and 700 ml tap water. The containers were then incubated in a hot water bath at 37°C and agitated with air for 12 to 16 hr.

After removing from the incubator the jars were allowed to sit 30 min at room temperature to allow the excysted larvae to settle to the bottom. Approximately two-thirds of the solution was carefully siphoned off the top and the jar filled with warm tap water.

After the solution had settled for 30 min, the top two-thirds was siphoned off and the remaining solution which contained the larvae was transferred to 400-ml beakers. The solution was again allowed to settle for 15 to 20 min and the water carefully siphoned off, leaving approximately 25 ml of water containing the larvae. This solution was then transferred to a 25-ml volumetric flask and brought to volume.

To determine the number of larvae per g of meat, the volumetric flask containing the larvae was vigorously shaken to distribute the larvae evenly throughout the

[a] Present address: Ralston Purina Company, St. Louis, Mo. 63199.

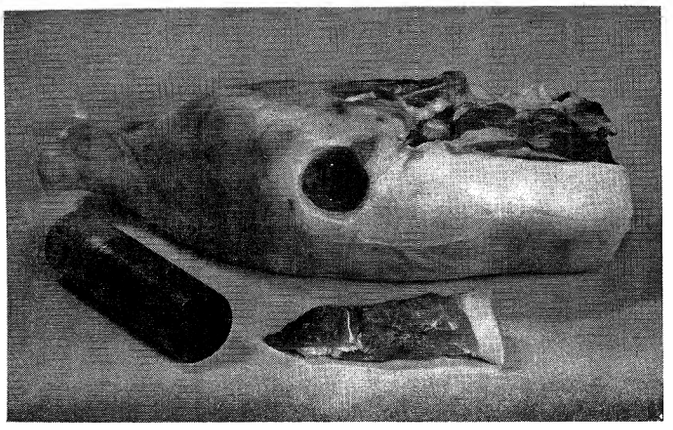

Fig. 1. Method of coring hams.

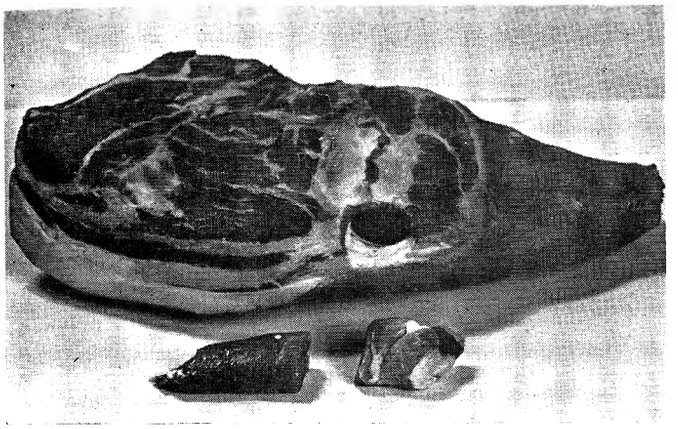

Fig. 2. Method of coring shoulders.

flask. A one ml aliquot was quickly withdrawn from the center of the flask and placed on a gridded glass plate. The larvae were then counted from three or more aliquots under a dissection microscope, using 10- to 20-power magnification.

Percent salt was determined by accurately weighing a lean sample of approximately 15 g and blending in a Waring Blendor with 150 ml distilled water for 3 min. The mixture was then filtered and the filtrate stored in 4-ml vials. Samples were analyzed on a Technicon Autoanalyzer according to an automated procedure of Zall *et al.* (1956). Moisture was determined by standard A.O.A.C. (1960) procedures.

RESULTS AND DISCUSSION

ANALYSIS OF FRESH SAMPLES of meat taken from each ham and shoulder showed an average of 192 and 175 trichina larvae per g of tissue respectively.

The mean values and standard deviations for percent salt and water, the water-salt ratio, and live trichinae per g muscle for the different sampling periods are given in Tables 1 and 2.

The viability of the larvae was not affected by salt concentration in the interior of the hams and shoulders during the 60 days of curing and salt equilization. In many instances, the number of live larvae during these periods was higher than those found in the fresh samples. This increase

Table 1. Mean values and standard deviations for ham samples.

Period	No. tested	Percent water	S.D.	Percent salt	S.D.	Water/salt ratio	S.D.	Live trichinae per gram muscle		
								Range	Mean	S.D.
Out of cure	8	70.5	1.0	0.7	0.3	116	3.4	28–298	180	86
1 week salt equalization	8	70.5	1.4	1.0	0.2	66	8.8	3–379	186	112
2 weeks salt equalization	8	69.1	2.1	1.1	0.3	66	18.7	71–434	241	111
3 weeks salt equalization	8	68.7	2.2	1.0	0.3	66	22.4	0–431	149	153
Before smoking	8	69.5	2.2	1.7	0.5	42	13.1	0–440	159	362
After smoking	8	68.4	1.5	2.1	0.7	36	12.2	0–259	140	103
1 week aging	2	70.3	0.5	2.1	0.1	33	0.6	25– 75	50	35
2 weeks aging	8	67.6	2.0	2.5	0.6	28	5.7	0– 75	34	35
3 weeks aging	1	67.0	.....	2.9	.....	23	.....	.....	25	.....
4 weeks aging	37	66.5	1.9	3.3	0.5	20	3.5	0	0	0

Table 2. Mean values and standard deviations for shoulder samples.

Period	No. tested	Percent water	S.D.	Percent salt	S.D.	Water/salt ratio	S.D.	Live trichinae per gram muscle		
								Range	Mean	S.D.
Out of cure	8	70.8	1.1	1.3	0.5	61	22.5	1–515	226	170
1 week salt equilization	8	69.8	1.3	1.9	0.8	42	16.8	<1–430	215	126
2 weeks salt equalization	8	68.8	0.6	2.8	2.0	26	8.1	<1–395	152	122
3 weeks salt equalization	8	68.4	2.1	2.6	0.8	28	7.1	0–187	114	62
Before smoking	8	68.4	1.6	2.6	0.7	27	10.3	1–232	110	83
After smoking	8	67.8	2.0	2.8	0.7	26	7.5	<1–132	58	38
1 week aging	3	64.2	1.5	3.4	0.3	19	2.0	0– 75	33	35
2 weeks aging	8	63.5	2.2	3.6	0.7	18	3.7	0– 25	10	9
3 weeks aging	2	64.7	0.2	4.2	1.3	16	0.3	3– 4	4	<1
4 weeks aging	35	62.7	2.7	4.4	0.7	15	2.6	0	0	0

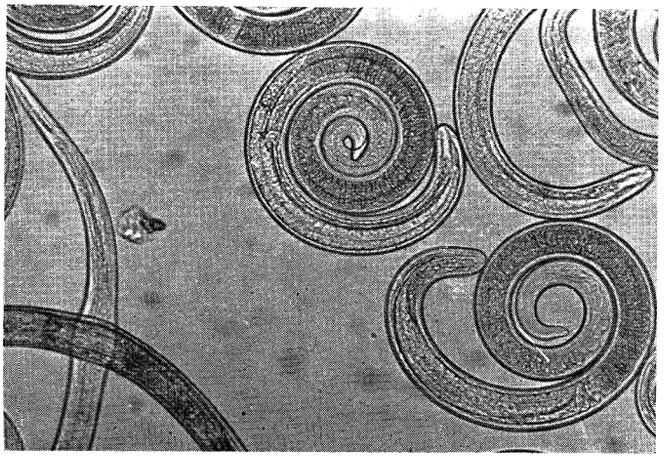

Fig. 3. Live trichinae excysted from pork muscle. × 27.

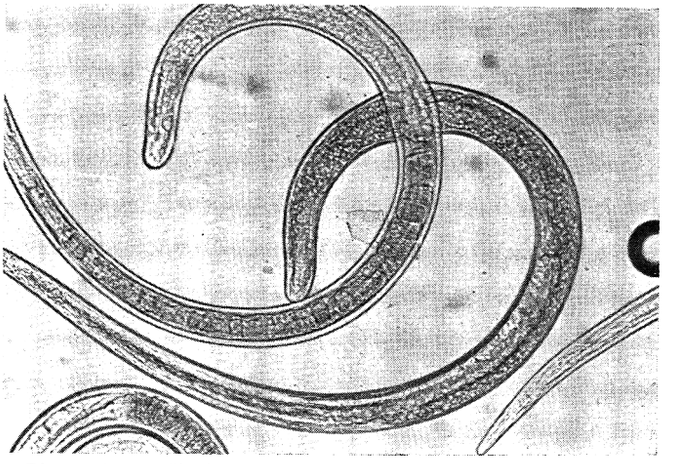

Fig. 4. Dead trichinae excysted from dry-cured hams and shoulders. × 27.

was most likely owing to the variation of infection in the different muscles by the larvae.

As indicated by Allen *et al.* (1962), Ransom *et al.* (1920) and Zimmerman (Personal Communication, 1965) these low temperatures do not appreciably decrease the viability of the larvae regardless of the salt concentration.

Shoulder samples taken after smoking showed a marked decrease in the number of live trichinae, while this decrease was not evident in the hams until one week later.

During smoking the hams and shoulders were subjected to temperatures between 90 and 100°F for 24 hr which gradually increased the internal temperature of the meat. Although there was a slight increase in percent salt, owing to loss of water, the marked decrease in number of live larvae after smoking was presumably due to the increased temperature or a combination of salt and temperature. While the decrease in the number of live larvae in the hams was not evident until one week after smoking, this may have been due to the lower internal temperature attained during smoking because of the greater thickness of the hams.

Trichinae larvae excysted from ham and shoulder samples from the periods prior to smoking were similar to the coiled larvae shown in Fig. 3. Immediately after digestion and washing in warm water, the larvae were quite active. When the larvae were allowed to cool they became tightly coiled and inactive, but when warmed to body temperature, they became active again.

Excysted larvae from samples taken after smoking were weak and had very little movement after digestion. Many of the larvae were coiled and could not be activated by using warm water or digestive fluid. The larvae were much paler than those of earlier periods. Also many were in the shape of a figure "6" as described by Ransom *et al.* (1920) and shown in Fig. 4. Meat samples from these hams and shoulders were fed to rats which all became heavily infected with trichinae.

The larvae became increasingly more dehydrated and degenerated during the latter part of aging. At 2 and 3 weeks of aging they were very weak. They were not tightly coiled, and none could be activated after coiling. Light infections were found in 8 out of 12 rats fed meat from hams and shoulders that had been aged for 2 or 3 weeks. After 4 weeks of aging, a total of 72 hams and shoulders were sampled, and no live or coiled larvae were found in the digested samples. The fluid did contain many pale fragments of digested larvae which were extremely dehydrated and degenerated. Meat samples were fed to 45 rats of which all were found to be free of trichinae larvae when examined.

REFERENCES

Allen, R. W. and Goldbert, A. 1962. The effects of various salt concentrations on encysted *Trichinella spiralis* larvae. *Amer. J. Vet. Res.* **92**, 580–585.

A.O.A.C. 1960. "Official Methods of Analysis," Washington, D. C.

Cecil, S. R. and Woodroof, J. G. 1954. Effect of storage temperatures on the aging of country-style hams. *Food Technol.* **8**, 216–219.

Christian, J. A. 1962. Country-cured hams boom in North Carolina. *National Provisioner* **146**(13), 10–11.

Hunt, W. E., Suplee, W. C., Meade, D. and Carmichael, B. C. 1939. Qualities of hams and rapidity of aging as affected by curing and aging conditions and processes. Bull. 428, Maryland Agr. Expt. Sta.

Kemp, J. D., Moody, W. G. and Goodlett, J. L. 1961. The effects of smoking and smoking temperatures on the shrinkage, rancidity development, keeping quality and palatability of dry-cured hams. *Food Technol.* **5**, 267–270.

Ransom, B. H. Schwartz, B. and Raffensperger, H. B. 1920. Effects of pork-curing processes on trichinae. Bull. 880, U. S. Dept. Agr., Washington, D. C.

Zall, D. B., Fisher, D. and Garner, M. 1956. Photometric determination of chlorides in water. *Anal. Chem.* **11**, 1665–1668.

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Chemical Induction of Mutation or Variation In Aflatoxin-Producing Cultures of Aspergillus Flavus

SUMMARY—The mutation or variation of a strain of *Aspergillus flavus* was induced chemically after six successive "generations" of exposures to barium ions. Colony isolates of *A. flavus*, developing from spores harvested from cultures grown in barium medium, were examined for mutation on the basis of morphology, loss of aflatoxin-producing capacity, and loss of ability to grow in basal medium. The new characteristics exhibited by the mutant of *A. flavus* are the inability to produce aflatoxins and yellow pigment, accompanied by the loss of fluorescence in the culture under ultraviolet light. These changes did not revert after more than eight successive transfers in a barium-free medium. Mutagenic action of barium ions on the fungus *A. flavus* was shown by the mutatation of colonies arising from single spores. The characteristics of the mutant are permanent and irreversible.

INTRODUCTION

CONSIDERABLE INFORMATION is available concerning the role of metals in the growth of fungi (Steinberg, 1956). Specific effects of metals on form development have been observed in various microorganisms (Nickerson, 1956). Chemically induced mutation was obtained by successive cultivation of *Aspergillus niger* in media containing metals (Yamagata *et al.*, 1956). It was suggested that the molds present two possibilities for attack (Thom *et al.*, 1939): (1) The spores or propagative cells may be treated, then inoculated into basal medium; (2) New mold colonies may develop from vegetative mycelia growing in medium containing chemical mutagen. From such colonies either spores or vegetative mycelia may be transferred to basal media. If mutation or variation occurred, the changes would appear in the new colony and could be carried through subsequent generations. In this study, the latter alternative was chosen for the chemically induced mutation of *Aspergillus flavus*.

Lee *et al.* (1966) showed that barium ions inhibit the production of aflatoxins by *A. flavus*. To ascertain whether this barium effect on *A. flavus* is temporary or permanent, the variability of several generations of barium treated fungus was observed. Schwartz *et al.* (1966) suggested three methods of assessing mutagenic action on molds: (1) Morphological variation; (2) Lack of ability to synthesize certain end-products, and (3) Nutritional mutants, i.e., mutants that are incapable of growing on basal medium.

The latter two of these methods are considered to be more objective and reliable in determining the degree of mutation.

Since the mutants of barium treated *A. flavus* did not show either morphological variation or incapability of growing on basal medium, each population of colonies was assessed for the presence of mutations on the inability to synthesize aflatoxins and other end-products.

MATERIALS AND METHODS

Organism and assays

The *A. flavus* parent culture and the chloroform extracts for aflatoxin assays were prepared as previously described by Lee *et al.* (1966).

A uniform suspension of spores from the original stock culture of *A. flavus* was prepared by suspending one inoculating platinum loop, 3 mm in diameter, of spores in 10 ml of sterile 0.01% of sodium lauryl sulfate solution. This suspending medium has no apparent harmful effect upon the molds under study. The resulting suspension was subsequently mixed in an Adams cyclomixer and diluted serially twice in sterile water, 1/10, v/v. From the highest dilution, one loop of spore suspension was examined under the microscope. Of the many repeated examinations, an average of 30 to 40 single spores per loop could be detected. Very rarely were spores found in pairs.

From this final spore suspension, a loop of single spores was plated out on an agar medium. After 24 hr of incubation at 35°C, approximately ten germinated single spores were picked randomly from each plate and transferred to separate agar slants for further growth and sporulation at 25°C for 5 days. All the single-spore cultures, including controls, were handled the same way.

Media and cultures

The solid medium used for isolation and sporulation of single spores of *A. flavus* contains, per liter, glucose, 165.0 g; Bacto-Peptone, 1.0 g; $MgSO_4 \cdot 7H_2O$, 0.050 g; KH_2PO_4, 0.060 g; KNO_3, 0.500 g; $Fe_2(SO_4)_3 \cdot nH_2O$, 0.040 g; and agar 30.0 g.

A total of 100 single spores, designated as culture "A," were isolated at random from the original stock culture. Each of the subsequent single-spore colonies was subcultured to 50 ml of basal medium contained in a 250-ml Erlenmeyer flask and was incubated under stationary conditions at 25°C. Each flask was shaken by hand once a day. Aflatoxins were assayed after 7 days.

From culture "A," two single-spore colonies originally producing 1.0 to 1.5 OD (chloroform extract measured at wavelength 362 mμ) units of aflatoxins per ml of unfiltered basal medium were taken and labelled "B" and "C." Both of these colonies were transferred into barium media. After each transfer to barium medium, 100 single spores were isolated randomly from each of the progenies of the two single-spore "B" and "C." Growth and aflatoxin determination were carried out as described previously. Thus, successive exposures of the progenies to the barium from two separate single spores, "B" and "C," continued until 100% mutants were obtained.

The techniques used to inhibit aflatoxin production, by the addition of barium ions as barium carbonate to the low zinc (0.8 ppm) basal medium containing 0.5 to 1.0 mg of barium acetate, were identical to those previously described (Lee *et al.*, 1966). A total of 1 to 2 g of barium carbonate per 100 ml of medium was added in aliquots of 0.75 g every 24 hr after germination of the conidia. The conidia from the barium-treated cultures were inoculated into the basal medium and the low zinc medium containing barium ions. The basal medium sub-culture was used to determine whether or not the organism was able to recover its ability to produce aflatoxins. These transfers were repeated for more than eight "generations." The term "generation" is considered as from spore germination through vegetative mycelial development to spore production (Thom *et al.*, 1939. p, 331). All transfers were duplicated.

RESULTS AND DISCUSSION

A. flavus lost its ability to produce aflatoxins and the yellow pigment after it had been subjected to barium treatment for six successive "generations" or more. Fig. 1 shows the aflatoxin absorption peak from chloroform extracts of *A. flavus* cultured in a basal medium as compared to the absence of this component in the extract of the mutant cultured in the same medium.

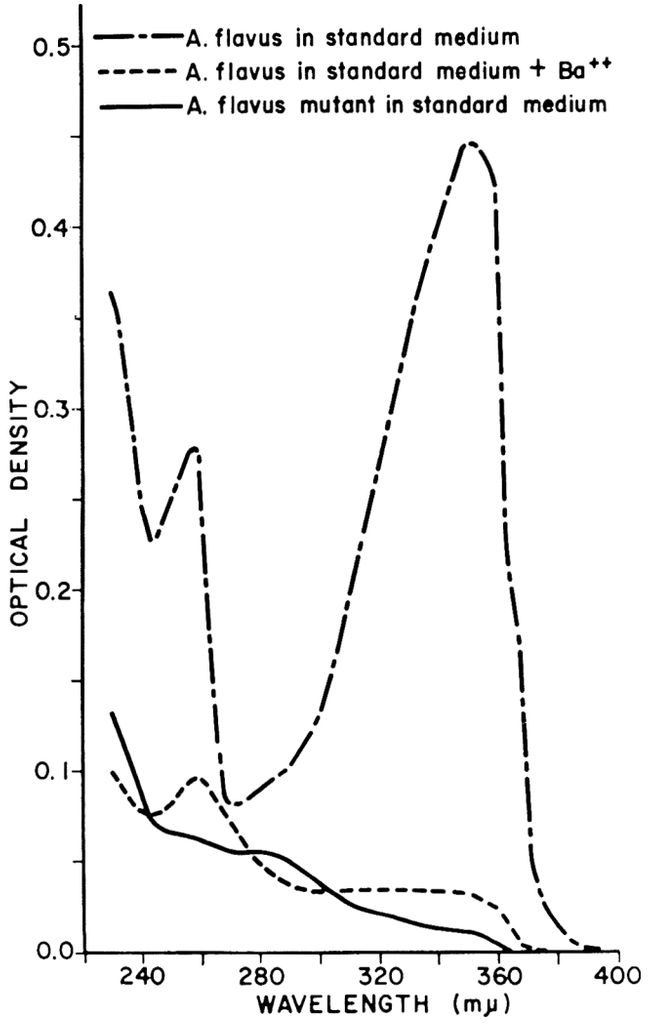

Fig. 1. Absorption spectra of chloroform extracts from various cultures of Aspergillus flavus.

The absorption of the chloroform extracts from the *A. flavus* mutant did not show the "characteristic plateau" at wavelength 262 mμ, as was always present in the extract from *A. flavus* which was exposed to barium for the first time. There was a slight variation, however, in the number of repeated barium treatments required for a complete transformation. Generally, at least six or more "generations" of barium treatments were required. The ability to produce aflatoxins was lost about one "generation" earlier than that of the yellow pigment. Once the yellow pigment and the absorption "characteristic plateau" of the chloroform extract at 262 mμ were not observed from the culture grown in a basal medium, the culture had achieved its stable and complete transformation.

There was no difference in the visible morphology between the aflatoxin-producing *A. flavus* and its mutant when both were grown in a barium-free medium. However, it was observed microscopically that the mycelia were smoother and more elongated for the *A. flavus*, both the parent and the mutant, grown in the presence of barium than those grown in a barium-free medium as shown in Figs. 2 and 3.

In the early stage of growth (24 hr incubation at 25°C) the mycelia in barium-free medium were as smooth and elongated as those in barium medium. After three to four days the morphology of the mycelia in barium-free medium changed to nodular, vacuolar, and budding, but those in barium medium remained unchanged. Upon the addition of barium carbonate, white crystals of unknown composition accumulated on the mycelium during incubation. From these observations, barium may have the ability to preserve the young morphology of the mycelia, possibly by precipitating or neutralizing certain toxic substances which may cause the change in morphology of the older cultures.

Under ultraviolet light the parent aflatoxin-producing *A. flavus* culture was strongly fluorescent in comparison to

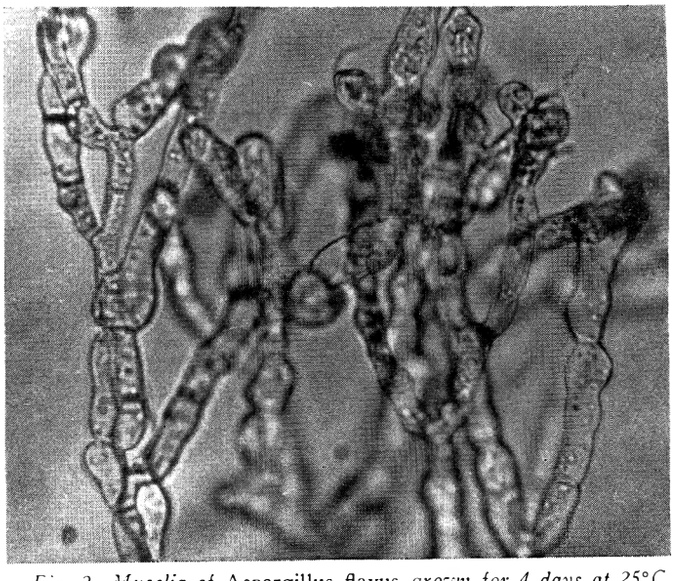

Fig. 2. Mycelia of Aspergillus flavus *grown for 4 days at 25°C in a barium-free standard basal medium (mycelium diameter 6 to 7.5 μ).*

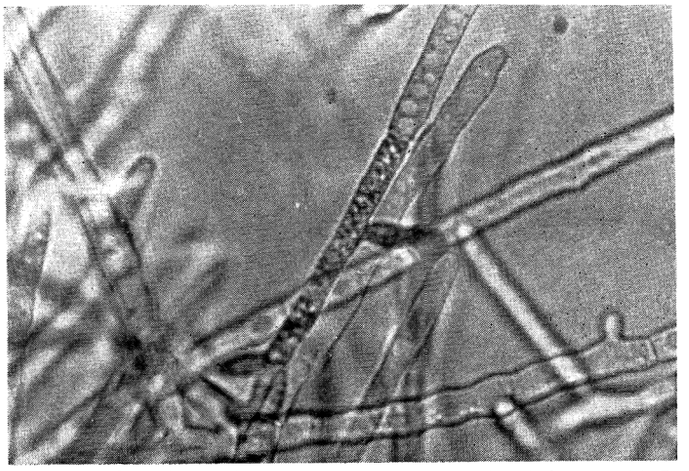

Fig. 3. Mycelia of Aspergillus flavus *grown for 4 days at 25°C in a standard basal medium containing barium ions (mycelium diameter 6 to 7.5 μ).*

the absence of fluorescence in the mutant culture as shown in Fig. 4. The degree of fluorescence was proportional to the amount of yellow pigment and aflatoxins produced.

In order to demonstrate that mutation was induced chemically in a pure culture, the effect of barium ions on aflatoxin production by the progeny of a single spore was performed. Table 1 shows the effect on aflatoxin production of successive transfers of *A. flavus* to barium medium.

The number of individual spores producing aflatoxins within a population of 100 single spores selected at random was proportionally decreased with the increased number of successive transfers to medium containing barium ions. Culture "A" was a group of single spores selected at random from the original stock culture to serve as control in producing aflatoxins after many transfers in a basal medium, under similar conditions as those of cultures "B" and "C." Cultures "B" and "C" were obtained from two separate single spores among the 100 single spores of culture "A," as indicated in Table 1. The first transfer of "B" to barium medium did not induce any mutants among the 100 single spores selected at random. However, upon the second transfer to barium, one spore of the 100 single spores selected at random was mutated. At the end of the third transfer, the rate of mutation increases by about 20 times that of the second transfer. The number of mutants obtained from each subsequent transfer was almost doubled from the last "generation." Finally, at the sixth transfer, mutation was found to be 100% in the population of culture "B."

Table 1. Effect on aflatoxin production of successive transfers of progenies from a single spore of *A. flavus* to basal medium containing Ba^{++}.

No. of transfers	Metal	Groups of 100 single spores	No. of spores producing aflatoxins (O.D. at 362 mμ per ml)							% of spores mutated
			0.0	0.05	0.1	0.1–0.5	0.5–1.0	1.0–1.5	1.5–2.0	
None	None	A[1]					19	80	1	0
1	None	A					20	78	2	0
	Barium	B				15	85			0
		C					13	86	1	0
2[2]	None	A				25	75			0
	Barium	B	1			96	3			1
		C				90	10			0
3	None	A					8	92		0
	Barium	B	23		2	63	12			23
		C				1	7	92		0
4	None	A					10	89	1	0
	Barium	B	64	10	2		2	22		64
		C					42	58		0
5	None	A					2	98		0
	Barium	B	85				13	2		85
		C			5	58	37			0
6	None	A					11	89		0
	Barium	B	100							100
		C	3	90	7					3
7	None	A					15	85		0
	Barium	C	28	68	4					28

[1] A. Group of single spores selected at random from original stock culture.
B and C. Two separate groups of single spores reproduced from two individual single spores of group "A" before Ba^{++} treatment, the progenies of each of these two single spores are capable to produce aflatoxine O.D. from 1.0–1.5 at 362 mμ per ml of medium after 7 days stationary incubation at 25°C.

[2] Cultures assayed for aflatoxins at 10 days old.

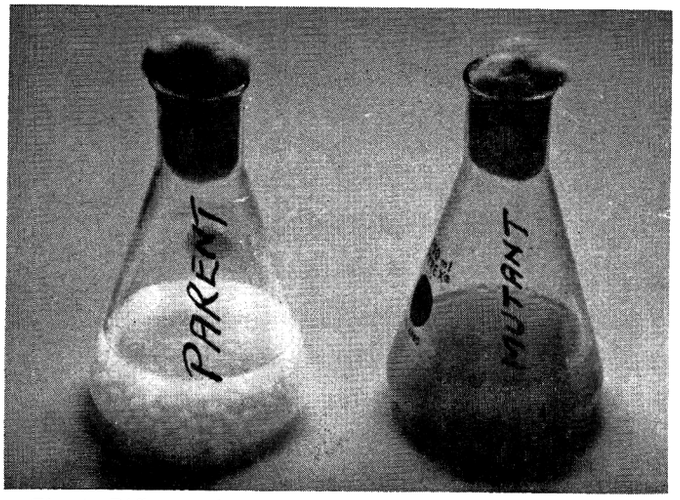

Fig. 4. Difference between parent and mutant Aspergillus flavus *in fluorescence under ultraviolet light.*

Fig. 5 sums up the entire process of mutation induced chemically in the progenies of a single spore "B."

As indicated in Table 1, culture "C" reacts to barium treatment somewhat differently from that of culture "B." No mutants were found after as many as five transfers. However, at the end of six transfers, three non-aflatoxin white mutants were detected among the 100 single spores selected at random. By the end of the seventh transfer, the percentage of non-aflatoxin-producing spores was increased from 3% to 28%. The whole population was eventually changed accordingly to non-aflatoxin-producing mutants upon the tenth successive subsequent exposures to medium-containing barium ions (not shown in Table 1).

The experiments described above have shown that mutation or variation had taken place in the barium-treated *A. flavus* culture. Since the resulting mutants did not revert after more than eight transfers in a basal medium without barium, the mutation "characteristics" expressed, were considered stable. The number and frequency of variants that had lost detectable ability to produce aflatoxins were obtained from the various spores germinated and developed in barium medium (Table 1). In addition, more than 800 colonies (group "A," Table 1) arising from non-barium medium were examined to obtain a measure of spontaneous mutation. This group did not produce a single variant colony significantly different from that of the parent culture as far as the ability to produce aflatoxins is concerned. Furthermore, the presence of barium in the medium did not appear to suppress spore germination or significantly inhibit or stimulate the growth rate.

This evidence indicates that barium does not select barium-resistant non-aflatoxin-producing spores during the process of growth, but rather chemically interferes with aflatoxin production. The *A. flavus* mutant obtained by growing the culture in contact with barium would agree with Thom *et al.* (1939) in the support of a chemical mutation. It is hoped that this evidence may serve for

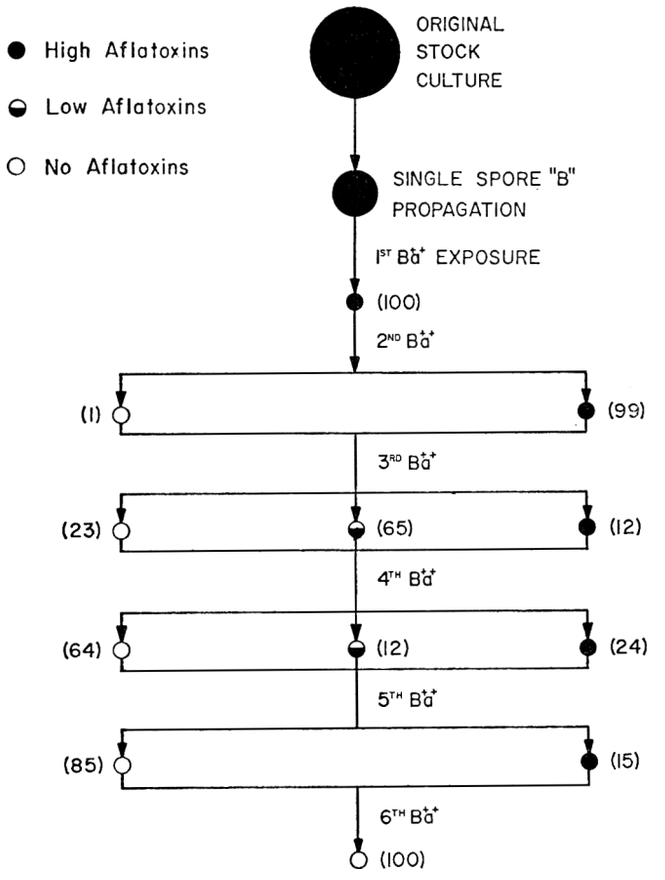

Fig. 5. Chemically induced mutation was obtained from single spore "B" by successive transfers of its progenies to medium containing Ba^{++}. *Arabic numerals in parentheses refer to the number or percentage of single spore cultures selected at random from the population.*

further studies concerning the role of bivalent metals in the biosynthesis of aflatoxins and their control of fungus metabolites produced in food.

REFERENCES

Lee, E. G.-H., Townsley, P. M. and Walden, C. C. 1966. Effect of bivalent metals on the production of aflatoxins in submerged cultures. *J. Food Sci.* **31**, 432.

Nickerson, W. J. 1956. Chelation and the development of form in fungi. National Academy of Sciences—National Research Council. Publication **514**, 15.

Schwartz, L. J. and Stauffer, J. F. 1966. Three methods of assessing the mutagenic action of ultraviolet radiation on the fungus *Emercellopsis glabra*. Appl. Microbiol. **14**(1), 105.

Steinberg, R. A. 1956. The role of metals in the growth of fungi. National Academy of Sciences—National Research Council. Publication **514**, 1.

Thom, C. and Steinberg, R. A. 1939. The chemical induction of genetic changes in fungi. *Botany* **25**, 329.

Yamagata, K., Oda, M. and Ando, T. 1956. Variation of aspergillus oryzae IX. The variation induced by chemicals. *J. Fermentation Technol.* (Japan) **34**, 254.

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Clostridium perfringens in Dehydrated Soups and Sauces

SUMMARY—Fifty-five samples of nationally advertised dehydrated sauce and gravy mixes, soup mixes, spaghetti sauce mixes, and cheese sauce mixes were examined for the presence of *Clostridium perfringens*. The organism was found in 18.2% of the samples. Spaghetti sauce mixes had the highest incidence of *C. perfringens* and the soup mixes had the lowest incidence. One strain possessed heat-resistant spores that were able to withstand boiling at 97.4°C for one hour prior to isolation. The presence of preservatives in the food products did not influence the presence of *C. perfringens* in these food preparations. No common ingredient was detected as the source of contamination. The general presence of this organism in dehydrated soups and sauces may have epidemiological significance in *C. perfringens* food poisoning, especially since these products are exposed to short heating periods.

INTRODUCTION

DURING THE PAST 10 YEARS considerable interest has developed on the nature of *Clostridium perfringens* food poisoning. The wide distribution of this organism results in the contamination of many foods and food products consumed by humans. The presence of *C. perfringens* in meat and meat products is well documented (Brown *et al.*, 1960; Canada *et al.*, 1964; Baltzer *et al.*, 1965; Hall *et al.*, 1965; Greenberg *et al.*, 1966; Barbe *et al.*, 1967). Strong *et al.* (1963) isolated the organism from a variety of American foods, and Goudkov *et al.* (1965) isolated *C. perfringens* from dairy products.

C. perfringens has been implicated in many outbreaks of food poisoning in the United States involving hundreds of persons (Browne *et al.*, 1962; Kemp *et al.*, 1962; Nelson *et al.*, 1966). Most of the outbreaks resulted from eating meat dishes, confirming the earlier reports of food poisoning in England (Hobbs *et al.*, 1953).

In recent years a wide variety of dehydrated soups and sauces has appeared on the market and has eased the task of the modern housewife. These products have a convenient envelope package, usually made of plastic coated aluminum, and need only rehydration and a brief heating period. The heating time for rehydrated soups is 5 to 15 min although the mean heating time is approximately 10 min. Other products require merely the addition of water with no heating. Some sauces and gravies are heated to boiling and simmered for a minute or until thick.

Although vegetative cells of *C. perfringens* are rapidly killed by heat (Nakamura *et al.*, in press), the spores of these organisms may be quite heat-resistant even after heating at 100°C for one or more hours (Hobbs *et al.*, 1953; Hobbs, 1965; Hall *et al.*, 1963; Weiss *et al.*, 1967; Nakamura *et al.*, 1967). Therefore, it is logical that dehydrated soups and sauces, if contaminated with *C. perfringens* spores, could be a source of food poisoning outbreaks. This is particularly true if leftover sauces or gravies are not properly refrigerated or properly reheated prior to consumption. This paper reports a survey of dehydrated soups and sauces for the presence of *C. perfringens*. Previous reports are unknown to the authors.

EXPERIMENTAL METHODS

Collection of samples

Samples of nationally advertised dehydrated soups and sauces were purchased from four supermarkets in the Missoula, Montana area. The samples were divided into four general categories: soups, sauces and gravies, cheese and cheese sauces, and spaghetti sauce mixes. All of the products contained a meat or dairy product with the exception of three spaghetti sauce mixes. Fifty-five samples from 15 major brands were purchased and studied.

Isolation of C. perfringens

Ten-g samples were aseptically removed from the package and placed in a sterile petri dish. This facilitated repeated sampling and reduced the possibility of contamination of the product. The samples in the commercial packages were enclosed in aluminum foil or plastic lined envelopes. A modification of the method of Hall *et al.* (1965) was used for the primary isolation of *C. perfringens*. One-g samples of the dehydrated food were introduced into each of six 25 × 200 mm culture tubes containing 30 ml of fluid thioglycollate medium (Difco). One of these thioglycollate tubes was incubated immediately at 46°C. Four of the tubes were heated to boiling (97.4°C at an altitude of approximately 3,223 feet elevation) for 5, 10, 20 and 60 min respectively, immediately cooled in running tap water, and incubated at 37°C. The sixth tube was heated at 80°C for 15 min to kill contaminant organisms, cooled, and incubated at 37°C.

The tubes were observed daily for gas production for up to a week after inoculation. The tubes in which gas production occurred were subcultured on lactose-egg yolk-milk agar (Willis, 1964). On this medium *C. perfringens* colonies produced lactose fermentation and opalescence but no proteolysis.

Biochemical tests were performed routinely on the strains isolated. Gas and acid production by the organisms was determined in lactose, glucose, maltose, sucrose, mannitol, inulin, salicin, and glycerol broth. Nitrate reduction, sulfide and indole production, motility, gelatin liquefaction, and milk fermentation were also determined. Hemolysis and ability to grow aerobically on horse blood agar were observed. The biochemical and physiological tests were recorded after 48 hr of incubation at 37°C.

Food samples that did not yield *C. perfringens* were tested further. One ml of a solution containing 1 g of the

food product and 9 ml of 0.1% peptone was inoculated into 10 ml of cooked meat medium (Difco). These cultures were incubated at 37°C for 3 weeks to allow outgrowth and sporulation. After incubation, 2 ml of the cooked meat medium culture were heated at 80°C for 15 min in 9 ml of 0.1% peptone water. Subcultures were made in duplicate on lactose-egg yolk-milk agar plates and in fluid thioglycollate broth. The plates were placed in Case anaero jars and flushed with pure nitrogen gas three times under vacuum at −25 lb of pressure and then the jars were sealed tight at +3 lb of pressure so that if a leak developed anaerobic conditions would be retained. Anaerobic indicator (Baltimore Biological Laboratory) was used to check anaerobiosis in the jar. The plates and tubes were incubated at 37°C for 48 hr.

To determine the extent of facultative anaerobic contamination of the food product, 1 g of the food was rehydrated in 9 ml of 0.1% peptone water, decimally diluted to 10^{-6} and inoculated onto lactose-egg yolk-milk agar. The cultures were incubated anaerobically at 37°C for 48 hr. The colonies were counted, and the number of organisms per g of dehydrated product was determined.

RESULTS

C. perfringens WAS ISOLATED from 10 out of 55 samples examined. The organism was isolated from 8 out of 15 national brands sampled. The results were tabulated in Table 1. The incidence of *C. perfringens* was highest in spaghetti sauce mixes and lowest in soup mixes. Total facultative anaerobic counts ranged from 1,000 to 2,500,000 organisms per g. Gram positive and gram negative rods and gram positive cocci were present. However, these were not further identified.

One of the seven strains isolated from spaghetti sauce mixes was able to withstand boiling (97.4°C) for 1 hr prior to isolation and produced both alpha and beta hemolysis on blood agar. Two of the strains required heating for 5 min at boiling temperatures before isolation. Another three strains were isolated after heating at 80°C for 15 min. Only one of the strains was isolated from unheated material. The spaghetti sauce mixes contained spices, salt, sugar and starch. All but one of the samples contained some combination of dehydrated vegetables; onion and garlic were the most common. Products that did not have dehydrated vegetables, however, did contain *C. perfringens*. Yeast and cheese were also common ingredients in these preparations, but dehydrated beef was present in only one of the mixes tested.

Table 1. Incidence of *C. perfringens* in dehydrated soups and sauces.

Item sampled	Number of samples examined	Number that yielded *C. perfringens*	Total facultative anaerobe contamination (number/g)
Spaghetti sauce mixes	13	7	2,000 to 800,000
Sauce and gravy mixes	8	1	1,000 to 500,000
Soup mixes	28	1	1,000 to 400,000
Cheese and cheese sauce	6	1	3,000 to 2,500,000
Total	55	10	1,000 to 2,500,000

The incidence of *C. perfringens* in dehydrated soup mixes was exceptionally low. Only one strain was isolated from 28 soup mixes sampled. These mixes, from 6 nationally advertised brands, all contained an animal protein source. The animal protein source consisted of dehydrated beef, chicken, turkey, bacon, and yeast.

Three of the 10 products that yielded *C. perfringens* contained preservatives. One of the soup mixes utilized sodium sulfite to preserve freshness and two spaghetti sauce mixes containing tricalcium phosphate. There was no consistent pattern with regard to the presence of preservatives or ingredients. Most of the preservatives are added to dehydrated foods to preserve the flavor of the product rather than to inhibit yeasts, molds, and bacteria, although these agents do control the microbial populations.

All of the strains of *C. perfringens* isolated from the dehydrated food products liquefied gelatin, produced sulfide from sulfite, reduced nitrate to nitrite, produced stormy fermentation in iron milk, fermented lactose and produced opalescence but no proteolysis on lactose-egg yolk-milk agar, were non-motile, failed to produce indol, and were unable to grow aerobically. Large amounts of acid and gas were produced from glucose, sucrose, lactose, and maltose. These strains produced varying quantities of acid and gas in mannitol, inulin and glycerol. All the strains, except for one, fermented salicin. The strains produced beta-hemolysis on blood agar. The heat resistant strain also produced alpha-hemolysis on blood agar.

DISCUSSION

IN GENERAL, DEHYDRATED and properly packaged food and food products are not subject to spoilage. In themselves, most of these dehydrated foods are not dangerous as sources of food poisoning. However, the method of handling of the food after rehydrating is important in human health. Many dehydrated foods may be potential sources of *C. perfringens* food poisoning if proper cooking and proper refrigeration are not included in the process of food preparation, food serving, and food storage.

In the present study 10 out of 55 of the dehydrated soups and sauces sampled contained *C. perfringens*. The products tested required boiling for no more than 30 min after hydration. Since certain strains of *C. perfringens* produce spores that resist boiling for several hours, the lethal time-temperature condition is not attained in the reconstitution and preparation of the dehydrated foods. Furthermore, *C. perfringens* is capable of growing at temperatures up to 55°C. Many of the products would cool to this temperature rather quickly, within half an hour, after heating.

Some of the gravy and sauce mixes require heating for less than 10 min. If these items are allowed to cool or are left at room temperature for several hours considerable vegetative reproduction of *C. perfringens* would be possible making this food a potential source of food poisoning.

These studies indicate that dehydrated soups and sauces

contain *C. perfringens* and that the methods of preparation of these foods are such that these foods may be involved in food poisoning outbreaks if proper refrigeration is not applied to left-over foods for future consumption.

REFERENCES

Baltzer, J. and Wilson, D. C. 1965. The occurrence of clostridia on bacon slaughter lines. *J. Appl. Bacteriol.* **28**, 119.

Barbe, C. D., Mandigo, R. W. and Hendrickson, R. L. 1967. Bacterial flora associated with rapid-processed ham. *J. Food Sci.* **32**, 988.

Brown, W. L., Vinton, C. A. and Gross, C. E. 1960. Heat resistance and growth characteristics of microorganisms isolated from semiperishable canned hams. *Food Research* **25**, 345.

Browne, A. S., Lynch, G., Leonard, A. R. and Stafford, G. 1962. A clostridial or enterococcal food poisoning outbreak. *Pub. Health Rept.* **77**, 533.

Canada, J. C. and Strong, D. H. 1964. *Clostridium perfringens* in bovine livers. *J. Food Sci.* **29**, 862.

Goudkov, A. V. and Sharpe, M. E. 1965. Clostridia in dairying. *J. Appl. Bacteriol.* **28**, 63.

Greenberg, R. A., Tompkin, R. B., Bladel, B. O., Kittaka, R. S. and Anellis, A. 1966. Incidence of mesophilic *Clostridium* spores in raw pork, beef, and chicken in processing plants in the United States and Canada. *Appl. Microbiol.* **14**, 789.

Hall, H. E. and Angelotti, R. A. 1965. *Clostridium perfringens* in meat and meat products. *Appl. Microbiol.* **13**, 352.

Hall, H. E., Angelotti, R. A., Lewis, K. H. and Foter, M. J. 1963. Characteristics of *Clostridium perfringens* strains associated with food and food-borne diseases. *J. Bacteriol.* **85**, 1094.

Hobbs, B. C. 1965. *Clostridium welchii* as a food poisoning organism. *J. Appl. Bacteriol.* **28**, 74.

Hobbs, B. C., Smith, M. E., Oakley, C. L., Warrack, G. H. and Cruickshank, J. C. 1953. *Clostridium welchii* food poisoning. *J. Hyg.* **51**, 75.

Kemp, G. E., Proctor, R. and Browne, A. S. 1962. Food-borne disease in California with special reference to *Clostridium perfringens (welchii). Pub. Health Rept.* **77**, 910.

Nakamura, M. and Converse, J. D. 1967. Heat resistance of spores of *Clostridium welchii. J. Hyg.* **65**, 359.

Nelson, K. E., Ager, E. A., Marks, J. R. and Emanuel, I. 1966. *Clostridium perfringens* food poisoning report of an outbreak. *Am. J. Epidemiol.* **83**, 86.

Strong, D. H., Canada, J. C. and Griffiths, B. B. 1963. Incidence of *Clostridium perfringens* in American foods. *Appl. Microbiol.* **11**, 42.

Weiss, K. F. and Strong, D. H. 1967. Some properties of heat-resistant and heat-sensitive strains of *Clostridium perfringens. J. Bacteriol.* **93**, 21.

Willis, T. 1964. "Anaerobic Bacteria in Clinical Medicine." Butterworths, London, England. p. 76.

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Nutritive Quality of Simulated Milk Mixtures Prepared from Tropical Plant Products

SUMMARY—Using products of tropical plants taro, soybeans and coconut for carbohydrate, protein and fat, mixtures to simulate cow's milk were prepared. These were tested on rats for the quality of protein, the utilization of calcium, the effect of vitamin B_{12} and the composition of blood and liver. Calcium retention was significantly better for the milk than for the mixtures. The quality of the protein was measured by the PER and the NPU. Coconut milk in the diet raised the NPU of the mixtures from 36.5 to 41.2 by raising the nitrogen storage in the body. Vitamin B_{12} raised the NPU of Poi-II from 40.4 to 50.0 by raising both the nitrogen intake and the nitrogen storage, the latter to a greater degree. The NPU of the mixtures Poi-II ranged from 50.0 to 51.1 and were similar to the NPU of 54.4 for Sobee, were lower than the NPU of 76.6 for Similac and 80.8 for milk. Blood hemoglobin and liver lipid and moisture of rats fed the mixtures Poi-II and milk were similar and within the normal range. It is concluded that the mixture Poi-II is similar to Sobee for protein quality, similar to milk for hemoglobin formation and deposition of lipid and moisture in the liver, and poorer than milk for the retention of calcium.

INTRODUCTION

IN COUNTRIES WHERE ARTIFICIAL FEEDING is practiced, the predominant food for infants is cow's milk, and the evaporated form is particularly suitable and well used. Upon developing sensitivity to cow's milk, replacement with simulated milk preparations from soy products or modified cow's milk is employed. In developing countries, proper feeding of the young remains a problem because of cost and inadequate supply of milk and, except in government-controlled operations, the unhygeinic dilution of milk by vendors. Breast feeding is prolonged until the second or third year of life and, upon weaning, children are exposed to a diet deficient in protein but adequate in calories. Mild or severe kwashiorkor is the result (Blix, 1962). An inexpensive substitute, in the form of beverage or crackers made from locally-available foods high in protein of satisfactory quality, could play a part in combating protein undernutrition.

Soybean milk was used for centuries in China as infant food (Piper *et al.*, 1943). Besides being high in protein, soybean was reported to be a good source of iron, calcium, phosphorus and of the B-vitamins (Payne *et al.*, 1944). Studies with soybeans in children's diet were summarized by Dean (1953). In India a spray-dried mixture of peanut protein isolate and soy flour, developed by Shurpalekar *et al.* (1964), gave a protein efficienty ratio (PER) of 2.34

on rats. This ratio was raised to 2.86 upon addition of methionine and approached the PER of 3.4 for milk.

Coconut milk was used for feeding infants who had gastric disorder and acute nutritional disturbances by Bejarano (1933) and Gesteira *et al.* (1932). Freudenberg (1948) successfully used the milk of jennies and mares to feed infants. Buffalo milk is more readily available in India and its preservation for infant feeding was studied by Subrahmanyan *et al.* (1958).

Preparation of simulated milk mixtures involves substituition for the milk components, chiefly the minerals, carbohydrate and protein. Calcium and iron salts are nearly always added to a vegetable preparation. The carbohydrate and protein substitutes should be very low in fiber and easily digestible. These conditions are met by the use of poi (fermented, pounded, steam-cooked taro roots) for carbohydrate and of tofu (soybean curd) for protein. The purpose of the study is to develop a product simulating milk from produce of Hawaii that are also available economically in developing countries. This product could be used instead of milk in milk allergies and milk shortage.

EXPERIMENTAL

Materials

Tofu is a soft white cheese-like product manufactured in Hawaii from soybeans according to Japanese methods (Standal, 1963; Miller, 1933). It has no crude fiber (Miller *et al.*, 1952) and the digestibility coefficient is high (Pian, 1930; Chiang *et al.*, 1941). Its protein quality is comparable to milk casein (Standal, 1963), and the trypsin inhibitor is destroyed during its preparation (Chang *et al.*, 1949). Poi has a pasty consistency with a color varying from greyish brown to dark brown. It has a high digestibility (Langworthy *et al.*, 1922) and it is a non-allergic food (Glaser, 1963; Derstine *et al.*, 1952). The taro paste is converted to poi by fermentation at room temperature. The fermentation could be stopped by canning, drying or freezing (Deurnberger, 1951).

For this study, poi was fermented at room temperature for one day and canned. Taro (*Colcasia esculenta* L., Schott) may mean either the cooked or the raw corms. In this study, for the sake of consistency, taro is considered to be the freshly made poi paste canned immediately without fermenting. The unfermented paste was darker in color and more viscous than the fermented paste. Coconut milk is prepared commercially by mascerating coconut meat with water and pressing out the liquid. It is rich in oil, which forms an emulsion containing most of the proteins and carbohydrates (Nicholls *et al.*, 1945). The raw materials were purchased in one large quantity from the same manufacturers.

Preparation of the mixtures

Four types of simulated milk mixtures were prepared (Table 1). The solids and water were mixed in a Waring blender and steamed cooked in a damp cooker with constant stirring until it started to boil. The amount of calcium lactate added to mixtures I was less than to mixtures II to compensate for the calcium from tofu. The control milk formula was prepared according to Jeans *et al.* (1947) for six-months-old infants (Table 1). The mixtures and the milk were fortified with $FeSO_4 \cdot 7H_2O$ to fulfill the recommended amounts for rats (Williams *et al.*, 1963). The four mixtures were designed to contain protein, calcium, and calories in the same amount as the milk formula to meet the requirements of infants up to six months of age (Jeans *et al.*, 1947; Recommended Dietary Allowances, 1963). All four mixtures were thicker than milk, had good appearance and flavor and were accepted by rats.

Animal experiments

Expt. 1. Quality of protein of the mixtures. Rats of 25 days old were divided according to sex, weight, and littermates into experimental and control groups. Four groups of 10 rats each were fed *ad libitum* on the four mixtures and four groups of four to five rats each received different amounts of milk formula, which provided the same amount of nitrogen as was consumed by rats on the test mixtures during the previous day. One control rat was paired with two or three experimental rats. One group of rats received a protein-free diet (Table 1) and served as non-protein controls. Vitamins A, D, E and B-complex were supplemented in liquid form in separate containers three times a week. Each rat received weekly 120 μg each of thiamine, riboflavin, folic acid and pyridoxine; 600 μg each of pantothenic acid and para-aminobenzoic acid; 400 μg of niacin; 12 μg of biotin; 15 mg of inositol; 30 mg of choline; 600

Table 1. Composition of simulated milk mixtures, milk formula, and protein-free diet.

Ingredients	Simulated milk mixtures				Milk formula (per l)	Protein-free diet (per 1000 g)
	Poi-I	Poi-II	Taro-I	Taro-II		
Poi (g)	167	150	.....	.....	.....	.....
Taro (g)	.....	.....	167	150	.....	.....
Tofu (g)	333	300	333	300	.....	.....
Coconut milk (g)	.....	50	.....	50	.....	.....
Cane sugar (g)	70	70	70	70	60	150
Calcium lactate (mg)	2729	3166	2729	3166	.....	.....
Water (ml)	500	500	500	500	569	.....
Evaporated milk (ml)	.....	.....	.....	.....	371	.....
Cornstarch (g)	.....	.....	.....	.....	.....	650
Primex (g)[1]	.....	.....	.....	.....	.....	150
Hubbell salt mix (9)[2]	.....	.....	.....	.....	.....	50

[1] Primex, a hydrogenated vegetable oil, Procter and Gamble, Cincinnati, Ohio.
[2] Hubbell *et al.* (1937).

USP of vitamin A; 60 USP of vitamin D; and 6 mg of vitamin E.

After 10 days on the diets the rats were sacrificed, the carcasses dried as described by Standal (1963) and the body nitrogen calculated from body water according to Miller *et al.* (1955). Previous study (Standal, 1963) showed a high correlation for the body nitrogen of rats by this method and that obtained by Kjeldahl analysis. To assess the quality of protein these measurements made were: the net protein utilization (NPU) and the net protein retention (NPR) according to Bender *et al.* (1957) and the PER, which is the gain in weight per gram of protein consumed.

Expt. 2. Availability of calcium from the mixtures. Five groups of eight weanling rats each were placed on one of the four mixtures or on the milk formula *ad libitum*. The rats were housed individually in wire cages with elevated bottoms. Fecal and urinary excretions were collected together on large filter paper and daily food intakes were recorded. Calcium retention was calculated from the difference between that consumed and that excreted over the total period.

Expt. 3. Effect of vitamin B_{12} on the quality of proteins. The rats on all diets reported above showed porphyrin on the fur of the neck and head beginning with the second or third week of the experiment. Rats on cow's milk with the highest weight gain showed the most porphyrin. Vitamin B_{12} was added to the vitamin mixture and was used in all subsequent experiments. The NPU of mixtures Poi-II, Poi-II without iron, and Poi-II with methionine was measured along with the NPU of Similac (a modified milk product, M and R Laboratories, Columbus 16, Ohio), Sobee (a soybean product, Mead Johnson and Co., Evansville 21, Indiana) and evaporated milk. No porphyrin was observed during the 10-day experimental period.

Expt. 4. Effect of the mixtures on the blood and liver. Weanling rats were placed on mixtures Poi-II, Poi-II with methionine, Poi-II without iron, Poi-II with methionine but without iron, and evaporated milk for 58 days. All rats received the vitamin mixtures containing B_{12}. Very slight porphydrin was observed on some rats after one month on all diets. At sacrifice, hemoglobin, hemotocrit, liver fat and water were measured.

Chemical analysis

Proximate analyses were made on all diets and their components using the methods described in AOAC (1960). Calcium on diets, carcasses and excreta was determined by the method of Greweling (1961) using a Beckman flame photometer after the samples were individually ashed. Hemoglobin was measured by the cyan-methhemoglobin method (Drabkin *et al.*, 1932) using Acuglobin (Ortho Pharmaceutical Corporation, Raritan, New Jersey) for standard. Liver water was determined as for the whole body, and fat by petroleum ether extraction of dried pulverized samples using the Goldfisch apparatus.

RESULTS AND DISCUSSION

Chemical data

The proximate composition of the mixtures and their ingredients are reported in Table 2. The water and calcium values for tofu were slightly lower than those reported by Miller *et al.* (1957), while protein, fat, and carbohydrates were higher. Mixtures I contained considerably less fat than milk and adjustments were made by the addition of coconut milk. The adjusted mixtures were designated mixtures II and the percentage of calories for fat from these mixtures was four times that from mixtures I. The protein calories, expressed as percent of total calories, were 15–16 for milk, Taro-II and Poi-II, and 19 for Poi-I and

Table 2. Proximate composition of the milk formula, the simulated milk mixtures, and of the ingredients of the mixtures.

Products	Water (%)	Protein[1] (%)	Fat (%)	Carbohydrate (%)	Ash (%)	Calcium (mg/100g)	Energy (Cal. per 100g)	Protein calories as percentage of total calories
Ingredients:								
Poi	80.86	0.47	0.01	18.38	0.28	13	76	2.5
Taro[2]	78.81	0.51	trace	20.38	0.34	12	83	2.4
Tofu	76.64	11.72	6.72	6.16	0.76	103	132	35.5
Coconut milk	71.00	2.49	21.80	4.71	0.45	16	225	4.4
Milk mixtures: wet								
Milk	84.65	2.49	1.32	10.92	0.62	98	66	15.2
Poi-I	84.94	2.87	0.35	11.55	0.29	88	61	18.9
Taro-I	84.00	3.14	0.35	12.19	0.32	96	64	19.5
Poi-II	85.86	2.56	1.46	9.84	0.28	89	63	16.3
Taro-II	84.90	2.50	1.50	10.80	0.30	88	67	15.0
Milk mixtures: dry								
Milk		16.00	8.60	71.08	4.04	635	429	14.9
Poi-I		19.06	2.32	76.69	1.93	581	404	18.9
Taro-I		19.63	2.19	76.19	2.00	600	403	19.5
Poi-II		18.10	10.32	69.57	1.98	631	444	16.3
Taro-II		16.55	9.93	71.50	1.99	583	442	15.0
Growing rat diets[3]		20	5			600	400	20

[1] Protein = 6.25 × N.
[2] Taro here is identical to poi except for the omission of fermentation.
[3] Nutrient requirements of laboratory animals (1962).

Table 3. Growth of rats raised on the milk and the simulated milk mixtures for 25 days (Expt. 2).

Mixtures	No. of rats[3]	Total intake: food (g)	Total intake: protein (g)	Total intake: energy (cal.)	Weight gain per 100 cal. (g)	Total[1] weight gain (g)	Significance of difference between group means[2]: Groups	Significance
Milk	8	1194	29.7	782	9.5	74	Milk—Poi-I	0.001
Poi-I	6	988	28.4	601	5.6	34	Milk—Poi-II	0.001
Taro-I	8	978	30.7	631	6.3	40	Milk—Taro-I	0.001
Poi-II	7	853	21.8	535	6.0	32	Milk—Taro-II	0.001
Taro-II	6	896	22.4	598	5.7	34	Taro-I—Poi-I	0.05
							Taro-I—Taro-II	0.05

[1] One way analysis of variance was applied. The variance estimate $F = S_b^2/S_w^2 = 76.6$. The treatment of rats affected the data at 0.001% level. S_b^2 and S_w^2 are the variance estimates between and within groups respectively.
[2] $t = (\bar{x}_a - \bar{x}_b)/\sqrt{S_w^2/n_a + S_w^2/n_b}$ $\bar{x}$ is a group mean, n is the number of rats per group.
[3] Originally 8 rats per group. Some escaped.

Taro-I. Tofu was the main source of protein for the mixtures contributing 94–98% of the total nitrogen. The amount of nitrogen from poi, taro, and coconut milk ranged from 1.9 to 3.4 percent. Upon expressing the composition of the formulas in terms of dry weights (Table 2) it was observed that the composition of the mixtures were in line with the requirement of nutrients by growing rats (Natl. Research Counc., Publ. 990, 1962).

Biological data

Caloric intake and growth. Weight gains and food intakes (expt. 2) for twenty-five days in the absence of vitamin B_{12} are shown in Table 3. The total amount of food intake was greatest for rats on milk formula, less for rats on mixtures I and least for rats on mixtures II. The caloric intake followed the same pattern despite the fat in mixtures II. The total food intake for rats on the four mixtures was approximately two-thirds as great as that of rats on milk while the weight gains were only half. The protein calories needed for maintenance (P_m) were calculated according to Miller *et al.* (1961) (Table 4) and were well within the available protein calories for all groups (Table 2). The difference between total weight gains for rats on the mixtures and those on milk was significant at 0.001 level (Table 3). Rats on Taro-I were heavier than rats on Poi-I and Taro-II at 0.05 level (Table 3).

Protein quality and the effect of vitamin B_{12}

In Expt. 1 the NPU values for the mixtures were lower than those for the milk (Table 4). Expressed in percent of respective controls, mixtures II were 69% of the milk and mixtures I were 48–52%. The coconut milk in mixtures II raised the NPU values by the same amount (4.65) while the lesser food intake for the milk pair-fed rats to mixtures II resulted in lower NPU of 59.7 for milk. The NPU of milk determined from pair-fed controls to mixtures I was 73.4. The ratio of carcass nitrogen to nitrogen intake was higher for mixtures II than for mixtures I and greater nitrogen retention was obtained by the addition of fat (coconut milk) to the almost fat-free mixtures I. The PER of 1.06 and 0.88 for the poi mixtures were lower than those for the taro mixtures (1.29 and 1.20), which were lower than the PER of milk (2.06 to 2.77). Compared to the respective controls, the PER of the mixtures was lower as a percentage of the controls (38.7% to 55.8%) than were the NPU. While more nitrogen and calories were consumed in the present study, the observed NPU and PER values were lower than for tofu as reported by Standal (1963).

The effect of vitamin B_{12} was studied on Poi mixtures, Similac, Sobee and evaporated milk (Table 5, expt. 3). Vitamin B_{12} improved the value of the NPU of Poi-II

Table 4. Quality of protein of the milk and the simulated milk mixtures in absence of vitamin B_{12}. (Expt. 1, 10 days).

Mixtures	No. of rats	Total nitrogen consumed per rat (10-day period) (g)	Carcass nitrogen (g)	Carcass N / N intake	Net protein utilization (NPU)[1]	% of control	Net protein retention (NPR)[2]	Protein efficiency ratio (PER)[3]	% of control	Percent protein calories needed for maintenance (Pm)[4]
Poi-I	10	1.86	1.933	1.04	35.8 ± 2.1[5]	47.5	0.61	1.06	38.7	11.18
Control	4	1.82	2.773	1.52	75.3 ± 2.1		2.30	2.74		5.31
Taro-I	10	2.12	2.056	0.97	37.2 ± 1.9	52.0	0.87	1.29	46.6	10.76
Control	4	1.98	2.823	1.43	71.5 ± 3.9		2.37	2.77		5.60
Poi-II	10	1.38	1.824	1.32	40.4 ± 2.3	68.8	0.24	0.88	43.7	9.89
Control	5	1.36	2.072	1.52	58.7 ± 1.1		1.48	2.06		6.81
Taro-II	10	1.39	1.851	1.33	41.9 ± 1.9	69.1	0.56	1.20	55.8	9.56
Control	5	1.36	2.164	1.59	60.6 ± 3.3		1.56	2.15		6.60

[1] $NPU = [B - (B_k - I_k)]/(I - I_k)$ where B is the body N of rats on the mixtures or milk
B_k is the body N of rats on the protein-free diet
I_k is the N intake by rats on the protein-free diet
I is the N intake by rats on the mixtures or milk.
[2] $NPR = \frac{\text{weight gained by rats on mixtures or milk} + \text{body weight loss by rats on protein-free diet}}{\text{protein intake by rats on the mixtures or milk}}$
[3] PER = grams gained/g protein eaten.
[4] $P_m = (4 \times 100)/NPU$. Where 4 is a percent of the net dietary protein value (N.D. — p.v.) calculated on the basis of food energy. N.D. — p.v. is a measure of utilizable protein (Miller *et al.*, 1961).
[5] Standard error.

Table 5. Change in the quality of protein of mixture Poi-II due to the addition of vitamin B_{12}. Comparison with Similac and Sobee (Expt. 3 10 days experimental period).

Mixtures	Protein content of mixtures g/100g	Number of animals	Nitrogen intake (g)	Carcass nitrogen (g)	Carcass N / N intake	PER	NPU[1]	Group differences which are significant[2]	
								Groups	Level of significance
Poi-II	3.63	6	1.739	2.129	1.22	1.86	50.0	Milk—Sobee	0.001
Poi-II no iron	3.65	6	1.650	2.105	1.28	1.75	51.1	Milk—Poi-II	0.001
Poi-II plus methionine	3.59	6	1.703	2.113	1.24	1.93	50.2	Similac—Poi-II	0.01 -0.001
Similac[3]	3.06	6	1.008	1.966	1.95	2.51	76.6	Similac—Sobee	0.01 -0.001
Milk	1.96	6	1.388	2.387	1.72	3.33	80.9		
Sobee[4]	2.22	7	1.891	2.286	1.21	1.94	54.4		

[1] One-way analysis of variance, F = 12.5. The treatment of the rats affected the data at 0.01 level.
[2] t-test to compare two means at a time using the variance estimate S_w^2.
[3] M and R Laboratories, Columbus 16, Ohio.
[4] Mead Johnson and Co., Evansville 21, Indiana.

from 40.4 (Table 4) to 50.0 (Table 5). This improvement was due both to enhanced appetite and greater nitrogen retention in the body. Neither the addition of methionine nor the removal of iron from Poi-II influenced the NPU, which remained at 50.0 to 51.1. All the NPU's for the three Poi mixtures were comparable to the NPU of 54.4 for Sobee and were lower than the NPU of 65.0 for tofu (Standal, 1963). The NPU of 76.6 for Similac and 80.8 for milk were greater than the NPU's of Poi-II and Sobee at a 99% level of significance (Table 5). The PER for the three Poi-II mixtures ranged from 1.75 to 1.93 and were higher than 0.88 for Poi-II without the vitamin B_{12}. Similar increase in the PER of legume protein due to this vitamin was observed by Fatterpaker *et al.* (1960) for peanuts and by Singh *et al.* (1960) for the pulse *arhar*.

Bricker *et al.* (1947) reported that the total requirement of food nitrogen per basal calorie for a nitrogen equilibrium of adult rats and humans was essentially the same for milk (3.34 and 2.76 mg, respectively). However, for soybean flour the requirement for rats was 6.07 mg and for man 2.88 mg. Adult rats require more sulfur-containing amino acids and this was limited in soybean. In this study, the addition of methionine to Poi-II did not alter the protein quality. The short duration of the experiment (10 days) and the growth rates of the rats might have overshadowed the need for more sulfur amino acids. The biological value of soybean flour protein was reported by Bricker *et al.* (1945, 1947) to be 65 when fed to humans and 52 when fed to rats. In the present study, the observed NPU of 50.0 to 54.4 for Poi-II mixtures and Sobee may present higher values when consumed by infants.

Calcium utilization

Calcium retention was calculated from the total intake and total excretion in urine and feces for 25 days (Table 6). Total retention was highest for milk and lowest for Poi-II. The retention for Taro-II was lower than for Poi-I and Taro-I. Upon calculating the retention as a percent of intake the values obtained were lowest for Poi-II (73 percent) and highest for milk (81 percent). Milk was significantly better than the mixtures. Of the mixtures, those containing coconut milk had less calcium retention. The retention of calcium by infants (Recommended Dietary Allowances, 1963) is 30 to 50 percent for cow's milk, and it is possible that similar percentages would be retained from the mixtures. The calcium intake of 617–1,161 mg for 25 days provided 25–45 mg per rat per day. This is within the recommendation of 0.6% in the diet, which provides a young rat with 30–60 mg of calcium per day for a 5–10 g food intake.

Blood and liver composition

All rats in this study received vitamin B_{12}. The weight gain for the 58 days was similar for all groups and was slightly higher for milk (Table 7). The hemoglobin and hematocrit values were all normal and slightly lower for

Table 6. Calcium retention[1] of rats raised for 25 days on the milk and simulated milk mixtures (Expt. 2).

Mixtures	No of rats[2]	Final body weight (g)	Calcium				Significance of the difference[4]	
			consumption (mg)	excretion (mg)	retention per rat (mg)	retention % of intake[3]	Groups	Level
Milk	8	114	1162	219	943	81	Milk—Poi-I	0.05
Poi-I	6	74	803	186	617	77	Milk—Poi-II	0.01
Taro-I	8	78	841	175	666	79	Milk—Taro-II	0.01
Poi-II	7	73	622	167	455	73	Taro-I—Poi-II	0.01
Taro-II	6	76	719	179	540	75	Taro-I—Taro-II	0.05
							Poi-I—Poi-II	0.05

[1] Calcium consumed—calcium excreted during the experimental period.
[2] Originally 8 rats per group but some were lost.
[3] The variance estimate F is 7 using one-way analysis of variance. The treatment of the rats affected the data at 0.01 level.
[4] t-test to compare two means at a time using the variance estimate S_w^2.

Table 7. Hemoglobin, hematocrit, liver fat and water of rats fed the supplemented mixture Poi-II (Expt. 4).

Mixtures	No. of rats	Total weight gain (g)	Hemoglobin (g/100 ml blood)	Hematocrit	Liver	
					water g/100g	fat g/100g
Poi-II	6	127	14.4	0.47	70.9	4.01
Poi-II, no iron	6	133	14.2	0.47	70.2	4.12
Poi-II plus methionine	6	129	14.6	0.50	70.4	4.40
Poi-II no iron plus methionine	6	124	14.2	0.49	70.4	3.95
Milk	5	137	13.7	0.44	69.5	4.58

milk (Table 7). The percentages of fat and water in the livers were all normal and similar for all groups. For blood formation and liver composition Poi-II appears to be the same as milk. Neither the removal of iron nor the addition of methionine had any effect on the quality of Poi-II.

REFERENCES

Assoc. of Offic. Agri. Chemists. 1960. "Methods of Analysis," ed. 9. Washington, D.C.

Bejarano, J. 1933. Treatment of dyspepsia in the newborn with coconut milk. *Rev. Fac. Med. Bogota* **2**.

Bender, A. E. and Doell, B. H. 1957. Biological evaluations of proteins: a new aspect. *Brit. J. Nutrition* **11**, 140.

Blix, G. 1962. Mild moderate forms of protein-calorie malnutrition. Symposia Swedish Nutrition Foundation I, Bastad, August 29–31.

Bricker, M., Mitchell, H. H. and Kinsman, G. M. 1945. The protein requirements of adult human subjects in terms of the protein contained in individual foods and foods combinations. *J. Nutrition* **30**, 269.

Bricker, M. L. and Mitchell, H. H. 1947. The protein requirements of the adult rat in terms of the protein contained in egg, milk, and soy flour. *J. Nutrition* **34**, 491.

Chang, I. C. L. and Murray, H. C. 1949. Biological value of the proteins and the mineral, vitamin and amino acid content of soybean milk and curd. *Cereal Chem.* **26**, 297.

Chiang, L. T., Li, H. C. and Lan, T. H. 1941. Biological values of soybean protein and mixed protein-pork and soybean-egg protein in human subjects. *Chin. J. Physiol.* **16**, 83.

Dean, R. F. A. 1953. Plant proteins in child feeding. Med. Research Council Special Report Ser. No. 279. London: Her Majesty's Stationery Office.

Derstine, V. and Rada, E. L. 1952. Poi in Hawaii. Some dietetic factors influencing the market for poi in Hawaii. Hawaii Agr. Expt. Sta. Agr. Econ. Bull. 3.

Drabkin, D. L. and Austin, J. H. 1932. Spectrophotometric studies I. Spectrophotometric constants for common hemoglobin derivatives in human, dog and rabbit blood. *J. Biol. Chem.* **98**, 719.

Duernberger, G. W. 1951. Processing on canning and quick-freezing of Hawaiian fruits and vegetables. Hawaii Agr. Expt. Sta. Ann. Rept. 1950–51.

Fatterpaker, P., Manjrekar, S. P., Marfatia, U., Molgaonkar, A. G., Rege, D. V. and Sreenivasan, A. 1960. Observations on the influences of vitamin B_{12} and folic acid on protein utilization in the growing rat. *J. Nutrition* **71**, 371.

Freudenberg, E. 1948. Über Säuglingsernährung mit Stutenmilch 2. *Ann. Paediat.* **171**, 49.

Gesteira, M. and Bahia, A. 1932. Coconut milk in nutritional disturbances in infants. *Brazil Med.* **46**, 169.

Glaser, J. 1963. Poi for pediatrics. *J. Am. Med. Assoc.* **183**(3), 28.

Greweling, T. 1961. Flame photometry of calcium and magnesium in ashed plant materials. *Chemist Analyst* **50**, 40.

Hubbell, R. B., Mendel, L. F. and Wakeman, H. J. 1937. A new salt mixture for use in experimental diets. *J. Nutrition* **14**, 273.

Jeans, P. C. and Marriott, W. M. 1947. "Infant Nutrition." C. V. Mosby, St. Louis, Mo.

Langworthy, C. F. and Deuel, H. J. 1922. Digestibility of raw rice, arrowroot, taro, tree-fern, and potato starches. *J. Biol. Chem.* **52**, 251.

Miller, C. D. 1933. Japanese foods commonly used in Hawaii. Hawaii Agr. Expt. Sta. Bull. 68.

Miller, C. D., Denning, H. and Bauer, A. 1952. Retention of nutrients in commercially prepared soybean curd. *Food Research* **17**(3), 261.

Miller, C. D. and Branthoover, B. 1957. Nutritive values of some Hawaii foods. Hawaii Agr. Expt. Sta. Circ. 52.

Miller, D. S. and Bender, A. E. 1955. The determination of the net utilization of proteins by a shortened method. *Brit. J. Nutrition* **9**, 382.

Miller, D. S. and Payne, P. R. 1961. Problems in the prediction of protein values of diets. *Brit. J. Nutrition* **13**, 11.

Nicholls, L. and Drummond, J. C. 1945. Nutritional value of coconut. *Nature* **155**, 392.

Nutrient Requirements of laboratory animals. 1962. A Report of the Committee on Animal Nutrition. Natl. Acad. Sci. Natl. Research Counc. Publ. 990, Washington, D. C.

Payne, D. S. and Stuart, L. S. 1944. Soybean protein in human nutrition. In "Advances in Protein Chem.," Vol. I. Academic Press, N. Y.

Pian, J. H. C. 1930. Biological value of the proteins of mung bean, peanut and bean curd. *Chin. J. Physiol.* **4**, 431.

Piper, C. V. and Morse, W. J. 1943. "The Soybean," p. 228. Peter Smith, Massachusetts.

Progress in Meeting Protein Needs of Infants and Preschool Children. 1961. Natl. Acad. Sci. Natl Research Counc. Publ. 843. Washington, D. C.

Recommended Dietary Allowances. Rev. 1963. Natl. Acad. Sci. Natl. Research Counc. Publ. 1146. Washington, D. C.

Shurpalekar, S. R., Korula, S., Chandrasekhara, M. R., Swaminathan, M., Chandrasekhar, B. S., Sreenivasan, A. and Subrahmanyan, V. 1964. Studies on a spray-dried infant food based on peanut protein isolate and full-fat soy flour and fortified with DL-methionine and certain vitamins and minerals. II. Protein efficiency ratio and over-all nutritive value. *Food Technol.* **18**(6), 110.

Singh, D. K., Gupta, Y. P. and Das, N. B. 1960. Effect of amino acids and vitamin B_{12} on the nutritive value of pulse protein. *Annals of Biochem. and Experi. Med.* **20**(1), 1.

Standal, B. R. 1963. Nutritional value of proteins of oriental soybean foods. *J. Nutrition* **81**(3), 279.

Subrahmanyan, V., Chandrasekhara, M. R. and Swaminathan, M. 1958. Buffalo milk—its nutritive value and use in infant feeding. *Indian J. Pediat.* **25**(123), 174.

Williams, M. A. and Briggs, G. M. 1963. An evaluation of mineral mixtures commonly used in diets for experimental aniamls. *Am. J. Clin. Nutrition* **13**, 115.

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Dilution of Cow's Milk and Egg Proteins with Glutamic Acid and the Effect on the Protein Efficiency Ratio

SUMMARY—The effect of dilution of egg proteins and cow's milk proteins with varying levels of L-glutamic acid (GA) on the growth of young rats and protein efficiency ratio of the blends was studied. Addition of glutamic acid to diets containing 8.5% to 5.0% egg proteins to maintain the nitrogen content of the diet constant at 1.6% (equal to 10% protein) did not cause any increase in the growth rate of rats as compared to that on corresponding diets without added glutamic acid. The protein efficiency ratios progressively decreased from 4.74 for 10% egg protein diet to 2.88 for 5% egg protein + 8.4% glutamic acid diet. Addition of glutamic acid to diets containing 8.5 to 5.0% milk proteins to maintain the nitrogen content of the diet constant at 1.6% level caused a significant decrease in the growth rate as compared to that on corresponding diets without added glutamic acid. The protein efficiency ratios also progressively decreased from 3.48 for 10% milk proteins to 1.46 for a mixture of 5% milk proteins + 8.4% glutamic acid. The results show that both egg proteins and milk proteins contain adequate amounts of non-essential amino acids for maximum utilization of essential amino acids present in them for the growth of young rats.

INTRODUCTION

IT IS NOW WELL RECOGNIZED that the nutritive value of a protein depends on the content, availability and balance of essential amino acids (Almquist, 1956). Since egg proteins possess the highest nutritive value among food proteins, it has been assigned the 'highest' chemical score of 100 by Block *et al.* (1946). Their system of chemical scoring for assessing the relative nutritive value of food proteins expresses the quantity of the limiting amino acids in different food proteins as a percentage of the same amino acid in egg proteins. They found a good correlation between the biological value of the proteins and the 'chemical scores'.

The FAO/WHO Expert Group on Protein Requirements (FAO/WHO, 1965) suggested that after the required pattern of the essential amino acids has been specified, the percentage of total nitrogen intake contributed by the essential amino acids must be indicated. They also stated that there is good reason to believe that in both human milk and egg proteins, the proportions of essential amino acids are higher than that required for older children and adults, although the physiological reason for this is not clear.

Snyderman *et al.* (1962) reported that in the case of human infant, the limiting factor at low levels of milk protein intake is non-essential nitrogen, rather than of essential nitrogen. Scrimshaw *et al.* (1966) observed that in adult human subjects receiving daily 0.36 to 0.34 g egg proteins per kg body weight, replacement of 30 to 40% of egg proteins with non-essential nitrogen in the form of glycine and di-ammonium citrate did not significantly alter N retention.

Venkat Rao *et al.* (1964) described the effects of dilution of egg proteins with glutamic acid on the protein efficiency ratio. The present paper deals with the effect of dilution of cow's milk protein with glutamic acid on the PER of the proteins.

MATERIALS AND METHODS

Diets

Spray-dried skim milk powder of good quality was incorporated into the diets at 10, 8.5, 7.5, 6.5 and 5% protein levels. Another batch of diets containing 8.5, 7.5, 6.5 and 5.0% milk proteins were diluted by the addition of L-glutamic acid at 2.5, 4.2, 5.9 and 8.4% levels, so that the nitrogen contents of these diets were nearly the same, i.e. about 1.6% N (equivalent to 10% protein).

Diets containing 5.0% egg proteins and a mixture of 5.0% egg proteins + 8.4% glutamic acid were also prepared. The composition of the experimental diets was similar to that described by Tasker *et al.* (1962). The contents of four essential amino acids of the diets (amino acids g/16 gN) calculated using the figures for the amino acid composition of the proteins of egg and cow's milk given by the FAO/WHO Expert Group (1965) are given in Tables 1 and 2.

Protein efficiency ratio

The protein efficiency ratio of the diets was determined by the rat growth method of Osborne *et al.* (1919). Freshly weaned male rats (21 days old) from the laboratory stock colony (Wistar strain) were allotted to different groups according to randomized block design. They were housed individually in cages fitted with raised wire mesh bottoms. The diets were mixed with twice their weight of cold water to prevent scattering.

Records of the weekly increase in body weight and daily food intake of the individual animals were maintained. The PER was calculated from the data obtained. Data reported earlier with 6.5 to 8.5% levels of egg proteins (Venkat Rao *et al.*, 1964) are also given for comparison.

RESULTS

Growth rates

The growth rates of rats fed on different levels of milk proteins (with and without added L-glutamic acid) are given in Table 3. The rats fed 10% milk proteins gained 98.4 g in 4 weeks; the groups receiving lower levels of protein gained less. The addition of L-glutamic acid to

Table 1. Essential amino acids (g/16 g N) E/TN ratios and chemical scores of egg proteins diluted with glutamic acid.

Amino acids	Hen's egg protein (HEP) only	HEP 8.5% + G.A. 2.5%	HEP 7.5% + G.A. 4.2%	HEP 6.5% + G.A. 5.8%	HEP 5.0% + G.A. 8.4%
Lysine	6.40	5.44	4.80	4.16	3.20
Total sulphur amino acids	5.50	4.68	4.13	3.58	2.75
Threonine	5.10	4.34	3.83	3.32	2.55
Tryptophan	1.60	1.36	1.20	1.04	0.80
Total essential amino acids	51.3	43.61	38.48	33.35	25.65
E/NA ratio	1.05	0.89	0.79	0.68	0.53
E/TN ratio	3.22	2.73	2.41	2.09	1.61
Chemical score (egg protein 100)	100	85	75	65	50

Table 2. Essential amino acids (g/16 g N) E/TN ratio and chemical score of cow's milk proteins diluted with L-glutamic acid.

Amino acids	Cow's milk protein only (CMP)	CMP 8.5% + G.A. 2.5%	CMP 7.5% + G.A. 4.2%	CMP 6.5% + G.A. 5.8%	CMP 5.0% + G.A. 8.4%
Lysine	7.80	6.63	5.85	5.07	3.90
Total sulphur amino acids	3.30	2.81	2.48	2.15	1.65
Threonine	4.60	3.91	3.45	2.99	2.30
Tryptophan	1.40	1.19	1.05	0.91	0.70
Total essential amino acids	50.3	42.76	37.73	32.70	25.15
E/NE ratio	1.0	0.85	0.75	0.65	0.5
E/TN ratio	3.15	2.68	2.36	2.05	1.58
Chemical score: (egg protein 100)	60	50	45	40	30

Table 3. Protein efficiency ratio of cow's milk proteins and egg protein diluted with varying levels of glutamic acid. (Period of experiment, 4 weeks) (10 male rats per group).

Group no. Source of protein	Level of protein in diet (N × 6.25)	Initial body weight (g)	Gain in body weight (g/4 weeks)	Protein intake (g)	PER
Series I:					
1. Cow's milk (10%)	10.2	37.4	98.4	28.32	3.49
2. Cow's milk (8.5%)	8.6	37.4	84.0	22.54	3.72
3. Cow's milk 8.5% + GA 2.5%	10.3	37.0	65.2	23.69	2.76
4. Cow's milk 7.5%	7.6	37.1	75.2	18.03	4.18
5. Cow's milk 7.5% + GA 4.2%	10.2	37.2	52.0	22.74	2.28
6. Cow's milk 6.5%	6.6	36.9	64.0	15.45	4.15
7. Cow's milk 6.5% + GA 5.8%	10.2	37.4	40.8	20.96	1.92
8. Cow's milk 5%	5.1	37.0	45.6	11.71	3.90
9. Cow's milk 5% + GA 8.4%	10.2	37.1	24.8	16.90	1.46
Standard error of the mean (72 df)			± 2.5		±0.08
Series II:					
10. Egg, 5%	5.18	37.7	68.5	14.12	4.84
11. Egg, 5% + L-glutamic acid 8.4%	10.22	37.5	67.5	23.47	2.88
12. Egg, 10%	10.24	37.8	125.7	27.28	4.60
Standard error of the mean (18 df)			± 3.6		±0.07

Table 4. The influence of added L-glutamic acid on the growth of rats and PER of diets containing 5 to 8.5% milk or egg proteins.

Level of egg or milk protein in diet (%)	Level of glutamic acid in diet (%)	Gain in weight (g/4 weeks)				PER			
		Milk protein only	Milk protein +G.A. to make total N equivalent to 10% protein	Egg protein only	Egg protein +G.A. to make total N equivalent to 10% protein	Milk protein only	Milk protein +G.A. to make total N equivalent to 10% protein	Egg [1] protein only	Egg protein +G.A. to make total N equivalent to 10% protein
10.0	.....	98.4	.....	132.4 [2]	.....	3.49	.....	4.68	.....
8.5	2.5	84.0	65.2	105.7	105.8	3.72	2.76	4.74	4.03
7.5	4.2	75.2	52.2	95.0	101.6	4.18	2.28	4.72	3.53
6.5	5.8	64.0	40.8	90.9	90.4	4.15	1.92	4.64	3.12
5.0	8.4	45.6	24.8	68.5	67.5	3.90	1.46	4.84	2.88

[1] Data for diets containing 6.5 to 8.5% protein from Venkat Rao *et al.* (1964).
[2] Mean values for 4 groups of 8 male rats each, one group run with each of the four levels of added G.A.

milk protein (5.0 to 8.5%) adversely affected the growth rates of rats.

Data previously published (Venkat Rao *et al.* 1964) for 6.5 to 8.5% egg proteins and obtained in the present study for 5% egg protein (with and without L-glutamic acid) (Table 4) indicated that the 4-week gains were proportional to the quantity of egg protein in the diet, but in this case, dilution of the protein with L-glutamic acid did not adversely affect growth rates.

Protein efficiency ratios

The PER of cow's milk proteins at 5.0 to 10.0% levels ranged from 3.49 to 4.18. Dilution of milk proteins (8.5% to 5.0%) with L-glutamic acid (to maintain the N equivalent of the diet to 10.0% protein) brought about a progressive decrease in the PER.

The PER data of Venkat Rao *et al.* (1964) for 6.5 to 8.5% egg proteins and obtained in the present study for 5.0% egg protein (with and without glutamic acid) indicated that the PER progressively decreased when the egg protein content decreased and the L-glutamic acid content increased in the diet.

DISCUSSION

The FAO/WHO Expert Group on Protein Requirements (FAO/WHO, 1965) suggested that the ratio between essential amino acids to total nitrogen (E/TN ratio) will be a good index of the nutritive value of proteins. The results presented in this paper indicate that though the E/TN ratio for milk and egg proteins (with and without added glutamic acid) is nearly equal, there is considerable difference in the PER of the blends. It is evident therefore that E/TN ratio is not a reliable index of the nutritive value of proteins. On the other hand, the 'Chemical Score' of the proteins (based on the limiting essential amino acids) diluted with different levels of L-glutamic acid is a reliable index of the nutritive value of proteins as seen from Table 5.

A mixture of 5.0% egg proteins + 8.4% glutamic acid containing 3.3 g lysine, 0.8 g tryptophan, 2.8 g threonine and 2.8 g total sulphur amino acids per 16 g N and having a chemical score of 50, possesses a PER of 2.88. PER of similar order has not so far been reported for other dietary proteins having similar amino acid compositions, and chemical score (Block *et al.*, 1946).

Table 5. Essential/non-essential amino acid ratio (E/NE ratio), E/TN ratio, chemical score and PER of egg and cow's milk proteins diluted with glutamic acid (level of protein in diet, 10%).

Protein source	E/TN ratio	E/NE ratio	Chemical score[1]	PER	PER (4 weeks) as % of PER of whole egg
Series I:					
Hen's egg 10%	3.22	1.05	100	4.74	100
Egg 8.5% + l-glutamic acid 2.5%	2.73	0.89	85	4.03	85
Egg 7.5% + l-glutamic acid 4.2%	2.41	0.79	75	3.53	74
Egg 6.5% + l-glutamic acid 5.8%	2.09	0.68	65	3.12	66
Egg 5.0% + l-glutamic acid 8.4%	1.61	0.53	50	2.88	61
Series II:					
Cow's milk 10%	3.15	1.0	60	3.49	74
Cow's milk 8.5% + l-glutamic acid 2.5%	2.68	0.85	50	2.76	58
Cow's milk 7.5% + l-glutamic acid 4.2%	2.36	0.75	45	2.28	48
Cow's milk 6.5% + l-glutamic acid 5.8%	2.05	0.65	40	1.92	41
Cow's milk 5.0% + l-glutamic acid 8.4%	1.58	0.50	30	1.46	32

[1] Taking the chemical score of whole egg as 100 according to Block *et al.* (1946).

The results further indicate that both egg and milk proteins contain adequate amounts of non-essential amino acids for the maximum utilization of the essential amino acids and addition of L-glutamic acid to egg or milk proteins does not bring about a higher growth rate. These results are in conformity with those of Stucki *et al.* (1962) who found that in diets based on a well-balanced mixture of amino acids, a mixture having E/NE ratios of 1.0 (similar to that in egg proteins) promoted the maximum growth and possessed the maximum PER. It is difficult to offer an explanation for the results of Snyderman *et al.* (1962) who report that addition of non-essential nitrogen such as urea and glycine improved N retentions in infants receiving 1.1 g/kg of milk proteins. These claims require confirmation.

REFERENCES

Almquist, H. J. 1956. In "Amino Acid Handbook," ed. Block, R. J. and Weiss, K. W. C. C Thomas, Springfield, Illinois.

Block, R. J. and Mitchell, H. H. 1946. Amino acid composition of proteins. *Nutr. Abst. Rev.* **16**, 249.

FAO/WHO. 1965. Protein Requirements, FAO Nutrition Meetings Rep. Ser. No 37, p. 39.

Osborne, T. B., Mendel, L. B. and Ferry, E. L. 1918. A method for expressing numerically the growth-promoting value of proteins. *J. Biol. Chem.* **37**, 223.

Scrimshaw, N. S., Young, V. R., Schewartz, R., Piche, M. L. and Das, J. B. 1966. Minimum dietary essential amino acid-to-total nitrogen ratio for whole egg protein fed to young men. *J. Nutr.* **89**, 9.

Snyderman, S. E., Holt, L. E., Dancis, J., Roitman, E., Boyer, A. and Ballis, M. E. 1962. Unessential nitrogen—a limiting factor for human growth. *J. Nutr.* **78**, 57.

Stucki, W. P. and Harper, A. E. 1962. Effects of altering the ratio of indispensable to dispensable amino acids in diets for rats. *J. Nutr.* **78**, 278.

Tasker, P. K., Joseph, A. A., Ananthaswamy, H. N., Indiramma, K., Narayana Rao, M., Swaminathan, M., Sreenivasan, A. and Subrahmanyan, V. 1962. Nutritive value of the proteins of groundnut flour and a 4:1 blend of groundnut flour and skim milk powder. *Food Sci.*, **11** 175.

Venkat Rao, S., Daniel, V. A., Joseph, A. A., Sankaran, A. N. and Swaminathan, M. 1964. The pattern of amino acid requirements of albino rat for optimal growth. *J. Nutr. Diet.* **1**, 103.

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RESEARCH NOTES

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Lemon Oil Analysis. 3. Rapid, Capillary Gas Chromatography With Combined Flow and Temperature Programming

SUMMARY—Without preliminary treatment, cold-pressed lemon oil was gas chromatographed on a 100-ft temperature and flow programmed capillary in 10 min. Twenty important volatile flavor constituents were identified and approximately 40 more were detected. The speed and convenience of this procedure suggest use in general survey applications.

INTRODUCTION

WITH THE DEVELOPMENT OF temperature-programmed capillary gas chromatography (GC), it became possible to separate more than 100 volatile components in a single sampling of cold-pressed lemon oil injected under mild conditions into a 300-ft column (MacLeod *et al.*, 1966b). The procedure required 2 hr of unattended operation which was acceptable for research purposes but somewhat lengthy for general survey applications. Zlatkis *et al.* (1965) have suggested combined flow and temperature programming for accelerating GC analysis at minimal cost in column resolution. Such a policy was employed here on a 100-ft capillary to provide a rapid, yet efficient GC method for analyzing lemon oil flavor volatiles.

EXPERIMENTAL

Apparatus

GC analysis was performed on a Perkin-Elmer hydrogen-flame ionization gas chromatograph, model 226, equipped with a 100-ft × 0.010-in.-ID stainless-steel capillary column coated with a mixture of 90% w/w decolorized Apiezon L and 10% w/w IGEPAL CO-880. Flow programming of the helium carrier gas was attained either manually by periodically increasing the inlet pressure or automatically by a Perco-Flo-trol programmer inserted in the carrier gas line just upstream from the injector block inlet.

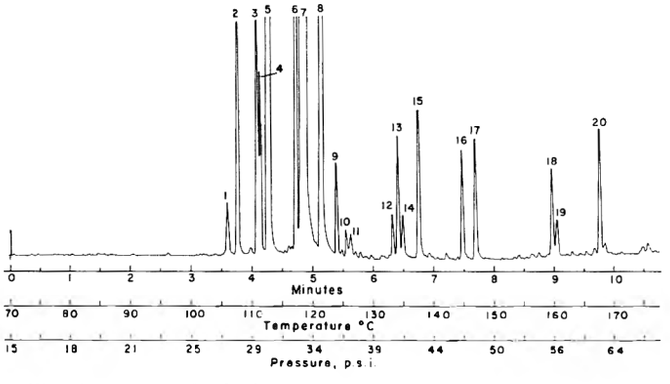

Fig. 1. Automatic dual temperature- and flow-programmed gas chromatogram of California cold-pressed lemon oil on a 100-ft, 0.010-in.-ID capillary coated with 10% w/w CO-880 in decolorized Apiezon L.

Sample injection

A 0.1 μl sample of USP, cold-pressed lemon peel oil was taken up in a Hamilton model 7101 microsyringe and injected into the GC inlet which was maintained at 120°. The sample was immediately vaporized in the carrier gas stream and split 100:1. The smaller portion was then swept with the carrier gas into the capillary column.

Automatic temperature and flow program

Simultaneously with sample injection the column temperature was linearly programmed at 10°/min from 70° to 175° during 10.5 min. Similarly, the inlet carrier gas pressure was programmed exponentially from 15 to 68 psig with the Flo-trol programmer.

Optional manual flow and temperature program

Upon sample injection the column temperature was maintained isothermally at 55° for 0.5 min, then successively linearly programmed at 50°/min for 0.5 min to 80°, 20°/min for 0.5 min to 90°, 10°/min for 8 min to 170° and then maintained at 170° for 1 min. The carrier gas flow was programmed quasi-exponentially by manual stepwise increase of the carrier gas inlet pressure from 16 to 18, 21, 24, 28, 32, 36, 42, 48, 56 and 64 psig, respectively, at 1-min intervals after sample injection.

Identification of flavor constituents

All compound identifications in Table 1 were based upon quantitative proportionality and retention time identity with compounds previously identified in cold pressed lemon oil by combined gas chromatography and mass spectroscopy (MacLeod *et al.*, 1966b).

Table 1. Identities of peaks in Fig. 1.

1.	α-thujene	11.	p, α-dimethylstyrene
2.	α-pinene	12.	terpineol-4
3.	sabinene	13.	neral
4.	myrcene	14.	α-terpineol
5.	β-pinene	15.	geranial
6.	ρ-cymene	16.	neryl acetate
7.	limonene	17.	geranyl acetate
8.	γ-terpinene	18.	α-bergmotene
9.	terpinolene	19.	caryophyllene
10.	citronellal	20.	β-bisabolene

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Hydrochloric Acid in Isolating Anthocyanin Pigments from Montmorency Cherries

INTRODUCTION

THE USE OF strong- and weak-acid ion-exchange resins during the isolation of anthocyanin pigments from fruits is a common technique. Hydrochloric acid is added to the elution solvent to facilitate removal of the pigment from strong-acid resins (e.g., Dowex 50) and is commonly added during elution of pigment from weak-acid resins (e.g., Amberlite CG50) to prevent formation of the anhydrobase.

During the isolation of pigments from Montmorency cherries, it was found that different resins yielded variable results associated with minor changes in technique. The purpose of this note is to demonstrate that the use of HCl in connection with resins may result in the isolation of pigments not naturally occurring, and in incorrect relative proportions.

EXPERIMENTS AND RESULTS

THE SOURCE OF pigment for the experiments with the strong-acid resin (Dowex 50W-4X) was a 5% acetic acid in methanol extract of lyophilized Montmorency cherries, whereas the source of pigment for the weak-acid resin (Amberlite CG50, Type I) was cherry juice applied directly to the resin. In both instances, the resins were washed with 20 volumes of distilled water to remove interfering substances. To accomplish the removal of pigment from strong-acid resin, 1% HCl in methanol was used. Pigment was removed from the weak-acid resin with 5% acetic acid in 95% ethanol.

The HCl-methanol eluate was collected and divided into two fractions. One fraction was dried under vacuum at 40°C without further treatment; the other was neutralized with sodium hydroxide, reacidified with acetic acid and concentrated under vacuum at 40°C. When nearly dry, 1 ml of 0.1% HCl in ethanol was added and the solution evaporated to dryness as above. The acetic acid-ethanol eluate from the weak-acid resin was treated in the same manner as the neutralized eluate above.

Separation of the individual pigments was accomplished by descending paper chromatography on Whatman 3-mm paper, using 1% HCl as solvent. Strips were cut from the dried chromatograms and scanned by an Analytrol densitometer, using a 550-mμ interference filter (Fig. 1).

Electrophoresis has been used previously to establish the relative amounts of the three naturally occurring anthocyanin pigments in Montmorency cherries (Schaller *et al.*, 1968) (Table 1). The relative proportions of pigments isolated by each procedure and determined by Analytrol traces are calculated in Table 1. It is apparent that no

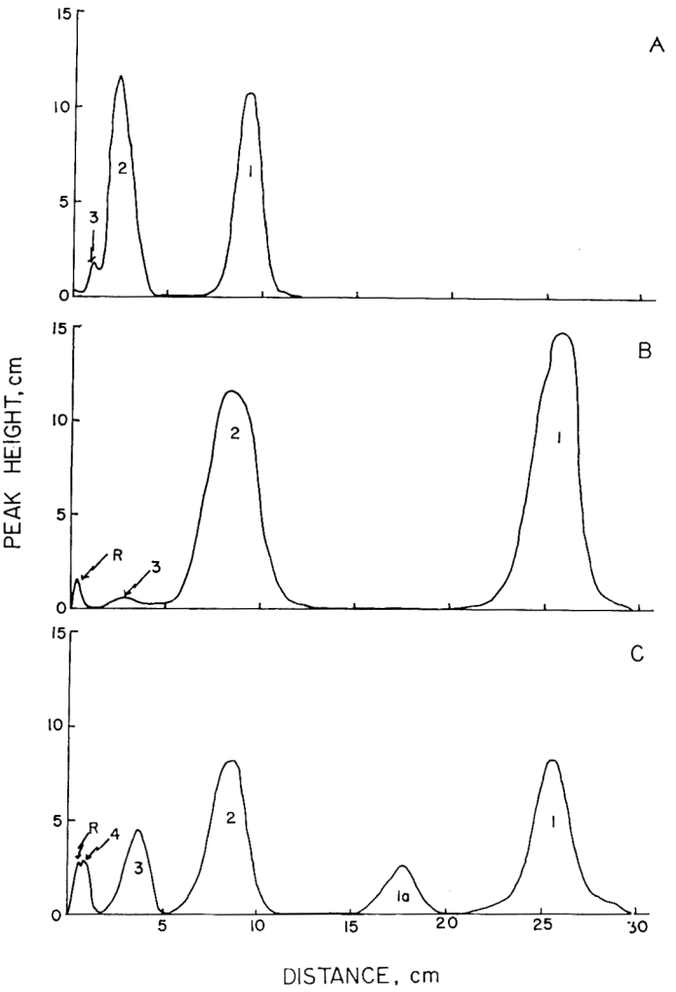

Fig. 1. Analytrol traces of cherry pigment obtained from paper chromatograms developed with 1% HCl.
A. Pigments obtained from Amberlite C650. Type I pigments removed with 95% ethanol.
B. Pigments obtained from Dowex 50-4X. Eluate neutralized to remove HCl prior to drying.
C. Pigments obtained from Dowex 50-4X. HCl remaining in eluate during drying.
Pigment 1—cyanidin 3-gentiobioside, Pigment 2—cyanidin 3-rhamnoglucoside, Pigment 3—cyanidin 3-monoglucoside, Pigment 4—cyanidin, R—residue.

method using resin reproduced the known pigment ratio, and the unneutralized eluate obtained from the strong-acid resin yielded two anomalous pigments (1a, 4) together with a significant increase in the amount of cyanidin-3-monoglucoside. During washing of the weak-acid resin, some loss of pigment was noted.

To demonstrate that the two anomalous pigments found

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Table 1. Relative amounts of cherry pigments obtained by various methods of isolation.

	Pigment	Electrophoresis %	Crude pigment obtained from weak-acid resin %	Crude pigment obtained from strong-acid resin (neutralized) %	Crude pigment obtained from strong-acid resin (non-neutralized) %	Crude pigment obtained from strong-acid resin (non-neutralized) reapplied onto weak-acid resin %
1	Cyanidin-3-gentiobioside	62	48	54	40	48
1a	Cyanidin-diglycoside	.....	.....	.....	9	2
2	Cyanidin-3-rhamnoglucoside	35	49	44	38	44
3	Cyanidin-3-monoglucoside	3	3	2	10	4
4	Cyanidin	.....	.....	.....	3	2

in the unneutralized fraction were not lost during washing of the weak-acid resin, an eluate containing these two pigments was reapplied to the weak-acid resin. The results of this experiment (Table 1) showed that the weak-acid resin would have retained these pigments if they had been present. Pigment 4 was shown to be cyanidin and pigment 1a a cyanidin-diglycoside. The complete identity of the latter is not known, and its occurrence cannot be attributed to a partial hydrolysis by HCl. It would seem, therefore, that using resin in the isolation of pigments cannot be relied upon to yield the natural relative proportions of pigments, and eluates containing HCl must be neutralized prior to drying to avoid the formation of anomalous pigments.

Titration of aliquots showed that the HCl concentration rose to 20% during the latter stages of drying. It seems logical, therefore, that the appearance of the two anomalous pigments (cyanidin and Band 1a) together with a significant increase in the amount of cyanidin-3-monoglucoside results from chemical degradation occurring during the drying step when HCl is present. This may explain why some workers have reported traces of anthocyanidins present in some investigations.

REFERENCES

Schaller, D. R. and J. H. von Elbe. 1968. The minor pigment component of Montmorency cherries. *J. Food Sci.* **33**, 442–443.
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Quantitative Determination of Diacetyl by Electron Capture

INTRODUCTION

THE IMPORTANCE OF DIACETYL as a flavor compound in foods is widely recognized. In some existing methods for the quantitative determination of diacetyl in foods, the diacetyl is purged from the sample with nitrogen gas, trapped as dimethylglyoxime in buffered hydroxylamine and converted to a colored complex by reaction with ferrous sulfate (Owades *et al.*, 1963; Pack *et al.*, 1964). The colored complex is then measured spectrophotometrically. A polarographic method for the determination of diacetyl in buttermilk has been described (Ferren *et al.*, 1967). These methods are suitable for measuring diacetyl in the parts per million range but not in lesser amounts. Since the average flavor threshold for diacetyl in milk has been found to be 10–20 parts per billion (ppb) (Bennett *et al.*, 1965; Hempenius *et al.*, 1966; Scanlan, 1967), a method capable of determining diacetyl in this range would be useful in flavor work.

A gas entrainment, on-column trapping procedure for gas chromatographic analysis of flavor volatiles designed by Morgan and Day (1965) has been used for the quantitative determination of volatile compounds in foods (Bills *et al.*, 1966; Langler *et al.*, 1967). As originally described, a flame ionization detector was employed for the detection of compounds. However, a recent report indicated that the electron capture detector is 1,200 times more sensitive to diacetyl than the flame ionization detector (Shilman *et al.*, 1966). This communication describes the quantitative determination of diacetyl in the parts per billion range using the gas entrainment, on-column trapping procedure with an electron capture detector.

EXPERIMENTAL

KNOWN AMOUNTS OF DIACETYL were added to samples of whole milk. Ten-ml samples of milk were pipetted into screw-capped vials (Kimble No. 60957, size 1) containing approximately 1 mg of tetradecanol to control foaming during collection of volatiles. The vials were sealed by

means of screw-caps fitted with silicone rubber liners and shaken vigorously to mix the milk and the tetradecanol.

The operating parameters for the analyses were:

Entrainment apparatus

Purge time and rate	10 min at 10 ml/min of N_2
Water bath temperature	37°C ± 2°C

Gas chromatographic conditions

Instrument	F & M Model 810
Detector temperature	200°C
Detector pulse interval	150 microseconds
Detector purge rate	140 ml/min of 5% methane in argon
Column	20% 1,2,3-*Tris*(2-cyanoethoxy) propane on 80–100 mesh Celite 545
Column dimensions	13.5 ft × ⅛-in. O.D.
Column temperature	isothermal 70°C
Flow rate	24 ml/min of 5% methane in argon

RESULTS AND DISCUSSION

Peak heights obtained for the various concentrations of diacetyl added to milk were plotted to obtain the standard curve shown in Fig. 1. Each point on the curve represents the mean of two determinations. Although not shown on the curve in Fig. 1, samples containing 80 and 160 ppb were also analyzed and the recorder response was also linear throughout this range.

Five replicate samples of heated milk were analyzed for diacetyl. The mean for these samples was 30.3 ppb, the range 29.0–31.5 ppb and the standard deviation 1.04.

The procedure described herein is a sensitive, rapid method for the quantitative analysis of diacetyl. Concentrations of diacetyl at the average flavor threshold for milk can easily be determined by this procedure. In our laboratory samples of milk containing as little as 2–3 ppb of diacetyl have been successfully analyzed. Although the procedure has been used only on milk, undoubtedly a variety of food products could be analyzed for diacetyl using this procedure.

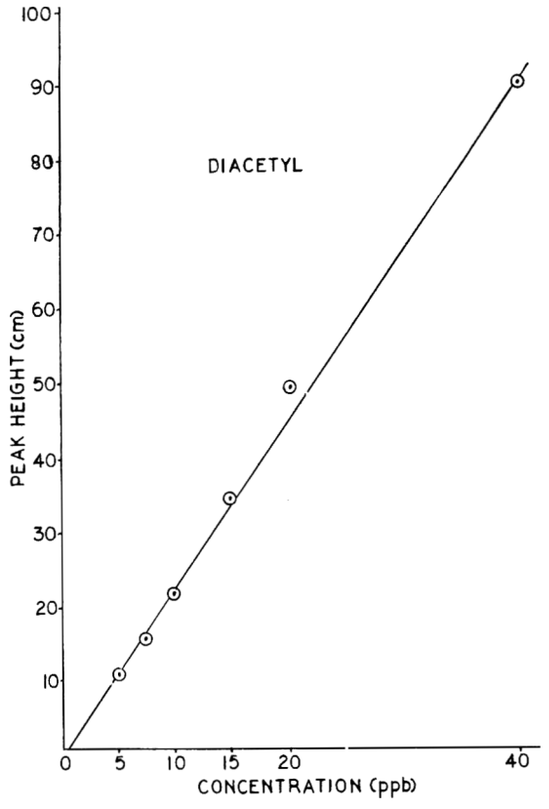

Fig. 1. Recorder response obtained with various concentrations of diacetyl in milk.

REFERENCES

Bennett, G., Liska, B. J. and Hempenius, W. L. 1965. Effect of other flavor components on the perception of diacetyl in fermented dairy products. *J. of Food Sci.* **30**, 35.

Bills, D. D., Willits, R. E. and Day, E. A. 1966. Determination of some neutral constituents in ten commercial cheddar cheese. *J. Dairy Sci.* **49**, 681.

Ferren, W. P., Shane, N. and Schweigerathi, R. 1967. Polarographic determination of diacetyl in buttermilk. *Food Tech.* **21**, 1265.

Hempenius, W. L., Liska, B. J., Bennett, G. and Moore, H. L. 1966. Analytical taste-panel studies on diacetyl flavor levels in creamed cottage cheese. *Food Tech.* **20**, 334.

Langler, J. E., Libbey, L. M. and Day, E. A. 1967. Identification and evaluation of selected compounds in Swiss cheese flavor. *J. Agr. Food Chem.* **15**, 386.

Morgan, M. E. and Day, E. A. 1965. Simple on-column trapping procedure for gas chromatographic analysis of flavor volatiles. *J. Dairy Sci.* **48**, 1382.

Owades, J. L. and Jakovac, J. A. 1963. Microdetermination of diacetyl in beer. *Proc. Am. Soc. Brewing Chemists.* pp. 22–25.

Pack, M. Y., Sandine, W. E., Elliker, P. R., Day, E. A. and Lindsay, R. C. 1964. Owades and Jakovac method for diacetyl determination in mixed-strain starters. *J. Dairy Sci.* **47**, 981.

Scanlan, R. A. 1967. Heat induced compounds in milk. Ph.D. Thesis, Oregon State University, Corvallis, Oregon.

Shilman, A. and Schaper, L. W. 1966. The analysis of small concentrations of biacetyl. *J. of Gas Chromato.* **4**, 486.

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The Minor Pigment Component of Montmorency Cherries

INTRODUCTION

THE MAJOR pigments in Montmorency cherries were identified as cyanidin-3-rhamnoglucoside and cyanidin-3-gentiobioside by Li *et al.* (1956). In an attempt to isolate these pigments for anthocyanin studies, a third naturally-occurring anthocyanin pigment was noted. The identification is herein described.

EXPERIMENTS AND RESULTS

BOTH FRESH and pitted frozen Montmorency cherries (*Prunus cerasus* L., var. Montmorency) were used in this study. Solvents used for paper chromatography are given in Table 1. The chromogenic spray reagent used for locating sugars in paper chromatography was that of Mukherjee *et al.* (1952). Isolation of the crude pigment was accomplished by applying cherry juice to the weak-acid resin column described by Sakamura *et al.* (1961). Separation of individual pigments was effected by successive paper chromatography using 1% HCl and 0.1% HCl (Table 1) as solvents.

The identification of the pigment was carried out using the following standard techniques. R_f values were obtained for the anthocyanin, its aglycone and sugar moiety using the respective solvent systems listed in Table 1. The position of the sugar moiety on the aglycone was verified by the hydrogen peroxide degradation (Chandler *et al.*, 1961). Visual spectral data were obtained to determine the maximum absorption peak and to calculate the E_{440}/E_{max} ratio. The results of these experiments are listed in Table 2. From these data it was concluded that the minor pigment component is cyanidin-3-monoglucoside.

Paper electrophoresis of skin sections from fresh fruit resulted in the separation of three pigments. The pigments were shown by paper chromatography to be cyanidin-3-gentiobioside, cyanidin-3-rhamnoglucoside, and cyanidin-3-monoglucoside. Analytrol traces of the paper strips (550 mμ filter) showed ratios of 62:35:3, respectively.

Separation of the pigments from the skin occurred within 0.5 hr at 3°C, and complete separation of the individual pigments within 5.5 hr. Individual purified pigments, when subjected to paper electrophoresis under the conditions used for fresh fruit skins, were observed to travel as a single band. It is, therefore, concluded that cyanidin-3-monoglucoside is a naturally occurring pigment present in small quantities.

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Table 1. Solvent systems used in paper chromatography of anthocyanins, anthocyanidins and sugar moieties.

Solvent notation	Composition	Solvent phase used for chromatography	Used for separation of
15% HAc	15% acetic acid by vol.	entire solution	anthocyanidins
BAW	n-butanol : acetic acid : water 4 : 1 : 5 by vol.	upper phase	anthocyanidins anthocyanins
BuHCl	n-butanol : 2 *N* HCl 1 : 1 by vol.	upper phase	anthocyanins
1% HCl	water : 12 *N* HCl 97 : 3 by vol.	entire solution	anthocyanins
0.1 HCl	water : 12 *N* HCl 99.7 : 0.3 by vol.	entire solution	anthocyanins
HAc : HCl	acetic acid : HCl : water 15 : 3 : 82 by vol.	entire solution	anthocyanins
Forrestal	water : acetic acid : HCl 10 : 30 : 3 by vol.	entire solution	anthocyanidins
Formic	formic acid : HCl : water 5 : 2 : 3 by vol.	entire solution	anthocyanidins
PEW	propanol : ethyl acetate : water 7 : 1 : 2 by vol.	entire solution	sugar
EAW	ethyl acetate : acetic acid : water 3 : 3 : 1 by vol.	entire solution	sugar

Table 2. Chromatographic and spectrophotometric data used to identify the minor pigment component.

	R_f value in solvents												
Moiety	BAW[1]	BuHCl	HAcHCl	1% HCl	15% HAc	Forrestal	Formic	PEW	EAW	E max ± 1 mμ	E_{440}/E_{max} (as %)	$AlCl_3$ Shift	Identification
Anthocyanin	0.38	0.31	0.23	0.05	.....	.....	.....	.....	.....	525	22	+	cyanidin 3-monoglucoside
Anthocyanidin	0.53	.....	.....	.....	0.14	0.50	0.24	.....	.....	535	....	+	cyanidin
Cyanidin	0.53	.....	.....	.....	0.14	0.50	0.24	.....	.....	535	....	+	.....
Sugar	.....	.....	.....	.....	.....	.....	.....	0.28	0.25	.....	....	....	glucose
Glucose	.....	.....	.....	.....	.....	.....	.....	0.28	0.25	.....	....	....	.....
H_2O_2 degradation	.....	.....	.....	.....	.....	.....	.....	0.27	.....	.....	....	....	glucose

[1] R_f values measured to center of spot.

REFERENCES

Chandler, B. V. and Harper, K. S. 1961. Identification of saccharides in anthocyanin and other flavonoids. *Australian J. Chem.* **14**, 486.

Li, K. C. and Wagenknecht, A. C. 1956. The anthocyanin pigments of sour cherries. *J. Am. Chem. Soc.* **78**, 979.

Mukherjee, S. and Srivastava, H. C. 1952. Improved spray reagent for the detection of sugars. *Nature* **189**, 330.

Sakamura, S. and Francis, F. J. 1961. Anthocyanin of the American cranberry. *J. Food Sci.* **26**, 318.

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NOTICE

Page Charges for Publication of Research Papers

☐ Publication costs and publication pressures have increased at a fast rate over the past few years. The result is problems for all publishers, including the Institute of Food Technologists. An example of publication pressure is the considerably higher rate of receipt of research manuscripts for *Food Technology* compared with the rate of publication in the journal. One solution to this problem is the publication of special or supplemental issues at great expense to the Institute.

At the October 1967 Executive Committee meeting, the Subcommittee on Publications recognized and acknowledged the problem posed by increased costs and pressures, and discussed the matter at length. The Executive Committee then voted unanimously—as a financial expedient—to establish a page charge for research articles of $30 per page printed in either *Food Technology* or the *Journal of Food Science*.

☐ The page charge will be effective for research manuscripts received after April 1, 1968. The page charge is subject to adjustment by the Executive Committee upon recommendation of the Subcommittee on Finance after suitable experience has been gained.

The page charge SHALL NOT *constitute a bar to the acceptance of research papers for publication because an author is unable to pay the charge.*

☐ It is the view of the Executive Committee that the page charge is a matter of policy in effective administration of the journals, so long as the page charge is normal for other similar journals, it is imposed at a reasonable level, it provides for hardship cases so that it is not a barrier to publication, and it is necessary and defensible for financial reasons. At the request of the Executive Committee, concurrence of the Council Policy Committee was obtained. This publication is to serve as notice that the $30 charge per printed page will become effective for research manuscripts received after April 1, 1968. •